STABLE ISOTOPE TRACER STUDIES FOR THE MEASUREMENT OF EQUINE GASTROINTESTINAL MOTILITY

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ABSTRACT

Abdominal disorders are a major cause of morbidity and mortality in horses, and abnormal gastrointestinal motility may be a significant factor in the aetiopathogenesis of many equine colic syndromes. The understanding of such conditions is hampered by the lack of suitable noninvasive tests for the quantitative measurement of intestinal transit. The overall objective of this work was to investigate the potential value of stable ^{13}C-isotope breath tests for the assessment of specific parameters of equine gastrointestinal motility. A new method developed for the collection of equine expiratory breath and measurement of its ^{13}C:^{12}C ratio was shown to have excellent repeatability. Assessment of peripheral blood ^{13}C tracer content was also performed and correlated significantly to that of concurrent breath samples.

In the first study, the ^{13}C-octanoic acid breath test (^{13}C-OABT) was evaluated for the measurement of solid phase gastric emptying rate in 12 healthy horses by direct comparison with the predicate method of gastric scintigraphy. Significant correlations ($P < 0.001$) were found between the two techniques for measurement of both gastric half-emptying time ($t_{1/2}$) and lag phase duration (t_{lag}). Constant real-time differences in $t_{1/2}$ measurement between the modalities were demonstrated to correspond to the post-gastric processing time of the ^{13}C tracer prior to its release from the bicarbonate pool. Having shown that the ^{13}C-OABT was a reliable diagnostic procedure for use in healthy horses, a further study was performed against scintigraphy in subjects with atropine-induced gastroparesis ($n = 8$) to determine whether the test remained accurate when emptying rate was markedly prolonged. Significant inter-modal correlations were again present for calculation of both $t_{1/2}$ ($P < 0.01$) and t_{lag} ($P < 0.05$), and a similar mean bias was found between the techniques for $t_{1/2}$ measurement, suggesting that the test was valid for detection of gastroparesis.

In study 3, the ^{13}C-OABT was applied to measure the relative and dose-related effects of common sedative agents on solid phase gastric emptying in 8 horses. Using a randomised study design, test meal ingestion was followed by intravenous administration of saline (control) or specific doses of xylazine, detomidine, detomidine/butorphanol or acepromazine. Detomidine/butorphanol combination (0.01/0.02 mg/kg) prolonged both $t_{1/2}$ and t_{lag} with respect to xylazine 0.5 mg/kg and the saline control ($P < 0.05$). Detomidine 0.03 mg/kg also delayed each parameter with respect to saline, acepromazine 0.05 mg/kg and xylazine 1.0 mg/kg ($P < 0.001$). The inhibitory effect of detomidine was dose-dependent in nature and greater than equipotent doses of xylazine. The study results may

have clinical significance for case selection when these agents are used for purposes of sedation and/or analgesia.

The ^{13}C-bicarbonate and sodium ^{13}C-acetate breath tests were investigated in study 4 for the assessment of equine liquid phase gastric emptying, and elucidation of the pattern of $^{13}CO_2$ recovery from the body bicarbonate pool. Using these tests, mean (± s.d.) $t_{1/2}$ for 250 ml labelled solution was shown to be 1.13 (± 0.35) h or 1.72 (± 0.21) h respectively. The mean difference in $t_{1/2}$ estimation was thought to correspond to the time required for oxidative metabolism of the ^{13}C-acetate tracer. Recovery of $^{13}CO_2$ from the ^{13}C-bicarbonate test accorded with a three-compartment model for the equine bicarbonate pool.

The lactose ^{13}C-ureide breath test (^{13}C-LUBT) was investigated in study 5 for estimation of orocaecal transit time (OCTT), and concurrent comparison made to the hydrogen breath test (H_2BT). The former proved to have greater repeatability than the latter for estimation of this parameter, and ingestion of the ^{13}C-tracer was followed in all individuals by a discrete peak in $^{13}CO_2$ output. *In vitro* bacterial incubation techniques were then used to validate the ^{13}C-LUBT for specific measurement of OCTT and to produce a test protocol for clinical use. Bacterial microbes with glucoseureide hydrolase activity were found to reside predominantly in the equine caecum, with minimal activity present in the small intestine. Microbial slurries from the caecal apex also digested the tracer significantly faster than those collected from the small intestine ($P < 0.05$) with a greater rate of $^{13}CO_2$ production, suggesting that the ^{13}C-LUBT was a valid tool for OCTT measurement. As bacterial ^{13}C-LU metabolism was maximised by prior exposure to unlabelled LU, an induced test protocol was developed for clinical application.

In study 6 the induced ^{13}C-LUBT was evaluated *in vivo* for the measurement of OCTT and a mean (± s.d.) time of 3.24 (± 0.65) h was gained. Prior exposure to ^{12}C-LU ('induction') was shown to be a vital part of the test protocol in the study animals, resulting in a greater and steeper generation of $^{13}CO_2$ and improved estimation of OCTT. In order to examine the relationship between gastric emptying of solid ingesta, small bowel transit and its arrival in the caecum, a combined test was developed and applied in study 7, incorporating both ^{13}C-OA and ^{13}C-LU. Mathematical modelling of ^{13}C recovery after ingestion of the dual test meal allowed calculation of small bowel half transit time, in addition to gastric and caecal parameters. Interestingly, initial small bowel transit was shown to be independent of gastric emptying rate and a significant negative correlation was present between gastric and small bowel $t_{1/2}$ times, consistent with a small intestinal negative feedback mechanism. A positive correlation ($P < 0.001$) existed between early small

intestinal and caecal transit parameters, suggesting that the equine small intestine has a major regulatory role in the delivery of nutrients to this structure.

Finally, minimised test protocols were developed for the ^{13}C-OABT and ^{13}C-LUBT in order to increase their clinical utility. The effects of decreasing the duration or frequency of breath collection on generation of intestinal transit parameters were assessed and linear regression models produced for each test based on the collection of 5 breath samples. Gastric $t_{1/2}$, t_{lag} and OCTT estimates from the reduced model and the full sampling protocols were highly correlated. However, in each case the reduced models were likely to underestimate these parameters when significantly prolonged, decreasing their sensitivity for the detection of delayed intestinal transit.

The stable isotope breath tests offer a novel means of investigating features of intestinal motility and physiology in the horse and have potential value as both diagnostic modalities and humane research tools in this species. As the tests are non-invasive, simple to perform and do not require extensive equipment, they may be performed on site and the samples then submitted for isotopic analysis. Unlike other techniques for assessment of equine gastrointestinal motility, the stable isotope breath tests also provide an indirect measure of the transit rate of ingesta itself, which is directly relevant to the clinical situation.

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Sutton, D.G.M., Preston, T., Christley, R.M., Cohen, N.D., Love, S. and Roussel, A.J. (2002) The effects of xylazine, detomidine, acepromazine and butorphanol on equine solid phase gastric emptying rate. *Equine vet J.* **34**, 486-492.

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Equis ubique prosit hoc opus

AUTHOR'S DECLARATION

The work presented in this thesis was performed solely by the author, except where the assistance of others is acknowledged.

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List of Abbreviations

AAT	acetaminophen absorption test
ABCA	automated breath ^{13}C analyser
^{13}C-ABT	^{13}C-acetate breath test
AP	action potential
APT	applied potential tomography
AUC	area under the curve
BIPS	barium impregnated polyethylene spheres
Bwt	bodyweight
CF-IRMS	continuous flow isotope ratio mass spectrometry
DIR	duodenal instillation and recovery
EGG	electrogastrography
EIT	electrical impedance tomography
ENS	enteric nervous system
g	gram
GE	gastric emptying
GEC	gastric emptying coefficient
GECS	gastroenterocolonic scintigraphy
GIT	gastrointestinal tract
GU	glucoseureide
h	hour
H_2BT	hydrogen breath test
HPLC	high performance liquid chromatography
^{111}In-DTPA	^{111}Indium diethyltriaminepentaacetic acid
LSB	long spike burst
LU	lactose ureide
^{13}C-LUBT	lactose ^{13}C-ureide breath test
MAPC	migrating action potential complex
MCFA	medium chain fatty acids
MCT	medium chain triglyceride
min	minute
MMC	migrating myoelectrical complex
MRI	magnetic resonance imaging
NMR	nuclear magnetic resonance spectroscopy
^{13}C-OABT	^{13}C-octanoic acid breath test
OCTT	orocaecal transit time

PDB	Pee Dee Belemnite
PDR	percentage dose recovery of isotope
PEG	polyethylene glycol
PPM ^{13}C	parts per million ^{13}C
RMS	root mean square
RQ	respiratory quotient
SA	surface area
SBTT	small bowel transit time
s.d.	standard deviation
SIBO	small intestinal bacterial overgrowth
SLZ/SP test	sulphasalazine/sulphapyridine test
SPECT	single-photon emission computed tomography (SPECT) imaging
^{13}C-SPT	^{13}C-*Spirulina platensis* breath test
SSB	short spike burst
SW	slow wave
^{99m}Tc-DTPA	^{99m}technetium diethyltriaminepentaacetic acid
^{99m}Tc-SC	^{99m}technetium sulphur colloid
$t_{10\%}$	transit time of 10% of content
$t_{1/2}$	half-emptying time
t_{lag}	duration of lag phase (prior to maximal emptying rate)
WGTS	whole gut transit scintigraphy
y	years

Chapter 1 MEASUREMENT OF EQUINE GASTROINTESTINAL MOTILITY: BACKGROUND LITERATURE AND GENERAL INTRODUCTION

1.1 Historical Perspectives

The gastrointestinal tract of the modern horse *Equus caballus* represents the end product of millions of years of evolutionary drive and adaptation. The horse is perhaps the ultimate extant grazing herbivore – a social creature built for speed and stamina, yet able to sustain life on a wide variety of forage materials. Although the evolution of the equid digestive system is not represented in the fossil record, the documented progressive adaptations of the brain, dentition and skeletal systems suggest radical shifts in dietary strategy (Simpson 1951) that must have been facilitated by gastrointestinal refinements. The earliest equine genus has been identified as *Eohippus*, a pad-footed, four-toed browser, of miniature pony size, living 55 million years ago (Simpson 1951). One of the greatest transformations in the subsequent lineages occurred during the Miocene, coincident with the appearance of steppes and prairies (Clabby 1976). *Parahippus* genera developed high-crowned, cement-covered teeth with complex enamel ridges that allowed life-long utilisation of abrasive grasses. The consequent increase in dietary fibre resulted in gastrointestinal adaptation, which necessitated a progressive increase in body size, balanced by the requirement for retention of agility to avoid predators. Both of these factors forced changes in the skeletal system, with extension of the lower limb, development of a spring mechanism, and reduced digit number, such that by the Pliocene, 10 million years ago, the one-toed *Pliohippus* genera were similar in morphology to the modern *Equus* genera (Simpson 1951).

Compared with grazing ruminants, equids represent an alternative evolutionary response by the ungulate order to the utilisation of herbaceous food resources. The relative wet mass of equid gastrointestinal contents (13 – 20% bwt) is considerably less than that of ruminants (Duncan 1992), facilitating athletic function. However, this has been gained at the cost of forage digestive efficiency, which is lower in ponies than ruminants, and poorer still in horses (Slade and Hintz 1969; Koller *et al.* 1978). The special feature of the equid digestive tract that makes such a system viable is its rapid rate of passage of ingesta, with particle retention time approximately half that of ruminants (Koller *et al.* 1978). This is achieved by the series of mixing chambers in the proximal colon (modified plug-flow reactor system) that enables maintenance of food intake as dietary fibre content increases (Hume 1998). This system allows equids to extract more nutrients than comparable ruminants from most natural forages, but requires higher input, with prolonged foraging

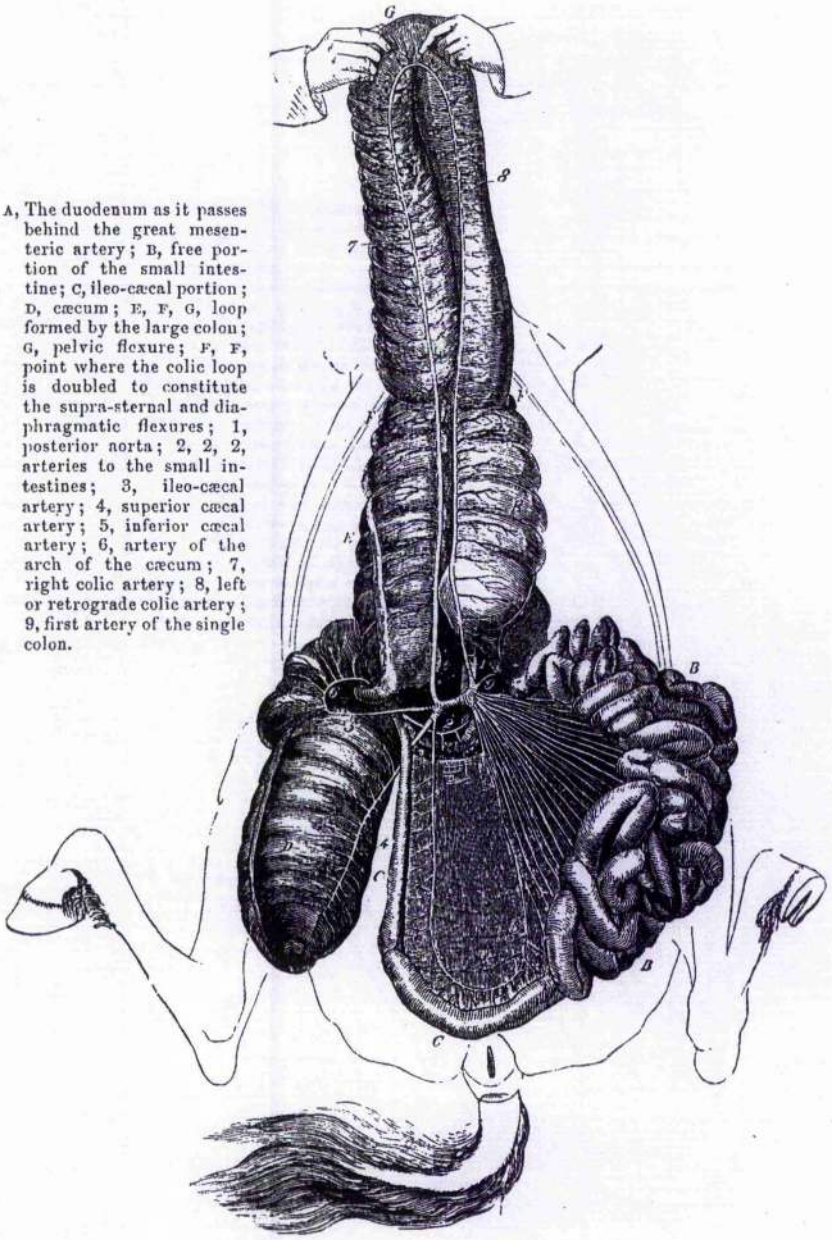
time (Duncan 1992). Feral horses and zebras forage for approximately 60% of the day, with peaks of activity organized around dawn and the afternoon, such that a high level of gut fill and maximal food assimilation is maintained (Duncan 1992).

Given these specific evolutionary adaptations of the equine gastrointestinal tract over millions of years, it should not be surprising that the regimen of twice daily feeds of concentrates and inadequate forage, as frequently given to the domesticated horse, can lead directly to a variety of physiological and mechanical disorders. The ancient Egyptians revered the gastrointestinal tract and appointed a deity as a guardian god of the embalmed guts of the deceased (Walker-Smith 2002). Whilst the custom of separate burial of the gastrointestinal tract has not been recorded in the veterinary field, a further degree of reverence for such a remarkable organ would not be amiss in equine gastroenterology.

1.2 Features of equine gastrointestinal anatomy

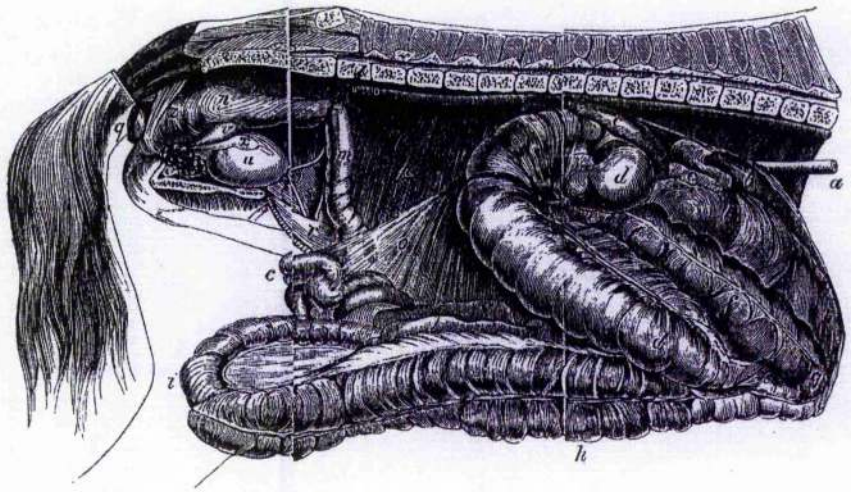
The gross anatomy of the equine gastrointestinal tract has been accurately characterised for well over a century, and the topographical illustrations of Chauveau (1891) reproduced in Figures 1.1 and 1.2 have scarcely been bettered. Detailed contemporary descriptions of the equine gastrointestinal tract have been provided by Sisson (1975) and by Nickel *et al.* (1979b). Giving a brief overview, the equine digestive system is defined by a small simple stomach with a maximum capacity of approximately 18 l (see Table 1.1), which is in sharp contrast to the mean capacities of the caecum and large colon (33 l and 81 l respectively). The J-shaped stomach is situated in the left dorsoproximal region of the abdomen, and empties into the moderately sized small intestine via a short horseshoe-shaped duodenum of approximately 1.5 m length. It is the jejunum that comprises the major part of the small intestine (17 – 28 m long), and this section of the gut is supported by mesentery in the dorsal aspect of the left half of the abdomen in a highly mobile system of coils that are susceptible to volvulus and intussusception. In contrast to the jejunum, the ileum is relatively fixed in position by the distal aspect of the mesentery and the ileocaecal fold that connects the antimesenteric ileal border to the dorsal band of the caecum. The ileum is very short (0.7 – 0.8 m long) and muscular, and enters the caecum at the lesser curvature of the caecal base, at the ileal ostium (Pfeiffer and MacPherson 1990). In accordance with its specialised role in digestive and absorptive functions the equine hindgut is much more elaborate, with a large caecum and large (ascending) colon, terminating in the small (descending) colon and rectum.

A, The duodenum as it passes behind the great mesenteric artery; B, free portion of the small intestine; C, ileo-cæcal portion; D, cæcum; E, F, G, loop formed by the large colon; G, pelvic flexure; F, F, point where the colic loop is doubled to constitute the supra-sternal and diaphragmatic flexures; 1, posterior aorta; 2, 2, 2, arteries to the small intestines; 3, ileo-cæcal artery; 4, superior cæcal artery; 5, inferior cæcal artery; 6, artery of the arch of the cæcum; 7, right colic artery; 8, left or retrograde colic artery; 9, first artery of the single colon.



GENERAL VIEW OF THE HORSE'S INTESTINES (THE ANIMAL IS PLACED ON ITS BACK, AND THE INTESTINAL MASS SPREAD OUT).

Figure 1.1 Topographical representation of the equine gastrointestinal tract showing the entry of the ileum into the caecum and the relatively huge volume of the large colon which occupies the major part of the abdomen. Reproduced from *The Comparative Anatomy of the Domesticated Animals*, A. Chauveau (1891), Ed. G. Fleming, J. & A. Churchill Press, London.



GENERAL VIEW OF THE INTESTINES OF THE HORSE (SEEN FROM THE RIGHT SIDE, WITH THE PELVIC FLEXURE AND A PORTION OF THE SMALL INTESTINE CARRIED BEYOND THE ABDOMINAL CAVITY).

a, Œsophagus; *b*, right sac of the stomach; *c*, small intestine, showing its origin or duodenal portion, encircling the base of the cæcum; *d*, cæcum; *e*, origin of the large colon; *f*, first portion of the large colon; *g*, supra-sternal flexure; *h*, second portion of the large colon; *i*, pelvic flexure; *j*, third portion of the large colon; *k*, diaphragmatic flexure; *l*, fourth portion of the large colon; *m*, termination of the free colon; *n*, rectum; *o*, mesentery proper; *p*, colic mesentery (meso-colon); *q*, anus; *r*, internal inguinal ring; *s*, spermatic vessels; *t*, deferent canal; *u*, bladder; *v*, vesiculæ seminales; *x*, pelvic enlargement of the vas deferens; *y*, prostate; *z*, suspensory ligament of the penis.

Figure 1.2 Sagittal view of the equine gastrointestinal tract demonstrating the topography of the caecum. Reproduced from *The Comparative Anatomy of the Domesticated Animals*, A. Chauveau (1891), Ed. G. Fleming, J. & A. Churchill Press, London.

Table 1.1 Relative dimensions and capacity of the equine gastrointestinal tract.

Intestinal region	Length (m) (Mean & Range)	Relative Length (%)	Mean Capacity (L)	Relative Capacity (%)
Oesophagus	1.25 - 1.50	-	-	-
Stomach	-	-	18.0 (8 - 20)	8.5
Small Intestine	22.4 (19.0 - 30.0)	75.0	63.8	30.2
Duodenum	(1.0 - 1.5)			
Jejunum	(17.0 - 28.0)			
Ileum	(0.7 - 0.8)			
Caecum	1.0 (0.0 - 1.3)	4.0	33.5	15.9
Large Colon (Ascending)	3.4 (3.0 - 4.0)	11.0	81.2 (55 - 130)	38.4
Small Colon (Descending)	3.1 (2.4 - 4.0)	9.0	14.8 (inc. rectum)	7.0
Rectum	0.3	1.0	-	-
Total	29.9	100.0	211.3	100.0

Data compiled from R.Nickel, A.Schummer and E. Seiferle (1979). The Viscera of the Domestic Mammals, 2nd Ed., New York, Springer-Verlag.

In many ways the equine stomach and small intestine are designed as conduits to facilitate the rapid passage of ingesta to the large bowel. Sufficient large intestinal content must be maintained by this rapid transfer mechanism or its digestive efficiency is reduced (Hume 1998).

In addition to being of large fermentative capacity, the caecum and large colon have special anatomical features called haustrae. These sacculations function to increase the mucosal surface area and to prolong the large intestinal residence time by initiating series of mixing contractions that serve to agitate the luminal contents. The caecum has four rows of sacculations (*haustra caeci*) formed by four longitudinal muscular taenial bands; the medial and lateral taeniae are joined by the ventral and dorsal bands before meeting at the apex of the caecum. The right and left ventral colon also has four taenial bands, which decrease to one band on the lesser curvature of the pelvic flexure. The left dorsal colon has one band initially, but gains a further two before becoming the right dorsal colon, which continues with three rows of sacculations (Nickel *et al.* 1979b).

Certain features of equine gastrointestinal physiology and motility should also be introduced briefly here, but will be expanded as appropriate at later stages in the text. A complex three-dimensional neuronal system exists throughout the intestinal wall, known as the enteric nervous system (ENS), which is intimately involved with the control of most gastrointestinal functions, including motility. Many authors consider the ENS to be the third component of the autonomic nervous system, after the sympathetic and parasympathetic nervous systems, which are excitatory and inhibitory respectively to intestinal contractile force. The neuronal network of the ENS is organised into the submucous plexus, located in the submucosa and the myenteric plexus, which lies between the circular and longitudinal intestinal muscle layers of the *muscularis externa* (Fenger *et al.* 1998). These plexi have synaptic input from both afferent intestinal fibres and efferent fibres that stimulate or inhibit tonic or propulsive contractions, such that they can both integrate luminal signals and initiate action. The intrinsic plexuses are therefore very important for the regulation of the reciprocal patterns of excitation and inhibition that are necessary for the migration of spiked potentials that constitutes 'motility' (Ruckebusch 1981). Indeed, the intrinsic plexuses can support propulsive motility in the absence of extrinsic neuronal input (Wood 1987).

Cholinergic neurones originate from the myenteric plexus and stimulate smooth muscle activity. Modulatory neurones coordinate this activity and the sympathetic nervous system provides inhibitory input by pre-synaptic α_2-adrenergic stimulation. Further modulating

neural inputs include cholinergic motor neurones and peptidergic interneurones. Vasoactive intestinal polypeptide (VIP) and nitric oxide (NO) are the main inhibitory neurotransmitters in this control system (Fenger *et al.* 1998). Extrinsic autonomic parasympathetic input is supplied by the vagus nerve from the oesophagus to mid-colon, whilst the sacral nerves supply the rectum. Pre-ganglionic parasympathetic fibres synapse on myenteric plexus ganglion cells. Sympathetic input to the intestine exits the spinal cord between T_8 and L_3, with synapse of pre-ganglionic fibres in the sympathetic chain of ganglia, and synapse of the postganglionic sympathetic fibres in the myenteric plexus (Nickel *et al.* 1979b).

The generation of slow wave depolarisation signals has been demonstrated in a number of different pacemaker regions in the equine gastrointestinal tract. Such pacemakers have been reported to exist on the greater curvature of the gastric antrum in the longitudinal muscle layer (King and Gerring 1988); ventral in the caecum, towards the apex (Sellers *et al.* 1982b; Ross *et al.* 1986); at the origin of the right ventral colon (Ross *et al.* 1986) and in the pelvic flexure (Sellers *et al.* 1979, 1982a, 1982b). The rhythmic pacemaker currents in these regions are generated by the interstitial cells of Cajal (Hudson *et al.* 1999) and have great importance in the regulation of intestinal motility and function. In the stomach, the antral pacemaker produces 2 to 5 depolarizations per minute, which sweep aborally over the fundus to the pylorus, with variable superimposition of spiking activity depending on the autonomic balance (Meyer *et al.* 1979). During progressive motility of ingesta towards the pylorus there is intermittent spiking activity, which may be followed by prolonged antral spiking activity if mixing of ingesta is required. Loss of gastric pacemaker activity may potentially be involved in the development of gastric dilatation.

The small intestinal migrating myoelectrical complex (MMC) is discussed in detail in Chapter 8. This pattern of electrical activity is cyclical, lasting approximately 90 min in the horse and unlike other species, is not disrupted by *ad libitum* feeding activity. The MMC ranges from a quiescent period of just slow wave activity (Phase 1), through a period of intermittent action potential generation (Phase 2) which results in peristalsis, to a short period of repetitive spiking activity (Phase 3) which correlates to a 'housekeeper' pattern of motility, which serves to remove residual (indigestible) ingesta. Duodenal myoelectrical activity has a complex but coordinated relationship with pyloric antral action potential activity, but does not arise continuously from it (Merritt 1999).

The pacemaker area found towards the caecal apex is thought to generate coordinated contraction of the caecum and right colon, such that it promotes caecocolic movement of

ingesta (Ross *et al.* 1986). Failure of this function may lead to caecal impaction. The right ventral colon however may continue to empty even in the absence of caecal pacemaker activity due to an additional intrinsic pacemaker area at its origin (Ross *et al.* 1986). The pelvic flexure pacemaker can generate bi-directional retropulsive-propulsive coordinated pressure peaks. Sellers *et al.* (1982a) suggested that this was responsible for controlling the retention of large particles of digesta being held in the caecum and proximal parts of the large colon for upto 48-72 h, whilst allowing the passage of better hydrolysed (smaller) particles. Damage to this area may lead to both pelvic flexure and caecal impaction (Sellers *et al.* 1982b).

As in other species, the horse has a vagus-mediated gastrocolic reflex, which results in stimulation of colonic motility following gastric distension (Ruckebusch 1981). This has direct relevance with regard to the orocaecal transit tests described in Chapters 7 and 8 of this thesis, as ingestion of a test meal itself may result in movement of residual ingesta into the caecum. A sympathetico-sympathetic intestinal reflex is also present, in which local distension results in reflex inhibition of intestinal motility due to increased adrenergic activity (Ruckebusch 1981). Inhibition of this reflex may be one of the mechanisms of action of lignocaine that contributes to its reported prokinetic activity in the horse (see section 1.7.3.7). Until recently the horse was thought to have little receptive relaxation of the stomach during swallowing. However, using an intragastric balloon barostat, Lorenzo-Figueras *et al.* (2002) have demonstrated recently the presence of a biphasic relaxation response in the proximal portion of the equine stomach in response to the ingestion of hay. This was considered to correspond to the processes of receptive then adaptive relaxation.

One further interesting anatomical phenomenon that has been discovered recently by the same research group, is the effect of exercise on equine gastric volume. Lorenzo-Figueras and Merritt (2002) showed that intense exercise in horses resulted in increased intra-abdominal pressure that caused gastric compression, with consequent direct contact between the proximal squamous mucosa area and acidic gastric contents. This was considered to be a possible factor in the development of squamous erosions and ulceration in horses undergoing intensive training programmes (Lorenzo-Figueras and Merritt 2002).

1.3 Equine gastroenterology: specific challenges for the clinician and research scientist

Despite advances made in the understanding of equine gastroenterology and nutrition over the last decade, equine colic remains a condition of high morbidity and mortality, with

significant welfare implications and large economic impact (Traub-Dargatz *et al.* 1991, 2002). In a huge study by the National Animal Health Monitoring System in 1998 of the incidence of colic in 28 states throughout America, an overall incidence of 4.2 colic events per 100 horses per year was recorded, of which 50% received veterinary treatment (Traub-Dargatz *et al.* 2002). The cause of the majority of colic events reported by owners was unknown, while gas colic and feed-related factors were reported to be the second and third most common (Traub-Dargatz *et al.* 2002). From the results of the same study it was estimated that the annual national cost of colic was 115 million dollars (Traub-Dargatz *et al.* 2002). The results of this national survey generated a similar colic incidence as that reported by Kaneene *et al.* (1997a) from the Michigan equine monitoring study. Although Kaneene *et al.* (1997a) found colic to be only the seventh most frequent health problem it was associated with the second highest mortality. In a prospective one-year study in Virginia and Maryland, Tinker *et al.* (1997) also reported colic to have the highest proportional mortality rate (28%) of any cause of equine death.

Huge challenges therefore remain in equine gastroenterology to improve this situation for the domestic horse. Further knowledge is required regarding the aetiology of undiagnosed colic and better identification of specific risk factors so that preventative measures can be implemented. Improved diagnosis in individual cases, with identification of the specific source of abdominal pain, would also be beneficial to treatment selection and to improved efficacy of long-term preventative plans. In the Michigan equine population study, the vast majority of colic cases were undiagnosed: Kaneene *et al.* (1997b) reported that 64% of cases were non-specific diagnoses, 17% were caused by impaction/acute intestinal obstruction, 9% were spasmodic colics, 5% were sand colic, 3% were gas colics, 1% were caused by verminous arteritis and the remaining 1% of cases were caused by mouldy food ingestion.

With growing recognition of the importance of evidence-based medicine in both human and equine clinical practice, a number of retrospective epidemiological studies have been performed to identify possible risk factors for colic (White and Lessard 1990; Cohen *et al.* 1995; Cohen and Peloso 1996; Kaneene *et al.* 1997b; Hudson *et al.* 2001; Little and Blikslager 2002). The major management factors that have been associated with colic are dietary changes (White and Lessard 1990; Cohen *et al.* 1995; Cohen and Peloso 1996; Hudson *et al.* 2001; Little and Blikslager 2002). Some specific dietary risk factors have been identified for certain types of colic such as the feeding of Coastal Bermuda hay for development of ileal impaction (Little and Blikslager 2002). Dietary change was not implicated in the development of simple colonic obstruction and distension colic in horses,

but stabling for 24 h/day, crib-biting/windsucking and travel in the previous 24 h were significant risk factors in the final model (Hillyer *et al.* 2002).

Sackett described evidence-based medicine as 'the conscientious, explicit and judicious use of current best evidence in making decisions about the care of patients' (Sackett *et al.* 1996). The current major obstacle to the instigation of this desirable approach in equine colic is the lack of knowledge of the actual effect on gastrointestinal function of apparent risk factors identified from retrospective studies. Generally, quantitative assessment of equine gastrointestinal transit has not been performed in clinical practice, and has been restricted to small research studies. In large part this is due to the lack of simple non-invasive test methods for the measurement of intestinal transit in clinical cases. In a recent evidence-based study, an attempt was made using transrectal ultrasonography to determine whether or not sudden dietary change caused an alteration of intestinal motility (Nicholls and Freeman 2002) and the preliminary results suggested that this was the case. The findings were perhaps not unexpected, but served to illustrate that accurate measurement of intestinal response to changes in specific factors such as frequency, composition, and variability of feeding pattern, would permit a much more rational, evidence-based approach to be made to the prophylaxis of colic. If such an approach could be adopted, with well-designed prospective studies, it is likely that more rapid and effective progress could be made in the prevention of colic than possible from repeated retrospective epidemiological studies.

The development of improved diagnostic techniques for the detection of equine gastrointestinal dysmotility would also offer important welfare benefits in terms of enhanced case management and treatment. Many equine gastrointestinal disorders may either cause impaired intestinal transit or involve a primary dysmotility in the aetiopathogenesis. In addition to post-operative ileus and equine grass sickness, such disorders may potentially include gastroduodenal ulceration syndrome, proximal duodenitis-jejunitis, gastric dilatation, chronic recurrent colic, ileocaecal intussusception, ileal hypertrophy, caecal and caeco-colic intussusceptions, and impactions of the ileum, pelvic flexure or caecum.

Because of the factors outlined above, the major objective of this study was the development of new, quantitative, non-invasive tests of equine intestinal transit that would be suitable for both diagnostic and research purposes. The relative merits and disadvantages of tests currently used in both human and veterinary gastroenterology for the measurement of gastrointestinal transit are discussed below and comprise the major subject

of this literature review. The diagnostic tests have been divided into those used for detection of specific transit parameters, starting with those used for the measurement of gastric emptying rate.

1.4 Measurement of gastric function

This is an exciting time to be involved in the field of equine gastroenterology. Whilst equine clinicians may not yet have access to the jellybean-sized wireless videocameras that have recently facilitated unprecedented visualization of the small intestine in children (Costamagna *et al.* 2002), within the last twenty years advances in equine abdominal ultrasonography, laparoscopy, fluoroscopy, manometry, scintigraphy and the development of 3 m gastroscopes have hugely enhanced the diagnostic armoury. However, equine gastroenterology currently lags behind the human field in the development and availability of effective tests for the measurement of gastrointestinal motility. This has consequently restricted understanding of specific areas of gastrointestinal physiology and pathophysiology. It has also limited the evaluation of equine patients with suspected motility disorders.

A spectrum of complementary modalities are used in human medicine, and it is necessary to distinguish between those that measure transit of ingesta (e.g. scintigraphy), those that measure contractile force (e.g. antroduodenal manometry) and those that monitor myoelectrical activity (e.g. electrogastrography). Whilst the latter two may be important diagnostically, it is the tests of transit rate that have been the best validated and offer the most clinically significant results (Camilleri *et al.* 1998), and this is also likely to be the case in equine gut disorders. Thus, in human gastroenterology, scintigraphy would perhaps be selected first to establish the presence of gastric motor dysfunction. Manometric and electrogastrographic procedures may then be performed to characterise the motility disorder as myopathic/neuropathic/obstructive or dysrhythmic in origin, respectively. The tests that have been reported for the measurement of equine gastrointestinal motility are discussed here, together with interspecies comparison and specific emphasis on the advantages and disadvantages of each. The modalities for assessment of intestinal transit are presented first, followed by those for the measurement of contractile force or myoelectrical activity.

1.4.1 Measurement of gastric emptying rate

1.4.1.1 Gastric radioscintigraphy

Gastric scintigraphy has been termed the 'gold standard' technique for quantitating gastric emptying in human medicine (Maurer 1995) and has been evolving since its first application 40 years ago (Griffith *et al.* 1966) to its current widespread use (Malmud *et al.* 1982; Collins *et al.* 1983; Maurer and Krevsky 1995). The main principle is simple: the patient ingests a test meal labelled with a radionuclide of specific energy, whose transit through the intestinal tract can then be charted using a gamma camera. Since the test makes use of a physiological meal, it is truly applicable to clinical disorders of gastric emptying, and is also accurate, non-invasive and easily quantitated as radioactive count rate is proportional to volume of labelled ingesta (Maurer 1995). Though relatively few equine studies have been performed by this technique (Neuwirth 1994; Ringger *et al.* 1996), it is also currently considered the optimum modality for measurement of gastric emptying rate in this species (Merritt 1997, 1999).

Several factors are vital for an effective radionuclide study of gastric emptying: a gamma emitting label is required, which must have high affinity for the food employed, such that it remains in the solid/liquid/oil phase of emptying, as required (Christian *et al.* 1983). A large field-of-view gamma camera is also required, linked to computing software that can process the radioactive counts in the regions of interest. Since the early use of chromium-51 (Griffith *et al.* 1966) a variety of tracers have been used: ^{99m}Technetium (bound to sulphur-colloid, pentetate, disofenin or mebrofenin) and ^{111}Indium diethyl-triaminepentaacetic acid (^{111}In-DTPA) are perhaps the mostly commonly used, and have decay half-times of 6 h and 2.2 d respectively, making them relatively safe (Parkman *et al.* 1995b). Newer, indigestible solid markers for intestinal transit measurement include ^{131}I-fibre (Malmud *et al.* 1982), ^{99m}Tc-labelled cellulose fibre and ^{111}In-labelled plastic particles (Madsen and Jensen 1989). ^{99m}Tc-SC and ^{111}In-DTPA are most commonly used to label the solid and liquid phases of gastric emptying, respectively and may be imaged simultaneously as they emit gamma radiation at different energies (Siegel *et al.* 1989). Although Siegel *et al.* (1989) suggested that liquid phase measurement alone was sufficient to detect delayed gastric emptying, this group did not report a close correlation between liquid and solid gastric $t_{1/2}$, and most other groups have suggested that solid phase measurement is preferable for clinical purposes (Minami and McCallum 1984; Chaudhuri and Fink 1991; Parkman *et al.* 1995b).

1.4.1.1.1 Equine gastric scintigraphic studies

Scintigraphic imaging may have been recognised as the optimum technique for quantitating the movement of labelled ingesta from the horse's stomach, but very few studies have been performed. In the majority of reported studies, liquid phase emptying (Lohmann *et al.* 2000, 2002), or a combination of liquid and solid phase emptying (Sojka and Cantwell 1988; Neuwirth 1994; Ringger *et al.* 1996) has been assessed after nasogastric administration of the labelled compounds. Until recently, solid phase studies were limited by the lack of a validated substrate that retained the radiolabel in the solid ingesta. Bahr *et al.* (2002b) measured the solid-phase label retention of ^{99m}Tc-mebrofenin bound to an extruded complete equine diet, and the more standard ^{99m}Tc-SC contained in baked egg yolk *in vivo*, in horses fitted with a gastric cannula. Both of these labelled meals demonstrated excellent solid phase retention of radioactivity for up to 6 h in the equine gastric environment (Bahr *et al.* 2002b), such that either would be suitable for further studies. Until recently, a further problem was that a standard protocol and test meal had not been established for equine gastric scintigraphy, prohibiting establishment of reference ranges and cross-study comparisons. As a small part of this study, attempts were made to develop a standard protocol for solid phase emptying assessment (as reported in Bahr *et al.* 2001).

Using a test meal of 1 kg ^{99m}Tc-mebrofenin-labelled horse pellets, Bahr *et al.* (2002a) reported a gastric half-emptying time ($t_{1/2}$) of 1.14 ± 0.48 h and 0.93 ± 0.37 h in 7 horses, before and after treatment for gastric ulceration. These solid phase transit measurements were similar to those recorded by Ringger *et al.* (1996) using a test meal of ^{99m}TC-SC-labelled egg albumen, administered by nasogastric tube. The latter group reported a gastric $t_{1/2}$ of 1.50 ± 0.17 h for emptying of solids in 4 horses, which was significantly reduced by prior administration of both bethanechol (0.50 ± 0.17 h) and erythromycin (1.01 ± 0.17 h). After feeding a test meal of 450 g grain labelled with ^{99m}Tc-SC to 8 adult horses, Levy and Sojka (1991) measured greater variation in the range of solid phase $t_{1/2}$ measurements (1.50 h ± 0.81 h, CV% = 53.92%). Levy and Sojka (1991) also found that cisapride at 1 mg/kg IV had no significant effect on GE in that group of normal horses ($t_{1/2}$ = 1.44 ± 0.96 h, CV% = 66.39%). These reports on equine solid phase GE suggest that there may be greater interindividual variation in $t_{1/2}$ in healthy horses, than the CV% of 15 – 20% noted in humans (Degen and Phillips 1996; Gryback *et al.* 2000). However, further application of a standardised test protocol with a standard test meal is required before further conclusions can be drawn.

Equine liquid phase GE also has been measured and requires nasogastric administration of the radionuclide solution. This in itself is undesirable as the processes of swallowing and pharyngeal stimulation are intimately related to gastric function (Lorenzo-Figueras *et al.* 2002). Again, a standard protocol has not been developed and administered volumes of labelled fluid have varied from 120 ml (Ringger *et al.* 1996) to 1 L (Lohmann *et al.* 2002). However, liquid phase emptying appears to be less variable than that of solids, with an average $t_{1/2}$ of approximately 0.50 h (Ringger *et al.* 1996; Lohmann *et al.* 2000).

The main advantages of gastric scintigraphy for equine use are its ease of quantitation (Maurer 1995), accuracy, non-invasiveness and the direct real-time imaging that it allows of the transit of gastric contents – the only modality to permit this in the horse. However, before scintigraphy becomes a true 'gold standard' for equine use, a number of problems must be addressed. Standardisation of the test meal, protocol and modelling techniques used for $t_{1/2}$ generation, would permit establishment of reference ranges. Since imaging of the equine gastric region is most accurate from the left side and variable from the right (Bahr *et al.* 2001; Lohmann *et al.* 2000, 2002), compensation for variable tissue attenuation of the isotope and movement of solids within the stomach is difficult (Tothill *et al.* 1978). Calculation of geometric mean counts is used to compensate for this in small animal (Caride *et al.* 1984; Goggin *et al.* 1998) and human (Moore *et al.* 1988; Siegel *et al.* 1989; Ghoos *et al.* 1993) studies. Indeed, Mistiaen *et al.* (2000) noted that anterior and geometrically corrected measurements differed significantly for solid food (but not liquid) in the whole gastric region and in the antrum in both dogs and human subjects. This suggests that acquisition of left-sided gastric images alone may cause reduced accuracy for GE measurement in the horse. Improved right-sided imaging may be possible, but only by increasing test meal radioactivity.

The further disadvantages of equine gastric radioscintigraphy include the radiation risk to personnel, which is considerable if the isotope is administered by nasogastric tube, even with appropriate precautions (Neuwirth and Romine 2000) and the requirement for prolonged occupation of a nuclear medicine facility. Both of these factors reduce the utility of the test for clinical diagnostics.

1.4.1.1.2 Human gastric scintigraphic studies

Multiple refinements have been introduced to human gastric scintigraphy, made possible by large trials and patient numbers. A standard procedures guideline for gastric emptying and motility has been issued by the Society of Nuclear Medicine (Donohoe *et al.* 2000),

and nationwide reference values have resulted from multicentre trials (Gryback *et al.* 2000). Furthermore, population reproducibility characteristics of scintigraphic transit measurements have been assessed as an aid to designing studies for experimental therapeutics (Cremonini *et al.* 2002). Less costly but accurate, reduced test protocols have also been devised (Camilleri *et al.* 1991; Halkar *et al.* 1999).

Whilst equine gastric scintigraphic techniques may lack some of the accuracy of human standard techniques, scintigraphy remains the optimum standard for solid phase gastric emptying measurement, for the reasons outlined. It was hence chosen here as the best technique against which to validate the new modality of the ^{13}C-octanoic acid breath test for equine solid phase GE rate.

1.4.1.2 Gastric ultrasonography

1.4.1.2.1 Human applications

With technical advances in the resolution and tissue penetration of real time ultrasonography, there has been increased use of this modality as an alternative to scintigraphy for the measurement of GE rate (Bolondi *et al.* 1985; Hveem *et al.* 1996; Darwiche *et al.* 2003). The method has several potential advantages as it does not require a radionuclide and the equipment is more widely available, and cheap to run. The ultrasound transducer is used to measure gastric antral diameter, in the sagittal plane, from which its cross-sectional area (CSA) is calculated. Repeat measurements are taken after ingestion of the test substrate and plotted against time, from which ultrasonographic $t_{1/2}$ and the time from ingestion to complete emptying can be calculated (Benini *et al.* 1999).

Most human ultrasonographic studies have assessed liquid phase emptying as the technique cannot discriminate between emptying rates of the liquid and solid phases of the meal (Hveem *et al.* 1996). However, the technique has now been validated against scintigraphy for liquid (Hveem *et al.* 1996), semisolid (Darwiche *et al.* 2003) and solid (Benini *et al.* 1999) test meals in both healthy subjects and those with diabetic gastroparesis (Darwiche *et al.* 2003). Highly significant correlations were found between these two techniques for the measurement of gastric $t_{1/2}$ for semi-solid meals and low and high nutrient liquids (Hveem *et al.* 1996; Darwiche *et al.* 2003). Benini *et al.* (1999) also found a good correlation between scintigraphy and ultrasonography for determination of total solid phase emptying time, but the concordance between these two modalities was much poorer for half emptying times. The time necessary for a 50% reduction of the postprandial antral dilation, as measured by ultrasound, was much longer that the $t_{1/2}$ of

scintigraphy. This was thought to be due to the important role of the fundus in solid meal gastric accommodation: antral size itself will not begin to decrease until approximately 40% of the meal has emptied. There is therefore a difference in the lag phase of solid phase emptying as measured by scintigraphy and ultrasonography (Benini *et al.* 1999).

However, recent advances in three-dimensional ultrasonography may further improve the accuracy of this technique for gastric emptying studies (Gilja *et al.* 1997; Tefera *et al.* 2002). Using 3D ultrasound with a magnetic scanhead tracking system, Gilja *et al.* (1997) showed excellent *in vitro* accuracy in estimation of porcine stomach volume, and improved accuracy for calculation of human gastric emptying rates, when compared with 2D ultrasound. This new technique has also been used clinically to assist in the diagnosis of reflux oesophagitis as it can measure the proximal/distal intragastric volume ratio (Tefera *et al.* 2002). It is therefore likely that scintigraphic studies may be superseded by ultrasonography in human gastric emptying studies.

1.4.1.2.2 Equine applications

Transrectal and transcutaneous abdominal ultrasonography is an increasingly useful tool in equine gastroenterology, and has been used both for estimation of motility (Freeman and England 2001; Nicholls and Freeman 2002) and for diagnostic purposes (Klohnen *et al.* 1996). Duodenal diameter measurements are also routinely assessed ultrasonographically from the right side as an indicator of small intestinal ileus. However, visualization of the equine pyloric antrum is particularly difficult due the situation of the stomach within the confines of the rib cage, surrounded by liver, spleen and anechoic gas-filled colon. In gross gastric distension, the pyloric antrum may be imaged on the right ventral midline, but gas in the caecal base is likely to prevent repeatable examination (White and Sullins 1990). The anatomical position of the horse's stomach, and the technical demands of ultrasonography (Proudman and Baker 1994) are therefore likely to prohibit this from ever becoming a useful technique for assessing gastric emptying rate in this species. However, further work with 3D ultrasonography in the horse may lead to improved measurement of intragastric volume of liquids. Choi and coworkers (2002) have recently adapted the technique of Bolondi *et al.* (1985) for measurement of liquid phase emptying in dogs, but this required positioning of the animals in ventral recumbency, which would require general anaesthesia in the horse.

1.4.1.3 *Magnetic resonance imaging*

Over the last 10 years, magnetic resonance imaging (MRI) has become widely available in both the medical and veterinary fields for high resolution, artefact free imaging. The development and application of specific contrast agents has enabled significant advances to be made in image contrast and the delineation of organs and vessels. This combination of improved spatial resolution and rapid image acquisition has allowed MRI to become a remarkable tool for the real-time imaging of physiologic processes (Schwizer *et al.* 2003). For assessment of GE by MRI, repeated acquisition is made of transaxial stacks covering the gastric region after a test meal. The total gastric and meal volumes may be identified by distinct positive contrast and then outlined. Volume is then calculated by multiplication of sum of these areas by the slice thickness. The modality offers extreme precision as compensation for gastric secretions may even be made, by reference to the signal intensity of an external standard. Gastric half-emptying time, and duration of the lag phase may then be determined in the standard way, by plotting volume against time.

Original MRI studies of GE validated the technique against a double indicator intubation technique for liquids (Schwize *et al.* 1992), but subsequently, it has been validated against scintigraphy for GE of both liquids (Feinle *et al.* 1999) and solids (Feinle *et al.* 1999). Furthermore, MRI studies have recently been used to investigate the mixing of foods with gastric secretions and the grinding mechanisms of trituration (Marciani *et al.* 2001). MRI is also useful for characterisation of gastric accommodation, but is less precise for the measurement of gastroduodenal motility as, similar to ultrasound imaging, it under-detects propagated events. Compared to scintigraphy and ultrasonography, MRI has the respective advantages of not requiring a radioactive source and improved image resolution.

Although MRI is being used increasingly and to great effect in equidae for the disentanglement of specific musculoskeletal conditions (Dyson *et al.* 2003) and cranial disorders (Spoormakers *et al.* 2003), at present the cost of building an MRI unit capable of imaging the equine abdomen would be prohibitive. This imaging modality could potentially offer a great deal of new information regarding equine gastric emptying, accommodation and motility, although this would have to be performed under general anaesthesia, which is known to inhibit motility (Lester *et al.* 1992). In addition to expense issues, programming the special imaging sequences needed for GE requires specialist knowledge and the reconstruction of gastric volume is time consuming (Schwizer *et al.* 2003). These constitute further reasons for this modality being unlikely to gain common usage in equine gastrointestinal diagnostics.

1.4.1.4 *Single-photon emission computed tomography (SPECT) imaging*

Single-photon emission computed tomography (SPECT) imaging is a highly advanced nuclear medicine technique that makes use of a rotating dual head gamma camera to formulate 3D images of the organ of interest after its uptake of a radioactive source. Because the gastric mucosa is able to take up and excrete ^{99m}Tc pertechnetate from the circulating blood pool, 360° rotating SPECT studies of the cranial abdomen after intravenous administration of this tracer allows 3D imaging of the gastric mucosa. This property has been used in human subjects to investigate the process of gastric accommodation, by comparing gastric volumes before and after ingestion of the test meal (Kuiken *et al.* 1999). The technique is so accurate that it has been able to semiquantitate pharmacological modulation of fasting gastric volume and postprandial accommodation in humans (Liau *et al.* 2001), providing useful clinical data. However, most horses would be too large for the SPECT analysis systems, and there are the obvious further diasadvantages of expense, high radiation exposure and the requirement for general anaesthesia, such that this system is unlikely to be adapted for use in the horse. Gastric accommodation has recently been characterised for the first time by Lorenzo-Figueras *et al.* (2002) using a traditional polythene intragastric barostat technique, as consisting of two distinct phases of receptive and adaptive relaxation.

1.4.1.5 *Acetaminophen absorption test*

Acetaminophen (paracetamol) is a pharmacological tracer that has been used for approximately 30 years as a marker of liquid phase gastric emptying rate (Kim *et al.* 2000). Heading *et al.* first suggested in 1973 that gastric emptying was the main rate-limiting step in the appearance of this compound in the plasma after its ingestion, and proposed that its plasma appearance formed an indirect marker of GE rate. Subsequently, pharmacokinetic modelling by Clements *et al.* (1978) confirmed that small intestinal absorption of this compound was rapid, whilst that from the stomach was negligible. Using scintigraphy, Clements *et al.* (1978) validated the acetaminophen absorption test (AAT) for determination of liquid phase emptying rate, which has been repeated subsequently by Maddern *et al.* (1985). The latter group also found a significant correlation between time to maximal saliva concentration of the tracer and scintigraphic gastric $t_{1/2}$.

The use of the AAT for investigation of semisolid and solid phase emptying has been more controversial, as the marker may separate in the liquid phase. Petring and coworkers (1986) did not find a significant correlation between the emptying of ^{99m}Tc-labelled resin/oatmeal and acetaminophen absorption. However, when acetaminophen solution was

taken orally immediately after ingestion of a radiolabelled omelette in 7 volunteers, a significant correlation was observed between the two techniques for gastric $t_{1/2}$ calculation (Naslund *et al.* 2000). Tracer absorption kinetics were also demonstrated to have low intraindividual variability when the acetaminophen was consumed with a semi-solid meal (Paintaud *et al.* 1998). The discrepancy in results between these validation studies is likely to result from the differing test meal composition, as a solid test meal high in energy, fat and/or fibre will empty from the stomach more slowly than one lower in these compounds (Malagelada *et al.* 1984; Chaudhuri and Fink 1991). In general, acetaminophen absorption rate only approximates that of solid phase gastric emptying if a very low calorie or soft meal is co-ingested, such that most workers restrict its use to assessment of liquid phase emptying (Medhus *et al.* 2001). By consensus, disorders such as antral dysmotility causing failure of trituration are unlikely to be detected using this test alone.

A further problem of the AAT noted from human studies is the wide inter-individual variation in first-pass metabolism, which varies between 10 and 40% at test doses when paracetamol is administered orally during fasting (Medhus *et al.* 1999). This complicates interpretation of gastric emptying rate as it is based on modelling of serum concentrations of this tracer. More importantly, acetaminophen bioavailability may be further reduced when gastric emptying is delayed due to less saturation of the metabolic pathways in the liver (Tone *et al.* 1990), leading to underestimation of emptying rate. Drug interactions may also alter the clearance of acetaminophen significantly (Miners *et al.* 1983). Finally, unequal distribution of acetaminophen in body fluids during periods of rapid changes in serum concentration may further confound estimation of emptying parameters (Medhus *et al.* 1999). This combination of individual differences in first-pass metabolism and elimination of acetaminophen has been demonstrated to reduce the value of inter-individual comparisons of gastric emptying parameters based on acetaminophen absorption test results (Medhus *et al.* 2001).

However, the major advantages of the test in human application are the relative simplicity of the protocol and its non-invasive, non-radioactive nature. Patients ingest a liquid meal containing 1 g acetaminophen prior to collection of sequential blood samples over a 5 h period. Serum concentration of the tracer is then measured by fluorescence polarization assay, and values modelled to yield an appearance rate constant (k_a), maximum serum concentration (C_{max}), time to reach maximum serum concentration (t_{max}) and the area under the curve (AUC). Because of the ease of application, the test has been adapted for equine use (Doherty *et al.* 1998, 1999b; Lohmann *et al.* 2000, 2002), where acetaminophen solution (20 mg/kg bwt in 200 - 1000 ml water) is administered by nasogastric intubation.

Using this test in 6 healthy adult horses on 2 occasions, Lohmann *et al.* (2000) reported a mean (± s.d.) t_{max} of 50.31 ± 19.95 min with a mean intra-individual CV% of 28.38%. This parameter was significantly correlated to simultaneous gastric $t_{1/2}$ as measured by scintigraphy, as was k_a. However, correlations between scintigraphic $t_{1/2}$ and peak C_{max} or AUC were not significant. In a further validation of this test in 6 horses with atropine-induced gastroparesis, acetaminophen t_{max} and scintigraphic $t_{1/2}$ were again significantly correlated (Lohmann *et al.* 2002) but 1 individual exhibited a flat acetaminophen absorption profile, perhaps caused by decreased bioavailability of the tracer, such that GE could not be measured. The test may therefore be of variable accuracy in equine subjects with delayed GE. Although the possible effects of drug interactions on acetaminophen metabolism have not been assessed in the horse, the AAT has been used to study the effect of phenylbutazone, cisapride, metoclopramide and yohimbine on endotoxin-mediated delayed GE (Valk *et al.* 1998a; Valk *et al.* 1998b; Doherty *et al.* 1999a; Meisler *et al.* 2000). Doherty *et al.* (1999b) also studied the effect of various sedative agents on equine GE function utilising the AAT.

As will be discussed in Chapter 5, the findings of Doherty *et al.* (1999b) based on AAT methodology were in disagreement with the findings of this present study, in which the effect of the same sedative agents on equine solid phase emptying was assessed. This may highlight the main limitation of the AAT for equine clinical use, as many equine disorders may potentially delay solid phase emptying rate whilst not affecting liquid phase emptying. For this reason, most human clinicians advocate measurement of solid phase emptying for diagnostic purposes (Camilleri *et al.* 1998). However, as inhibition of gastric resting tone may also accelerate liquid phase emptying whilst delaying solid phase emptying, there are grounds for suggesting that measurement of both functions may be necessary (Parkman *et al.* 1995).

In summary, the AAT is potentially a valuable test for the measurement of equine liquid phase GE and is simple, non-invasive and relatively cheap to perform. Further evaluation of equine bioavailability of this compound should be performed before its general clinical application. Further disadvantages of the procedure appear to include inter-individual variation in absorption/first pass metabolism, and the diagnostic limitations that are true of any test that is restricted to measuring the liquid component of GE. The requirement for nasogastric administration of the test solution is also considered to be an important limitation as this procedure may in itself alter gastric function. Finally, the test protocol requires the collection and analysis of multiple blood samples, with a subsequent prolonged delay in generation of the requisite GE data.

1.4.1.6 *Marker dilution techniques*

Measurement of intragastric marker dilution forms a basic technique by which liquid phase emptying rate may be assessed, and has been in limited use for about 30 y (George 1968; Hunt and Stubbs 1975). The protocol requires dual intubation of the subject: 1 tube must be located with its tip in the fundus for marker infusion, and the second, used for gastric sampling, with its tip in the antrum. The stomach is lavaged and emptied prior to ingestion/administration of the test solution, which is labelled with either a dye or polyethylene glycol (PEG) of known concentration (Naslund *et al.* 2000). Aliquots of gastric content are then aspirated from the antral tube at 10-min intervals after ingestion of the test solution. The continuous decrease of PEG/dye in the samples with time is expressed as the % emptied from the stomach, and taken as a measure of gastric emptying. In the slightly more complex double dye dilution technique, measurement of progressive reduction in gastric volume is made by aspiration, coupled with instillation of further boluses of phenol red of specific concentration (Baker and Gerring 1994a). The ratio of phenol red concentration in the instilled to aspirated fluid is used to calculate the residual gastric volume.

Using the double dye dilution test in pony foals, Baker and Gerring (1994a) demonstrated that mare's milk emptied more slowly than a saline solution of equivalent lipid content, with a specific lag phase. A volume of 15 ml/kg milk was also calculated to have a gastric $t_{1/2}$ of 13.51 min compared with 4.05 min for an equivalent volume of water. Sosa Leon *et al.* (1997) also used this procedure to investigate the effects of temperature, tonicity, glucose content and exercise at 70% of VO_{2max} on liquid phase emptying of 8 L of solution in Standardbred horses. In the latter experiments, no treatment condition or test solution was found to produce a significant change in gastric emptying rate, and 90% of fluid was emptied by 15 min after administration in each case (Sosa Leon *et al.* 1997). However, the authors reported a high variance in estimation of residual gastric volume for all treatment conditions, and concluded that there was large measurement error. Experimental error is likely to have been caused by several factors. Sosa Leon *et al.* (1997) did not lavage the stomach before administration of the test solution, which may have caused retention of phenol red by residual particulate matter (George 1968). Dye dilution also requires frequent mixing and aspiration of gastric contents, leading to increasing error in volume estimation during the course of the test. This is compounded by possible flaws in the spectrometric measurement of phenol red concentration, which has optimum precision only in transparent media (Williams *et al.* 1986). It was noted also by Billeaud *et al.* (1990)

that the assumption of homogeneity of the marker in gastric contents may breakdown with the coagulation of milk.

As marker dilution is relatively easy, non-invasive and cheap to perform in the horse, it is likely to see continued application for the measurement of equine liquid phase GE. However, it is important to note that the method has unavoidable error caused by incomplete mixing of the marker with gastric contents, large variation and has never been validated against an optimum technique. Moreover, this method again depends on the nasogastric administration of a labelled solution, and therefore does not provide a physiological means of studying the complex process of GE and influences of receptive and adaptive relaxation thereon (Lorenzo-Figueras *et al.* 2002).

1.4.1.7 Contrast radiographic techniques

By taking sequential radiographs of the stomach after ingestion of radiopaque fluid or ingesta qualitative information can be gained regarding the rate of gastric emptying. Although this technique is still used widely in canine and feline gastroenterology, it has been superseded in human clinics by the techniques outlined earlier in this section. Contrast radiographic studies provide useful morphological information but the emptying rate of liquid barium from the stomach may not be physiologically relevant to the emptying of food or calorific liquids (Hinder and Kelly 1977) and is difficult to quantify.

Radiographic mapping of the transit rate of barium impregnated polyethylene spheres (BIPS) or indigestible markers has been investigated recently as a possible quantitative measure of solid phase GE in both cats (Chandler *et al.* 1997) and dogs (Guilford *et al.* 1997; Lester *et al.* 1999). Chandler *et al.* (1997) showed that 1.5 mm BIPS left the stomach more rapidly than those of 5 mm, and that both were affected by drug administration. Guilford *et al.* (1997) also concluded that transit of 1.5 mm markers accurately reflected test meal transit in 17 healthy dogs. However, in a simultaneous comparison of the BIPS technique with scintigraphy, Lester *et al.* (1999) found significant differences between the estimates of gastric $t_{1/2}$ and gastric t_{lag} generated by these two modalities. The variability observed with BIPS is likely to depend on the selected particle size: small indigestible particles (< 1 mm) empty with the digestible meal components in the fed pattern of motility, whilst larger (> 2mm) indigestible units generally empty in late phase II or phase III of the interdigestive MMC (Hinder and Kelly 1977; Meyer *et al.* 1985, 1988). The transit of indigestible solids is also influenced by sphere density, and size and viscosity of

the test meal (Meyer *et al.* 1985; Smith and Feldman 1986). The use of BIPS for assessment of GE rate thus has many problems that confound easy interpretation.

In addition, the likelihood of developing a test for equine GE rate based on the transit of BIPS is limited by the difficulty of radiographing the horse stomach. Using a powerful radiographic unit at high mAs, penetration of the cranial abdomen is possible but radiographic definition is likely to be poor. Nonetheless, contrast radiography has been used to demonstrate partial pyloric obstruction with gastric retention in 3 equine subjects (McGill and Bolton 1984; Church *et al.* 1986). Baker and Gerring (1994c) used radiography in ponies to monitor the transit of 1 cm long strips of tube of 6 mm diameter, which were weighted to increase radiodensity. Not surprisingly, given the large size of these markers, the recording gastric half-emptying times were extremely variable, ranging from below 1 h to greater than 24 h (Baker and Gerring 1994c). In view of the poor physiological basis of this technique, and difficulty of radiographing the equine stomach, this technique should not be pursued further for the measurement of equine GE.

1.4.2 Assessment of gastric contractility

1.4.2.1 Antroduodenal manometry

Antroduodenal/jejunal manometry is an invasive technique that uses either solid-state pressure transducers or impedance sensors mounted on a catheter, or multi-lumen perfused catheters to measure intraluminal pressure changes. The catheters are passed through the nose and positioned in the antropyloric region or small intestine, and can provide information on the frequency, pattern and amplitude of gastric contractions. The technique is considered most useful for demonstrating the presence or absence of normal motility in this region (Camilleri *et al.* 1998). Qualitative information that can be derived from manometric analysis includes the pattern of the 3 phases of the MMC; the presence of clustered contractions, which may postprandially be related to a mechanical obstruction (Frank *et al.* 1994); sustained (> 30 min) incoordinated pressure activity; and prolonged (> 8 s) intestinal contractions, which correlate to the myoelectrical migrating action potential complex form of a prolonged contraction. More quantitative motility indices gained from manometric studies include the number of MMCs; the average amplitude of intestinal contraction; propagation velocity of the MMC; and the postprandial distal antral motility index (equivalent to ln(Amplitude x number of contractions + 1)).

1.4.2.1.1 Human applications

Specific manometric observations have been correlated to clinical conditions in human studies. For example, non-propagated, prolonged contractions in small bowel have been demonstrated to have a predictive value of 82% for mechanical obstruction, and manometric detection of clustered contractions has also proved useful (Frank *et al.* 1994). Using a combination of manometry and scintigraphy, Vassallo *et al.* (1992) showed that an antral pressure transducer was able to semiquantitate the axial forces occurring in this region during both liquid and solid phase emptying. In a subsequent pharmacological study, antral phasic pressure activities were found to correlate significantly with scintigraphic GE, suggesting that measurement of antral axial forces provided an assessment of overall gastric propulsion during the emptying of solids (Prather *et al.* 1993). Furthermore, reduced manometric contraction amplitude is consistent with myopathic disorders, whilst neuropathic disorders are marked by antral hypomotility, abnormal MMC coordination or bursts of sustained pressure fluctuation (Greydanus and Camilleri 1989).

However, antroduodenal manometry does have limitations, even in the most experienced hands. For example, many gastrointestinal conditions have both neuro- and myo-pathic components, reducing the diagnostic impact of manometric investigation. Camilleri *et al.* (1998) noted that diabetes mellitus, inflammatory bowel syndrome and post-gastric surgery cases may all share manometric features of autonomic denervation. Several artefacts may confound interpretation, particularly the 'common cavity phenomenon' in which sensors along the same section of intestine may detect the same pressure wave, leading to the erroneous impression of multiple simultaneous contractions. Authorities in the field also note that the significance of detected patterns can be difficult to determine and that manometry has not been validated for directing therapy.

1.4.2.1.2 Equine applications

The application of this technique in equine gastroenterology has been limited, and is likely to be subject to the same interpretative difficulties as noted above. As equine intra-abdominal pressure and intra-gastric pressure both fluctuate markedly in response to respiration and exercise (Lorenzo-Figueras and Merritt 2002), one might expect interpretation of manometric data to be further complicated in this species. Merritt, Burrow and Hartless (1998) used a triple sensor manometric array in 4 horses to determine the effect of specific drugs on duodenal motility. The procedure required previous gastric cannulation of the horses, and the sensors were passed via the cannula 50 cm beyond the pylorus. This equine system was able to detect the 3 phases of the MMC and showed the

relative inhibiting effects of different α_2-agonists (Merritt *et al.* 1998). However, the apparent inhibitory effects of xylazine on equine duodenal activity in this study were contradictory to a previous publication by the same group in which a serosal myoelectric technique had suggested stimulation of duodenal phase III MMC activity by xylazine (Merritt *et al.* 1989).

In summary, manometry has proved a useful diagnostic tool in human gastroenterology and may improve selection of specific therapy. However, it is technically demanding and requires special expertise in interpretation. Although equine oesophageal manometry has been performed by nasal intubation (Stick *et al.* 1983; Clark *et al.* 1987; Wooldridge *et al.* 2002), duodenal pressure measurements presently require gastric cannulation, prohibiting clinical application. It is also doubtful that current interpretation of equine manometric data is sufficiently robust to apply the modality for diagnostic use in this species.

1.4.2.2 Myomechanical strain gauge recordings

In several equine pharmacological studies, an invasive variant of antroduodenal manometry has been used to assess gastric and aborad intestinal motility indices – myomechanical strain gauge transduction (Davies and Gerring 1983; Adams *et al.* 1984; Lamar *et al.* 1984; Hunt and Gerring 1986; Sojka *et al.* 1986; Clark *et al.* 1988; Rutkowski *et al.* 1991; Singh *et al.* 1997; Sasaki *et al.* 2000). This technique requires surgical preparation of the subject, with serosal implantation of paired strain gauge transducers at the region of interest during ventral laparotomy. Strain gauge instrumentation was honed by Clark *et al.* (1988) for measurement of caecal mechanical activity, and the force transducer circuit is completed using a physiograph transducer. Calibration is performed prior to implantation by hanging known weights from a surface to which the strain gauges are sutured, and an *in vivo* contraction is then defined as a deflection exceeding 5 g.

The following have been calculated from equine myomechanical studies: contractile force versus time AUC; mean amplitude of contractions (g) and the total duration of contractile activity (s) in each recording period (Clark *et al.* 1988; Rutkowski *et al.* 1991). As with manometry, a motility index is calculated as being the product of the mean amplitude (g) and total duration of contractile activity (s). The contraction product / min (contraction frequency x amplitude (mm)) has also been measured. The majority of myomechanical studies have investigated isolated Thiry-Vella loops of small intestine (Davies and Gerring 1983) or *in vivo* small and large intestinal motility. The findings of several of these pharmacological studies are discussed further in Chapter 5. The stomach has been little

investigated (Hunt and Gerring 1986) due to the difficulty of accessing the pyloric antrum for transducer placement. However, in their classic experiment, Hunt and Gerring (1986) showed that the contraction product/min of the equine gastric fundus was decreased significantly by adrenaline, xylazine, isoprenaline and clenbuterol, but increased by bethanechol. Phenylephrine did not have a significant effect on fundic activity, and Hunt and Gerring (1986) concluded that α_2, β_1 and β_2 receptors were the most important in controlling equine gastric motility.

In addition to assessing the effects of autonomic transmitters on equine small and large intestinal activity (Hunt and Gerring 1986), myomechanical techniques have been used to measure the action of xylazine and butorphanol on jejunal, ileal, caecal and pelvic flexure mechanical contraction (Adams *et al.* 1984; Clark *et al.* 1988; Rutkowski *et al.* 1991; Sojka *et al.* 1986; Singh *et al.* 1997; Sasaki *et al.* 1998; Sasaki *et al.* 2000); and the relative effects of neostigmine (Adams *et al.* 1984), metoclopramide (Adams *et al.* 1986) and yohimbine (Eades and Moore 1993) on small and large intestinal activity. Given the large number of studies performed, and the extrapolation made from these towards clinical prokinetic therapy, it is concerning that the technique has not been validated against the transit of ingesta in this species. Focal contractions in isolated areas of intestine do not necessarily correspond to the coordinated activity required for the movement of digesta. Similarly, a reference range for the motility indices generated by myomechanical studies in healthy animals has not been published, and these are less well known than myoelectrical values for the frequency and duration of MMC phases.

The value of the pharmacological information derived from the above studies must therefore be questioned. Furthermore, the technique is clearly not suitable for equine clinical application as it is invasive and has been associated with catastrophic post-operative complications.

1.4.3 Gastric myoelectrical activity

1.4.3.1 Electrical Impedance tomography

Electrical impedance tomography (EIT) is a non-invasive technique that images the distribution of the tissue resistivity within a body region, and can be used to measure GE by determining the change in resistivity of the gastric contents after ingestion of a liquid or semisolid meal (McClelland and Sutton 1985). The most commonly used EIT procedure for measurement of GE is currently applied potential tomography (APT). In human APT studies, 16 silver/silver electrogastrography electrodes are used to encircle the upper

abdomen, at the level of the gastric fundus or body. These are attached to a data collection system that emits and records electrical potential. Sequential currents of 1 mA are sent through adjacent electrode pairs at 50 Hz and the potential difference measured (van Berge Henegouwen *et al.* 1997). During testing, impedance measurements are made at 1 min intervals for 2 h, and these measurements can then be computed as relative values of the preprandial data to produce an impedance curve, corresponding to the gastric contents. Acid secretion must be inhibited by cimetidine, but provided that this is performed, then the technique has been validated against scintigraphy for the emptying of both liquids and solid test meals (Mangnall *et al.* 1991; Avill *et al.* 1997). As the technique is both non-invasive and non radioactive, it has also been utilised in neonates to study GE rate, and validated against aspiration techniques (Nour *et al.* 1995). This modality has proved sufficiently sensitive in human studies to quantitate the reduction in gastric lag phase time caused by octreotide administration (van Berge Henegouwen *et al.* 1997) and the reduction in GE caused by increased gastric acidity (Chaw *et al.* 2001).

Baker and Gerring (1994b) performed a simplified version of this technique in 3 foals, to assess its potential for measuring the emptying rate of liquids. Using a simple 2-channel impedance epigastrograph and a pair of silver/silver electrodes placed on the flanks over rib 14, Baker and Gerring (1994b) measured the change in gastric impedance produced by nasogastric administration of 1.5 l water. Exponential curves were produced, with an apparent mean $t_{1/2}$ for GE of 4.9 min. Although liquid phase GE has not been reported in foals, this would seem to be an exceptionally low value when compared to current adult scintigraphic values (Lohmann *et al.* 2000). The major problem noted with the technique in this study was extreme sensitivity to movement artefact, such that the foals had to be placed in stocks supported by a sling. However, given that the technique is non-invasive and relatively cheap to perform, further investigations of this technique using a multi-array electrode system (which is less susceptible to movement artefacts) may be indicated. Despite its accuracy and non-radioactive nature, APT has largely been superseded by gastric scintigraphy for human studies.

1.4.3.2 Cutaneous Electrogastrography

Electrogastrography (EGG) is a non-invasive technique in which the electrical activity of the smooth muscular layer of the stomach is recorded percutaneously through the abdominal wall. Using electrocardiographic leads in a 3-lead configuration centred between the xiphoid and umbilicus, gastric slow wave activity and electrical response activity has been measured in human subjects (Chen *et al.* 1996; Riezzo *et al.* 2000, 2001).

Computed power spectral analysis of the raw signal is performed to determine the frequency at any given point during the recording, and the change in signal power and amplitude after meal ingestion. The clinical applications of electrogastrography have been reviewed by Chen and McCallum (1993) and reference ranges have been established for specific parameters in healthy human subjects (Pfaffenbach *et al.* 1995).

Using prolonged ambulatory EGG recording and simultaneous manometric analysis, Chen *et al.* (1994) showed that an unstable EGG peak power was indicative of gastric contractions in the fasting state. It was also concluded that an increase in EGG peak power and/or dominant frequency after a solid test meal suggested a normal postprandial gastric motility (Chen *et al.* 1994). In a large clinical study, human patients with delayed GE (n = 97) had a reduced percentage of normal gastric slow waves in the EGG and a lower postprandial increase in the dominant power (Chen *et al.* 1996). These factors were able to predict delayed GE with aaccuracies of 78% and 75% respectively, suggesting abnormalities in the postprandial EGG to be a useful clinical indicator (Chen *et al.* 1996). A group of dyspeptic children (n = 52) was also found to have a higher percentage of tachygastria, a higher instability of gastric power, and a lower post/preprandial ratio than healthy children (Riezzo *et al.* 2000).

Despite the emergence of this technique in gastric motility studies in man, equine EGG has not been described. Equine caecal electrical activity has been monitored percutaneously in a similar manner and shown to correlate to myomechanical activity (Sasaki *et al.* 1998). However, the position of the equine stomach within the abdomen, the difficulty of imaging the antrum, and the large distance between the gastric wall and skin electrodes would be likely to preclude cutaneous measurement of low amplitude electrical acitivity in this region. In addition, the equipment costs for this procedure are high, and the significance of specific dysrhythmias and amplitude variation not clear. As with EIT, movement artefact also complicates interpretation. In summary, it is unlikely that EGG techniques could be used to contribute significantly to the understanding of equine gastric physiology or measurement of GE rate.

1.4.3.3 Serosal myoelectrical recordings

Serosal myoelectrical recordings have been used to provide the bulk of information currently available on equine gastrointestinal physiology and pharmacology. This technique also requires the prior surgical implantation of intestinal electrodes, and has often been performed in conjunction with myomechanical studies. The modality is better

established than myomechanical strain transduction, and equine caecal myoelectrical activity has been correlated significantly to drug-induced changes in the scintigraphic emptying rate of labelled markers (Lester *et al.* 1998b). Bipolar Ag-AgCl electrodes are incorporated into acrylic before being sutured to the serosal surface, such that a pin tip protrudes 2.5 mm into the intestinal surface. The electrodes are positioned at 5 cm intervals along the region of interest, and electrical signals recorded and analysed by computer (Lester, Bolton and Thurgate 1992). After filtering, the electrical signal can be processed to identify myoelectric spike burst activity and duration, the specific phases of the MMC and the propagative patterns of intestinal motility by comparison between electrodes (Lester *et al.* 1992).

Using this technique, mean equine antral slow wave frequency has been measured at approximately 2.5/min (Merritt *et al.* 1989a), compared with 12-14/min for the duodenum and proximal jejunum (Davies and Gerring 1983; Adams *et al.* 1984; Merritt *et al.* 1989a). Gastric spiking activity is superimposed upon 90% of gastric slow waves (Hunt and Gerring 1985) which is greater than in other species, and displays a syncopated interaction with the duodenum. Periods of equine gastric repetitive spiking activity occur simultaneously with duodenal Phase II (intermittent action potential (AP) activity) and Phase I (no AP activity) of the migrating myoelectrical cycle (MMC). Small intestinal Phase III MMC activity (intense AP activity) commences in the equine duodenum, and is coincident with cessation of gastric antral AP activity, which does not recommence until shortly before the appearance of Phase II in the duodenum (Merritt *et al.* 1989a; King and Gerring 1992). Although gastric and duodenal patterns are tightly coordinated overall, the onset of duodenal phase III activity does not result from migration of the antral electrical activity, but from a separate small intestinal focus (Baker 1992). Unlike carnivora and primates that switch to a postcibal pattern of prolonged phase II intestinal MMC activity, the horse maintains the above pattern of cyclical activity as long as it is allowed to feed *ad libitum* (Hunt and Gerring 1985; Baker 1992).

Using myoelectrical recording, Merritt *et al.* (1989a) measured duodenal MMC periodicity to be approximately 125 min in both fed and food-deprived states, and suggested that xylazine administration triggered Phase III activity, with resetting of the duodenal MMC. Further work with this modality has shown that α_2-agonists, opioids, endotoxin and anticholinergic agents decrease spiking activity and increase MMC duration, with a probable resultant decrease in transit rate (Hunt and Gerring 1985; Merritt *et al.* 1989b; King and Gerring 1991; Merritt *et al.* 1998); conversely, neostigmine (Adams *et al.* 1984),

cisapride (King and Gerring 1988) and metoclopramide (Adams *et al.* 1986) have been shown to increase small intestinal myoelectrical activity.

However, although useful information has been gained from such studies, a direct correlation between transit of ingesta and myoelectrical activity has been demonstrated only for the equine caecum (Lester *et al.* 1998b), and GE of solid ingesta cannot be quantitated by this method. In addition, it may be optimistic to expect a high correlation between proximal intestinal myoelectrical activity and transit rate (Merritt 1999). As with most of the equine techniques that have been reported previously, the further disadvantage of this modality is its invasive nature, which prohibits diagnostic application in clinical cases. Serosal implantation of electrodes may itself alter intestinal electrical activity. In the absence of formal corroboration of the modality for assessment of pharmacological intervention, the results of such studies should therefore be considered with a questioning mind.

1.4.4 Invasive tests of equine gastric function

A number of invasive studies have been performed in horses to measure gastric function. Argenzio *et al.* (1974) administered PEG and radiopaque markers of 2 mm diameter in ponies, and followed the transit of these along the intestinal tract by *post mortem* examination of intestinal contents at specific times after dosing. This technique demonstrated the differences in liquid and solid phase gastric emptying rate in the horse. While 75% of the liquid marker had already left the stomach by 0.5 h, 75% of the particulate markers were still present in the gastric contents. By 1 h after administration most of the liquid marker had shifted to the terminal third of the small intestine (Argenzio *et al.* 1974). Using a similar method, Adams and MacHarg (1986) studied the effect of neostigmine on equine gastric function, and concluded, unexpectedly, that this agent delayed GE. However, Adams and MacHarg (1986) used plastic beads of 6 mm diameter to monitor solid phase GE, which would have been too large to pass through the pylorus (Becker and Kelly 1983), invalidating the study results.

Further equine gastric work has required the surgical creation of a gastric fistula (Healy *et al.* 1993) or implantation of a gastric cannula (Campbell-Thompson and Merritt 1990; Merritt *et al.* 1998; Berschneider *et al.* 1999), and has provided information on the nature and pattern of duodenogastric reflux. At best these techniques form useful research tools. They are not relevant to diagnostic investigation, and may on occasion provide erroneous results due to non physiological approaches to study design. Gastric cannulation alters the

gastric contour, and may possibly alter motility and relaxation of the antral region. Alternative methods are therefore required to replace these procedures.

1.4.5 Stable isotope breath tests for measurement of gastric emptying rate

1.4.5.1 ^{13}C-octanoic acid breath test

Breath tests incorporating meals labelled with the stable non radioactive isotope, ^{13}C, are based on the principle that the ^{13}C-component empties simultaneously with the rest of the test meal, and that it is representative of overall emptying rate. After leaving the stomach the ^{13}C-substrate disintegrates in the small intestine. The ^{13}C-compound is then rapidly absorbed and metabolised with the consequent production of $^{13}CO_2$, and its expiratory exchange from the bicarbonate pool (Kim *et al.* 2000). The choice of ^{13}C-labelled substrate determines the specific physiologic process that is studied, and indirectly determines the rate of increase in expiratory ^{13}C:^{12}C ratio. An ideal marker for GE rate must have specific properties: it should not be absorbed from the stomach; must be retained in the requisite phase of the test meal; should not adhere to the gastric mucosa, and must be absorbed rapidly and completely from the small intestine, with minimal interindividual variation (Swart and Van den Berg 1998). Substrates that have been used to assess liquid phase GE include ^{13}C-bicarbonate (Bjorkman *et al.* 1991), sodium 1-^{13}C-acetate (Braden *et al.* 1995; Gatti *et al.* 2000) and ^{13}C-glycine (Macs *et al.* 1994), and these are discussed in more detail in Chapter 6. ^{13}C-octanoic acid was selected as being a suitable marker for solid phase gastric emptying by Ghoos *et al.* (1993) based on its known properties as a medium chain fatty acid. As the compound is lipophilic it can be readily incorporated (cooked) into a solid phase test meal, and is also rapidly absorbed in the small intestine and metabolised by the liver. *In vitro* validation studies performed by Ghoos *et al.* (1993) showed that octanoic acid retention in egg yolk, incubated in gastric juice, was very high with greater than 95 % solid phase retention after 1 h and more than 90 % after 2 h.

1.4.5.1.1 Principle of the ^{13}C-octanoic acid breath test

The underlying principle of the ^{13}C-octanoic acid breath test (^{13}C-OABT) is that, following ingestion of a ^{13}C-octanoate labelled test meal, the rate of increase in expiratory ^{13}C:^{12}C ratio forms an indirect measure of the rate of solid phase gastric emptying of that meal (Ghoos *et al.* 1993). As stated above, for this to be true it must be assumed that gastric emptying is the main rate-limiting step in this process, and that the post-gastric handling of ^{13}C-octanoic acid proceeds at a constant rate with little variation, and without additional rate-limiting steps. The absorption and hepatic metabolism of medium chain triglycerides

(MCTs) has been well studied and comprehensively reviewed by Bach and Babayan (1982).

After leaving the stomach, MCTs are hydrolysed rapidly both by intraluminal pancreatic lipase and enterocytic lipase to form medium chain fatty acids (MCFAs) which are absorbed. As these 8-carbon compounds do not easily bind fatty-acid-binding protein they are not esterified into chylomicrons, but rapidly leave the intestine as fatty acids, via the portal venous system (Bach and Babayan 1982). The majority of MCFAs is retained in the liver and metabolised such that appearance in the peripheral blood is minimal. Furthermore, because the MCFAs do not conjugate the fatty-acid-binding-protein, they are not incorporated into hepatic cytosolic lipids, but rapidly cross the double mitochondrial membrane (McGarry and Foster 1980). On the outer mitochondrial membrane, in the presence of ATP and octanoyl-CoA synthetase, the MCFAs are activated first to MCFacyl adenylates and then to MCFacyl-CoAs, which pass into the mitochondrial matrix (Stryer 1995a). Once in the matrix, the MCFacyl-CoAs undergo β-oxidation with the production of acetyl-CoA and successively shorter acyl-CoAs. In the case of octanoic acid -1-^{13}C, the first acetyl-CoA produced from each molecule will carry the ^{13}C moiety, and will enter one of the following pathways: Krebs cycle after combining with oxaloacetate, ketogenesis, FA elongation (all in the mitochondria) or cytosolic new FA synthesis.

The key concerns for the ^{13}C-OABT are that there are no further rate-limiting stages in the production of $^{13}CO_2$ from the tracer, other than GE, and that there is a good yield of $^{13}CO_2$ for maximal test accuracy. The liver is known to produce higher ratios of CO_2 from C8:0 FAs than from C16:0 structures, but the capacity of the Krebs cycle is limited (Bach and Babayan 1982). MCTs have also been demonstrated to be ketogenic (Bach *et al.* 1977). Therefore, recovery of $^{13}CO_2$ from octanoic acid-1-^{13}C will not be 100% as ^{13}C-acetyl-CoA will also be directed towards ketogenesis. However, an initial peak in $^{13}CO_2$ expiration would be expected after its ingestion due to rapid transport to the liver, β-oxidation and entry into the Krebs cycle. For the ^{13}C-OABT a very small quantity of ^{13}C-OA is required, such that it is unlikely to deplete oxaloacetate supplies sufficiently to result in inhibition of the Krebs cycle.

Finally, the $^{13}CO_2$ generated from the Krebs cycle equilibrates with the body bicarbonate pool and undergoes respiratory pulmonary exchange. The rate of increase in expiratory $^{13}CO_2$ production is determined by serial collection of the subject's breath after ingestion of the test meal, and analysis of its ^{13}C:^{12}C ratio by isotope ratio mass spectrometry. This modern technique has an analytical precision of 1 part per million (ppm) $^{12/13}$C (see section

2.5.1.3) and is highly sensitive to small changes in ratio. The rate of ^{13}C production is then plotted against time and modelled by non-linear regression to provide an indirect measure of indices of solid phase GE rate.

1.4.5.1.2 Validation studies of the ^{13}C-OABT in Man

Since the initial study of Ghoos *et al.* (1993) the ^{13}C-OABT has attracted a great deal of interest in both clinical and research fields, as it potentially has several advantages to existing techniques for the measurement of GE rate. Unlike gastric scintigraphy, real-time ultrasonography, MRI, SPECT, EIT or EGG assessment of gastric transit of ingesta, the ^{13}C-OABT requires little equipment, and may be performed on site with relatively low set-up expense. All that is required at the study site is the test meal and the breath collection system. Samples of expired air may then be sent directly to the analytical centre/isotope laboratory for the requisite ^{13}C:^{12}C measurements. As discussed further in section 9.3.4, the processing of stable isotope samples is also becoming quicker and cheaper due to the development of new instrumentation such as non-dispersive isotope-selective infrared spectroscopy (Braden *et al.* 1994, 1996). For the patient, the ^{13}C-OABT has the advantages of being non invasive, non radioactive and simple to perform. Contrary to most of the direct or indirect imaging techniques described earlier in this chapter, implementation of the ^{13}C-OABT does not require specific skills or training, and is also quantitative and non subjective in its estimation of gastric emptying parameters.

As the ^{13}C-OABT provides an indirect measure of the transit of solid ingesta, the results should be directly relevant to clinical investigation, unlike those of the myoelectrical and myomechanical modalities that provide less specific information regarding intestinal contractility. Since the measurement of solid phase GE is usually preferable for clinical purposes (Parkman *et al.* 1995), the ^{13}C-OABT also has potential advantages to the liquid phase tests of dye dilution and impedance epigastrography.

In view of the potential benefits of the ^{13}C-OABT, several large validation studies have been performed to corroborate the initial findings of Ghoos *et al.* (1993) who validated the test against scintigraphy for GE measurement in 36 subjects. Choi *et al.* (1997) reported contradictory results to those of Ghoos *et al.* (1993) in 15 normal patients, and found no significant correlation between the ^{13}C-OABT and scintigraphy for generation of GE indices. It was suggested by Choi *et al.* (1997) that the lack of correlation was caused by wide inter-individual variation in the absorption, metabolism and excretion of the ^{13}C marker. However, the conclusions of Choi *et al.* (1997) were based on investigation of

insufficient subjects (Maes *et al.* 1998b) and did not consider methodological problems with the scintigraphic technique (Perri *et al.* 1998). Subsequently, the ^{13}C-OABT has been closely correlated against scintigraphy in several large trials in both control subjects and patients, although it overestimates emptying parameters in real time (Duan *et al.* 1995; Pfaffenbach *et al.* 1995; Ziegler *et al.* 1996; Choi *et al.* 1998; Perri *et al.* 1998; Delbende *et al.* 1998, 2000). A similar close relationship has been demonstrated between the ^{13}C-OABT and ultrasonography for assessment of solid phase GE rate (Cappello *et al.* 2000).

In order to determine the cause of the real-time lag between the parameters of GE determined by the ^{13}C-OABT and scintigraphy, Bluck *et al.* (2002) performed GE tests using both ^{13}C-octanoate and ^{2}H-octanoate tracers, and compared the results to simultaneous scintigraphy. As the majority of the deuterium would pass to the body water after β-oxidation, it was hypothesised that the kinetics observed with the deuterated material would be more closely related in time to scintigraphy than with the ^{13}C-analogues. As predicted, Bluck *et al.* (2002) demonstrated that whilst both ^{13}C-OABT and ^{2}H-OABT GE indices were correlated significantly to scintigraphy, the deuterated material also produced equivalent temporal values. Bluck *et al.* (2002) thus confirmed that the time lag observed with the ^{13}C-OABT was associated with hepatic processing and recovery of the ^{13}C label from the bicarbonate pool.

In a large multi-centre European trial in 69 healthy subjects and 54 patients, Delbende *et al.* (2000) reported a highly significant correlation between ^{13}C-OABT and scintigraphy for solid phase $t_{1/2}$ estimation (r = 0.744, P < 0.001). When compared to scintigraphy, the breath test detected an abnormal GE rate with 67% sensitivity and 80% specificity. Ziegler *et al.* (1996) had previously found a sensitivity and specificity of 75% and 86% for ^{13}C-OABT compared to scintigraphy for estimation of this parameter in diabetic patients.

1.4.5.1.3 Equine studies with the ^{13}C-OABT

Use of the ^{13}C-OABT for the assessment of solid phase gastric emptying has been investigated recently in the mouse (Symonds *et al.* 2000), dog (Wyse *et al.* 2001c, 2003) and horse (Wyse *et al.* 2001a, 2001b), but prior to the start of this thesis work the breath test had not been validated against a standard method in any of these species. A comparison of the rate of recovery of $^{13}CO_2$ in exhaled breath with the appearance of ^{2}H in body water was performed in the dog, following ingestion of ^{2}H/^{13}C-octanoic acid (Wyse *et al.* 2003). A similar relationship was found in the dog between ^{2}H/^{13}C recovery as reported in man (Bluck *et al.* 2002; Wyse *et al.* 2003). Initial work with the ^{13}C-OABT in

ponies produced highly repeatable data with low intra-individual coefficients of variation for gastric emptying parameters (Wyse *et al.* 2001a). The breath test also proved sufficiently sensitive to detect reductions in GE caused by the addition of high calorie soya oil to the test meal (Wyse *et al.* 2001b).

Further details of the advantages and disadvantages of the ^{13}C-OABT for equine use are given in Chapters 3-5 of this thesis. In summary, initial data with this modality were extremely promising, and the test has the benefits of being non-invasive, non radioactive and relatively simple to perform (Wyse *et al.* 2001a, 2001b). As a particular advantage in the horse, the procedure does not require inherently difficult imaging of the abdomen to be performed, and offers quantitative data that can only otherwise be gained by scintigraphy in specialist centres equipped with a nuclear medicine facility.

1.4.5.2 ^{13}C-Spirulina platensis breath test

Spirulina platensis is an edible blue-green alga that has been used as a dietary protein source. When grown in a closed hydroponics chamber purged with $^{13}CO_2$ it is possible to produce cultures uniformly labelled with ^{13}C, such that metabolism of each constituent part gives rise to respiratory $^{13}CO_2$. ^{13}C-*S. platensis* has been used as a novel substrate for stable isotope breath tests, and offers the advantage of easy incorporation into a solid phase test meal as the contents of algal cells are not freely diffusible (Lee *et al.* 2000b). After incorporation into egg white, algal ^{13}C is released only after GE of the test meal, digestion of the algal cells and absorption of their components (Viramontes *et al.* 2001).

Lee *et al.* (2000b) showed a significant correlation between the ^{13}C-*S. platensis* test (^{13}C-SPT) and scintigraphy in healthy subjects for estimation of both t_{lag} and $t_{1/2}$. A reduced breath sampling protocol developed by linear regression was shown also to give close approximation to scintigraphic measurement of GE. To validate the ^{13}C-SPT further, Viramontes *et al.* (2001) compared it with scintigraphy for detection of delayed or accelerated GE in subjects that had received atropine or erythromycin respectively. Although there was moderate overestimation of $t_{1/2}$ by the breath test model in patients that had received erythromycin, a close correlation was noted (r = 0.88, $P < 0.0001$) between the two modalities for $t_{1/2}$ estimation in the 57 volunteers in this study (Viramontes *et al.* 2001).

The ^{13}C-SPT has not been reported in the horse but, based on human study results, the test would have potential value for solid phase GE measurement. As with the other stable isotope tests, the ^{13}C-SPT avoids radiation, is non-invasive and can be performed in the

field due to remote processing of the generated samples. It also provides an indirect measure of the intestinal transit of solid ingesta, which is the most useful parameter for diagnostic purposes. Although the differences in parameter estimation between the ^{13}C-OABT and the ^{13}C-SPT have not been established, further investigation of the ^{13}C-SPT may be of value in equine clinical gastroenterology.

1.5 Measurement of equine whole gut transit time

1.5.1 Indigestible/radiopaque marker techniques

Many of the early studies performed in both human and equine gastroenterology have assessed the whole transit time of ingested markers by measuring the time from ingestion to appearance of the markers in the faeces. Selected markers have included radioactive or heavy metals such as chromium, cobalt EDTA and ytterbium chloride (Uden *et al.* 1982; Milne *et al.* 1996; Holland *et al.* 1998; Pagan *et al.* 1998); radio-opaque plastic markers of variable size (Read *et al.* 1980; Adams and MacHarg 1985; Metcalf *et al.* 1987; McGreevy *et al.* 2001) which can be counted using faecal radiography; polystyrene spheres (Milne *et al.* 1996) and specific dyes such as carmine red (Read *et al.* 1980). Although mouth-to-anus transit times do have some value in the diagnosis of diffuse gastrointestinal motor disorders such as equine dysautonomia (Milne *et al.* 1996), the passage of such markers is likely to be dominated by the slow rate of transit through the large intestine.

Using polystyrene spheres of 6mm diameter and 1.05 specific gravity, Milne *et al.* (1996) reported a mean mouth-to-anus transit time of 29 h in healthy horses eating alfalfa forage diets. The mean time to peak excretion of CoEDTA-labelled liquid in the same individuals was 31 h (Milne *et al.* 1996). These values were in close agreement to those of Pearson and Merritt (1991) who reported a mean retention time of 29.9 h and 31.3 h for chromium-mordanted fibre and CoEDTA-labelled liquid in ponies on a hay diet. The same authors found that the rate of passage of both solid particles and the liquid phase of digesta was considerably slower in donkeys maintained on the same diet (37.9 and 36.9 h respectively). In both of these species, rate of mouth-to-anus passage of solid and liquid phases was reduced when the subjects were maintained on a straw diet. Uden *et al.* (1982) placed chromium-fibre and CoEDTA solution directly into the caeca of 6 equines, and reported mean retention times of 23 h and 18 h respectively, consistent with whole gut transit data. Of physiological interest in these studies was the finding that both solid particles and labelled liquid phases had similar mean retention times in ponies, horses and donkeys.

Liquid flow and capacity of the caecum and right colon also has been measured using intracaecal infusions of chromium EDTA, and collection via an egress cannula, but this technique requires surgical preparation (Simmons and Ford 1990). Based on 4 determinations in ponies, Simmons and Ford found liquid flow from the caecum and right dorsal colon to approximate 54.2 and 49.4 litres per day.

In addition to gut residence times, whole transit studies have been used to gain information about equine faecal kinetics in relation to diet (Holland *et al.* 1998), but specific information regarding gastric emptying or small intestinal transit times cannot be derived. As dysmotility of these specific sections of the GIT may be involved in the actiology of many abdominal disorders, more specific tests of small intestinal transit rate and small intestinal residence times have been sought. Furthermore, the intestinal passage of inert, indigestible markers may not be truly representative of the movement of normal ingesta. The further disadvantage of such markers in equine gastroenterology is that the abdomen of most equidae cannot be penetrated sufficiently by x-rays to permit *in vivo* assessment of marker number and location. Useful techniques involving ingestion of multiple radiopaque markers have been developed in human medicine for measurement of both OCTT and mean colonic transit times (Metcalf *et al.* 1987) but require radiography of the abdomen, and are therefore unsuitable for equine use.

1.6 Measurement of Orocaecal transit time

The symptoms of many gastrointestinal disorders may be secondary to small intestinal dysmotility, and techniques have been developed to assess orocaecal transit time (OCTT) as an indirect measure of small intestinal transit. Traditional radiologic techniques for measurement of this parameter involved the sequential imaging of a barium meal (Hirakawa *et al.* 1988). However, the principle clinical tests currently used in man are the hydrogen breath test (H_2BT) after lactulose administration, and radionuclide gastroenterocolonic scintigraphy. The advantages and disadvantages of the currently available tests for OCTT measurement and their applications in veterinary, specifically equine, gastroenterology are discussed here. The case for development and validation of a new diagnostic test for equine OCTT is presented.

1.6.1 The Hydrogen Breath Test

1.6.1.1 Principles

The end expiratory H_2 content of breath was first investigated 40 y ago by Nielsen (1961), and reported by Calloway in 1966, as part of an investigation into the 'gas production'

caused by indigestible feeds. Certain feeds resulted in an acute postprandial rise in expiratory hydrogen content, which was shown in ileal/colonic stomata patients to correspond to the arrival of undigested carbohydrate in the colon. H_2 is not liberated by mammalian metabolic activity, and its production (together with methane (CH_4) and CO_2) results from microbial fermentation in the intestinal tract (Calloway 1966; Levitt 1969). Clostridial species have been specifically identified as H_2-emitting (Roberts *et al.* 1968). Once produced, approximately 14% of the luminal H_2 diffuses into mucosal capillary blood (Levitt 1969), and is then excreted via the lungs in expiratory breath. Sequential measurement of end-expiratory H_2 is simple to perform and its rate of increase after ingestion of an indigestible carbohydrate forms the principle of the H_2BT for OCTT assessment. Lactulose (β-1,4-galacto-fructose) was suggested as a suitable indigestible substrate by Bond and Levitt in 1975 and since that time, a number of studies have been performed to optimise the protocol for OCTT measurement by the lactulose H_2BT.

The definition of OCTT using the lactulose H_2BT has not been universally agreed, but is generally accepted to be that time after ingestion when an increase of 5 (Miller *et al.* 1997) to 10 (La Brooy *et al.* 1983) parts per million (ppm) H_2 excretion over baseline first occurs in the breath, sustained over successive time points. This represents arrival of the head of the lactulose column in the colon. Inter-individual variation in colonic H_2 perfusion and subsequent exhalation rate appears small (Bjorneklett and Jenssen 1980). However, jejunal H_2 excretion is variable, with an efficiency one magnitude greater than that of the colon, perhaps due to its higher relative mucosal surface area and blood flow (Perman *et al.* 1981). This factor can lead to interpretative difficulties in the presence of small intestinal bacterial overgrowth (Taylor *et al.* 1981; Karcher *et al.* 1999) although the early expiratory H_2 peak seen in this latter condition tends to be short-lived rather than sustained. The accuracy of the lactulose H_2BT for OCTT measurement has been correlated to several different techniques in man.

1.6.1.2 Validation studies for measurement of OCTT

Using a combined test meal of polyethylene glycol (PEG) and lactulose, Bond and Levitt (1975) found that PEG appeared in the terminal ileum 7 min before the appearance of H_2 in the breath, and concluded that a real-time lag of about 5 min existed between caecal lactulose arrival and microbial H_2 production. Similarly, close temporal associations for OCTT assessment have been demonstrated to exist between the lactulose H_2BT and barium meal studies (Hirakawa *et al.* 1988) or sulphasalazine/sulphapyridine test (Staniforth 1989). Simultaneous comparison with the predicate method of gastrocentrocolic

scintigraphy (GECS) has also been performed in man. Taking first appearance of ^{99m}technetium in the caecum to represent OCTT, excellent correlations have been found between the lactulose H_2BT and scintigraphy for estimation of this parameter ($r = 0.88$, $P < 0.01$, Read *et al.* 1986; $r = 0.90$, $P < 0.001$, Sciarretta *et al.* 1994; $r = 0.95$, $P < 0.001$, Miller *et al.* 1997). However, although there is good linear correlation between these modalities for OCTT, the lactulose H_2BT may variably overestimate OCTT when compared to scintigraphy by up to 30 min in some individuals (Madsen *et al.* 1991; Pressman *et al.* 1987). This error is minimised by using the lowest threshold of H_2 production (5 ppm) to represent OCTT, and is negatively correlated to the subject's total H_2 production after lactulose ingestion (Sciarretta *et al.* 1994). Some authors have further recommended that visual assessment of individual H_2 output is the most reliable means of determining OCTT (Sarno *et al.* 1993).

1.6.1.3 Further applications of the H_2BT

In small intestinal bacterial overgrowth (SIBO), colonisation of the proximal gut may result in rapid fermentation of ingested mono- and di-saccharides. Whilst this may potentially complicate interpretation of OCTT data, attempts have been made to positively identify SIBO by the rapid appearance of H_2 after carbohydrate ingestion (Taylor *et al.* 1981). O'Connor *et al.* (1987) found the glucose H_2BT to have a sensitivity and specificity of 91 and 75% respectively for SIBO diagnosis in man, and a mixed sugar H_2BT has been reported to aid in the presumptive diagnosis of SIBO in dogs (Rutgers *et al.* 1995). Early transient H_2 peaks after both lactulose and xylose ingestion have also been described in the horse in a confirmed case of pyloric obstruction, consistent with gastric microbial substrate fermentation (Murphy *et al.* 1994), although SIBO has not specifically been recognised in the horse.

Extensive investigation has also been made into use of the H_2BT for detection of carbohydrate malabsorption (Levitt and Donaldson 1970) and its quantification (Bond and Levitt 1972). Indeed, the lactose H_2BT has been described as an ideal population screening test for lactase deficiency (Newcomer *et al.* 1975; Flatz *et al.* 1984), and the xylose H_2BT has been reported to have a specificity of 1.00 and sensitivity of 0.86 for monosaccharide malabsorption (Casellas *et al.* 1993). The advantages of the H_2BT for all of these purposes are manifest: it is cheap, easy to perform and entirely non-invasive, without requirement for radiopharmaceuticals. However, before detailing the veterinary applications of this test, several disadvantages must be highlighted, as reported in the human literature.

1.6.1.4 Disadvantages of the H_2BT

1.6.1.4.1 Low H_2 producers

The majority of reports on the lactulose H_2BT detail subjects termed low or 'non-producers' of H_2 in which the test is ineffective for determination of OCTT. Depending on the criteria used, this ranges from approximately 5% of the population (Levitt and Donaldson 1970; Bond and Levitt 1974; Pressman *et al.* 1987; Cloarec *et al.* 1990) to 27% (Corazza *et al.* 1993; Hammer *et al.* 1996). Much work has been performed to elucidate this interesting phenomenon. Rather than low production, certain individuals may have increased colonic consumption of H_2, resulting from high numbers of sulphate-reducing bacteria or methanogens (Miller and Wolin 1983; Gibson *et al.* 1988). These microbes compete for H_2, as do acetate-forming organisms (Lajoie *et al.* 1988), reducing its subsequent expiration.

The production of H_2 from carbohydrates by colonic bacterial flora is also reduced by an acidic colonic microclimate (Perman *et al.* 1981). This can be reversed in some individuals by the prior ingestion of magnesium sulphate to increase faecal pH (Vogelsang *et al.* 1988), increasing sensitivity of the H_2BT. Fasting basal H_2 production is lower in CH_4 producers than non-CH_4 producers (Cloarec *et al.* 1990; Corazza *et al.* 1993), and an acidic environment may favour the growth of methanogens. In addition, H_2 consumption is dramatically increased and absorption decreased at high faecal H_2 tension, such as occurs with decreased luminal stirring (Strocchi and Levitt 1992; Hammer 1993). Each of these factors may contribute to the lack of H_2 expiration in certain individuals after the ingestion of an indigestible carbohydrate.

1.6.1.4.2 Intrinsic effect of lactulose on gastrointestinal transit times

A major disadvantage of the lactulose H_2BT is that lactulose itself causes a dramatic reduction in OCTT (Miller *et al.* 1997; Yin *et al.* 1998), inversely correlated to the dose used (Bond and Levitt 1975). By performing a simultaneous GE study, duodenal manometry and GECS, Miller *et al.* (1997) showed that lactulose slowed GE, but caused a significant increase in small intestinal motility and overall decrease in OCTT. These effects may have been due to increased gastric viscosity/osmolality, followed by osmotic distension of the small intestine and stimulation of increased peristalsis. Lactulose also decreases the compliance of the proximal colon, allowing increased colonic transit of ingesta (Barrow *et al.* 1992). These factors caused Lembcke (1997) to dismiss the lactulose H_2BT as of greater scientific interest than clinical value. However, the test has proved useful clinically to confirm delayed OCTT in both pregnancy (Wald *et al.* 1982) and

anorexia nervosa (Kamal *et al.* 1991) and to measure pharmacological effects on this parameter (Staniforth 1987).

1.6.1.4.3 Inter-individual variability

Although intra-individual repeatability for OCTT measurement is good, inter-individual variation is high, and increases with lactulose dose (La Brooy *et al.* 1983). High fasting basal H_2 production can reduce the sensitivity of the test (Flatz *et al.* 1984), though this may be improved by more prolonged abstention from carbohydrate (Kotler *et al.* 1982; Perman *et al.* 1984) or glycoproteins (Perman and Modler 1982). Attempts to reduce variability have included substrate (Casellas and Malagelada 1998) and test meal (La Brooy *et al.* 1983) manipulation, and improved breath collection technique (Strocchi *et al.* 1991). However, intra-individual repeatability of OCTT measurement is poorest in those individuals with prolonged transit times (Camboni *et al.* 1988), and variability may also be caused by the specific phase of the interdigestive migrating motor complex in which the test is applied (Di Lorenzo *et al.* 1991).

1.6.1.4.4 Physiologic relevance of OCTT measurement

The lactulose H_2BT measures arrival of the head of the column of ingesta in the caecum only, which is dependent on both GE rate and the rate of small intestinal transit (Miller *et al.* 1997). These latter factors may be largely independent of one another (Read 1984; Malageleda *et al.* 1984). Upon entry into the small intestine, the chyme spreads out such that first arrival of chyme into the caecum may not be an accurate measure of the subsequent transit of the majority of the ingesta. The H_2BT cannot be used to assess the small intestinal or orocaecal transit rate of a whole meal, as total hydrogen production has been shown not to correlate to the scintigraphic rate of colonic filling (Read *et al.* 1985; Madsen *et al.* 1991). Hence, a single measure of OCTT is an over-simplification of a complex physiological process in which there may be continuous GE with variable small intestinal transit rates (Malagelada *et al.* 1984). However, as discussed above, this simple test does appear to have diagnostic value. Possible means of improving the physiologic relevance of indirect measures of small intestinal transit rate are discussed and applied in chapter 8 of this thesis.

1.6.1.5 Veterinary applications of the H_2BT

Lactulose administration resulted in H_2 production in preruminating calves, although the test has not been validated specifically for OCTT measurement in this species (Holland *et al.* 1986). The same authors also reported that H_2 production after xylose or milk ingestion

was sufficiently sensitive to detect bovine small intestinal malabsorption induced by either chloramphenicol treatment or cryptosporidiosis respectively (Holland *et al.* 1986 and 1989). Lactulose administration has also been shown to result in subsequent H_2 expiration in the cat and dog (Washabau *et al.* 1986; Muir *et al.* 1991) and was highly correlated to the sulphasalazine method for measurement of OCTT in canines (r = 0.94, P = 0.016, Papsouliotis *et al.* 1995). Daily fluctuations in canine intra-individual H_2 output are low (Ludlow *et al.* 1994) and the H_2BT is suitable for clinical studies (Papasouliotis *et al.* 1993a). Similarly in cats, the lactulose H_2BT has demonstrated a significant effect of hyperthyroidism on OCTT (Papasouliotis *et al.* 1993b) and the xylose absorption test had sufficient sensitivity to detect antibiotic-induced malabsorption (Muir *et al.* 1996).

1.6.1.6 Efficacy of the H_2BT for measurement of equine OCTT

A thorough evaluation of the use of this test in equidae was performed by Murphy (1997). In agreement with the results of Bracher and Steiger (1995), Murphy found that lactulose only rarely resulted in an increase in H_2 expiration in this species, making the lactulose H_2BT unsuitable for OCTT measurement. Attempts to identify the fate of oral lactulose in the horse were unsuccessful (Murphy 1997) as ingestion was not followed by significant urinary excretion, nor significant alteration of plasma ammonia levels. Longterm lactulose administration did not improve the H_2 signal, although colonic flora adaptation to lactulose has been reported following its longterm ingestion in man (Florent *et al.* 1985).

However, a complete ration high fibre, pelleted test meal has been reported to result in a repeatable H_2 response in ponies, which was sufficiently sensitive to detect a reduction in OCTT caused by cisapride (Murphy and Love 1995). Both oats and wheat flour also produced a repeatable H_2 response in ponies, although wide inter-individual variation in OCTT measurement was noted using this technique (Murphy *et al.* 1998). H_2 response to test meal ingestion was further affected by basal diet, with greatest H_2 expiration occurring when ponies were maintained on a hay ration (Murphy *et al.* 1998).

Attempts have been made also to quantify carbohydrate malabsorption in the horse using xylose/glucose/lactose H_2BTs (Bracher *et al.* 1995; Murphy *et al.* 1998). Interpretation of such results is far from clear, as Breukink (1974) suggested that partial small intestinal malabsorption of an oral glucose load may be normal, and increased by prolonged fasting. Breukink (1974) reported in addition that 50% of horses may be lactase deficient by the age of 2.5 y. The recent work of Dyer *et al.* (2002) has demonstrated that D-glucose is transported across the equine small intestinal brush border membrane by a high affinity,

low capacity Na^+/glucose transporter, that may be subject to over-saturation. However, specific lactase activity remained present in adult horse small intestine, albeit it at reduced levels in animals over 4 y (Dyer *et al.* 2002).

It is important to note that the H_2BT has not been validated for the measurement of equine OCTT. Bracher and Baker (1994) opined that further information about the microbial composition of the equine gastrointestinal tract, and the sites and fate of H_2 and CH_4 production were required before further use of the test. Diet is known to have a dramatic effect on the bacterial populations in the equine large intestine (Kern *et al.* 1973; Garner *et al.* 1978; Murphy 1997) and most recently equine expiratory H_2/CH_4 production has been used in an attempt to quantify this phenomenon (Sasaki *et al.* 1999; Pratt *et al.* 2002). However, the combination of a high proportion of H_2 'non-producers' (Sasaki *et al.* 1999) and the marked variability of equine H_2 production is likely to result in a test of poor sensitivity. In summary, several of the disadvantages of the H_2BT reported in man appear to exist in the equine population, and are complicated further by the lack of a reliable response to lactulose ingestion. A validated, non-invasive test for OCTT measurement is therefore still required for use in this species.

1.6.2 The sulphasalazine/sulphapyridine test

Sulphasalazine (SLZ) is produced by the azo-bond coupling of sulphapyridine with 5-aminosalicylic acid, and cannot be absorbed in the small intestine as it requires bacterial cleavage of the azo bond in the large intestine before liberation of the individual components. Once released in the large intestine, the sulphapyridine is rapidly absorbed and undergoes hepatic conjugation to a variety of metabolites. This property of the SLZ compound has been utilised in a simple test for orocaecal transit time. Kellow *et al.* (1986) reported that the first plasma appearance of sulphapyridine in man after oral ingestion of SLZ was closely correlated ($r = 0.82$, $P < 0.01$) to the simultaneous measurement of OCTT by gastroenterocolonic scintigraphy. Measurement of OCTT by the SLZ/SP test has also proved very similar to that of concurrent lactulose/H_2BT (Staniforth 1989).

Use of the SLZ/SP test in dogs ($n = 6$) also showed that OCTT estimation was closely correlated to simultaneous H_2BT measurements, although SLZ/SP OCTT values exceeded those of the H_2BT by 15-45 min (Papasouliotis *et al.* 1995). The authors of the canine study suggested that this difference between the techniques was due to insufficient sensitivity of the spectrophotometric technique used for detection of first SP plasma appearance. High performance liquid chromatography (HPLC) may allow earlier plasma

detection of SP (Staniforth 1989). McGreevy and Nicol (1998) adapted the SLZ test for measurement of equine OCTT and reported its use in 12 individuals. Using this test, they reported that OCTT was considerably prolonged in crib-biting individuals if these individuals were prohibited from performing their sterotypical behaviour. Equine OCTT was shown also by the SLZ/SP test to be relatively reduced in crib-biting horses (105 ± 29 min) compared with normal individuals (135 ± 57 min; McGreevy *et al.* 2001).

However, the major disadvantage of the SLZ/SP test for equine use is that it has not been validated against a standard technique for OCTT measurement. As intestinal bacteria capable of cleaving the azo-bond are widespread, it is possible that they may not be restricted to the caecum in this species. In addition, the possible effects of inter-individual variation in gastrointestinal bacteria, and of specific drugs on these populations, have not been investigated in the horse. The test requires multiple blood samples to be taken, and their subsequent analysis by HPLC. Furthermore, the pharmacokinetics and therapeutic indices of SP have not been established in the horse, and SP has been reported to have many adverse effects in humans, and may cause keratoconjunctivitis sicca in dogs (Sansom *et al.* 1985; Knoll *et al.* 2002).

1.6.3 Gastroenterocolonic scintigraphy

Unlike the H_2BT and SLZ/SP test, gastrointestinal scintigraphy allows separate analysis of gastric and small bowel transit, and this constitutes one of its chief advantages as it permits specific diagnosis of small bowel dysmotility (Camilleri *et al.* 1998). In human gastroenterology this procedure is the next diagnostic step of choice in gastrointestinal patients with persistent symptoms despite a normal GE test result, as small bowel transit time (SBTT) and small intestinal residence times may be determined in isolation to GE rate. Whole gut transit scintigraphy (WGTS) allows the measurement of upper GIT motility, and determination of colonic filling rate and transit times by simultaneous monitoring of the movement of labelled liquids (^{111}In-DTPA) and solids (^{99m}Tc-sulphur colloid) with a gamma camera (Bonapace *et al.* 2000). WGTS has improved both clinical diagnosis and patient management in human gastroenterology (Bonapace *et al.* 2000).

Scintigraphic determination of OCTT provides one of the simplest direct measures of small intestinal motility (Bennink *et al.* 1999). The first detectable arrival of a ^{99m}Tc-labelled meal in the caecum has been used to signify OCTT, and this parameter is correlated to the results of H_2BTs (Caride *et al.* 1984). However, small intestinal motility is complex, and OCTT provides, at best, information regarding movement of the head of the column of

ingesta. After entering the small intestine, some particles will move faster than others, producing a spectrum of transit times (Read *et al.* 1986). Read *et al.* (1986) accounted for this by deconvolving the rate of colonic filling with the rate of GE, to produce the mean small bowel transit time. However, using ^{99m}Tc-DTPA and ^{131}I-fibre, Malagelada *et al.* (1984) found no significant difference for SBTT between the labelled liquids and solids. Simpler derivation of SBTT, such as subtracting gastric emptying $t_{10\%}/t_{50\%}$ from colonic filling $t_{10\%}/t_{50\%}$ may therefore be sufficient (Malagelada *et al.* 1984), although this technique does not allow for bolus movements or plateaux of activity (Camilleri *et al.* 1998). The rate of accumulation of radioactive ingesta in the terminal ileum has also been used to measure small intestinal motility (Bonapace *et al.* 2000).

SBTT has a wide inter-and intra-individual range in healthy human subjects (Argenyi *et al.* 1995), but significant reduction has been noted in patients with diarrhoea (Read *et al.* 1986). Although the time of appearance of jejunal phase III has been directly correlated to GE $t_{1/2}$ (Read *et al.* 1984), SBTT is independent of the rate of GE (Read *et al.* 1980, 1986) and specific investigation of this discrete segment of bowel may be useful. When compared to the H_2BT, scintigraphy gives superior specificity due to correction for variable GE rate, and its interpretation is not complicated by intestinal bacterial overgrowth. Gastroenterocolonic scintigraphy has been adapted for use in dogs (Iwanaga *et al.* 1998) but technical complications limit its clinical utility in the horse. The equine gastric region and proximal small intestinal tract can be imaged after ingestion of a ^{99m}Tc-labelled meal (Bahr *et al.* 2001), but poor resolution of the jejunum, ileum and large intestine confound interpretation. Lester *et al.* (1998) used scintigraphy to study equine caecal emptying rate, via right-sided images, but this necessitated direct caecal instillation of the radiolabel rather than oral ingestion. Alternative methods must therefore be sought for the non-invasive measurement of equine OCTT and SBTT, so as to aid diagnostics and the preclinical testing of prokinetic agents.

1.6.4 Use of the lactose ^{13}C-ureide breath test for OCTT measurement

1.6.4.1 Background and principles

Lactose ^{13}C-ureide is one of the glycosylureide molecules, consisting of a sugar moiety (galactose-glucose) bound to a ^{13}C-urea molecule. The lactose ^{13}C-ureide breath test (LUBT) was first proposed to be a suitable test for human OCTT measurement by Heine and co-workers in 1994 (Heine *et al.* 1994,1995). This group demonstrated that bolus ingestion of lactose or cellobiose ^{13}C-ureide was followed by a discrete $^{13}CO_2$ signal in the breath, the appearance of which was enhanced or delayed by prior medication with

metoclopramide or loperamide respectively. Heine *et al.* concluded that the glycosyl ^{13}C-ureide breath test reflected intestinal transit time, and that it acted as a specific marker for the action of colonic microbial flora (Heine *et al.* 1995). The principle of the test is shown in Figure 1.3:

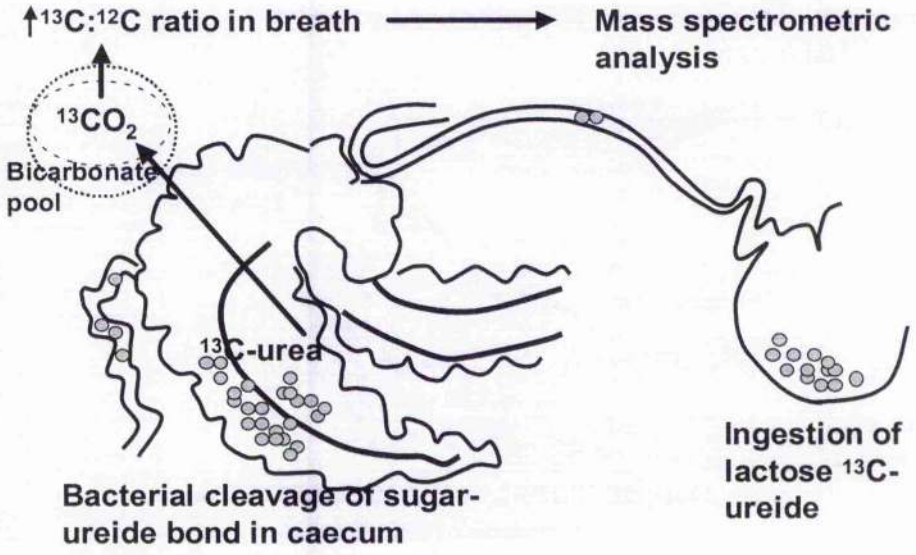


Figure 1.3 Chart demonstrating the principles of the lactose ^{13}C-ureide breath test for the potential measurement of equine orocaecal transit time.

Lactose ^{13}C-ureide is synthesised by the acid-catalysed condensation of lactose and urea, with different techniques resulting in yields of 50 – 70% from equimolar amounts of the two components (Merry *et al.* 1982a; Morrison *et al.* 1998; Morrison 2000). The lactose moiety may be cleaved in the small intestine to yield galactose and glucose ^{13}C-ureide, but the glucose-urea bond itself cannot be cleaved by intestinal brush border enzymes (Ruemmele *et al.* 1997). Ruemmele and coworkers demonstrated that homogenised human small and large intestinal material did not possess glucoseureide hydrolase enzymic activity. Rather, the bond is cleaved by intestinal microflora in ruminants (Merry *et al.* 1982b), humans (Heine *et al.* 1994) and also horses (Sutton *et al.* 2000). Using faecal bacterial cultures, Heine *et al.* (1994) reported high $^{13}CO_2$ release from L^{13}C-U added to cultures of the following intestinal bacteria: aerobic streptococci, *Staphylococcus aureus*, *Proteus*, *Klebsiella*, *Pseudomonas aeruginosa* and *Peptostreptococcus productus*. However, this early study of Heine *et al.* (1994) was flawed as it assumed incorrectly that allantoin-degrading microbes were capable also of cleaving glucoseureide, and the purity of the specific microbial cultures was also questionable. Later work by the same research group showed that the ability to cleave GU was much more exclusive than previously

thought (Mohr *et al.* 1999). Using thin layer chromatography to assess the capacity for GU digestion of cultures of speciated faecal microbes, Mohr *et al.* (1999) demonstrated clearly that *Clostridium innocuum* was the only microbe of 174 intestinal species studied that cleaved GU. Mohr and coworkers hypothesised that this common intestinal organism synthesised a glucoscureide hydrolase enzyme, resulting in release and oxidation of the ^{13}C-urea moiety in the colon. The organism was found to be present in the faecal flora of all individuals evaluated by Mohr *et al.* (1999) and this correlated to the ubiquity of $^{13}CO_2$ production after faecal incubation with ^{13}C-LU.

In order to use the LUBT for OCTT measurement, a number of criteria must be satisfied. In particular, it must be demonstrated that the target microflora are restricted to the large intestine; that glucoseureide hydrolysis and subsequent ^{13}C-urea oxidation proceed at a constant rate; and finally, as for all stable isotope breath tests, that the dynamics of the bicarbonate pool do not form a variable rate-determining step in the expiratory recovery of $^{13}CO_2$. Research on glycosylureide metabolism has been centred upon ruminal flora (Merry *et al.* 1982b, 1982c) and human faecal culture results (Mohr *et al.* 1999; Morrison 2000). These experiments have helped to elucidate the pharmacokinetics of the microbial hydrolytic process, but have not identified the specific locus of the relevant microbes in the human gastrointestinal tract. Subsequent acceptance of the LUBT for measurement of human OCTT has depended on correlation with the results of gastroenterocolonic scintigraphy as described below.

1.6.4.1.1 Ruminant studies

Merry *et al.* (1982b) investigated the use of glycosylureides as alternate, less toxic sources of non-protein nitrogen in sheep and steers using rumen contents collected via cannulation. The effects on *in vitro* ureide hydrolysis of adding ureide to the diet for different periods (adaptation) and of subsequently withdrawing it (de-adaptation) were studied. *In vitro* hydrolysis of both glucoseureide (GU) and lactoseureide (LU) was significantly enhanced by the prior addition of GU to the diet. Maximal rate of GU hydrolysis was achieved after at least 8 days of dietary adaptation, and given its extended duration, was hypothetically caused by proliferation of the GU-fermenting bacteria rather than enzymic induction (Merry *et al.* 1982b). De-adaptation produced a detectable reduction in GU fermentation rate within 7 days of dietary exclusion. In both sheep and steers, very little degradation of bound urea occurred when LU was incubated with rumen contents from unadapted animals. Merry *et al.* (1982b) also noted that dietary supplementation leads to increased rates of degradation of free lactose and urea. Addition of LU to the rumen cultures resulted

in a steady bacterial proliferation, rather than the rapid rise and fall of numbers seen after the addition of free lactose and urea.

Repetition of the above studies *in vivo* produced similar results (Merry *et al.*1982c). Sheep and steers required a dietary adaptation period of 7-10 days before maximising GU/LU hydrolysis rate. In unadapted individuals, there was little hydrolytic activity, but when fully adapted, cleavage of the sugar-ureide bond was complete in 2 – 4 h (Merry *et al.* 1982c).

1.6.4.1.2 Induction of glucoseureide hydrolase activity

In view of the findings of Merry *et al.* (1982b, 1982c), the effects of induction doses of unlabelled LU on the results of LUBT results have been assessed (Wutzke *et al.* 1997a, 1997b; Geypens *et al.* 1999; Morrison 2000). Defining OCTT as the first significant rise in expiratory $^{13}CO_2$ afer the ingestion of L^{13}C-U, Wutzke *et al.* (1997a, 1997b) reported that OCTT was shortened in 12 subjects by the prior feeding of unlabelled substrate. In these subjects, premedication with unlabelled LU at five times the test dose in the 24 h preceding the test, resulted in an earlier and steeper rise in $^{13}CO_2$. Cumulative $^{13}CO_2$ excretion remained constant in both studies, reaching a plateau of 36% at approximately 22 hours. LU did not exert an apparent osmotic effect in either set of studies, and OCTT results using the LUBT were significantly slower than those of the lactulose H_2BT (3.02 ± 1.4 h, 1.84 ± 0.5 h, $P < 0.005$), due to the osmotic effects of the latter (Miller *et al.* 1982c). Using *in vitro* stool studies in young children, Van den Driessche *et al.* (2000) also found that L^{13}C-U hydrolysis rate was enhanced by prior induction with unlabelled substrate.

In subsequent studies involving the LUBT for measurement of OCTT, relatively arbitrary induction doses of unlabelled LU have been given prior to administration of the labelled test substrate (Geypens *et al.* 1999; Van den Driessche *et al.* 2000). Morrison (2000) attempted to clarify the effect of the 'induction' process. Using *in vitro* faecal culture techniques, it was shown that optimal LU fermentation occurred approximately 22 h after induction with unlabelled substrate, with an optimal ratio of unlabelled:labelled LU exceeding 2:1 (Morrison 2000). The relatively short duration of induction in this study suggests that upregulation of bacterial enzyme synthesis may be involved, in addition to likely multiplication of the selected bacterial populations.

1.6.4.2 *Validation of the ^{13}C-LUBT for OCTT measurement*

Geypens *et al.* (1999) sought to validate the LUBT for OCTT measurement by simultaneous comparison with the predicate method of GECS (Camilleri *et al.* 1998). Volunteers ingested 1 g unlabelled LU three times the day before the test, and then ingested a meal containing 500 mg L^{13}C-U and ^{99m}Tc. Geypens *et al.* (1999) reported an excellent correlation between the LUBT and scintigraphy for OCTT measurement (292 ± 58 min and 283 ± 53 min; P = 0.0001), and concluded that the breath test was a valid alternative to scintigraphy for OCTT measurement. Wutzke *et al.* (1997b) also compared the LUBT to the older lactulose H_2BT, and reported that the former had both greater sensitivity and specificity for OCTT measurement.

1.6.4.3 *Advantages and Disadvantages*

The advantages of the LUBT over existing methods of OCTT measurement include its safety, inert nature, non-subjective interpretation (first significant rise in $^{13}CO_2$ equals OCTT), and its relative ease of application. Although many studies have described GECS as the optimal modality for assessment of OCTT (see Camilleri *et al.* 1998 for review), loops of radioactive small intestine may complicate scintigraphic assessment of colonic/caecal arrival of ingesta, and this parameter is particularly difficult to determine in the horse. The LUBT may therefore offer certain further advantages to scintigraphy. Recent combination of the LUBT with GE stable isotope tests has also produced the possibility of a simultaneous non-invasive, non-radioactive method for calculation of GE rate, SBTT and OCTT (Van den Driessche *et al.* 1997). The LUBT has been used clinically to show that OCTT may be delayed in paediatric Crohn's disease, and that the majority of children with severe functional gastrointestinal symptoms have prolonged small bowel transit (Van den Driessche 2001). This may lead to improved therapeutic modulation of these disorders.

Potential disadvantages of the LUBT include the nature of the induction process, and the time required for induction before performing the test. Failure to optimise induction may lead to overestimation of OCTT and diagnostic error (Wutzke *et al.* 1997a). In addition, the effects of dietary change, administration of antibiotics, and of intestinal bacterial overgrowth on the results of the test have still to be assessed. Heine *et al.* (1995) noted that individuals treated with an antibiotic regimen routinely used before intestinal surgery lost the ability to cleave L^{13}C-U. In horses, certain antibiotics, such as erythromycin (Gustafsson *et al.* 1997), lincomycin (Staempfli *et al.* 1992) and oxytetracycline (White and Prior 1982) have been shown to cause marked disruption of colonic flora, but are not

widely used for that very reason. The most commonly administered oral antibiotic, trimethoprim/sulphadiazine, causes little change in the bacterial flora. Gustaffsson *et al.* (1999) found that trimethoprim/sulphadiazine caused an initial reduction in faecal coliforms in 6 horses, but no further significant changes. The equine hindgut flora undergo population modulation during sickness, with proliferation of specific clostridial species prior to the onset of diarrhoea (Wierup and DiPietro 1981). Although the characteristics of *Clostridium innocuum* have not been documented in the horse, it is most likely that capability of the hindgut for GU hydrolysis would be retained under such conditions.

Heine *et al.* (1995) reported that the LUBT had good repeatability within individuals, and suggested that daily dietary variation had little effect on test results. Once again, this may require further investigation if the test is to be widely used in the horse, although dietary variation is relatively small in this species.

The LUBT has proved useful in paediatric gastroenterology, but Van den Driessche *et al.* (2000) discovered that subjects aged below 6 months (prior to weaning) were unable to hydrolyse LU. The authors of this study concluded that colonisation of the colon with the necessary bacteria occurred only at the time of weaning, and that this capability was only reliably present after the age of 8 months (Van den Driessche *et al.* 2000). Because of the limitations of the LUBT in neonates, Van den Driessche (2000) suggested that the development of ^{13}C-labelled inulin or oligofructose breath tests might be the optimum diagnostic tool for OCTT measurement in this age group. Potential application of the LUBT to equine neonates prior to weaning would therefore require specific investigation of the colonic microflora in this age group.

1.6.4.4 Veterinary applications of the lactose ^{13}C-ureide breath test

As detailed in the preceding sections, both the H_2BT and the SLZ/SP test have been used for the measurement of equine OCTT, but both have a number of potential problems, and require further validation. Although GECS has been adopted as the standard technique for OCTT assessment in humans, the technique has limited accuracy in the horse, and a true 'gold standard' for the non-invasive measurement of equine OCTT has yet to be universally accepted. Hence, one of the major aims of this present research is the development and potential validation of a LUBT for equine OCTT estimation. As described in chapter 7, the results of equine LUBTs will be compared with those of the H_2BT. In order to specifically validate the LUBT for the measurement of OCTT, *in vitro* bacterial incubations will be used to identify the GU hydrolytic activity of enteric microbes

in different regions of the equine gastrointestinal tract. If successfully validated, the applications of the LUBT in equine medicine might include improved diagnostic investigation of animals with abdominal pain; preclinical testing of potential prokinetic drugs, and advancement of the knowledge of gastrointestinal pathophysiology.

1.7 Prokinetic therapy

1.7.1 Indications for prokinetic therapy

Gerring (1992) defined ileus as a disruption of coordination and reduction of gastrointestinal motility resulting in failure of propulsion of intestinal contents through the alimentary tract. Equine ileus may develop for a variety of reasons such as intestinal ischemia and distention, endotoxaemia, surgical trauma to the intestine, peritonitis, electrolyte imbalances, damage to the intrinsic or extrinsic nervous supply or secondary to the administration of specific analgesic or anaesthetic regimens (Adams 1988; Rakestraw 2003). Clinically, ileus is usually characterised by one or several of the following signs: a reduction in intestinal borborygmi, loss of appetite, depression, decreased faecal output and abdominal pain caused by progressive intestinal distension. Depending on the severity of the condition, further examination may then reveal excessive gastric fluid on nasogastric intubation and distended loops of small intestine. The site and length of intestine affected by ileus determines the clinical signs and rate of progression, and may be focal as in recurrent caecal impaction (Schusser *et al.* 2000) or more generalised as seen in acute grass sickness (Doxey *et al.* 1991). Initial treatment must be directed towards correction of any predisposing causes such as electrolyte abnormalities, inflammation, pain, infection or endotoxaemia. Fluid therapy, administration of non-steroidal anti-inflammatory drugs, and surgical removal of an endotoxic focus and/or treatment with endotoxin-binding compounds are the primary strategies for treatment. However, prokinetic agents are also given frequently in an attempt to decrease the severity and duration of ileus. Although large-scale clinical trials have been performed with certain compounds such as cisapride (Gerring *et al.* 1991) and metoclopramide (Dart *et al.* 1996) to determine their efficacy in the prevention or treatment of equine ileus, much of the use of prokinetics in equine gastroenterology is based on small retrospective clinical studies or extrapolated from myoelectrical experimental studies. An evidence based medicine approach is required with properly conducted prospective studies to determine the value of these compounds in equine medicine and the most effective dosage regimens. Such research has been hindered by the lack of availability of suitable non-invasive tests for the quantitative measurement of gut motility in clinical cases of ileus before and after prokinetic therapy.

Post-operative ileus following small intestinal surgery may be the commonest indication for administration of prokinetic agents in clinical practice (Dart and Hodgson 1998). As the the techniques for equine intestinal sugery have advanced, the relative post-operative survival rate has inceased, but the proportion of deaths attributable to post-operative ileus (POI) has also increased, demonstrating that this remains one of the most important causes of unsuccessful outcome (Edwards and Hunt 1986). Hunt *et al.* (1986) reported POI to be the cause of death in 43% of 84 postoperative equine fatalities. In the multiple logistic regression model of Morton and Blikslager (2002) for horses undergoing small intestinal resection and anastomosis, POI was identified as the greatest single risk factor for non-survival with a final model odds ratio of 29.7. Of those horses developing POI, 37.5% died, but the use of prokinetic agents was associated with a slightly improved survival (odds ratio of 0.6). The dual centre study of Smith *et al.* (2002) also provided evidence to support the hypothesis that use of prokinetic agents improved the prognosis of horses recovering from pedunculated lipoma obstruction with POI.

In addition to POI, specific prokinetics have been recommended for use in treatment of pelvic flexure impactions (Steinebach and Cole 1995) and of chronic grass sickness (Milne *et al.* 1996). However, care must be exercised in the selection of appropriate cases as prokinetic regimens have resulted in intestinal rupture in cases of caecal and large colon impactions (Rakestraw 2003).

1.7.2 Pathophysiology of equine ileus

1.7.2.1 Colonic and caecal impactions

Large colon impaction is relatively common, occurring in 13.4% horses referred with colic in one large hospital-based retrospective study (Dabareiner and White 1995), and may be more frequent still in the general population. A contributory factor in many uncomplicated cases is likely to be the dehydration of ingesta, with resultant impaction at the pelvic flexure, an area of naturally increased resistance to transit. The vast majority of these cases resolve with medical treatment alone, consisting of pain relief and oral isotonic fluid and cathartic therapy. However, up to 16% of cases may require surgical treatment (Dabareiner and White 1995) and cisapride therapy has proved useful in refractory cases (Steinebach and Cole 1995). Certain subjects suffer recurrent or chronic colonic impactions and Schusser and White (1997) reported that these individuals had a significant reduction in neurone density in the colonic myenteric plexus compared to normal subjects. It was also shown that enteroglial cell numbers were increased in myenteric plexuses of horses with acute and chronic obstruction (Schusser and White 1997), suggesting the

presence of inflammation. The presence of an inflammatory infiltrate in the myenteric plexus has also been linked to dysmotility of the equine small colon (Burns *et al.* 1990). Recurrent small colon impaction in a single horse was associated with mononuclear cell infiltration of the myenteric plexus, followed by intraganglionic and fascicular fibrosis with neuronal degeneration (Burns *et al.* 1990). These histological changes noted in equine impactions may be analogous to those found in idiopathic pseudo-obstruction in man (White 2000).

Chronic recurrent caecal impaction occurs less commonly than colonic impaction, but may have a similar aetiopathogenesis. Schusser *et al.* (2000) showed that the linear neuron densities of all caecal base areas and of the caudal body region and apex were significantly lower in euthanased horses with chronic recurrent caecal impaction than in clinically normal controls. The circular muscle layer of all caecal regions was also hypertrophied, probably as a result of chronic uncoordinated motility associated with the neuron deficit in the myenteric plexus of the caecal base (Schusser *et al.* 2000). Treatment with non-steroidal anti-inflammatory drugs has also been linked to the development of both caecal and colonic impactions. Campbell and Blikslager (2000) demonstrated that treatment with flunixin meglumine resulted in delayed return of transepithelial resistance after intestinal ischaemia, and linked this to blockade of cyclo-oxygenase-1 (COX-1) activity and reduced mucosal prostaglandin levels. Work is currently being performed to investigate the possible benefits of specific COX-2 inhibition for maintenance of contractility in equine abdominal disorders. Flunixin meglumine was shown to result in an increased mucosal permeability in bile-injured colonic tissue, whilst a selective COX-2 inhibitor had no significant effect on this parameter (Campbell *et al.* 2002). Selective inhibition of COX-2 may therefore reduce the incidence of adverse effects in horses requiring NSAID therapy.

1.7.2.2 Equine grass sickness

The precise aetiopathogenesis of equine grass sickness (EGS) has yet to be proven, although it is thought to be multifactorial, likely involving toxicoinfection with *Clostridium botulinum* type C (Hunter and Poxton 2001; McGorum *et al.* 2003a), with oxidative stress (McGorum *et al.* 2003b) and previous ingestion of cyanogenic glycosides from white clover (McGorum and Anderson 2002) acting as possible contributory factors. Equine grass sickness is a primary dysautonomia that is characterised by histopathological lesions in the autonomic ganglia, enteric plexi and specific nuclei in the CNS (Griffiths *et al.* 1993). Recent work has also shown that the interstitial cells of Cajal (ICC), responsible for the generation of pacemaker activity in gastrointestinal smooth muscle, are

significantly decreased in the myenteric plexus and circular muscle regions of both ileum and pelvic flexure in EGS cases, compared to normal animals (Hudson *et al.* 2001). Although ICC-mediated pacemaker activity remains intact in EGS, it has been demonstrated that ileal membrane potential oscillations are reduced significantly in both frequency and duration (Hudson *et al.* 2002). The loss of synaptic contact between neurones and ICC in EGS, either because of destruction of the neurones or a decrease in ICC, may be crucial in the subsequent disruption of normal intestinal motility seen in this condition (Hudson *et al.* 2002).

Overlapping clinical syndromes have been described in EGS, ranging from peracute with rapid death from intestinal distention and gastric rupture, to chronic EGS in which there is variable reduction in motility of specific regions of the intestinal tract with reduced feed intake and reduced faecal output. More is being discovered about the histopathological differences between these clinical variants of the disease. Gilmour (1973) reported pathological changes in the extrinsic neurones of both acute and chronic grass sickness cases. This was subsequently suggested to be retrogradal damage, occurring secondary to toxic damage to the enteric plexus, with further non-specific changes reported in various brainstem nuclei (Wright and Hodson 1988). Scholes *et al.* (1993) found reduced normal and increased numbers of abnormal neurones in the ileal submucosal and myenteric plexuses of both acute and chronic grass sickness cases, but was able to correlate severity of damage to severity of clinical presentation. Doxey *et al.* (1995) also reported significantly less neuronal damage in the intrinsic nervous system of the jejunum and small colon in chronic when compared with acute EGS cases. Most recently, Murray *et al.* (1997) found that acute grass sickness cases had lower numbers of neurones than chronic cases in all regions of the duodenum and ileum, with equal damage to the submucosal and myenteric plexuses. Interestingly from the clinical standpoint, a good correlation was also shown between functional cholinergic responses to physostigmine and the extent of neuronal damage (Murray *et al.* 1997). In simplified terms, the variable disruption to intestinal motility seen in the different forms of EGS may thus reflect the extent of neuronal damage in the enteric plexuses and the length of intestine that has been affected. The presence of functional, albeit reduced, numbers of ICC in the condition suggests that further investigation of potential prokinetic agents may be beneficial in the treatment of this condition (Hudson *et al.* 2002).

Damage to the central nervous system nuclei is also likely to be involved in the loss of intestinal motility seen in EGS. Many central nuclei that govern the final motor pathways of the parasympathetic nervous system may be affected (Barlow 1969). Damage to the

vagal nuclei will result in loss of extrinsic control of the contractility of the upper alimentary tract, loss of tone and a failure of vagally mediated communication between intestinal compartments (Cottrell *et al.* 1999). Neuronal damage to cranial nerves V, VII, X and XII has also been reported (Gilmour 1973) and may be linked to the dysphagia and swallowing difficulty frequently observed in this condition.

Altered production and release of intestinal neurotransmitters has also been implicated in the pathogenesis of the ileus observed in EGS, with particular interest in the inhibitory compounds vasoactive intestinal peptide (VIP) and nitric oxide (NO). Electron microscopic investigation of ileal sections from EGS cases showed a depletion of neuronal vesicles from nerve endings in the myenteric and submucous plexuses, including those with VIP immunoreactivity (Cottrell *et al.* 1999). As VIP and NO are involved in the inhibition of the descending components of the peristaltic arc, it has been suggested that decreased release results in a relative increase in sympathetic stimulation or sympathicotonus, with consequent increased tone and blood flow, and loss of effective peristaltic activity (Cottrell *et al.* 1999).

1.7.2.3 Equine postoperative ileus

Many horses have an uncomplicated recovery following intestinal surgery, but others develop clinical signs consistent with one of two forms of postoperative ileus (Dart and Hodgson 1998). Some exhibit a transient period of ileus, with reduced intestinal borborygmi, delayed transit of ingesta and delayed gastric emptying, marked by the retrieval of small quantities of gastric reflux on nasogastric intubation. This condition is similar to the very common transient ileus observed in humans postoperatively, caused by reversible electromechanical disturbance of the stomach and large colon (Condon and Sarna 1982). In horses, sympathetic hyperactivity has been suggested as a factor, with dopamine infusion shown to cause a dose-dependent inhibition of gastric and small intestinal myoelectrical activity (King and Gerring 1988). Circulating endotoxin may also be implicated (King and Gerring 1989, 1991), one if its many actions being to disrupt endogenous prostaglandin production. Hunt and Gerring (1991) demonstrated that intravenous prostaglandin E_1 infusion resulted in a significant decrease in gastric and colonic electrical spiking activity, with a lesser effect on the small intestine. Most horses will make a relatively rapid recovery from this transient form of POI with routine medical management.

The second, more serious form of equine POI is analogous to 'adynamic ileus' in humans, and has an incidence of approximately 10% after intestinal surgery, with a similar mortality rate (Freeman *et al.* 2000; Proudman *et al.* 2002). Morton and Blikslager (2002) reported a much higher incidence of POI in 92 horses following small intestinal resection, with a relatively higher survival rate of 62.5%, but this was due to a different definition of ileus based on retrieval of just 2 l of nasogastric reflux. Severe POI is recognised as the most influential factor in determining short-term survival in horses after small intestinal surgery (Morton and Blikslager 2002). Risk factors for the development of POI itself include surgery of greater than 2 h duration, lesions localised to the small intestine and resection of small intestine, whilst pelvic flexure enterotomy has been calculated to reduce the risk of POI (Roussel *et al.* 2001). French *et al.* (2002) also identified strangulating pedunculated lipoma lesions as carrying a separate increased risk for development of POI.

The exact mechanisms of this persistent form of POI are not fully elucidated, but are thought to include persistent endotoxaemia, shock, electrolyte imbalances and intestinal distension, ischaemia-reperfusion injury or inflammation with chemotactic neutrophil recruitment and subsequent release of destructive proteases and oxygen free radicals (Gerring and Hunt, 1986; Hunt *et al.* 1986; Moore *et al.* 1995). Direct mechanical irritation of the peritoneal membranes at surgery is also thought to be a major risk factor for the development of POI, and serosal friction has been used as a standard experimental technique for the induction of transient ileus (Gerring and Hunt 1986; Gerring and King 1989). Much research is continuing to elucidate further factors involved in the development of POI. In the meantime, in view of the prevalence and mortality rate of this condition, clinicians have turned to the administration of prokinetic agents as a further therapeutic tool, often based on minimal, poor or even misleading experimental evidence of efficacy. The mechanisms of action of prokinetic agents and the methods used for examination of their potential value in equine gastroenterology are presented in the subsequent sections.

1.7.3 Mechanisms of action of prokinetic agents

A number of therapeutic prokinetic compounds have been developed that alter interaction between specific gastrointestinal neurotransmitters and their receptors, either by affecting ion channels and/or inducing the release of calcium from the endoplasmic reticulum, mediated via secondary messenger systems.

1.7.3.1 *Cholinomimetic agents*

Acetylcholine is released from parasympathetic postganglionic cholinergic axon terminals and exerts its excitatory effect on smooth muscle cells by binding to M_2-receptors and stimulating contraction. Stimulation of the M_2-receptors is thought to regulate the phasic movements of the bowel in the inter-digestive period (Demol *et al.* 1989). The quantity of acetylcholine released is controlled by stimulation of prejunctional receptors by compounds including histamine and serotonin, or by inhibition via interaction with α_2-receptors. Acetylcholine also acts at the ganglionic level through nicotinic receptors and muscarinic M_1 receptors (Demol *et al.* 1989). Bethanechol is a specific muscarinic cholinergic agonist, devoid of nicotinic action, which has been investigated as a potential prokinetic agent in the horse (Roger and Ruckebusch 1987; Ringger *et al.* 1996; Lester *et al.* 1998) and cow (Steiner *et al.* 1995). The modalities used to investigate this compound and the study results are summarised in Table 1.2. The gastrointestinal effects of neostigmine, an anti-cholinesterase with motor-stimulating properties, have also been examined in the horse (Adams *et al.* 1984; Adams and MacHarg 1985; Lester *et al.* 1998b) as given in Table 1.2. It should be noted that this compound is not gut specific (Steiner and Roussel 1995).

1.7.3.2 *Adrenoceptor-blocking agents*

As the majority of sympathetic postganglionic nerve endings are adrenergic, exerting their effect via α- and β-adrenoceptors (Virtanen 1986), adrenergic receptor antagonists have been examined for their potential use as modulators of intestinal motility. Noradrenaline exerts an inhibitory influence on intestinal contractility by activation of presynaptic α_2-receptors. At higher concentrations, there may also be stimulation of postsynaptic α_1 and β receptors with consequent hyperpolarization of postsynaptic membrane potential (Virtanen 1986; Rang *et al.* 1999). This mechanism is discussed further in Chapter 5. As the presynaptic α_2-receptor mediated mechanism appears to be the most important for inhibition of gastrointestinal motility, equine studies have examined the potential value of specific α_2-antagonists (e.g. yohimbine) in the treatment of ileus (Gerring and Hunt 1986; Roberts and Argenzio 1986; Eades and Moore 1993; Lester *et al.* 1998b; Malone *et al.* 1999; Meisler *et al.* 2000; Re *et al.* 2001). Yohimbine has been shown to ameliorate the disruptive effects of endotoxin on both GE rate (Meisler *et al.* 2000) and small intestinal electromechanical activity (Gerring and Hunt 1986). The results of these equine studies are summarised in Table 1.2.

1.7.3.3 *Antidopaminergic agents*

Although the existence of specific intestinal dopamine receptors is still a matter of controversy in some species (Demol *et al.* 1989), dopamine has been shown to have a physiological role in the modulation of gastrointestinal motility (Steiner and Roussel 1995). The presence of dopaminergic receptors on postganglionic myenteric nerves of the antroduodenal area has been postulated in several species, including the horse (Gerring and Hunt 1986), and these are thought to mediate inhibition of coordinated gastric motility. However, dopamine is also known to be a non-specific adrenoceptor agonist, and may reduce intestinal motility by stimulation of presynaptic α_2-receptors. Dopamine antagonists such as metoclopramide and domperidone have been shown in humans to increase the tone of the lower oesophageal sphincter, increase fundic tone and to increase antral peristalsis. The net effect of these actions is to decrease gastro-oesophageal reflux, accelerate liquid phase emptying and to increase GE of solid ingesta (Demol *et al.* 1989). Dopamine antagonists also regulate coordination between the antrum and duodenum and decrease duodeno-gastric reflux (Demol *et al.* 1989).

In addition to antagonising dopamine, Albibi and McCallum (1983) showed that metoclopramide increased acetylcholine release from postganglionic cholinergic nerve terminals and increased smooth muscle response by sensitising muscarinic receptors. Further actions of metoclopramide that may account for its prokinetic properties include blockade of inhibitory 5-HT_3 receptors, and activation of excitatory 5-HT_4 receptors (Steiner and Roussel 1995).

Metoclopramide has been reported to stimulate gastric emptying of liquid in ponies (Doherty *et al.* 1998) and to ameliorate the effects of endotoxin on gastric emptying of acetaminophen solution (Doherty *et al.* 1999). Nieto *et al.* (2000a) also demonstrated that metoclopramide caused a dose-dependent increase in contractile amplitude *in vitro* of equine muscle strips taken from the pyloric antrum, proximal duodenum and mid-jejunum. However, electromechanical recordings taken from the jejunum and pelvic flexure after administration of metoclopramide suggested that it had no significant effect on the contractility of these intestinal regions (Sojka *et al.* 1988). Similarly, Ruckebusch and Roger (1988) reported that metoclopramide did not have a true prokinetic effect on the ileocaecal-colonic region of fasted conscious ponies. One might therefore conclude that metoclopramide treatment is beneficial only in the management of equine proximal small intestinal lesions. This may not be true though because in a retrospective study on the incidence of POI in 70 horses after small intestinal resection and anastomosis, Dart *et al.*

(1996) concluded that metoclopramide given by continuous infusion decreased the incidence and severity of ileus. Therefore, more objective, quantitative studies in both health and disease may be required on the efficacy of this compound on specific regions of the equine intestinal tract. Methods and results for metoclopramide studies in the horse are summarised in Table 1.2.

1.7.3.4 5-Hydroxytryptamine receptor agonists

At least 4 families of 5-Hydroxytryptamine (5-HT) receptors have been described, and 5-HT_1, 5-HT_3 and 5-HT_4 receptor types are widely considered to be the major receptors involved in regulation of intestinal motility (Taniyama *et al.* 1991; Steiner and Roussel 1995). Activation of the 5-HT_1 and 5-HT_3 receptors is thought to decrease acetylcholine release from the postganglionic cholinergic axon, thus inhibiting smooth muscle contraction. By contrast, stimulation of 5-HT_4 receptors on ganglionic cells in the myenteric plexus results in an increased release of acetylcholine by the postganglionic axon and a larger excitatory postsynaptic membrane potential in the smooth muscle (Taniyama *et al.* 1991). However, the effects of 5-HT receptors may vary according to the species and particular section of the gastrointestinal tract examined. In an excellent *in vitro* study with equine jejunum, Nieto *et al.* (2000) reported that contraction of jejunal muscle in response to 5-HT was mediated by 5-HT_2 and 5-HT_3 receptors. Incubation of equine muscle strips with a specific 5-HT_4 antagonist had no effect on contractions elicited by 5-HT (Nieto *et al.* 2000), suggesting that results should not be extrapolated from other species to the horse.

Cisapride is a 5-HT receptor agonist devoid of antidopaminergic or direct cholinergic properties that has been studied for its potential prokinetic properties in a number of species. The effects of cisapride on equine gastrointestinal motility are not completely understood, partly because several different experimental models have been used. Cisapride administration reduced the incidence of equine POI in both clinical trials (Gerring and King 1989; Gerring *et al.* 1991; Van der Velden and Klein 1993) and experimental models of ileus (Gerring and King 1989), and tended to decrease whole gut transit time in cases of chronic grass sickness (Milne *et al.* 1996). Endotoxin-mediated delays in GE were attenuated by cisapride (Valk *et al.* 1998b), but liquid phase emptying was not enhanced in healthy foals (Baker and Gerring 1994a). Ruckebusch and Roger (1988) demonstrated that cisapride induced migrating spoke bursts in the equine colon associated with subsequent contractions of the caecal body and base, and high doses of this compound also induced jejunal migrating contractions (Sasaki and Yoshihara 2000). The

studies of Nieto *et al.* (2000) suggest that these excitatory effects of cisapride are principally mediated by stimulation of equine 5-HT_2 receptors. Cisapride is one of the most studied prokinetic agents for equine use, and the methods and results of specific reports are given in Table 1.2.

1.7.3.5 Opioid receptor antagonists

As discussed further in section 5.1.4.2, opioids exert an inhibitory effect on intestinal motility by inducing neuronal hyperpolarization, which prevents the threshold for neuronal excitation from being reached. Naloxone is an opioid antagonist with μ-receptor affinity, which has been investigated as a potential equine prokinetic agent (Ruckebusch and Roger 1988) as shown in Table 1.2. However, this compound could only be expected to induce contractility if inhibitory opioid receptors were being stimulated by endogenous opioids (Steiner and Roussel 1995).

1.7.3.6 Erythromycin

Erythromycin is a macrolide antibiotic with known prokinetic activity, and was shown recently to exert part of its actions in the horse secondary to binding on intestinal motilin receptors (Koenig *et al.* 2002). Erythromycin was demonstrated to compete with ^{125}I-labelled porcine motilin for binding to motilin receptors. Koenig *et al.* (2002) also found that motilin receptors were at greatest abundance in the equine mid-duodenum and jejunum, with lower levels in the large colon and then caecum. Interestingly, intravenous injection of motilin has also been shown to induce strong phase-3 contractions in the equine jejunum, but to have no effect on the caecum (Sasaki and Yoshihara 1999). Roussel *et al.* (2000) also found that erythromycin stimulated postoperative myoelectrical actitivity in the ileum and pelvic flexure of healthy horses but not in the caecum. This was in contrast to the findings of Lester *et al.* (1998c), who demonstrated that erythromycin increased scintigraphic caecal emptying rate and caecocolic myoelectric activity of healthy ponies. Given the findings of Lester *et al.* (1998c) it is possible that erythromycin has more than one mechanism of action in the horse.

This prokinetic agent may also affect gastric emptying rate in the horse. Ringger *et al.* (1996) demonstrated scintigraphically that erythromycin caused a significant increase in the rate of solid phase gastric emptying in healthy individuals. These study findings are summarised in Table 1.2.

1.7.3.7 *Lignocaine*

The prokinetic effect of lignocaine has not been proven in the horse, but lignocaine ('lidocaine') infusion has been used with apparent success for the treatment of horses with postoperative ileus of at least 24 h duration or duodenitis-jejunitis (Malone *et al.* 1994). Proposed mechanisms of action of lidocaine in POI include direct stimulation of smooth muscle, blockade of sympathetic inhibitory reflexes, reduction of concentrations of circulating catecholamines and a decrease in inflammation (Nieto *et al.* 2000). In intestinal muscle strips collected from healthy horses, Nieto *et al.* (2000) demonstrated *in vitro* that lignocaine increased contractile activity of the proximal duodenum, but did not have an effect on the pyloric antrum or mid-jejunum. Again, these study results are summarised in Table 1.2.

1.7.4 Clinical relevance of equine prokinetic studies

As can be seen from examination of Table 1.2, in the vast majority of studies investigating the action of potential prokinetic agents on the equine gastrointestinal tract, healthy animals have been used and very few clinical trials have been performed. Studies have also generally involved small groups of animals of less than 6 in number. The four particular exceptions to this are the larger scale prospective trials of metoclopramide (Dart *et al.* 1996) and cisapride for the prophylaxis of POI (Gerring *et al.* 1989; Van der Velden and Klein 1993) or treatment of chronic grass sickness (Milne *et al.* 1996). Even in these large clinical trials, there are some limitations to the conclusions reached. Gerring *et al.* (1989) concluded that cisapride was effective in the prophylaxis of POI based on the uncomplicated recovery from colic surgery of 57/63 individuals that had received cisapride (6/63 developed POI). However, no controls were used in this multi-centre trial, and as the general incidence of POI in the study populations was not measured either, the conclusions of the study may be flawed. Milne *et al.* (1996) quantified intestinal WGTT in a total of 14 individuals with chronic grass sickness before, during and after treatment with cisapride by measuring the oroanal transit rate of polystyrene pellets. Although the study individuals showed a significantly faster transit rate after cisapride therapy than before, once again a case-control study was not performed. It is thus possible that the findings of Milne *et al.* (1996) reflected natural improvement of intestinal motility in those individuals rather than effective response to cisapride therapy.

Table 1.2 Prokinetic studies in the horse: summary of the disease models used, together with the study technique and the recorded mechanism of action.

Drug	Dose (mg/kg)	Disease Model	Study Technique	Action	Refs.
Cholinomimetics					
Neostigmine	0.022 IV	Healthy horses	Electro- and myo-mechanical activity	↑ MMC duration and ↑ pelvic flexure contractility	1
Neostigmine	0.022 SC	Healthy horses	GE of solid metal markers (radiography)	↓ GE of metal markers	2
Neostigmine	0.025 SC	Healthy ponies	Caecal scintigraphy & myoelectrical activity	↑ Caecal emptying rate & ↑ ileocaecocolic activity	3
Bethanechol	0.020 IV	Healthy horses	Serosal myoelectrical activity	↑ Myoelectrical activity of stomach and jejunum	4
Bethanechol	0.014 - 0.022 SC	Horses after trauma to jejunal serosa	Electromechanical & oroanal transit spheres	Enhanced prokinetic action of yohimbine	5
Bethanechol	0.025 IV	Healthy horses	Dual phase gastric scintigraphy	Significant ↑ in solid and liquid phase GE rate	6
Bethanechol	0.025 IV	Healthy ponies	Caecal scintigraphy & myoelectrical activity	↑ Caecal emptying rate & ↑ ileocaecocolic activity	3
Adrenoceptor-blocking agents					
Yohimbine	0.15 IV	Horses after trauma to jejunal serosa	Electromechanical & oroanal transit spheres	Effective restoration of small intestinal motility	5
Yohimbine	0.075 IV	Healthy horses after given endotoxin	Strain gauge force transducers	Restoration of caecal mechanical activity	7
Yohimbine	0.075 IV	Healthy ponies	Caecal scintigraphy & myoelectrical activity	↑ Caecal emptying but not significant effect	3
Yohimbine	0.250 IV	Healthy horses after given endotoxin	Acetaminophen test for GE rate of liquids	Reduction of endotoxin-mediated delay in GE	8
Antidopaminergic agents					
Metoclopramide	0.125/0.250/0.500 given per h IV	Healthy horses	Strain gauge force transducers	↑ Myomechanical activity of jejunum and ileum	9
Metoclopramide	0.250/0.500 given per h IV	Horses after trauma to jejunal serosa	Electromechanical & oroanal transit spheres	Complete restoration of small intestinal motility	5
Metoclopramide	2.00 IV	Healthy ponies	Electromyography of ileum, caecum and RVC	Weak unspecific motor effects	10
Metoclopramide	0.125 in 1litre of saline in 60 min	Healthy ponies	Acetaminophen test for GE rate of liquids	Stimulated GE rate of liquids in fasted ponies	11
Metoclopramide	0.125 in 1litre of saline in 60 min	Healthy horses after given endotoxin	Acetaminophen test for GE rate of liquids	Significant amelioration of endotoxin-delayed GE	12

1. Adams *et al.* 1984; 2. Adams *et al.* 1985; 3. Lester *et al.* 1998b; 4. Hunt and Gerring 1985; 5. Gerring and Hunt 1986; 6. Ringger et *al*. 1996; 7. Eades and Moore 1993; 8. Meisler *et al.* 2000; 9. Hunt and Gerring 1986; 10. Ruckebusch and Roger 1988; 11. Doherty *et al*. 1998; 12. Doherty *et al.* 1999a.

Prokinetic studies in the horse (Continuation of Table 1.2).

Drug	Dose (mg/kg)	Disease Model	Study Technique	Action	Refs.
5-Hydroxytryptamine-receptor agonists					
Cisapride	0.05/0.10/0.25 IV	Healthy ponies	Strain gauge force transducers	↑ contractility in stomach, S.I., LDC and small colon	13
Cisapride	0.100 IV	Healthy ponies	Electromyography of ileum, caecum and RVC	Stimulated coordinated motility of caecum & RVC	10
Cisapride	0.100 IV	Horses after trauma to jejunal serosa	Electromechanical & oroanal transit spheres	Restored WGTT and ↑ gastric/S.I. coordination	14
Cisapride	0.100 IM	Clinical Trial	Prevented POI in 22 clinical cases	?↓ Incidence of POI after surgery. No case-control.	14
Cisapride	0.100 IM	Clinical Trial	Prevented POI in 57 out of 63 horses	?↓ Incidence of POI after surgery. No case-control.	15
Cisapride	0.100 IM every 8 h	Randomized blind trial after S.I. surgery	Clinical observation	↑Restoration of bowel motility↓POI ↑ Recovery	16
Cisapride	0.100 IM or 0.800 *per os*	Clinical Trial in grass sickness cases	Oroanal transit of polystyrene spheres	Apparent beneficial effect ↑ intake of dry matter	17
Cisapride	0.750 or 1.00 *per os*.	Healthy horses	Electro- and myo-mechanical activity	↑ migrating contraction of jejunum	18
Opioid receptor antagonist					
Naloxone	0.050 IV	Healthy ponies	Electromyography of ileum, caecum and RVC	Coordinated motility of caecum & RVC	10
Motilin agonists					
Erythromycin lactobionate	0.100 or 1.00 IV	Healthy horses	Dual phase gastric scintigraphy	Significant ↑ in solid phase GE and ↑ liquid transit rate	6
Erythromycin lactobionate	0.010 - 10.00 IV 1.00 IV optimum	Healthy horses	Caecal scintigraphy & myoelectrical activity	Significant ↑ in caecal half-emptying & ↑contractility	19
Erythromycin lactobionate	0.500 IV	Horses after implant of electrodes	Electromyography of ileum, caecum and RVC	Variable effect on ileum ↑Activity of pelvic flexure	20
Lignocaine					
Lignocaine	1.300 as bolus IV then 0.050 per min as infusion	Cases of POI or proximal duodenitis-jejunitis	Clinical assessment	Possible ↓ in duration and quantity of gastric reflux	21
Lignocaine	*In vitro* study	Isolated smooth muscle *in vitro*	Isometric force gauge to measure contractility *in vitro*	↑ Contraction amplitude in the proximal duodenum	22

13. King and Gerring 1988; 14. Gerring and King 1989; 15. Gerring *et al*. 1991; 16. Van der Velden and Klein 1993; 17. Milne *et al*. 1996; 18. Sasaki and Yoshihara 2000; 19. Lester *et al*. 1998c; 20. Roussel *et al*. 2000; 21. Malone *et al*. 1994; 22. Nieto *et al*. 2000

Perhaps the most statistically robust prokinetic study to date in equine gastroenterology has been based on metoclopramide administration (Dart *et al.* 1996), in which a case-control study was performed in a relatively large number of horses. Dart *et al.* (1996) quantified ileus in horses after small intestinal resection by measuring the volume, duration and rate of gastric reflux. Horses that received a continuous intravenous metoclopramide infusion postoperatively had significantly reduced volume, duration and rate of gastric reflux than untreated controls, and a shorter duration of hospitalisation (Dart *et al.* 1996). The randomized blind trial of cisapride in 70 horses surgically treated for colic also offered good proof of the potential efficacy of this agent in the post-operative treatment of horses after small intestinal surgery (Van der Velden and Klein 1993).

The major problem in extrapolating results of prokinetic studies in healthy animals to animals with abnormal gastrointestinal motility is that pathophysiological changes may alter the action or efficacy of the pharmaceutical agent. Two recent studies have demonstrated this clearly in the horse. Using *in vitro* techniques, Nieto *et al.* (2002) measured the contractile response of equine jejunum to cisapride, metoclopramide and erythromycin infusion before and after temporary forced distension of the tissue. Compared with responses in control specimens, distension decreased the contractile response induced by all 3 compounds (Nieto *et al.* 2002). This was thought to result from increased mucosal permeability after distension, and Nieto *et al.* (2002) concluded that horses with intraluminal distension may have a decreased response to prokinetic agents.

Altered intestinal responses to erythromycin in the immediate and longer-term post-operative periods were also noted by Roussel *et al.* (2000) in a study of myoelectrical activity. The authors concluded that caution should be exercised when applying results of prokinetic studies from healthy animals to those with abnormal intestinal motility (Roussel *et al.* 2000). Conversely, certain prokinetic agents may only show effect in clinically abnormal animals, and have no action on healthy tissue. If it is assumed that lignocaine reduces the severity of ileus by suppressing inhibitory reflexes that are active only with bowel and peritoneal inflammation, then the effects of this agent would have to be evaluated using an effective model for inflamed intestine, or in clinical cases *in vivo*. This is also likely to be true for the mechanism of action of α_2-adrenergic antagonists and opioid antagonists.

Having discussed the advantages and disadvantages of the different techniques used for measurement of intestinal motility in sections 1.4 to 1.6, it is also known that many of the

techniques used in the prokinetic studies have not been correlated to the actual transit of ingesta, and may not have clinical relevance. The scintigraphic gastric and caecal emptying studies are the main exceptions to this, but these have not been repeated in clinical cases of ileus for technical reasons. In summary, it is difficult from the reported studies to draw firm conclusions about the efficacy of the listed prokinetics in clinical cases of equine ileus. Larger, quantitative case-control studies are needed to determine whether or not specific prokinetic agents are beneficial in horses suffering from ileus. It is anticipated that the ^{13}C-OABT and ^{13}C-LUBT may prove particularly useful tests for the performance of such studies. These stable isotope breath tests have the advantages of being non-invasive, quantitative and safe and provide directly relevant information, as they measure the actual transit rate of solid ingesta. In addition, they may be repeated in clinical cases before and after prokinetic therapy without detrimental effect.

1.8 Introduction to study

In the preceding review it has been established that new, non-invasive tests are required for the measurement of equine gastrointestinal transit rate. Such tests would permit improved examination of the physiological control factors of gut motility in healthy individuals, and also facilitate improved diagnostic investigation of clinical cases. Potentially, this would allow detailed investigation of a variety of feeding and managemental factors that have been identified as possible risk factors for the development of colic. Furthermore, it has been demonstrated that much of the information upon which equine prokinetic therapy is currently based may not be applicable to the clinical scenario, in which there is likely to be pathophysiological change in the intestinal wall. Quantitative, real-time measurements of the transit rate of ingesta would be the most effective means of determining the efficacy of prokinetics in clinical cases presenting with ileus. Scintigraphy may currently provide the most accurate means of assessing this function, but is generally limited to research application due to its radioactive nature and the requirement for a nuclear medicine facility.

The ^{13}C-OABT and ^{13}C-LUBT were identified as having potential value for the measurement of equine solid phase GE and OCTT, respectively. It was therefore the aim of this study to investigate the validity of these tests for quantification of these parameters in horses by comparison with existing optimum techniques. Having validated the breath tests, it was then aimed to investigate the effect of specific sedative agents on equine GE rate, and to develop a combined stable isotope test for the measurement of small intestinal transit time. The combined ^{13}C-OABT/^{13}C-LUBT test was then used to investigate the physiological relationship between equine GE and OCTT.

In Chapter 2, the physical principles of stable isotope technology are described, together with a preliminary analysis of the repeatability of the developed techniques for the measurement of ^{13}C:^{12}C ratio in equine expiratory breath. In addition, a technique is presented for the analysis of ^{13}C in equine peripheral blood. The mathematical methods used to fit the expiratory isotopic recovery data, and to determine the indices of gastric emptying and orocaecal transit are also presented. Having presented the general materials and methods used in this study in Chapter 2, the validation studies and pharmacological studies involving the ^{13}C-OABT will be presented in the subsequent 3 chapters. These studies are followed by separate investigations into the use of stable isotope tests for liquid phase GE, and then validation and application of the ^{13}C-LUBT for the measurement of orocaecal transit parameters.

Chapter 2 GENERAL MATERIALS AND METHODS

2.1 Study Animals

Stable isotope breath tests were performed on a total of 48 healthy adult equidae (4 ponies, 44 horses) of age range 3 to 25 years old. The specifics of each study group are presented in the relevant experimental sections of the thesis. Many of these animals were sampled on multiple occasions as part of repeatability studies or to enable measurement of the effect of pharmacological agents on gastrointestinal transit. A minimum interval of 6 days was allowed between tests to ensure that the basal bicarbonate pool ^{13}C:^{12}C ratio had returned to pre-test level.

2.1.1 Animal Selection

Adult horses or ponies were included in the studies only if in good body condition, and if free from any historical or physical evidence of gastrointestinal disease. Blood samples were collected by jugular venepuncture into potassium ethylenediaminetetraacetic acid (EDTA), serum and lithium heparin tubes (Vacutainer®, Becton Dickinson) for total cell counts and fibrinogen estimation, bile acid determination, and biochemical analysis respectively. Plasma biochemical parameters were measured on the Technicon AXON (Bayer Diagnostics) and Ektachem® (Johnson & Johnson Clinical Diagnostics) clinical chemistry analysers and included concentrations of urea, creatinine, total protein, albumin, globulin, total bilirubin, gamma glutamyltransferase, alkaline phosphatase, aspartate aminotransferase and electrolytes. Potential subjects were rejected if biochemical or total blood cell parameters were found to lie outside the reference range.

2.1.2 Management of test subjects

Dental examinations were undertaken in each subject at the start of each study, and routine dental floating provided when required. Ivermectin anthelmintic (Eqvalan®, Merial Animal Health Ltd.) was administered orally to each horse at approximately 8-week intervals, but not within 2 weeks of a gastrointestinal transit study. At Glasgow University Veterinary School (GUVS), all subjects were maintained in individual loose boxes, with daily turnout in an indoor school. These horses were bedded on wood-shavings or shredded paper during study periods, and on straw for the remaining period. At Texas A & M University Veterinary School (TAMU), horses were brought in from pasture at least 2 weeks prior to each experiment, and kept on concrete yards in groups of 4 – 5 individuals. In each setting, vigorous attempts were made to ensure a regular daily routine, with set feeding times

(07:30 and 16:00 at GUVS; 06:00 and 15:00 at TAMU) and regular exercise, and to minimise external stressing factors. At TAMU, this included daily hosing in hot weather, fly control and separation of aggressive dominant individuals. Although little investigated in equidae, many external factors have been reported to disrupt human and canine gastric activity.

Subjects were maintained on forage alone, which was fed twice daily, the daily total comprising approximately 30 g good quality ryegrass seed hay per kg bodyweight at GUVS or 25 g premium alfalfa hay per kg at TAMU. Horses were weighed fortnightly, and dietary intake adjusted accordingly to maintain constant body mass. Both ryegrass and alfalfa are C_3 plants which are relatively low in ^{13}C, and were selected to ensure a constant basal metabolic production of $^{13}CO_2$ in the test subjects. The traditional forage of coastal grass hay, made from Bermuda grass (*Cynodon dactylon*), was removed from the diet of the TAMU subjects at least two weeks before the start of each study, as this C_4 plant has relatively high ^{13}C content. Similarly, concentrate feeds were removed from the diet of all the horses as these may contain C_4 plants such as corn, maize, millet and sugar cane, which are also enriched in ^{13}C. The differences in isotopic ratios between these plants stem from differences in the photosynthetic processes by which they convert CO_2 into carbohydrate (Waller and Lewis 1979). Many central American and tropical plants (C_4 plants) convert atmospheric CO_2 to carbohydrate via 4-carbon intermediates, in the Hatch-Slack pathway (Hatch and Slack 1966), prior to transfer to sugars. This C_4 pathway provides a photosynthetic advantage over the C_3 plants, which can only fix CO_2 via a 3-carbon intermediate in the Calvin-Benson reaction (Quale *et al.* 1954). The difference in ^{13}C:^{12}C ratio between these plant groups results from dissimilar isotopic discrimination by the two pathways against ^{13}C in atmospheric CO_2. The ^{13}C content of ryegrass and alfalfa is approximately 1.0949 (Amelung *et al.* 1999) and 1.0943 atom % ^{13}C (Svejcar *et al.* 1993), whilst that of Bermuda grass hay exceeds 1.1020 atom % ^{13}C (Waller and Lewis 1979).

2.1.3 Ethics and welfare

For each *in vivo* procedure detailed in this thesis, advance approval was sought and gained from the University of Glasgow Ethics and Welfare committee. Further specific approval for individual experiments was received from the TAMU Laboratory Animal Care Committee: (i) 'Validation of the ^{13}C-OABT against radioscintigraphy for the measurement of equine solid phase gastric emptying' – Animal Use Protocol (AUP) 2000-112; (ii) 'Detection of atropine-delayed gastric emptying using the ^{13}C-OABT' – AUP 2001-35; (iii) 'Effects of xylazine, detomidine, acepromazine and butorphanol on equine

GE as determined by the ^{13}C-OABT' – AUP 2001-60. As the major aim of the presented work was the development of non-invasive tests for the measurement of equine gastrointestinal transit, all of the experiments were designed specifically to be non-invasive.

2.2 Test meal selection

2.2.1 Required criteria

The selection of a suitable test meal is critical in the validation of stable isotope breath tests (Boutton 1991). In addition to being palatable, the test meal should be of such a volume that it is rapidly and completely consumed. The meal must also be equivalent in carbon isotopic ratio to the habitual diet, so that the measured enrichment of the bicarbonate pool is dependent only on the metabolism of the chosen tracer substrate (Schoeller *et al.* 1980). Above all, in order to establish reference ranges for GE rate, the meal composition, nutrient value, volume and consistency must be kept constant. Classic experiments have shown the effect of meal energy density and volume on human GE rate (Hunt and Stubbs 1975), and in the horse, changes in test meal energy density (Sojka and Cantwell 1989), and oil content (Geor *et al.* 2001; Wyse *et al.* 2001b) have also been suggested to cause modulation of GE rate.

2.2.2 Test meal composition

Crimped oats and wheat bran were the principal components of the selected meal. These grains have ^{13}C contents of approximately 1.0922% and 1.0930% ^{13}C respectively (Morrison *et al.* 2000), which is considerably lower than that of corn/maize (1.1092%), for example, but close to that of the habitual alfalfa and ryegrass hay diets of the test subjects. For each horse, the test meal comprised: 150 g crimped oats, 100 g wheat bran, 200 ml water and two baked egg yolks containing the ^{13}C-octanoic acid tracer. Based on digestible energy (D.E.) contents of 12.1 and 10.8 MJ / kg dry matter for oats and wheat bran (Frape 1998), this provided a total D.E. value of approximately 3.234 MJ. Two egg yolks were required to ensure retention of the entire dose of the lipophilic tracer in the solid phase of gastric emptying (Ghoos, pers. comm.). Following addition of the isotope, the egg yolks were homogenised and then baked in a microwave oven for approximately 40 s, until firm. *In vitro* validation studies have shown that the retention of octanoic acid by cooked egg yolk, when incubated in gastric juice is very high (Ghoos *et al.* 1993). After cooking, the ^{13}C-labelled egg substrate was finely chopped (particle size 1-2 mm) before thorough mixing with the rest of the test meal.

For lactose ^{13}C-ureide breath tests, and dual isotope studies, the lactose ^{13}C-ureide was added to one egg white, which was whisked and then cooked in a microwave oven until firm. Again, this procedure was designed to ensure retention of the moderately hydrophilic tracer in the solid phase of gastric emptying. After cooking, the labelled substrate was finely chopped and incorporated into the test meal. In the combined gastric emptying and orocaecal transit time breath tests, the substrates were cooked separately, chopped and mixed together before addition to the test meal. Prior preparation and refrigeration of the cooked substrates was found to enhance palatability. In ponies, the test meal was halved in size (75 g oats, 50 g bran, 100 ml water, one egg yolk/white) but was otherwise prepared in the same manner. Preliminary work at GUVS by Wyse *et al.* (2001a) showed that the basal metabolic production of $^{13}CO_2$ in ponies maintained under identical conditions, after consumption of the same unlabelled test meal, remained constant over a 12 h period. This indicated that the given test meal was suitable for ^{13}C enrichment studies under the study conditions outlined above.

2.3 Stable Isotope Tracers

2.3.1 Gastric emptying studies

For each test, approximately 1 mg/kg ^{13}C-octanoic acid was used (Octanoic acid-1-^{13}C, Isotec Inc., Ohio, USA). Quality control ensured that this was of below 0.5% water content, of minimum 99% chemical purity and of at least 99 atom% ^{13}C. The cost of substrate per 450 kg horse was approximately 35.85 GBP.

2.3.2 Orocaecal transit tests

Lactose ^{13}C-ureide was synthesised at Bell College of Technology, Hamilton (Morrison *et al.* 2001) by the acid-catalysed condensation of lactose and ^{13}C-urea (Merry *et al.* 1982a). Substrate analysis was performed using standard analytical techniques and combustion isotope ratio mass spectrometry for carbon and nitrogen content and ^{13}C-enrichment. This showed that the crystalline compound used was lactose ^{13}C-ureide dihydrate, with a minimum 99% chemical purity and of at least 99 atom% ^{13}C at the carbonyl carbon. A dose of 2.7 mg/kg lactose ^{13}C-ureide was sufficient to produce a repeatable increase in the expiratory ^{13}C:^{12}C ratio, at a cost of approximately 72.90 GBP per 450 kg horse.

2.3.3 Safety considerations

Although the ^{13}C-tracers used in this study are not specifically licensed for use in animals, at the doses given they were considered to present a negligible risk to the subjects. Stable

isotopes are stable atoms of an element that contain different numbers of neutrons in the nucleus. The natural occurrence of ^{13}C is high, representing 1.11% of the total carbon pool, and significant physicochemical differences between ^{13}C and the more abundant ^{12}C have not been demonstrated (Wolfe 1992a). Indeed, by feeding ^{13}C-labelled algae, Gregg *et al.* (1973) increased the ^{13}C content of mice to 60-70% of the total body carbon pool without discernible effect. It has therefore been concluded in tracer studies, that there is no identifiable risk to (human) subjects even at the highest levels of enrichment that may be achieved.

Octanoic acid itself is a medium chain fatty acid that is found in materials such as coconut oil and milk. Gastric emptying studies using ^{13}C-octanoic acid have been performed in a number of species, including preterm neonatal children (Veereman-Wauters *et al.* 1996), without adverse effect. Lactose ureide is a synthetic glycosyl ureide compound that was first investigated as a possible improved source of non-protein-nitrogen (NPN) for ruminants (Merry *et al.* 1982a). *In vitro* and *in vivo* studies in ruminants have shown that this compound is cleaved to galactose and glucose-ureide, before microbial enzymic hydrolysis causes the production of glucose and urea (Merry *et al.* 1982b, 1982c). The low quantity of the molecule required for these tracer studies was insufficient to cause excessive generation of urea, nor was it sufficient to have an osmotic effect on gastrointestinal motility (Ruemmele *et al.* 1997).

2.4 Sample collection for carbon isotope analysis

2.4.1 Equine breath collection

Expiratory air collection for breath tests in equidae has previously been achieved using a nasal prong, inserted into the ventral meatus (Murphy *et al.* 1998; Wyse 1999). Repetitive use of this technique for serial breath collection was found at the start of this study to be poorly tolerated by some horses, and a non-invasive technique was developed, similar to that of Metges *et al.* (1990). Breath collection was performed using a modified equine Aeromask® (Trudell Medical International, Ontario, Canada), which is supplied commercially fitted with two expiratory and one inspiratory one-way valves. One of the expiratory valves was sealed and the second was replaced with a two-way valve, connected to a gas-tight aluminium coated polyethylene bag (QuinTron®, QuinTron Instrument Company, Wisconsin, USA) (Figure 2.1). The QuinTron® bag has an integral unidirectional valve so that expiratory breath is stored after entry. The horse was allowed to breathe several times through the mask before attachment of the 250 ml collection bag towards the end of expiration (Figure 2.2).

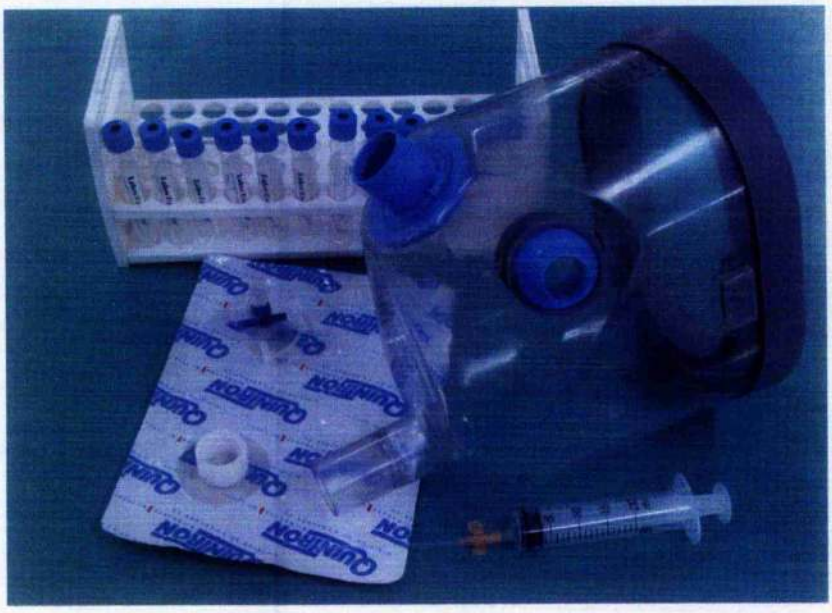


Figure 2.1 Equipment used for equine expiratory breath collection: a modified Aeromask® with a single two-way expiratory valve; 250 ml QuinTron® expiratory breath collection bag with 3-way tap; 20 ml syringe and catheter/needle; 10 ml Exetainers® for duplicate storage of serial breath samples.

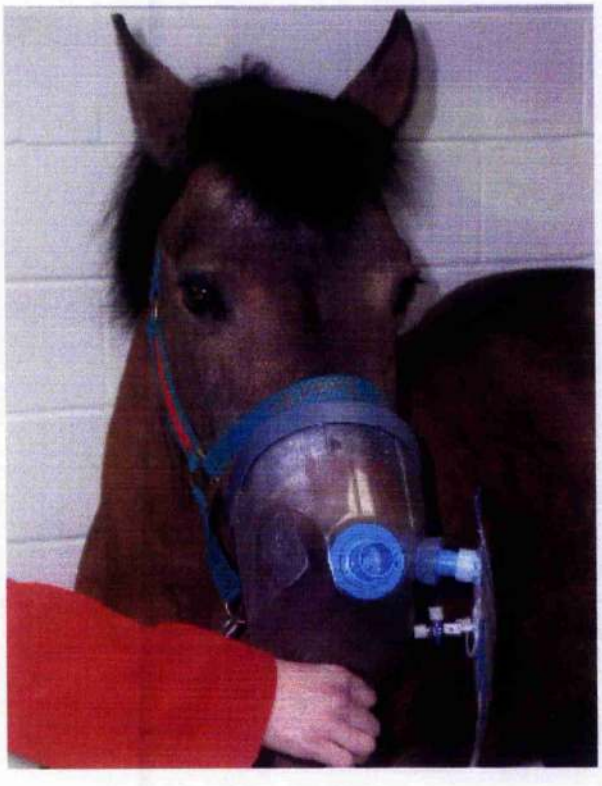


Figure 2.2 Each subject was allowed to breathe several times through the mask before attachment of the QuinTron® bag to the two-way valve towards the end of expiration. Sample collection was well tolerated and took approximately 2 min to perform. Duplicate breath samples were transferred to evacuated glass containers prior to isotopic analysis.

Sample collection was well tolerated even in 'head shy' subjects, and took approximately 2 min to perform. The mask was removed between each sampling event. Aliquots of expiratory breath were collected from the aspiration port of the bag using a 20 ml syringe and transferred in duplicate to 10 ml evacuated containers (Exetainer®, Labco Ltd., Buckinghamshire, UK), using the method described by Schoeller and Klein (1978).

This collection technique provided samples of 3-5% CO_2, consistent with expiratory breath. Any specimens containing below 0.5% CO_2 were not considered to be expiratory, and isotopic analyses of such samples were not included in the final results. In contrast to respiratory physiology experiments, involving air-tight collection systems and pneumotachograph readings (see Gauvreau *et al.* 1996; Hopkins *et al.* 1998), measurement of absolute expiratory CO_2 production over the course of each experiment was not required, as the relative ratio of $^{13}CO_2$:$^{12}CO_2$ production over time was the parameter of interest. Samples were stored in Exetainers® until automated analysis of ^{13}C:^{12}C content could be performed. Samples may be stored for at least 3 months (Schoeller and Klein 1978) and even up to 12 months (pers. obs.) prior to analysis.

2.4.2 Venous blood collection

A Teflon coated catheter (Angiocath, Becton Dickinson, Utah, USA) was placed aseptically in the left jugular vein prior to the start of each study. Venous blood samples were withdrawn anaerobically at each sampling point, and 1 ml of blood was injected into a 10 ml evacuated glass tube (Vacutainer®, Becton Dickinson, NJ, USA). This sample was hydrolysed immediately or after storage by the addition of 2 ml of 6 molar hydrochloric acid. As metal is corroded by 6 M HCl, the acid was added using a plastic pipette, and the procedure was performed anaerobically under a fume hood. Samples were then vortexed for 60 s, tube contents allowed to settle, and the Vacutainer septum cleaned with alcohol prior to mass spectrometric analysis of the resulting headspace gas. This technique is similar to that described by Cornetta *et al.* (1998) and Moeller *et al.* (2001). The effect of immediate processing of the blood, versus storage prior to processing and analysis was investigated (see Section 2.7).

2.4.3 Sample collection protocol

Basal ^{13}C production was established by collection of at least two breath (and blood, if required) samples before test meal ingestion i.e. –60 min and 0 min. Thereafter, samples were collected in duplicate at 15-min intervals for 6 h, then at 30-min intervals for a further 4-24 h, as dictated by the specific test protocol. An important principal of stable isotope

breath tests is that subjects should be maintained at rest and fasted during each study, in order to limit alterations in VCO_2 caused both by changes in respiratory rate and/or metabolic energy source. Exercise and/or feeding of the subject during a test also has been shown to result in substantial excursions of background ^{13}C in breath (Schoeller *et al.* 1984; Wolfe *et al.* 1984). Fluctuations in VCO_2 during a test become particularly important in digestion and metabolic studies when it is necessary to calculate the % dose recovery (PDR) of the administered isotope. Abnormal patient VCO_2 or increases in VCO_2 during the procedure may lead to significant underestimation of PDR (Kalivianakis *et al.* 1997; Amarri *et al.* 1998). Some calibrated correction for exercise-induced fluctuations in VCO_2 can be made from concurrent heart rate measurement (Slater 2002). However, in these studies, it was simpler to minimise likely changes in VCO_2, and to predict VCO_2 based on resting metabolic rate. In practical terms, the horses were allowed to move about a small stable or concrete yard during the test, and were fasted after ingestion of the test meal but given free access to water.

2.5 Measurement of sample ^{13}C:^{12}C ratio

2.5.1 Continuous flow isotope ratio mass spectrometry

2.5.1.1 Principles and background

Breath analysis and headspace gas analysis of hydrolysed whole blood samples was performed by continuous flow isotope ratio mass spectrometry (CF-IRMS). In mass spectrometry, molecules are converted into ions, and separated in a vacuum according to their mass-to-charge ratio (m/z) (Matern and Magera 2001). The principal components of a mass spectrometer are the inlet system, ion source, mass analyser, detector and vacuum system. Prior to 1985, stable isotope mass spectrometry was mostly performed in the geological sciences, for the measurement of isotope ratios of samples at natural abundance, using dual inlet gas IRMS. Such machines provided a very accurate measure of minute differences in isotope abundance, but were not amenable to rapid analysis of the multiple samples generated in breath tests, due to the extensive off-line preparation needed to isolate CO_2 from the sample, and its subsequent manual injection into the IRMS.

Rapid increases in sample analysis rate were facilitated by the interface of an elemental analyser with an IRMS by Preston and Owens (1985). This continuous flow technique permits internal combustion of the sample, producing CO_2, water and nitrogen, in a helium carrier gas. After removal of the water vapour, the CO_2 and N_2 are separated by gas chromatography and the quantity and enrichment of the CO_2 then determined by IRMS.

Initial use of this technique required manual injection of the breath samples into the elemental analyser (Preston and McMillan 1988), but the process was further accelerated by the subsequent addition of an autosampler, allowing full automation (Prosser *et al.* 1991). Individual sample analysis can now be completed in 2-3 min.

2.5.1.2 *Experimental Hardware*

Two dedicated breath test CF-IRMSs were used for analysis of the samples generated in this research: an AP2003 mass spectrometer with breath sample preparation module and Gilson autosampler (Analytical Precision Products Ltd., Northwich, Cheshire); and an Automated Breath ^{13}C Analyser (ABCA), v500.1.17 (PDZ Europa, Northwich, Cheshire) with autosampler (Figure 2.3). Both of these machines are computer controlled with sophisticated data processing software, and have the capacity to analyse up to 200 samples in one programmed run. Breath samples are aspirated from the relevant Exetainer via a side-bore needle, and swept into the IRMS in a stream of helium carrier gas. This sample is dried by an online Nafion membrane (AP2003) or magnesium perchlorate water trap (ABCA), before separation of the CO_2 component in a gas chromatography (GC) column, and removal of the waste gases. CO_2 content is determined by comparison to an online CO_2 standard reference gas, of 3% or 5% CO_2 (AP2003 and ABCA respectively). In each machine, the pure CO_2 is then introduced to the IRMS for analysis of the ^{13}C:^{12}C ratio.

The CO_2 is ionised by high sensitivity electron impact from an iridium filament ion source, and then deflected under vacuum through a magnetic sector analyser in order to separate the $^{12}CO_2$ and $^{13}CO_2$ ion beams. A 90° ion optics with 20 cm radius magnetic sector analyser is used in the AP2003, while the ABCA incorporates a 120° extended ion optics with 11 cm radius magnetic sector. In both, the deflected CO_2 molecular ion beams are detected by dedicated Faraday cups configured to measure atomic masses 44, 45 and 46: $^{12}C^{16}O^{16}O$ (44), $^{13}C^{16}O^{16}O$ & $^{12}C^{17}O^{16}O$ (45) and $^{12}C^{17}O^{17}O$ & $^{12}C^{16}O^{18}O$ (46). The Faraday detectors generate a signal corresponding to the detection of molecular ions over time, until the entire sample has passed through the IRMS. Signals are amplified and beam areas produced for each atomic mass. Comparison of the relative beam areas is used to calculate the isotopic ratio of $^{13}CO_2$:$^{12}CO_2$. The Craig correction factor (Wolfe 1982b) is used to correct for the influence of $^{12}C^{17}O^{16}O$ at mass 45, by consideration of the isotopic ratios of mass 45/44 and 46/44, with subsequent generation of the actual ^{13}C:^{12}C ratio.

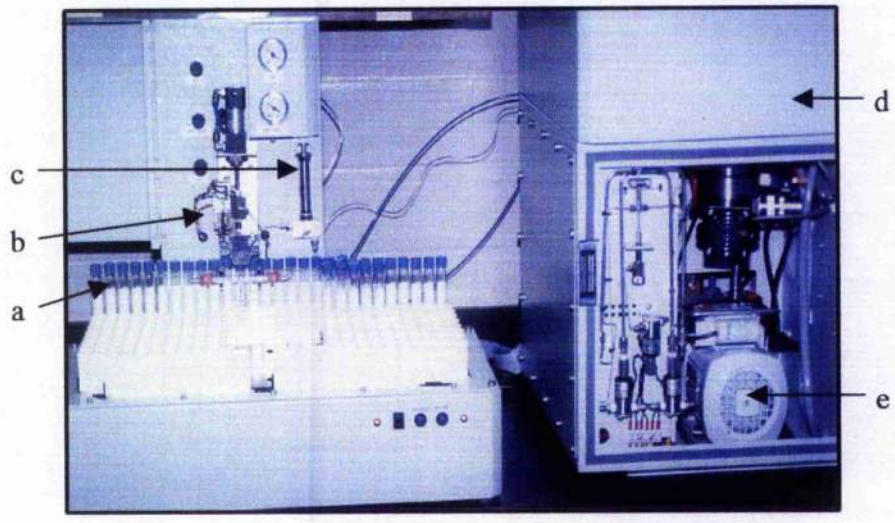


Figure 2.3 Continuous flow isotope ratio mass spectrometry, with labelling of constituent components. a. exhaled breath samples; b. autosampler; c. water trap; d. magnetic sector and Faraday cups; e. vacuum turbo pump.

2.5.1.3 Analytical precision and quality control

IRMS has an analytical precision of approximately 0.0001 atom % for carbon, or 1 part per million (ppm) $^{12/13}C$. Both machines use an online internally calibrated CO_2 reference gas, which can be measured after each breath CO_2 sample. In addition, in-built quality control checks were completed before the start of each batch analysis. External gas standards were also used during each run on the ABCA, with an acceptable standard deviation of ±2.0 ppm ^{13}C for 10 standard gas measurements of natural abundance.

2.5.2 Calculation and expression of sample ^{13}C:^{12}C ratio

IRMS measures the absolute ^{13}C:^{12}C ratio of the sample relative to the internal standard gas, and then expresses this in delta notation, relative to that of the international standard. The new standard for carbon isotopes is the Vienna Peedee Belemnite limestone fossil, which has an actual isotopic ratio of 0.0112372.

$$\delta^{13}C_{PDB} = ((Rx - Rs)/Rs) \times 1000 \qquad \textbf{Equation 2.1}$$

where Rs and Rx are the ^{13}C:^{12}C atomic ratio of the standard and sample respectively. Following current recommendation (Slater *et al.* 2001), S.I. notation has been used

throughout this thesis. To convert $\delta^{13}C_{PDB}$ into the S.I. units of atom % ^{13}C (AP ^{13}C), the standard formula was used:

$$AP\ ^{13}C = \frac{100}{1+1/((\delta/1000)+1)*R_{PDB}} \qquad \textbf{Equation 2.2}$$

where δ = measured $\delta^{13}C_{PDB}$ and R_{PDB} is the isotope ratio of PDB = 0.0112372. Parts per million (ppm) excess ^{13}C of each sample were then calculated from the formula:

$$\text{ppm excess } ^{13}C = ((\text{atom \%})_E - (\text{atom \%})_B) \times 10^4 \qquad \textbf{Equation 2.3}$$

where $(\text{atom \%})_E$ is the measured abundance of the enriched sample and $(\text{atom \%})_B$ is the average measured abundance of the baseline samples (i.e. predose samples collected before ingestion of tracer). Finally, stable isotope data were also expressed as percentage dose recovery (PDR) of the administered isotope per hour. This latter parameter, and the related cumulative PDR, is an aid in understanding the fate of the chosen tracer in the body. In order to calculate PDR/h, it is necessary to determine not only the ppm excess ^{13}C in the administered dose of tracer, but also the total recovery of ^{13}C in labelled CO_2, based on estimation of resting VCO_2. The ppm excess ^{13}C provided by the tracer dose is shown:

$$\text{ppm excess } ^{13}C \text{ in dose} = (\text{atom \% excess}/100) \times \text{dose(mmol)} \times 10^6 \times n \qquad \textbf{Equation 2.4}$$

where n = number of ^{13}C-labelled atoms in substrate (1-^{13}C-octanoic acid, n = 1)

$$PDR/h = \frac{\text{ppm excess}^{13}C_t \times VCO_2\ (\text{mmol/h}) \times 100}{(\text{atom \% excess}/100) \times \text{dose(mmol)} \times 10^6} \qquad \textbf{Equation 2.5}$$

Resting VCO_2 is a variable parameter that is likely to differ both between horses and ponies, and also according to the collection system used for its measurement (Evans and Rose 1988). Equine VCO_2 is rarely measured at rest due to variability caused by external factors and fluctuations in breathing patterns, tidal volumes and flow rates (Slocombe *et al.* 1992). Equine respiratory gas exchange kinetics is generally considered to fit a monoexponential function in the transition from rest to submaximal exercise levels (Powers *et al.* 1987), and VCO_2 is more regularly measured at steady-state exercise levels (see Rose *et al.* 1990). It was not possible or practical to measure resting VCO_2 in each animal used in this study, but in order to allow estimation of PDR/h, two formulae were

used to estimate VCO_2. For the ponies, resting VCO_2 was estimated using the formula of Orr *et al.* (1975) based on resting measurements made in non anaesthetised ponies of mean mass 147 kg, as previously described by Wyse *et al.* (2001). Orr *et al.* found mean resting VCO_2 to be equivalent to 0.156 (± 0.026) L/min/m^2, where pony body surface area = (10.5 x (Bwt (kg) x 1000)$^{2/3}$) / 10000. In the horses, the formula of Gallivan, McDonell and Forrest (1989) was used: VCO_2 = 2.84 ml/kg/min. This formula was generated from resting measurements made in fasted standardbreds and quarter horses of mean body weight 402 kg, using a similar mask and test conditions to those employed throughout this study (Gallivan *et al.* 1989). Provided that VCO_2 remains constant during a stable isotope breath test, its precise measurement does not affect the calculation of gastrointestinal motility indices. However, it is recognised that underestimation of VCO_2 results in an underestimate of the cumulative dose recovery of the ^{13}C-isotope, which has particular importance in studies measuring digestion (Amarri *et al.* 1998). The influence of VCO_2 estimation on equine stable isotope breath test results is discussed further in Chapter 6.

Cumulative PDR (cum PDR) of the tracer was calculated in the following manner:

$$\text{cum PDR} = [(PDR_t + PDR_{t-1}) \times \Delta t\ (h)/2] + \text{cum PDR}_{t-1} \qquad \textbf{Equation 2.6}$$

2.6 Calculation of gastric emptying indices

2.6.1 Background

Gastric emptying data may be analysed by fitting mathematical functions that permit characterisation of specific indices describing the time course of emptying. The method of analysis should incorporate consideration of the whole gastric emptying curve, rather than specific time points, and generate parameters that reflect the physiology of gastric emptying (see Elashoff *et al.* 1982 for review). However, gastric emptying patterns are determined by the interaction of a number of biological factors, such as meal density and energy content, antropyloric propulsion, small intestinal inhibitory feedback, central nervous control and many more. Consequently, models described for the analysis of gastric emptying data have been based on the principle of mathematical curve fitting rather than physiological modelling. Not surprisingly, this has resulted in the publication of many different functions and specific measuring indices, many of them based on the exponential loss of radioactive material from the stomach during scintigraphic studies (See Dugas *et al.* 1982; Siegel *et al.* 1988). Most recently, new indices for ^{13}C-OABT data have been proposed by Schommartz *et al.* (1998) and a new asymmetric triangle model for gastric emptying data proposed by Bluck *et al.* (2002).

According to Elashoff's criteria, any mathematical curve used to analyse gastric emptying data should satisfy five basic properties: (1) The curve should have the minimum number of parameters, which (2) have clear graphical interpretations; (3) the fitted curve should be sufficiently flexible to provide a good fit to the data points observed for a wide range of subjects and meal types; (4) the curve should always give a value between 0.0 and 1.0 for fractional gastric meal retention, and (5) this value for fraction remaining should be 1.0 at time zero. Following these criteria, and considering the biphasic exponential shape of most gastric scintigraphic emptying studies, these authors (Elashoff *et al.* 1982) proposed a power exponential in the form: $f = 2^{-(t/t_{1/2})^{\beta}}$, where $t_{1/2}$ is the time from the start of the test until 50% of the meal has emptied (gastric half-emptying time), and the parameter β is a constant determining shape of the curve. This formula has formed the basis for many of the gastric emptying formulae currently described, and allows modelling of a sigmoidal data set with initial lag phase as seen in solid phase gastric emptying.

2.6.2 *Definition of gastric emptying parameters*

Accurate mathematical definition of gastric emptying parameters is critical when reporting the results of such studies. The purpose of these parameters is to allow direct statistical comparisons to be made between normal and abnormal populations, and between studies, which can only be achieved with accurate description of the specific model and parameters used. The definition of 'time zero' is also important: in this study, time zero was taken as that time immediately after complete ingestion of the labelled meal. As the meal used for both GE and OCT studies was small, ingestion time was rapid, with an average of 5 min. This reduced the likelihood of significant movement of labelled meal from the stomach before its complete ingestion. Throughout this thesis, three major terms have been derived and used to describe the gastric emptying data:

2.6.2.1 *Gastric half-emptying time ($t_{1/2}$)*

The gastric half-emptying time ($t_{1/2}$) is that time after time zero when half of the ingested meal remains in the stomach, and half has entered the small intestine. In terms of the ^{13}C-OABT, $t_{1/2}$ is defined as that time after time 0 when half of the total cumulative recovered dose of isotope has been recovered in the breath. This is not directly equivalent to that estimated by gastric scintigraphy, as the appearance of ^{13}C in breath CO_2 after leaving the stomach is delayed in the bicarbonate pool, and recovery will be incomplete due to incorporation into products of the tricarboxylic acid cycle (van Hall 1999). As these latter factors have been reported to occur at a constant rate, the 'half-dose recovery time' is

considered to be directly proportional to $t_{1/2}$ since gastric emptying is the rate-determining step.

2.6.2.2 *Gastric lag phase (t_{lag})*

Most solid phase scintigraphic GE studies have demonstrated a delay period after time 0 before exponential emptying of the radiolabelled meal starts to occur, and this has been termed the lag phase (t_{lag}). The actual existence of a physiological lag phase has been debated, but this period is thought to correspond to the time taken for antral grinding to reduce the food into sufficiently small particles to pass through the pylorus (Siegel *et al.* 1988). Flow and shear forces are maximal in the antrum and pylorus, and the pressures exerted by the antrum during trituration have now been characterised in man by echo-planar magnetic resonance imaging (Marciani *et al.* 2001). Dense foods of higher fracture strength are likely to require more prolonged trituration than softer materials and liquids, thus resulting in prolongation of the lag phase.

Critically, this is one of the most variable parameters described in the literature, with different formulae proposed by certain American (Choi *et al.* 1998) and European (Ghoos *et al.* 1993) gastroenterology groups, prohibiting direct comparison. In this study, the duration of the lag phase, t_{lag} (h), is defined as being equivalent to the time of the inflexion point of the cumulative dose recovery curve, and is calculated in accordance with Ghoos *et al.* (1993), Schommartz *et al.* (1998) and Bluck *et al.* (2002), as shown below. This is effectively the time of maximal excretion rate of $^{13}CO_2$ and has previously been termed t_{max} (Maes *et al.* 1994).

2.6.2.3 *The Gastric Emptying Coefficient (GEC)*

This is a rather poorly defined parameter, introduced by Ghoos *et al.* (1993), and proposed to be a universal index of gastric emptying rate, reflecting the shape of the ^{13}C-OABT tracer recovery curve. Its derivation is described below, which reveals it to be related to both $t_{1/2}$ and t_{lag}, increasing in amplitude with rapid emptying, and becoming negative with delayed emptying. It is included here to allow comparison of equine data with that derived from man, but major conclusions about GE are based on modulations of $t_{1/2}$ and t_{lag} rather than GEC.

2.6.2.4 *Orocaecal transit time measurement*

Orocaecal transit time, when measured using the lactose ^{13}C-ureide breath test, has been defined as that time point at which 3% of the cumulative dose recovery of the administered

isotope has occurred in the breath. As detailed in Chapters 7 and 8, this is modified slightly from the work of Geypens (2000) and Morrison (2000). When measured using the hydrogen breath test, OCTT has been defined as that time at which end expiratory hydrogen increases by 5 ppm over previous measurements and continues to increase. These parameters are considered to be representative of arrival of the head of the bolus of ingesta into the caecum.

2.6.3 Gastric emptying formulae

Two principal power exponential formulae have been used in this work to derive indices of equine gastric emptying. These have been shown previously to provide good fits for scintigraphic and stable isotope GE studies in man.

2.6.3.1 Siegel's formula

This function was originally developed by Siegel *et al.* (1988) to fit solid phase scintigraphic emptying data:

$$y(t) = 1 - 1(1 - e^{-kt})^{\beta} \qquad \textbf{Equation 2.7}$$

where *y(t)* is the fractional meal retention at time *t*, *k* is the gastric emptying rate per h, *t* is the time interval in hours and β is a rate constant. The derivation of the constants is simple: when fractional retention is plotted on a logarithmic scale, *k* is calculated from the gradient of the exponential rate of decay. The y-intercept of this extrapolated gradient line = β, which will be >1.0 with an initial delay (lag phase) in GE, and <1.0 if emptying is rapid. In breath test studies, the cumulative ^{13}C dose recovery curve is inversely analogue to the scintigraphic GE curve, and therefore the function was modified as follows:

$$y(t) = m(1 - e^{-kt})^{\beta} \qquad \textbf{Equation 2.8}$$

where *y(t)* is the cumulative % ^{13}C excretion in breath at time *t*, and *m* is the total cumulative ^{13}C recovery when time is infinite. The first derivative of this formula allows a curve to be fitted to the rate of ^{13}C dose recovery:

$$dy/dt = mk\beta e^{-kt}(1-e^{-kt})^{\beta-1} \qquad \textbf{Equation 2.9}$$

where *dy/dt* is the recovery rate of the ^{13}C tracer at time *t*. From equation 2.8, the gastric half-emptying time ($t_{1/2}$) and the lag phase (t_{lag}) may be simply calculated:

$$t_{1/2} = -\ln[1-2^{-1/\beta}]/k \qquad \textbf{Equation 2.10}$$

$$t_{lag} = (\ln \beta)/k \qquad \textbf{Equation 2.11}$$

T_{lag} is numerically equivalent to the time (h) when the second derivative of the function is equal to zero.

2.6.3.2 Maes' formula

The second power exponential used to model the GE data in this thesis was elaborated at the University of Leuven by Maes (Maes *et al.* 1994; Ghoos *et al.* 1993) and is based on the chi-square distribution, with the production of a skewed bell-shaped curve:

$$y(t) = at^b e^{-ct} \qquad \textbf{Equation 2.12}$$

where *y(t)* is the % $^{13}CO_2$ excretion in breath per h, *t* is time (h) and *a*, *b* and *c* are constants. From this formula, $t_{1/2}$ is derived again from that point under the modelled dose recovery curve corresponding to cumulative recovery of 50% of the total recovered dose at infinity. This can be calculated from the integral of the model, and was performed using the iterative Gammainv (probability, alpha, beta) function in Microsoft Excel 9.0 software (Microsoft Corporation, Redmond, USA):

$$t_{1/2} = \text{Gammainv}\,(0.5;\, b+1;\, 1/c) \qquad \textbf{Equation 2.13}$$

T_{lag} is equivalent to that time at which GE or recovery of the ^{13}C label in the breath becomes maximal in rate, and is simply derived:

$$t_{lag} = b/c \qquad \textbf{Equation 2.14}$$

The Gastic Emptying Coefficient as defined by Ghoos *et al.* (1993) is equivalent to the natural logarithm of *a*: GEC = ln a **Equation 2.15**

2.6.4 Mathematical curve fitting

The models outlined above were fitted to the actual data by non-linear regression analysis using the least-squares method (Figures 2.4 and 2.5). The formulae were entered as functions into an Excel 9.0 spreadsheet, and the Excel Solver function used to minimise the difference between each experimental data point and that predicted from the model. The

Solver function was set to produce the global minimum root mean square (RMS) value for the model: RMS = √(sum of squares/degrees freedom) **Equation 2.16**

where the sum of squares (SS) = ∑(model prediction – observed data)^2 and degrees freedom (df) = (number of data points – number of model variables).

The Solver function was programmed to perform up to 300 iterations, using automatic scaling, to find the global minimum quadratic estimate for the given model. Both models (Equations 2.8 and 2.12) were found to fit equine gastric emptying data closely, and to produce similar estimates of both $t_{1/2}$ and t_{lag}. The fit of each model to ^{13}C-OABT dose recovery (PDR/h) and cumulative dose recovery data in a typical subject is illustrated in Figure 2.4.

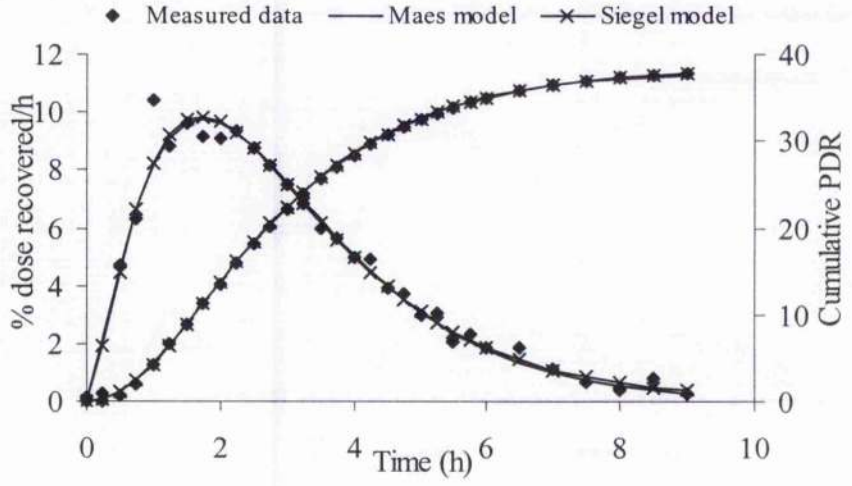


Figure 2.4 ^{13}C-octanoic acid breath test data from 1 subject, showing excellent fit of the experimental data by both the Maes and Siegel power exponential formulae. The rate of tracer recovery (PDR/h) is shown on the left *y*-axis, and the cumulative ^{13}C-dose recovery is plotted on the right *y*-axis.

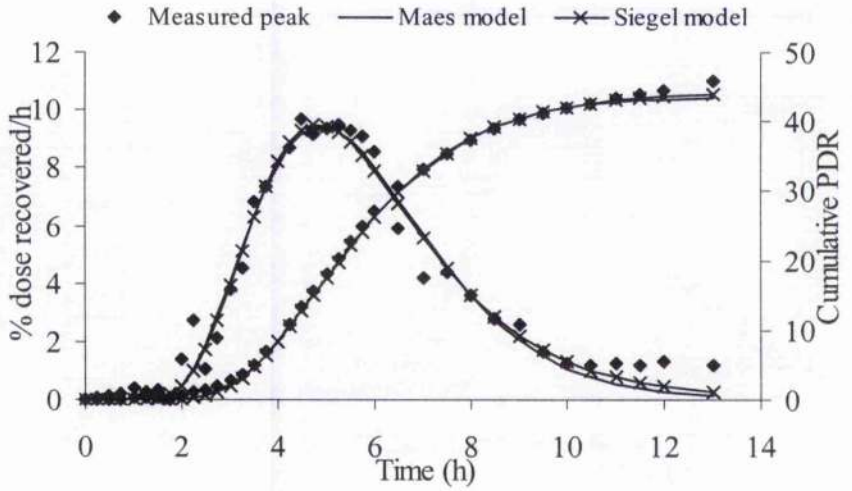


Figure 2.5 Induced lactose ^{13}C-ureide breath test from 1 subject showing good fit of the experimental data by both the Maes and Siegel formulae. The 'd' term is incorporated into each formula as shown in Equation 2.17. The rate of tracer recovery (PDR/h) is shown on the left *y*-axis, and the cumulative ^{13}C-dose recovery is plotted on the right *y*-axis.

2.6.5 Delayed and double peak fitting

Lactose ^{13}C-ureide breath test data were fitted in a similar manner to GE data. However, due to the prolonged delay from ingestion to arrival of the tracer in the caecum and appearance of the first signal in exhaled breath, curve fitting was improved by the introduction of a delay term (*d*) as developed by Morrison (2000). This factor improved fit by permitting the modelled peak to remain at zero (*t-d*) until the start of tracer recovery, at which point *t* exceeded *d*:

$$y(t) = a(t-d_1)^b e^{-c(t-d_1)} \qquad \textbf{Equation 2.17}$$

Orocaecal transit time data from an induced LUBT in a single individual, as modelled by equation 2.17, is shown in Figure 2.5.

Occasional ^{13}C-OABT dose recovery curves displayed more than one peak, attributable to discrete gastric emptying events or, possibly, to the kinetics of ^{13}C recovery from the bicarbonate pool (Van Hall 1999). However, in the absence of proof of a second GE event, these data sets were modelled with the best single peak, to facilitate comparison of $t_{1/2}$ and t_{lag} between individuals. A compartmental model for ^{13}C tracer recovery after ingestion, based on the physiological mechanisms considered to underlie the processing of ^{13}C-labelled meal, is under development at Glasgow but has not been fully validated. Whilst a new physiological rather than empirical model may be desirable, the models and parameters used in this work have followed those recommended by the Stable Isotopes in Gastroenterology and Nutrition (SIGN) organisation, which has formed a European concerted action plan (BIOMED1- project PL93 –1239) to promote the development of stable isotope breath tests for clinical diagnostics.

In order to fit combined GE and OCTT data, a model capable of fitting at least 2 discrete peaks was required (see Figures 2.6 and 2.7).

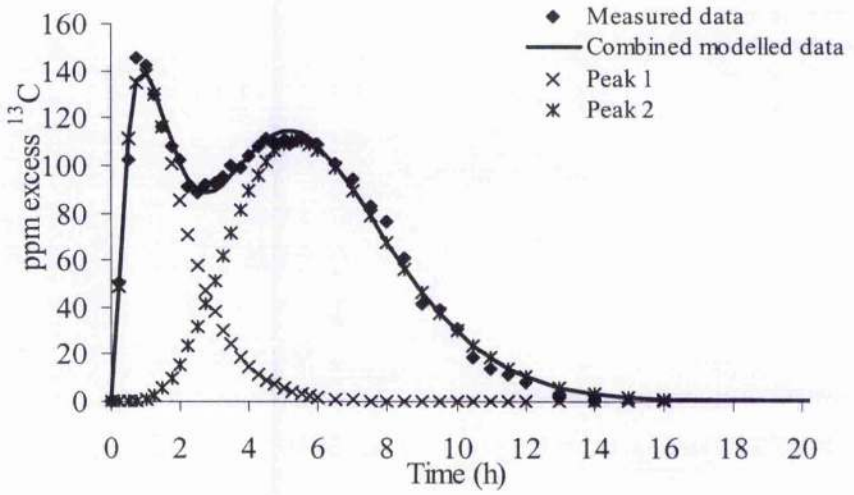


Figure 2.6 Combined ^{13}C-octanoic acid breath test and induced lactose ^{13}C-ureide breath test in one subject, modelled using a dual peak Maes formula, as described in Equation 2.18. The combined peak model has an excellent fit to the measured data (RMS = 5.23 ppm excess ^{13}C). Exhaled breath ppm excess ^{13}C after meal ingestion at time 0 is plotted on the *y*-axis against time (h).

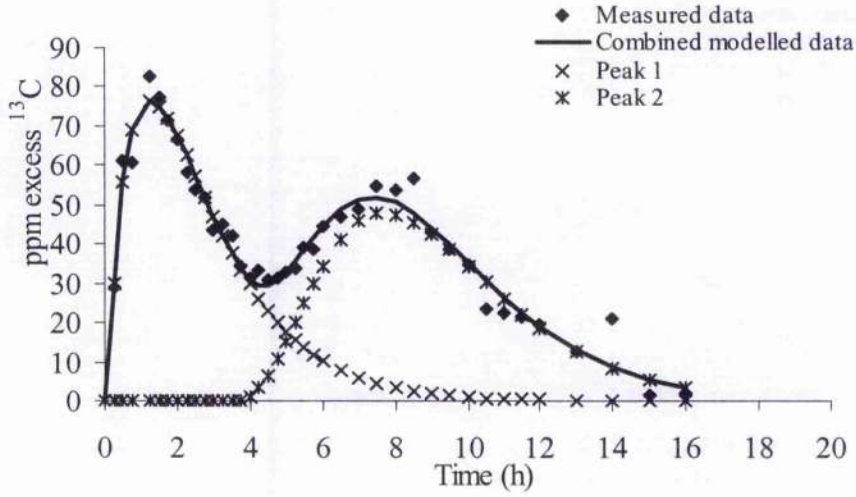


Figure 2.7 Combined ^{13}C-octanoic acid breath test and induced lactose ^{13}C-ureide breath test in a different subject, modelled using a dual peak Siegel formula. Once again, there is excellent fit of the combined modelled peaks to the measured data (RMS = 4.04 ppm excess ^{13}C). Exhaled breath ppm excess ^{13}C after meal ingestion at time 0 is plotted on the *y*-axis against time (h).

Combined GE and OCTT breath test data displayed at least 2 peaks, as shown in Figures 2.6 and 2.7, and were fitted using multi-peak modifications of equations 2.8 and 2.12, as described by Morrison *et al.* (1998):

$$y(t) = a_1(t-d_1)^{b1}e^{-c_1(t-d_1)} + a_2(t-d_2)^{b2}e^{-c2(t-d_2)} + a_n(t-d_n)^{bn}e^{-c_n(t-d_n)} \quad \textbf{Equation 2.18}$$

The discrete double peaks in these dual tests were thought to correspond to GE of both tracers, followed by caecal fermentation of the lactose ^{13}C-ureide.

2.7 Comparison of tracer recovery rates in breath and blood

2.7.1 Development of blood processing procedure

In order to maximise the clinical utility of stable isotope-based tests in equine veterinary medicine, a comparative study was performed of the ^{13}C:^{12}C profile of peripheral venous blood and expiratory breath samples collected concurrently during ^{13}C-OABTs. Although plasma and whole blood ^{13}C analysis has been performed in a number of different species (Metges *et al.* 1990; Cornetta *et al.* 1998; Moeller *et al.* 2001), a technique has not been reported previously for use in horses. After preliminary experiments, an acid:blood ratio of 2ml 6M HCl:1ml blood was selected (section 2.4.3) as this produced an average % CO_2 of approximately 4.65% in the headspace gas of the 10 ml Vacutainers used. This was in the optimum range for mass spectrometric analysis of the sample as the IRMS used a 5% CO_2 standard reference gas. A collection vessel of at least 10 ml volume was necessary to avoid contamination of the mass spectrometer autosampler needle during sample aspiration.

Total carbon dioxide carriage in venous blood under standard conditions (Lumb 2000) is reported to be approximately 23.30 mmol/l. It is transported as dissolved CO_2, H_2CO_3 and HCO_3^- in the plasma, and as dissolved CO_2, HCO_3^- and carbamino CO_2 by the erythrocytes. In this study, the average yield of CO_2 after acid treatment was consistent with CO_2 liberation of approximately 17.60 mmol/l blood. The discrepancy between this figure and total blood CO_2 carriage is likely to have been due to redissolution of CO_2, particularly given the enhanced solubility of CO_2 at lower temperatures (Lumb 2000). Escape of sample gas from the Vacutainer is also possible, although stable isotope samples stored in Vacutainers have been shown to remain suitable for analysis for periods of at least 3 months (Schoeller and Klein 1978).

2.7.2 Comparison of ^{13}C tracer recovery from blood and breath

The correlation between peripheral venous blood, after immediate acid hydrolysis and analysis, and expiratory breath sample ppm excess ^{13}C is shown in Figure 2.8 (left). The best-fit linear regression line and its 95% confidence intervals (Bland 1995) are plotted. As anticipated, there was a close linear correlation between the data sets ($r = 0.983$, n = 75, $P < 0.001$) with minimal scatter. In order to assess agreement, rather than simple linearity, the concordance correlation, ρ_c, was also calculated, as described by Lin (1989). This parameter is superior to tests such as paired *t*-tests and Pearson's correlation for showing agreement between alternative methods or instruments (Shoukri and Pause 1999; Sutton *et al.* 1999; Dawson *et al.* 2000). When the ppm excess ^{13}C of the immediately processed blood was compared with that of the corresponding breath sample, $\rho_c = 0.941$ (95% C.I. 0.908 – 0.962), which was consistent with almost perfect agreement (Shoukri and Pause 1999).

To assess any effects of enrichment magnitude on inter-method difference, a Bland Altman plot was constructed (Bland and Altman 1995; Bland and Altman 1999), as shown in Figure 2.8 (right). It can be seen that there is a slight tendency towards an increased difference in (breath – blood) ppm excess ^{13}C with increasing enrichment. However, the mean difference (4.59 ± 8.49, 95% C.I. 2.63 – 6.55 ppm excess ^{13}C) was small, with 95% of the sample differences falling within the range –12.05 to 21.23 ppm excess ^{13}C. It was therefore concluded that, when processed and analysed in the manner described, peripheral blood formed a viable alternative medium to breath for ^{13}C:^{12}C measurement in equine stable isotope studies.

In all subjects, the peak in breath % ^{13}C-dose recovery / h after ingestion of the test meal exceeded that of the corresponding blood sample. Blood dose recovery curves were generally wider and of lower amplitude than the matching breath recovery curves. Hence, the mean (± s.d.) difference between breath and blood techniques for estimation of gastric $t_{1/2}$ in 8 horses was 0.39 ± 0.26 h (95% C.I. 0.17 – 0.60 h; $P < 0.01$). Maximal blood ppm excess ^{13}C values were considered to be lower than those of the corresponding breath samples due to the kinetics of gaseous exchange in the pulmonary capillaries. Blood samples were collected from the jugular vein, such that ^{13}C tracer therein would already have cycled via the liver and heart to the lungs, before being returned from the heart to the body. The constant difference shown in estimates of blood/breath $t_{1/2}$ also reflected the anatomical location of sample collection.

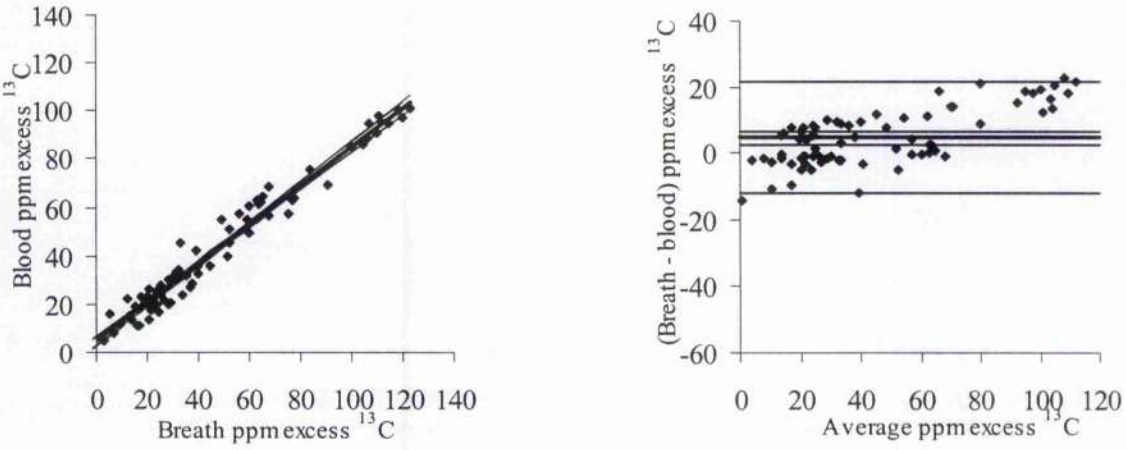


Figure 2.8 Comparison of ^{13}C recovery in concurrent equine breath and blood samples, following immediate acid hydrolysis of the blood samples. The left Figure displays the linear regression line and 95% confidence intervals: Pearson correlation coefficient = 0.983, *P* < 0.001. The right Figure displays the mean difference (breath minus blood) versus average ppm excess ^{13}C plot of the data presented; the 95% upper and lower confidence intervals and limits of agreement are shown.

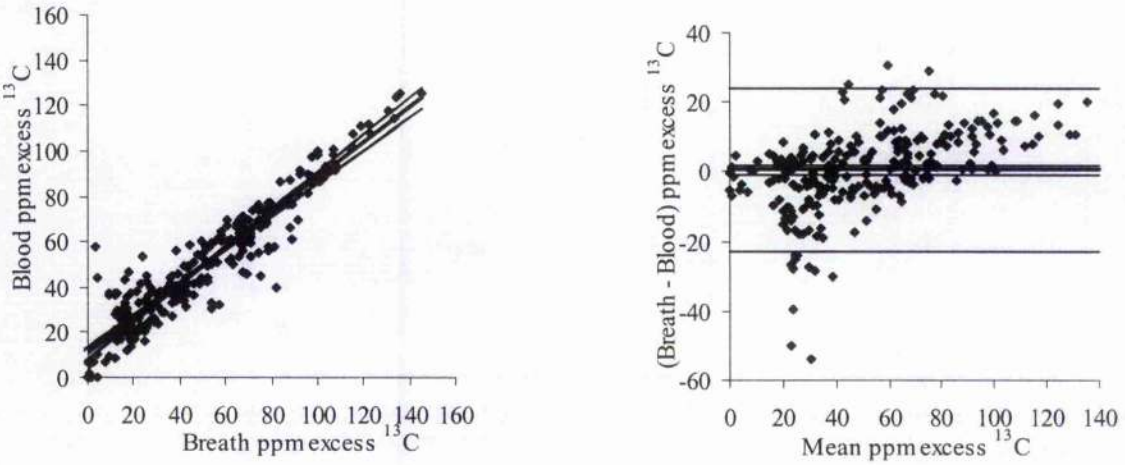


Figure 2.9 Comparison of ^{13}C recovery in concurrent equine breath and blood samples after storage of the blood samples prior to hydrolysis with 6M HCl. The left Figure shows the best linear regression line and 95% confidence intervals: Pearson correlation coefficient = 0.936, *P* < 0.001. The right Figure plots the mean difference (breath minus blood) versus average ppm excess ^{13}C of the combined data. The 95% upper and lower confidence intervals and limits of agreement are shown.

2.7.3 Effect of blood storage prior to analysis

If not processed and analysed immediately, blood samples were refrigerated and stored for up to 6 weeks, prior to acid extraction of CO_2. The correlation between the ppm excess ^{13}C of these stored blood samples, and the corresponding expiratory breath samples, is shown in Figure 2.9 (left). There was a good linear correlation between the data sets (r = 0.936, n = 257, P < 0.001), although there was more scattering of data at lower enrichment levels than seen with immediate sample processing. The plot of difference (breath minus blood) versus average ppm excess ^{13}C (Figure 2.9 (right)) again revealed a trend towards an increased difference in (breath – blood) ppm excess ^{13}C with increasing enrichment. However, the mean difference in sample ppm excess ^{13}C was small (0.56 ± 11.97, 95% C.I. –0.91 – 2.03 ppm excess ^{13}C). The 95% limits of agreement for blood/breath difference, after storage of the samples, were slightly increased at –22.95 to 24.07 ppm excess ^{13}C. The concordance correlation for this data set, ρ_c, = 0.924 (95% C.I. 0.904 – 0.940), which was again consistent with an excellent agreement. The correlation between the blood and breath data sets was not found to be significantly different following storage of the blood samples prior to analysis. These results suggested that equine blood samples may be stored for up to 6 weeks without significant detriment to the accuracy of subsequent ^{13}C:^{12}C analysis.

2.8 Repeatability of exhaled breath collection and tracer measurement

In order to test the overall repeatability of the method used for measurement of equine expiratory breath ^{13}C:^{12}C ratio, breath samples were collected in duplicate and analysed in separate batch runs by CF-IRMS. A total of 425 duplicate breath samples from 3 tests in each of 4 horses were included. The repeatability of the combined collection and analysis procedure was found to be extremely high. A linear regression plot showing the 2 data sets and the line of best fit is given in Figure 2.10. The correlation was excellent: r = 0.995 (95% C.I. 0.994 – 0.996, P < 0.001). The concordance correlation, ρ_c, as described by Lin (1989), was also determined: ρ_c = 0.993 (95% C.I. 0.991 – 0.994). As described by Shoukri and Pause (1999), this was consistent with almost perfect correlation between the data sets. A Bland Altman plot of the difference versus the combined average (ppm excess ^{13}C) of the 2 sample sets is presented in Figure 2.11. The mean difference between the data sets was –0.080 ± 3.568 ppm excess ^{13}C (95% C.I. –0.421 – 0.261), with 95% limits of agreement falling between –7.074 and 6.914 ppm excess ^{13}C. Increased sample enrichment was not found to have a significant effect on the repeatability of sample analysis.

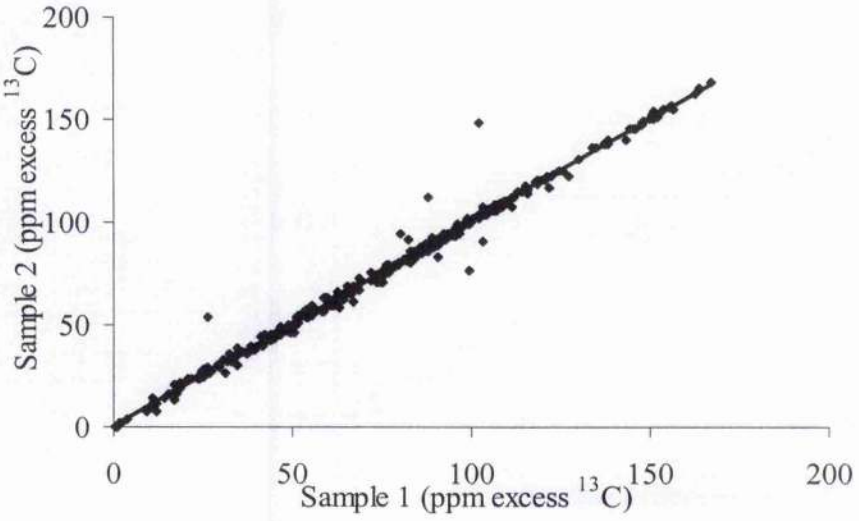


Figure 2.10 Linear regression plot comparing the ^{13}C enrichment in duplicate breath samples, analysed in separate batches by continuous flow isotope ratio mass spectrometry. The line of best fit is plotted and approximates the line of equivalence ($y = 0.996x + 0.365$). Pearson correlation coefficient, r, = 0.995 (95% C.I. 0.994-0.996), n = 425, $P < 0.001$.

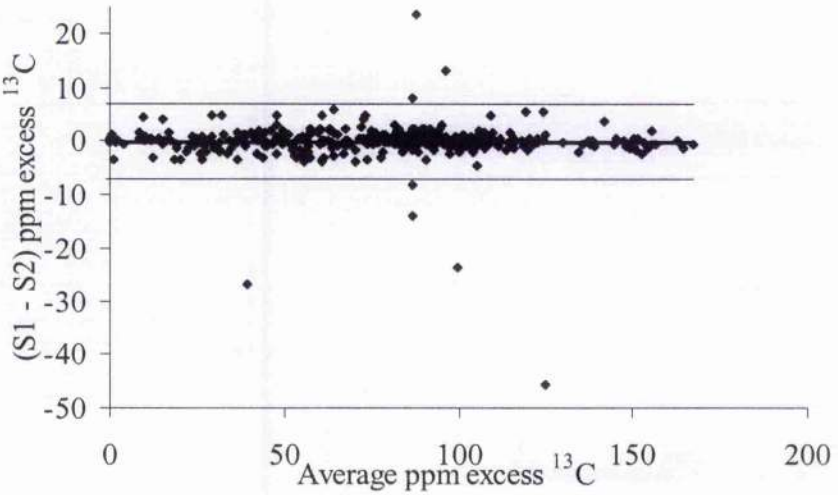


Figure 2.11 Bland Altman plot showing the difference (S1 – S2) in ppm excess ^{13}C plotted against average value for the 425 duplicate breath samples shown in Figure 2.10. The mean difference (-0.080 ± 3.568 ppm ^{13}C) between the sample sets and the 95% limits of agreement (-7.074 – 6.914 ppm ^{13}C) are plotted. There is excellent repeatability, which is not affected by level of breath ^{13}C enrichment.

Hence the repeatability of the combined breath collection procedure and isotopic ratio measurement used in this work was found to be excellent throughout the required range of breath ^{13}C enrichment.

2.9 Data organisation and Statistical Analysis

Organisation and presentation of this thesis was facilitated by use of document templates created in Microsoft (Microsoft Corporation, Redmond, USA) Word for Windows 98 version 9.0. In addition, Figures and tables were constructed using Microsoft Excel for Windows version 9.0 and Microsoft Powerpoint for Windows 98 version 9.0. Mathematical curve-fitting was performed using manually programmed functions in Microsoft Excel, and the Solver function of this software package was used to determine the model with the global minimum root mean square value.

Statistical analyses were performed using a combination of the data analysis components of Microsoft Excel version 9.0 and Minitab for Windows, release 13 (Minitab Inc., State College, PA, USA). Sensitivity and specificity analyses were performed in Microsoft Excel, with the aid of software developed by Greiner (Greiner 1995) as available at http://city.vetmed.fu-berlin.de/~mgreiner/TG-ROC/tgroc.htm.

Chapter 3 VALIDATION OF THE ^{13}C-OCTANOIC ACID BREATH TEST FOR THE MEASUREMENT OF EQUINE SOLID PHASE GASTRIC EMPTYING RATE

3.1 Introduction

3.1.1 Background information

Recent advances in stable isotope tracer production and analytical capacity have resulted in a rapid expansion in the use of stable isotope breath tests in clinical medicine for the investigation of physiological and metabolic functions, replacing previous radioisotopic tests (Swart and Van den Berg 1998; Klein 2001). The ^{13}C-octanoic acid breath test (^{13}C-OABT) was first validated for the measurement of gastric emptying of solids in humans in 1993 using radioscintigraphy (Ghoos *et al.* 1993). Subsequently, it has been validated by further research groups (Duan *et al.* 1995; Ziegler *et al.* 1996; Choi *et al.* 1998) and by a European multicentric trial (BIOMED 1 – Project PL93-1239; Delbende *et al.* 1998, 2000) and is now the diagnostic modality of choice for disorders of the upper gastrointestinal tract in children (Van Den Driessche *et al.* 1999), pregnant women, and critical care patients (*Ritz et al.* 2001). Most recently, a deuterated octanoate test (^{2}H-octanoate) has been shown to give real-time equivalent results to gamma scintigraphy, providing further proof that the ^{13}C-OABT may be used as an indirect test for measuring gastric emptying of the solid phase (Bluck *et al.* 2002). Although the ^{13}C-OABT has been used for the assessment of solid phase gastric emptying in the dog (Wyse *et al.* 2001), mouse (Symonds *et al.* 2000) and horse (Wyse *et al.* 2001a), it has yet to be validated against a standard method for any of these species.

As mentioned in Chapter 1, the *rationale* of the technique is that following ingestion of a ^{13}C-octanoate-labelled meal, the tracer will leave the stomach without being metabolised. After disintegration in the duodenum, the ^{13}C-octanoic acid is absorbed rapidly by the small intestine before undergoing hepatic oxidation, leading to the production of $^{13}CO_2$ and its subsequent exhalation from the bicarbonate pool (Ghoos *et al.* 1993). Since these post-gastric events are constant in rate, the change in $^{13}CO_2$:$^{12}CO_2$ ratio in expiratory breath is dependent on the rate of gastric emptying of the tracer as this is the rate-limiting step (*Ghoos et al.* 1993). Hence, the rate of appearance of $^{13}CO_2$ in the breath after ingestion of a solid phase-labelled test meal may be used to measure the rate at which solid material leaves the stomach.

Disordered gastric motility may be a significant factor in the pathogenesis of conditions such as gastric ulceration, equine dysautonomia, post-operative ileus, gastric impaction and

idiopathic recurrent colic. Previous methods of assessing solid phase gastric emptying in the horse have included transit measurements of administered plastic beads (Adams and MacHarg 1985) or polystyrene spheres (Milne *et al.* 1996), radiographic tracking of radiopaque markers (Baker and Gerring 1994) and scintigraphy following nasogastric intubation with radiolabelled solids (Sojka and Cantwell 1988; Levy and Sojka 1991; Neuwirth 1994; Ringger *et al.* 1996). The ^{13}C-OABT offers several advantages to all of these techniques: it is non-invasive, safe (non-radioactive), easy to perform, free from user interpretation and requires minimal equipment at the site of application. If validated for the measurement of equine gastric emptying of solids, the ^{13}C-OABT would have potential value as both a sensitive diagnostic tool and a humane research tool for future motility studies. The non-invasive nature of the test also means that it can be applied to normal individuals for use in larger scale epidemiological and pharmacological surveys.

3.1.2 Possible limitations of the ^{13}C-OABT

Despite the initial validation of the ^{13}C-OABT against scintigraphy for solid phase emptying in 36 subjects (16 normal, 20 with dyspepsia) by Ghoos *et al.* in 1993, diagnostic use of the test for the measurement of this parameter has not been without controversy. Choi *et al.* (1997) reported a lack of significant correlation between the gastric emptying indices obtained from the ^{13}C-OABT and scintigraphy in 15 healthy volunteers, and suggested that that this may have been caused by inter-subject variability in the absorption, metabolism and excretion of octanoic acid. However, having repeated its work in 30 volunteers, and extended the duration of breath collection, the same research group found a significant correlation between $t_{1/2}$ measured by the two tests ($P < 0.005$), although not for lag phase measurement (Choi *et al.* 1998). In the latter study, Choi *et al.* again reported that inter-modal differences in $t_{1/2}$ measurements were different between subjects, but highly reproducible within subjects.

Before further use of the ^{13}C-OABT in horses it was necessary to establish whether there was marked inter-individual variation in the post-gastric handling of octanoic acid by comparison with the predicate method for measuring solid phase emptying. Having investigated the ^{13}C-OABT in healthy horses, further investigation in horses with delayed gastric emptying would be required (Horowitz *et al.* 2002), and this is addressed in Chapter 4. It may also be necessary to further validate the test in diseased individuals. Correction factors, such as the bicarbonate factor and more recently the acetate factor (Van Hall 1999) have been introduced to correct for that component of ingested ^{13}C that may be incorporated into other products, particularly of the tricarboxylic acid cycle, rather than

being expired. However during acidosis, for example, there may be reduced elimination of CO_2 from the bicarbonate pool, due to reduction in pool size (Leese *et al.* 1994). Different physiological states may also change the enrichment of expired CO_2 significantly, by altering both metabolic rate and substrate oxidation (Wolfe *et al.* 1984). It has also been noted that changes in metabolic rate induced by hormonal changes may affect both these correction factors (Van Hall 1999). Therefore, after validation of the ^{13}C-OABT for solid phase measurement in healthy horses with both normal and delayed GE rate, further validation may be prudent in horses with different disease states.

3.1.3 Aims of the validation study

The ^{13}C-OABT has been investigated previously in ponies (Wyse *et al.* 2001a, b), but its use has not been validated for gastric solid phase measurement in this species, nor has it been used in horses. Wyse showed that British native ponies maintained on a grass hay diet produced a constant basal expiratory output of $^{13}CO_2$ after ingestion of an unlabelled test meal comprising oats and wheat bran, confirming the potential for enrichment with a ^{13}C-tracer (Wyse 1999). Furthermore, after ingestion of the same test meal containing baked egg yolk labelled with ^{13}C-octanoic acid, all ponies produced an increase in expiratory ^{13}C:^{12}C ratio, consistent with the patterns previously reported in man for solid phase gastric emptying (Ghoos *et al.* 1993). Before developing possible diagnostic applications of the test for horses, a pilot study was performed to determine whether horses showed similar patterns of expiratory $^{13}CO_2$ enrichment to ponies after ingestion of a ^{13}C-octanoate-labelled test meal. Test meal volume and composition was also optimised for use in horses.

Given the possible limitations of the technique listed in section 3.1.2, the major aim of the research presented in this Chapter was the validation of the ^{13}C-OABT for the measurement of equine solid phase emptying, by direct comparison with the predicate method of gastric scintigraphy (Merritt 1999). Radioscintigraphic imaging of the equine stomach is the only modality available that permits direct visualisation and quantitation of the movement of labelled ingesta from the horse's stomach, and has gained favour for equine pharmacological studies (Neuwirth 1994; Ringger *et al.* 1996). Gastric emptying scintigraphy has become accepted as a gold standard in human medicine, particularly after the development of alternate food labelling techniques for maintaining the radioactive label in the solid phase (Malmud *et al.* 1982; Maurer 1995). Aside from the requirement for a radiopharmaceutical and consequent radiation exposure, the use of a physiological meal, ease of quantitation, accuracy, and non-invasiveness have all made it acceptable to both patients and physicians (Parkman *et al.* 1995). However, the protocol for an equine gastric

scintigraphic examination has not been standardised and, therefore, a standard technique was also developed and reported for this procedure (Bahr *et al.* 2001). In addition, a method was developed for the analysis of ^{13}C:^{12}C ratio in equine peripheral blood samples, and a comparison performed between the results gained by breath or blood analysis (see section 2.7.2). This was performed so as to maximise the clinical utility of the stable isotope tests for equine practice.

By direct comparison of the results of the ^{13}C-OABT with those of concurrent gastric emptying scintigraphy, it was hoped to establish whether or not the factors listed in section 3.1.2 would prove prohibitive to the successful application of the ^{13}C-OABT for the measurement of equine solid phase gastric emptying rate in healthy adult horses.

3.2 Equine ^{13}C-OABT pilot study

3.2.1 Aims

The main aim of the pilot study was the development of a palatable test meal, of equivalent ^{13}C:^{12}C ratio to the basal diet, that allowed repeatable, discrete measurement of a single gastric emptying event. The test meal needed to be of sufficient energy content to exert an influence on gastric emptying rate, whilst being small enough to permit rapid and complete ingestion. In addition, initial ^{13}C-OABTs were performed to ascertain whether the pattern of ^{13}C-isotopic recovery in horses mirrored that previously reported in ponies (Wyse *et al.* 2001a), prior to scintigraphic validation.

3.2.2 Materials and methods

3.2.2.1 Subjects

Four adult, healthy horses of average age 9.25 y (3 thoroughbreds, 1 mixed breed) with no physical or historical evidence of gastrointestinal disease were selected for the pilot study. These horses were maintained on a restricted diet of mixed alfalfa/ryegrass hay *ad libitum* to minimise fluctuations in baseline $^{13}CO_2$ production. Exercise was maintained at constant levels, and was restricted on the day prior to a gastric emptying test. Food was withheld for 12-14 h before ingestion of the test meal to ensure an empty stomach, and exercise was restricted during each test. Basal breath sample collection started at the same time (0700 h) on each occasion.

3.2.2.2 *Selection of test meal*

In the only previously reported use of the ^{13}C-OABT in equidae, Wyse (1999) used a test meal of 75 g crimped oats, 50 g wheat bran and 100 ml water in native ponies of body mass 150 – 250 kg, with the ^{13}C-octanoic acid label contained within 1 baked egg yolk. This was reported to produce repeatable patterns of expiratory $^{13}CO_2$ production after test meal consumption, which could be modelled using human gastric emptying non-linear regression formulae (Wyse *et al.* 2001a). The relative volume of the equid stomach in relation to body mass has not been well documented. In view of this, different sizes of test meal were tried, with the following objectives: (i) complete and rapid consumption was required (less that 10 min); (ii) to establish that quantity of feed that produced an easily modelled monophasic peak of $^{13}CO_2$ recovery; (iii) to optimise test meal consistency such that gastric emptying of the labelled egg yolk was representative of the passage of ingesta; and (iv) to maximise intra-individual repeatability of the test. In order to reduce complexity of the test protocol, a standard test meal size was used for all horses, without attempt to calibrate for relative body size / gastric volume, as this relationship remains poorly known. Based on the work of Wyse (1999) the individual test meal components of crimped oats and wheat bran were retained.

3.2.2.3 *Dose of ^{13}C-octanoic acid*

To improve the accuracy of data modelling, a maximal breath enrichment of at least 100 ppm excess ^{13}C over baseline was required. As octanoic acid-1-^{13}C (Isotec Inc., Ohio, USA) is relatively expensive and unpalatable, it was necessary to find the minimum dose needed to produce this level of expiratory enrichment.

3.2.2.4 *Breath sampling protocol*

At least 2 expiratory samples were collected for basal ^{13}C:^{12}C analysis prior to ingestion of the labelled test meal. Initially, serial breath collection was extended for 12 h after ingestion of the test meal, but this interval was reduced to 10 h when it became apparent that this was sufficient to allow accurate modelling of the ^{13}C-dose recovery curve.

3.2.2.5 *Breath sampling technique*

During the pilot studies, the breath sampling technique detailed in section 2.4.1 was developed and successfully validated for the collection of equine expiratory air.

3.2.3 Results of the pilot studies

3.2.3.1 Test meal and tracer

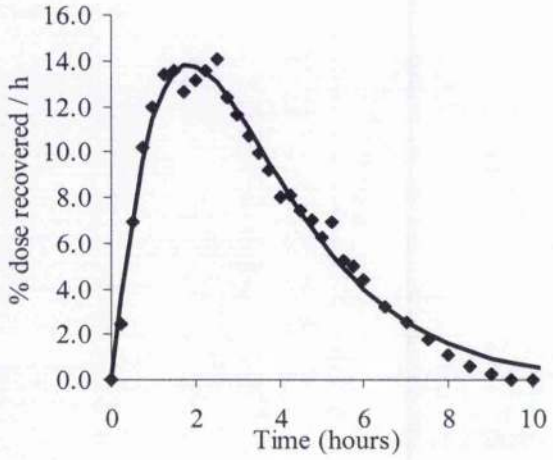
Some examples of the % dose recovery curves of the test subjects, following ingestion of the labelled test meals used in the pilot studies are shown in Figures 3.1 to 3.4. The optimum test meal, in terms of production of a discrete bell-shaped ^{13}C expiratory dose recovery curve, was found to consist of 250 g crimped oats, 100 g wheat bran and 200 ml water (calorific value 3.234 MJ). The minimal dose of ^{13}C-octanoic acid necessary to consistently produce an enrichment of at least 100 ppm excess ^{13}C was 1 mg/kg. This dose of ^{13}C-octanoic acid was tolerated by each subject, and its palatability enhanced by refrigerating the labelled baked egg yolk prior to consumption. The quantity of egg yolk required to carry the tracer was also examined. It was suspected that a single baked yolk was insufficient to bind the tracer necessary for an adult horse in the solid phase, as exponential isotope recovery curves (Figure 3.2) were noted, suggesting escape of tracer into the liquid phase. This theory was supported by Ghoos (*pers. comm.* 1999) and thereafter a maximum of 250 mg ^{13}C-octanoic acid was added to each egg yolk prior to thorough blending, baking and incorporation into the test meal.

3.2.3.2 Modelling of % dose expiratory recovery curves

A monophasic, skewed bell-shaped distribution was the commonest pattern of expiratory isotope recovery (Figure 3.1). However, biphasic patterns of recovery were frequent as demonstrated in Figures 3.3 and 3.4, and were best modelled by the dual peak Maes' model (equation 2.18). The cause for an early second peak (e.g. Figure 3.4) was considered to be discrete emptying events of the triturated test meal. A late peak (Figure 3.3) in the recovery of the isotope was common, and this phenomenon may have been linked to the pharmacokinetics of CO_2 expiration from the bicarbonate pool. This hypothesis is explored further in Chapter 6, using the ^{13}C-bicarbonate breath test.

3.2.3.3 Initial repeatability of the ^{13}C-OABT

Using the final test meal on 2 occasions in each of the 4 subjects, mean (s.d.) values for $t_{1/2}$, t_{lag} and GEC were 2.64 (± 0.60) h, 1.42 (± 0.80) h and 2.80 (± 0.31) respectively. The intra-individual coefficients of variation (CV%) for $t_{1/2}$, t_{lag} and GEC were 17.79 (± 18.56) %, 41.67 (± 24.53) % and 10.20 (± 10.01) % respectively, which compared favourably with the work of Wyse *et al.* (1999).



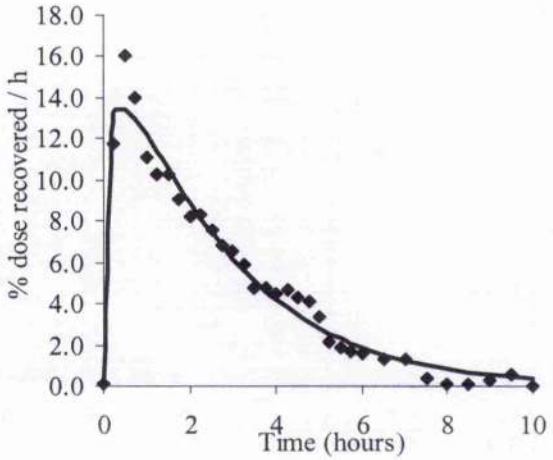
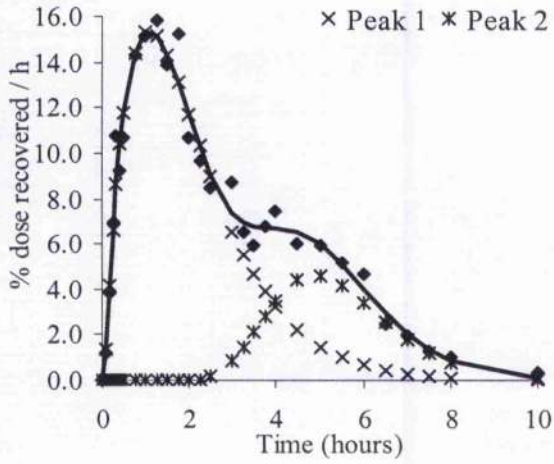


Figure 3.1 (left) Typical equine gastric emptying profile (subject 107) using the ^{13}C-OABT and the final developed test meal, showing % dose recovery of the ^{13}C-isotope in the breath, plotted against time. The modelled curve (Maes' formula) describes a skewed bell-shaped distribution. Gastric half-emptying time ($t_{1/2}$) = 2.93 h; gastric lag phase (t_{lag}) = 1.86 h.

Figure 3.2 (right) Exponential recovery of the ^{13}C-isotope in the breath in subject 102 after ingestion of the ^{13}C-octanoate-labelled test meal, consistent with early rapid gastric emptying, or possible escape of the ^{13}C-tracer into the liquid phase: $t_{1/2}$ = 1.98 h; t_{lag} = 0.41 h.



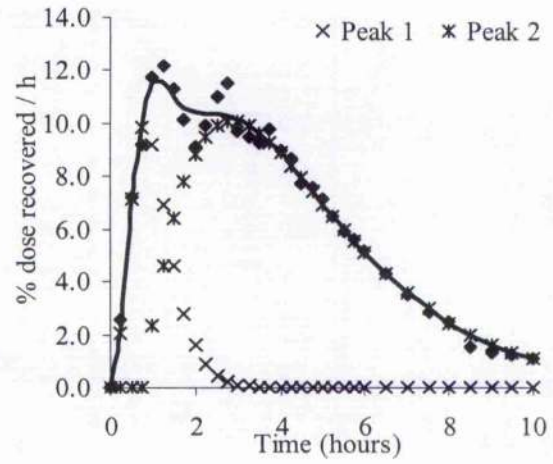


Figure 3.3 (left) Biphasic expiratory recovery of the ^{13}C-isotope in subject 103 after ingestion of the ^{13}C-octanoate test meal, consistent with a possible second gastric emptying event. These data have been modelled using the dual peak Maes' model, given in equation 2.18: $t_{1/2}$ = 2.47 h; t_{lag} = 1.08 h.

Figure 3.4 (right) Biphasic expiratory recovery of $^{13}CO_2$ in subject 104 after a ^{13}C-OABT. There is an early small peak, consistent with early rapid emptying of a small quantity of test meal from the stomach, followed by a second larger gastric emptying event. Gastric $t_{1/2}$ = 3.59 h; t_{lag} = 1.73 h.

3.2.4 Preliminary conclusions

The results of the pilot study suggested that, if using the final developed test meal and a dose of 1 mg/kg ^{13}C-octanoic acid (at a maximum of 250 mg/baked egg yolk), then the ^{13}C-OABT was potentially suitable for the indirect measurement of solid phase gastric emptying in horses. Based on these results, a validation study was performed, directly comparing the results of the ^{13}C-OABT with concurrent gastric scintigraphy.

3.3 Comparison of the ^{13}C-OABT with concurrent scintigraphy for the measurement of solid phase gastric emptying rate

3.3.1 Materials and Methods

3.3.1.1 Subjects

12 adult horses from the Texas A&M University research herd were used in accordance with Animal Use Protocol 2000-112 approved by the University Laboratory Animal Care Committee. These animals had no known history or physical evidence of gastrointestinal disease. Potential subjects were rejected if biochemical or total blood cell parameters were found to lie outside the reference range. The horses (4 mares, 8 geldings) were of mixed breed (7 Quarter Horses, 3 Quarter Horse cross, 1 Thoroughbred, 1 Arabian), with a median age of 8 years (mean 11.1, range 2 to 25 years), and median body weight of 467.2 kg (mean 469.4, range 347.0 to 563.4 kg). Ivermectin anthelmintic (Merial Animal Health Ltd, Essex, UK) was administered orally at least 2 weeks prior to the procedure, and all subjects were maintained on *ad libitum* alfalfa hay only for at least 2 weeks prior to each experiment, having previously grazed Coastal Bermuda grass at pasture.

3.3.1.2 Test meal composition

The test meal comprised 150 g crimped oats, 100 g bran, 200 ml water and two dual-labelled egg yolks. For each test, approximately 1mg/kg ^{13}C-octanoic acid (Octanoic acid-1-^{13}C, Isotec Inc., Miamisburg, OH, USA) and 14-19 mCi ^{99m}technetium sulphur colloid (CIS-US Inc., MA, USA) was added to two egg yolks, baked in a microwave oven until firm and thoroughly mixed into the test meal.

3.3.1.3 Study design

Following a 14 h overnight fast, the test meal was ingested voluntarily in the nuclear medicine room. Gastric radioscintigraphy and expired breath collection for isotope analysis were performed concurrently, and the horse was restrained manually until the protocols for

both procedures were completed. For all experiments, the protocol, equipment and personnel were the same. Each horse was measured once only.

3.3.1.4 ^{13}C-octanoic acid breath test

3.3.1.4.1 Breath collection

Equine expiratory breath samples were collected as detailed in section 2.4.1 using the modified Aeromask® (Trudell Medical International, Ontario, Canada) and Exetainer® tubes (Labco Ltd., Buckinghamshire, UK). Three basal breath samples were collected 60, 15 and 0 min before test meal ingestion (-60, -15, 0 min), and thereafter at 15-min intervals for 6 h, then 30-min intervals for a further 4 h.

3.3.1.4.2 Blood collection

Venous blood samples were collected from 6 individuals for comparison of ^{13}C:^{12}C ratio with simultaneous breath samples, and processed according to the method given in section 2.7.1. At each time point, 1 ml aliquots of bloods were collected from a pre-placed jugular catheter and acidified after anaerobic introduction into an evacuated glass container (Vacutainer®, Labco Ltd., Buckinghamshire, UK).

3.3.1.4.3 ^{13}C abundance

The ^{13}C:^{12}C ratio of each breath/blood sample was determined by automated continuous flow isotope ratio mass spectrometry (PDZ Europa Ltd ABCA analysis, Cheshire, UK), and expressed relative to the international PDB standard (δ_{PDB}). This ratio was converted to parts per million (ppm) ^{13}C, and expressed as ppm excess ^{13}C, after subtraction of the average ^{13}C-abundance of the three baseline (predose) breath samples. The percentage dose recovery (PDR) of the administered isotope in the breath was also calculated, and plotted against time, as described in more detail in section 2.5.2.

3.3.1.5 Gastric radioscintigraphy

A large field-of-view gamma camera with a low energy all purpose collimator linked to a nuclear medicine computer was utilized to acquire each image in a 64 x 64 matrix. Serial left and right lateral 30 s gastric scintigraphs were obtained at 15 min intervals, until the radioactive counts per 30 s interval in the region of interest had decreased to less than 10 % of that at time zero (immediately after test meal ingestion). Each region of interest was drawn freehand around the gastric activity and the total counts in the region were obtained using Nuclear Mac software (Microsoft Corporation, Redmond, USA). Since

^{99m}technetium has a short $t_{1/2}$ of approximately 6 h, counts were corrected for radioactive decay of the isotope using the formula given in equation 3.1:

$$A = A_0 e^{-\lambda t} \qquad \textbf{Equation 3.1}$$

where A = corrected count at time t, A_0 = initial count, λ = decay constant ($0.693/t_{0.5}$ with $t_{0.5}$ = half-life of isotope), and t = image acquisition time in minutes after time 0. The left and right-side counts and their geometric mean (square root of the product of left and right counts), thought to correct for tissue attenuation, were calculated at each time point (Halkar *et al.* 1999). Examples of the digital images gained using this technique are shown in Figures 3.6 – 3.9.

3.3.1.6 Effect of diet on basal ^{13}C expiratory output

In a preliminary experiment to assess the stability of basal metabolic ^{13}C production under test conditions, this parameter was measured in 4 of the 12 horses maintained on the test diet (alfalfa hay alone). To determine dietary influence on basal ^{13}C output, the results were compared with those in 5 separate horses maintained on a diet of Coastal Bermuda grass (*Cynodon dactylon*). The latter is a C_4 plant and relatively enriched with ^{13}C (section 2.1.2; Waller and Lewis 1979). The same test protocol and test meal as described above were used but without the addition of either isotope to the meal. Breath samples were collected and analysed according to the given protocol and ^{13}C abundance expressed as ppm ^{13}C against time.

3.3.1.7 Calculation of gastric-emptying indices

3.3.1.7.1 Radioscintigraphic test data

The counts in the gastric region of interest from the left and right series, and the geometric mean, were plotted separately against time as the fraction of retained radioactivity after decay correction. Siegel's formula (Siegel *et al.* 1988) was used to fit a modelled curve to the data, from which scintigraphic indices of gastric emptying ($t_{1/2s}$ and t_{lags}) were calculated as described previously in section 2.6.3.1.

3.3.1.7.2 ^{13}C-octanoic acid breath/blood test data

Expiratory breath samples containing less than 0.5% CO_2 were rejected to minimize analytical inaccuracies. Jugular venous blood samples were collected and processed as detailed in section 2.4.3. Data were plotted against time as either the ppm excess ^{13}C, or the percentage of the isotopic dose recovered per hour (PDR / h). The modified version of

Siegel's formula, as given in equation 2.8, was used to model the data and to determine the stable isotope half-emptying time ($t_{1/2b}$) and lag phase (t_{lagb}) duration. The gastric emptying coefficient (GEC), reflecting the gradient of the emptying curve, was calculated from the breath test data using the formula described by Ghoos *et al.* (1993), as given in equation 2.15.

3.3.1.8 Pharmacokinetic data on $^{13}CO_2$ appearance in breath

In order to determine the pharmacokinetics of ^{13}C-octanoic acid absorption and metabolism in the horse ('post-gastric handling'), the dynamics of $^{13}CO_2$ appearance in the breath after direct intraduodenal administration of ^{13}C-octanoic acid were determined also in three of the horses used for the validation study. Food was withheld for 14 hours prior to sedation with xylazine (0.5 mg/kg). After gastroscopic location of the pyloric antrum using a 3 m endoscope (Olympus America Inc., Melville, NY, USA), a polythene cannula was passed into the duodenum as shown in Figure 3.5. Octanoic acid-1-^{13}C (1 mg/kg) was instilled through this cannula, using 10 ml vegetable oil of low natural ^{13}C abundance (canola oil) as a carrier. Breath/blood samples were collected immediately before instillation, and then afterwards at 5 min intervals for the first hour, 10 min intervals for the second hour, and then 15 min intervals for a further 4 h. Each horse was maintained in stocks throughout, and allowed access to water but not to feed. The ^{13}C:^{12}C ratio of these samples was determined and processed as described in section 3.3.1.4.3. The aim of this part of the project was to measure the half-life time for the post-gastric handling of this medium chain fatty acid in the horse, and to assess whether its absorption, metabolism and expiration proceeded at a constant rate.

3.3.1.9 Statistical evaluation of results

3.3.1.9.1 Effect of diet on basal ^{13}C expiratory output

Two-tailed *t*-tests were used to compare the mean basal output of ^{13}C at each time for the two groups of horses maintained on different diets. The relationship of this parameter with time was studied by linear regression analysis.

3.3.1.9.2 Validation of ^{13}C-OABT versus scintigraphy

The relationships between the gastric emptying indices obtained by scintigraphy ($t_{1/2s}$, t_{lags}) and the data gained from the breath/blood test analyses ($t_{1/2b}$, t_{lagb} and GEC) were evaluated by Pearson correlation and linear regression.

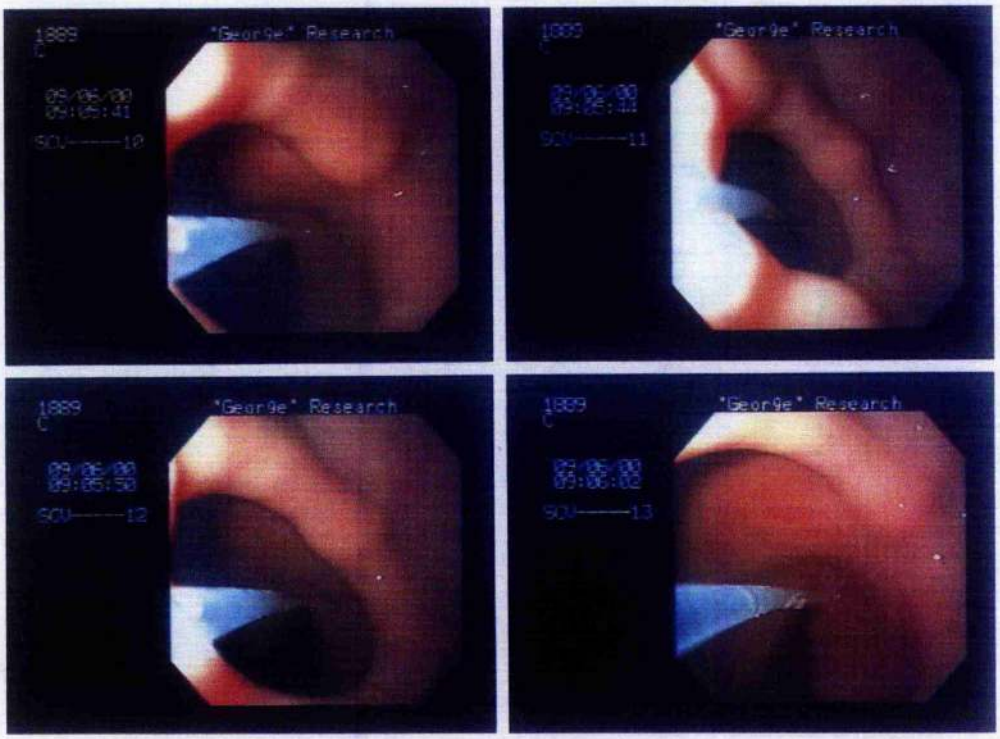


Figure 3.5 Intraduodenal administration of ^{13}C-octanoic acid in a carrier of canola oil, in an adult horse (subject 1889) using a 3-metre gastroscope and polythene cannula. The cannula was passed between successive waves of pyloric sphincter contraction.

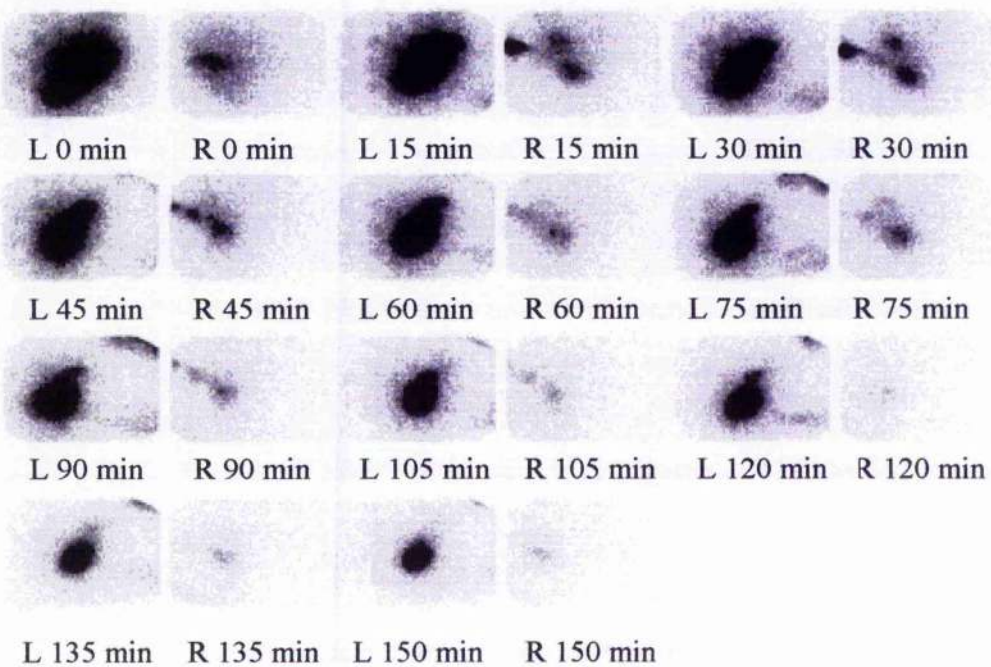


Figure 3.6 Paired gastric scintigraphic images (left and right) from horse with rapid gastric emptying rate (scintigraphic $t_{1/2}$ = 0.70 h) after ingestion of ^{99m}Technetium-sulphur colloid-labelled test meal. Serial images are illustrated, each collected for 30 s at 15 min intervals. The right-side region of interest loses definition at 105 min post ingestion of the radioisotope in this subject.

Bland Altman statistics were used to demonstrate the effect of measurement magnitude on the correlation between the two different methods (Bland and Altman 1995; Bland and Altman 1999) and 95% confidence limits were established. The relationship between the three scintigraphic data sets (left, right and geometric mean) was also evaluated by correlation and linear regression.

Univariate analysis was performed on each of the scintigraphic and breath test parameters in the 12 subjects to establish a reference range for each parameter. In addition, the coefficient of variation (standard deviation / mean x 100%) was calculated for each variable.

3.3.2 Results

3.3.2.1 Basal expiratory $^{13}CO_2$ production

The dietary studies showed that the horses maintained under test conditions (on an alfalfa hay diet) prior to assessing basal ^{13}C output had a very stable ^{13}C:^{12}C expiratory ratio in the measured period after unlabelled test meal consumption. The collated mean (± s.d.) ppm excess ^{13}C in this group after test meal consumption was small (1.54 ± 6.21 ppm excess ^{13}C). In contrast, the horses maintained on coastal grass had a marked reduction in basal ^{13}C output over time (Figure 3.10) with a Pearson correlation coefficient of -0.782, although this did not reach statistical significance. The coastal grass group also produced a significantly higher mean basal ^{13}C output than the alfalfa test subjects (10893.05 ± 10.21 versus 10829.14 ± 6.17 ppm ^{13}C; $P < 0.0005$) over the 10 h test period.

3.3.2.2 Scintigraphic gastric emptying technique

Scintigraphic measurement of gastric emptying and determination of the gastric region of interest was most easily and accurately performed from the left-sided gamma camera images. Radioactive counts per 30 s interval were on average 6.86 times greater for the left region of interest than the right side at time zero (s.d. 1.72, range 4.30 to 9.50). This ratio was not correlated to either body mass or body surface area. In addition to the low counts, accurate measurement of the right side region of interest often was made more difficult by superimposition of radioactive small intestine or ascending colon during the test (see Figures 3.7 to 3.9). The correlation between gastric half-emptying times measured from the left and right regions of interest was relatively low ($r = 0.714$, $P < 0.05$). Therefore, gastric emptying indices calculated from the left side alone were used for validation of the ^{13}C-OABT data.

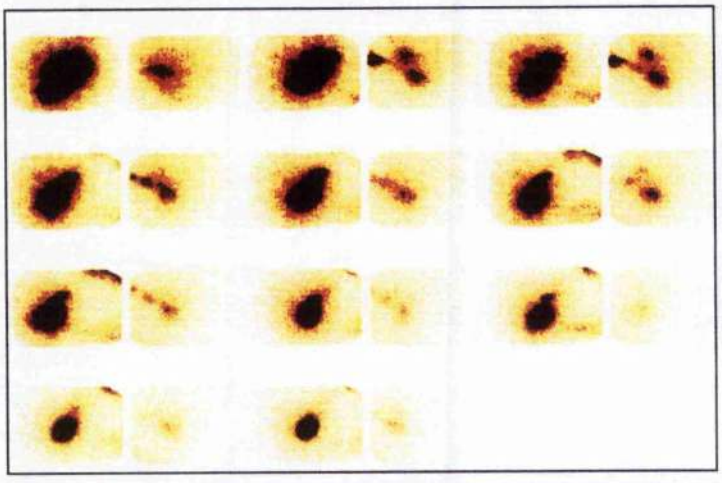


Figure 3.7 Example of rapid scintigraphic gastric emptying in subject 1929. Sequential left and right lateral images are shown, acquired at 15 min intervals. The pyloric region and proximal duodenum are visible in the right lateral images.

Gastric $t_{1/2}$ = 0.704 h; no lag phase detected.

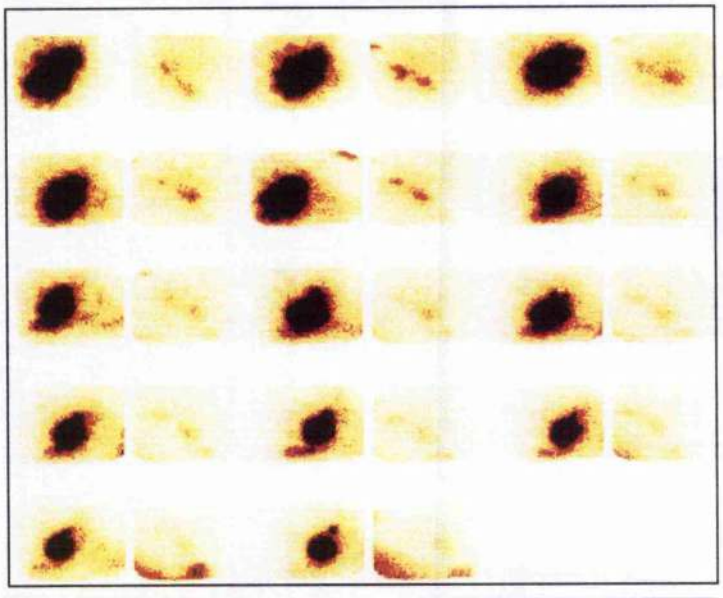


Figure 3.8 Moderate gastric emptying rate, as determined by gastric scintigraphy in subject 1927. Right lateral images lose definition at approximately 75 min after test meal ingestion.

$t_{1/2}$ = 1.130 h; t_{lag} = 0.174 h.

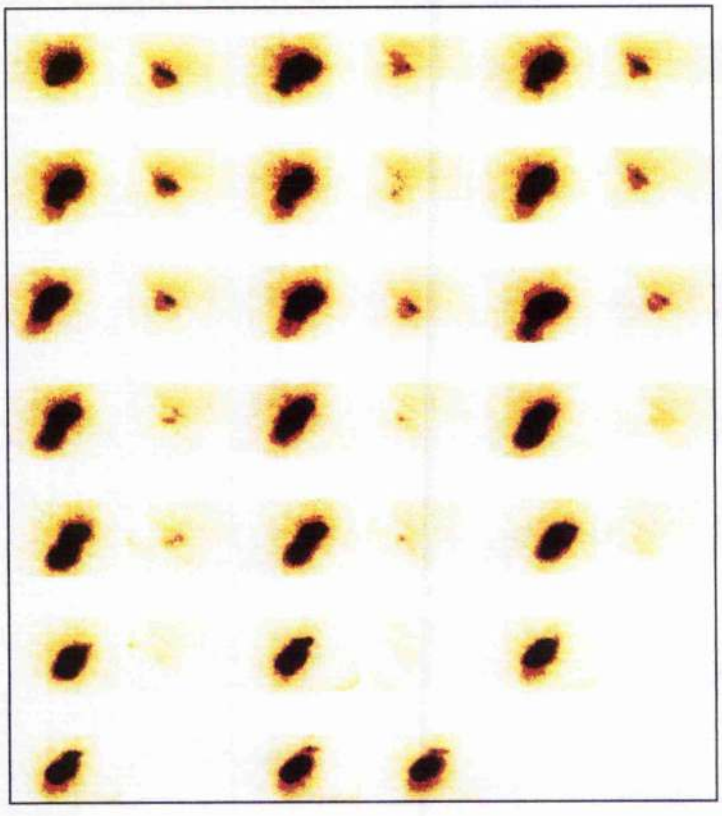


Figure 3.9 Slow gastric emptying rate as shown by scintigraphy in subject 1889. There is loss of radioactivity in the right lateral images at 150 min, but radioactive ingesta continues to be detectable in the left lateral gastric region at 300 min after test meal ingestion (final image – bottom right).

$t_{1/2}$ = 4.212 h; t_{lag} = 3.862 h.

All images are shown in 'hot body' colour scale.

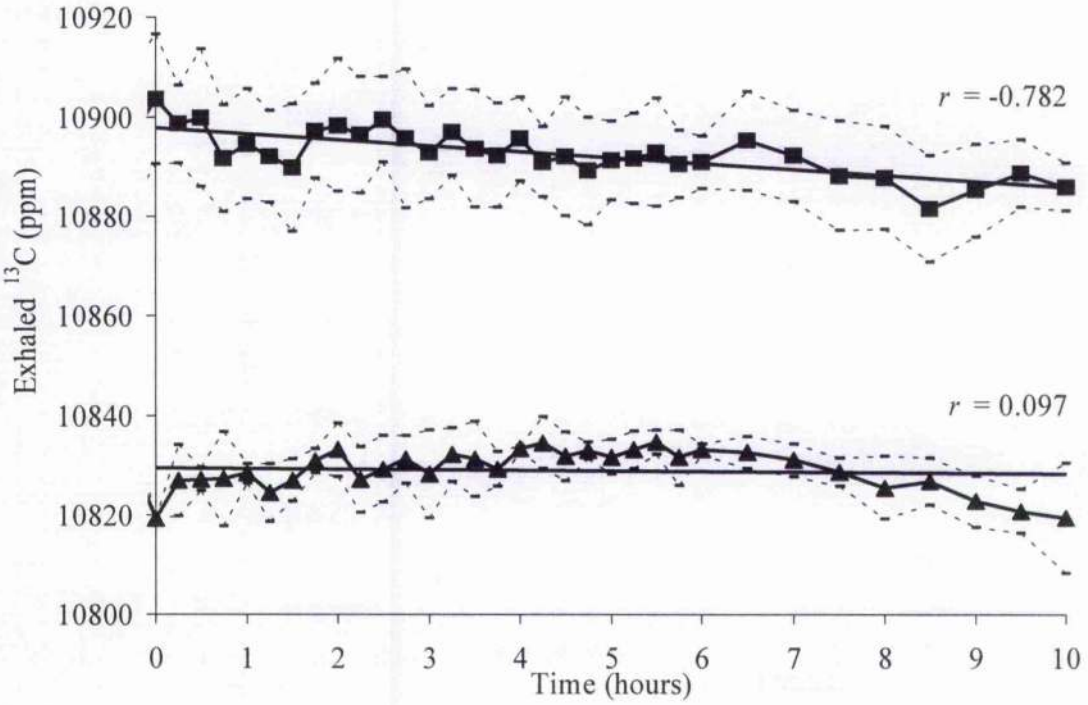


Figure 3.10 Comparison of mean (± s.d.) basal ^{13}C output in horses maintained on coastal Bermuda grass hay (■), n = 5, with those maintained on alfalfa hay (▲), n = 4. Mean expiratory ^{13}C values are plotted against time over the 10 h period after ingestion of the unlabelled test meal at time 0. ^{13}C ppm is significantly higher at each time point for the Bermuda grass group ($P \leq 0.0025$). The *r* values represent the Pearson correlation coefficient with time.

There is a negative correlation between $^{13}CO_2$ production and time in the horses maintained on coastal hay, which is not seen in the control group. This does not reach statistical significance due to the relatively small sample size.

Table 3.1 Univariate analysis showing mean, standard deviation (s.d.), range and CV% (coefficient of interindividual variation) for parameters of solid phase gastric emptying rate derived by radioscintigraphy and ^{13}C-octanoic acid breath test in 12 healthy horses.

Modality	*Parameter*	*Mean*	*s.d.*	*Range*	*CV%*
Gastric Scintigraphy	Left side $t_{1/2}$ (h)	1.56	1.08	0.56 - 4.21	69.23
	Right side $t_{1/2}$ (h)	1.16	0.69	0.18 - 2.19	60.09
	Geo. Mean $t_{1/2}$ (h)	1.27	0.76	0.43 - 2.74	59.84
	Left side t_{lag} (h)	0.36	1.37	-2.24 - 3.86	380.56
^{13}C-octanoic acid breath test	$t_{1/2}$ (h)	3.79	1.53	1.99 - 6.74	40.37
	t_{lag} (h)	2.32	1.14	1.16 - 5.52	49.14
	GEC	2.09	1.27	-1.5 - 3.35	60.48

$t_{1/2}$ = gastric half-emptying time (h); t_{lag} = duration of triturative lag phase (h), equivalent to ln β/k, negative if $\beta < 1$ i.e. when rapid early emptying occurs; GEC = gastric emptying coefficient.

3.3.2.3 *Comparison of diagnostic modalities*

Figure 3.11 illustrates solid phase gastric emptying as measured by simultaneous radioscintigraphy and ^{13}C-octanoic acid breath test. Three typical examples are shown, ranging from rapid (top) to typical (middle) to slow (bottom) gastric emptying rate. It can be seen that rapid loss of radioactive material from the stomach into the small intestine was matched by a relative increase in the rate of appearance of the ^{13}C tracer in the breath. Similarly, in subjects with prolonged retention of gastric radioactivity, there was a delay in the appearance of expiratory $^{13}CO_2$. The complete set of gastric emptying data in the 12 subjects is represented graphically in appendix I. The mean gastric emptying indices in the 12 subjects as measured by the two different techniques are shown in Table 3.1. With both scintigraphic and ^{13}C-OABT assessment, there was considerable range in individual $t_{1/2}$ values (0.56 – 4.21 h, and 1.99 – 6.74 h respectively). This parameter was not correlated to gender, age of animal, body weight or to body surface area. The coefficient of interindividual variation was therefore relatively high for both scintigraphic $t_{1/2}$ ($Lt_{1/2s}$) and breath test $t_{1/2}$ ($t_{1/2b}$), and higher for the established technique of scintigraphy (69.23% and 40.37% respectively).

There was a strong positive correlation between the gastric half-emptying times, as determined by scintigraphy and ^{13}C-OABT with a Pearson correlation coefficient of 0.944 ($P < 0.001$). The best linear regression line between these parameters, together with its 95 % confidence intervals, is demonstrated in Figure 3.12. The numerical relationship between mean $Lt_{1/2s}$ and $t_{1/2b}$ was best defined by equation 3.2:

$$y = 1.3365x + 1.7107 \qquad \textbf{Equation 3.2}$$

where y = breath test $t_{1/2b}$ and x = scintigraphic $Lt_{1/2s}$. Bland Altman statistics were used to investigate the observed trend in the difference in $t_{1/2}$ between the two methods with increasing measurement magnitude. The mean (± s.d.) bias between the techniques for estimation of gastric $t_{1/2} = 2.18 \pm 0.59$ h. Mean difference and the 95% C.I. and 95% limits of agreement were plotted against average value for $t_{1/2b}$ and $Lt_{1/2s}$ (Figure 3.13). Mean inter-modal difference in $t_{1/2}$ increased significantly with increasing measurement magnitude ($P < 0.01$). The relationship between mean bias and measurement amplitude was investigated further by linear regression, and the line of best fit and its 95% confidence intervals are shown in Figure 3.14. The 95% limits of agreement for this mean difference were calculated as follows: $D = 1.288 + 0.354A \pm 1.96$ x s.d. where D = Difference, A = Average, and s.d. = standard deviation of the mean difference (± 0.621).

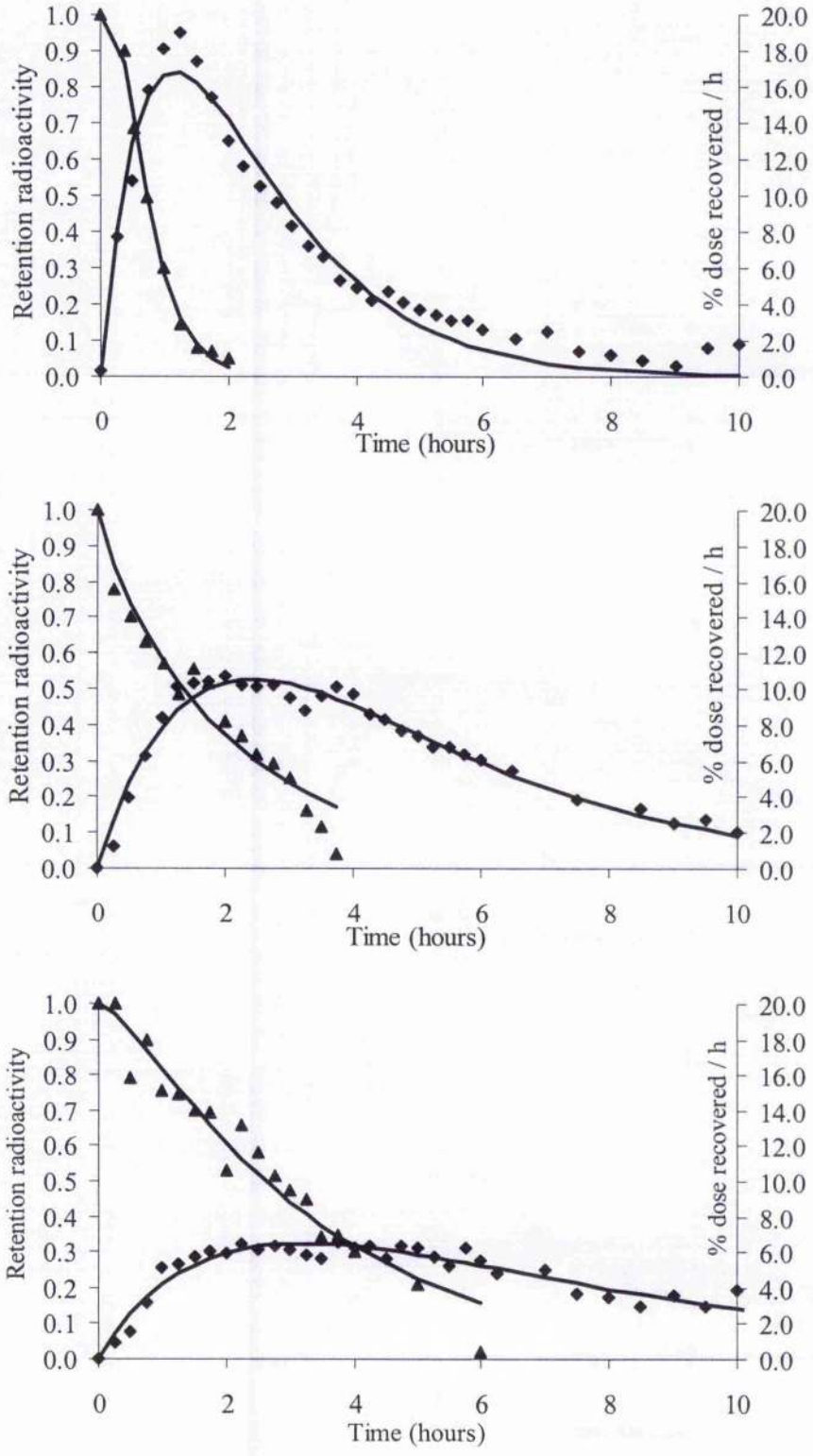


Figure 3.11 Results of simultaneous ^{13}C-octanoic acid breath test and gastric radioscintigraphy in 3 typical cases: (Top) Rapid gastric emptying rate in subject 1920 (scintigraphic $t_{1/2}$ = 0.73 h; t_{lag} = 0.55 h); (Middle) Modal gastric emptying rate in subject 1928 (scintigraphic $t_{1/2}$ = 1.34 h; no lag phase); (Bottom) Slow gastric emptying pattern in subject 1894 (scintigraphic $t_{1/2}$ = 2.62 h; t_{lag} = 0.96 h). Scintigraphic data (▲) on the left *y*-axis and breath test data (♦) on the right *y*-axis are plotted against time. The continuous lines represent the modelled data, calculated using the non-linear regression formulae described in section 2.6.3.

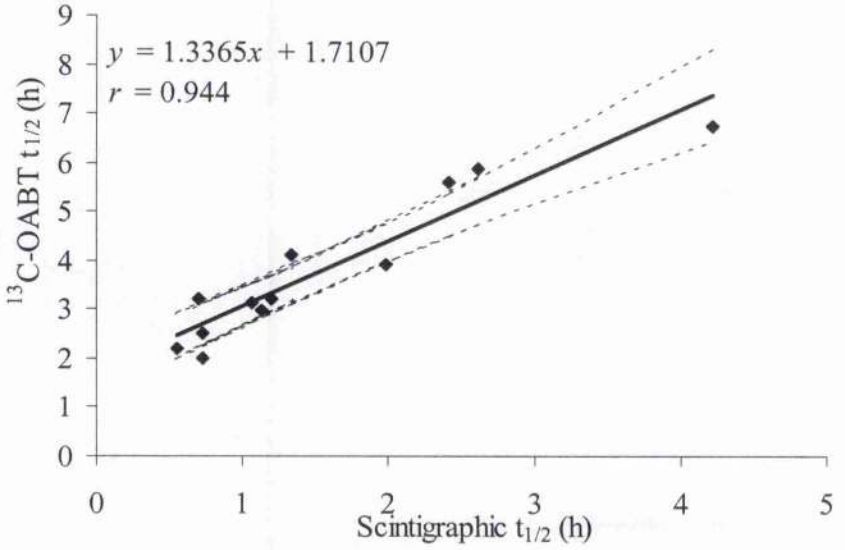


Figure 3.12 Comparison of gastric half-emptying times ($t_{1/2}$) determined by ^{13}C-octanoic acid breath test with those determined by scintigraphy in all individuals (n = 12). The best-fitting linear regression model is shown, together with its 95% confidence intervals. There is a close correlation between the modalities for calculation of $t_{1/2}$: $r = 0.944$; $P < 0.001$.

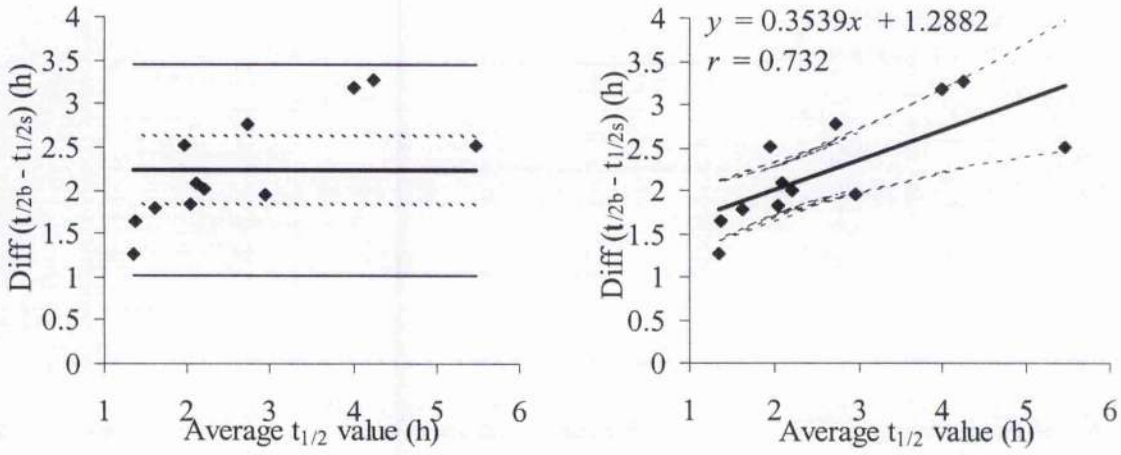


Figure 3.13 (left) Traditional Bland Altman plot showing inter-modality difference in $t_{1/2}$ estimation (breath $t_{1/2}$ – scintigraphic $t_{1/2}$) against mean value. The mean difference in $t_{1/2}$ estimation is plotted, together with the 95% confidence intervals (dotted lines) and 95% limits of agreement (continuous lines).

Figure 3.14 (right) The data presented in Figure 3.13 are re-plotted, highlighting the positive correlation observed between mean inter-modal bias ($t_{1/2b} - t_{1/2s}$), and increasing magnitude of $t_{1/2}$. A significant correlation is shown between these parameters: $r = 0.732$, $P < 0.01$. The equation of the best regression line is given, and its 95% confidence intervals. Mean bias is shown to increase significantly with increasing measurement magnitude.

The correlation between the two techniques for determination of lag phase duration was not significant when the results from all individuals were compared. However, when those scintigraphic curves (3/12) with $\beta < 1$ (indicating rapid early gastric emptying) were excluded, there was a highly significant correlation between the techniques for estimation of this parameter (n = 9, $r = 0.932$, $P < 0.001$). The best line of linear correlation between scintigraphic and ^{13}C-OABT for estimation of gastric lag phase, and its 95% C.I., is shown in Figure 3.15. A significant negative correlation was also found between breath test GEC and $Lt_{1/2s}$ (n = 12, $r = -0.930$, $P < 0.001$), as shown in Figure 3.16. Further significant correlations were found between $t_{1/2b}$ and scintigraphic geometric mean (GM) $t_{1/2}$ (n = 12, $r = 0.89$, $P < 0.001$), and between GEC and $GMt_{1/2s}$ (n = 12, $r = -0.81$, $P < 0.001$).

3.3.2.4 *Duodenal instillation and recovery (DIR) of ^{13}C-octanoic acid*

The effect of bypassing the gastric emptying stage in the production of $^{13}CO_2$, by intraduodenal gastroscopic administration of ^{13}C-octanoic acid, is shown in Figures 3.17 and 3.18. Following DIR, $t_{1/2}$ for absorption and metabolism of the isotope is similar in each case, despite the large difference in $t_{1/2b}$ (gastric emptying) observed in each subject; mean (± s.d.) DIR $t_{1/2} = 1.70 \pm 0.10$ h. In the third DIR subject, $t_{1/2}$ for recovery of the isotope was more prolonged ($t_{1/2} = 2.28$ hours), but the recovery curve described a gastric emptying pattern, suggesting misplacement or retrogradal movement of the isotope rather than DIR. As anticipated, the given $t_{1/2}$ for post-gastric absorption and metabolism of the isotope was therefore close to the point of intersection of the *y* axis presented in Figure 3.12.

3.3.2.5 *Deconvolution of the gastric emptying function*

A mathematical model ('deconvolution') has been described by which it is possible to separate the postgastric processing of octanoic acid after ingestion of a standard solid test meal from the rate-limiting gastric emptying component (Ghoos 1996; Maes *et al.* 1998; Geypens 2000). Using the deconvolution process, real-time gastric emptying curves may be obtained from ^{13}C-OABT isotope recovery curves, provided that comparable DIR data are also available. This approach has the advantage of allowing evaluation of the gastric emptying rates, rather than the amount of food emptied, as a function of time (flow curves). In those subjects with both ^{13}C-OABT and DIR data, the gastric emptying components were separated out by deconvolution, and compared to those gained from scintigraphy.

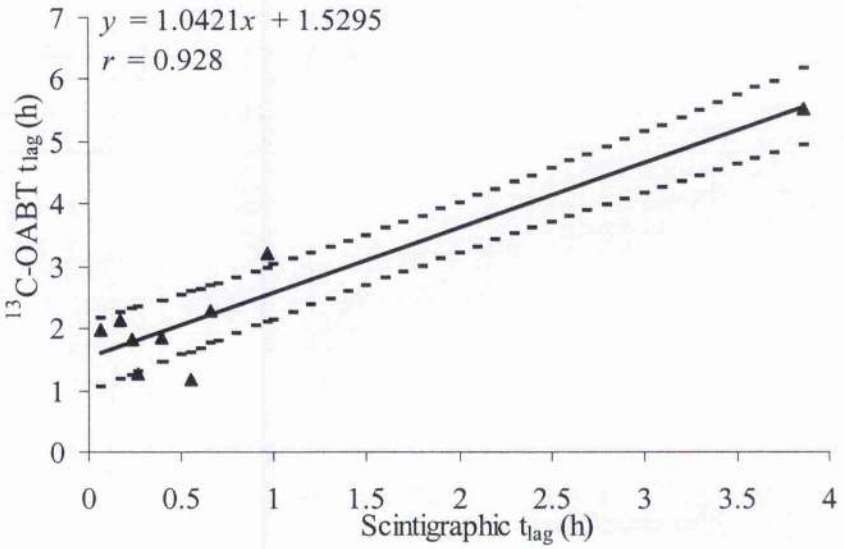


Figure 3.15 Comparison of gastric lag phase estimates (t_{lag}) determined by ^{13}C-octanoic acid breath test with those determined by scintigraphy. Those individuals that had immediate gastric emptying, without discernible scintigraphic lag phase, have not been included in the analysis (n = 3/12). The best-fitting linear regression model is shown, together with its 95% confidence intervals: r = 0.928; P < 0.001.

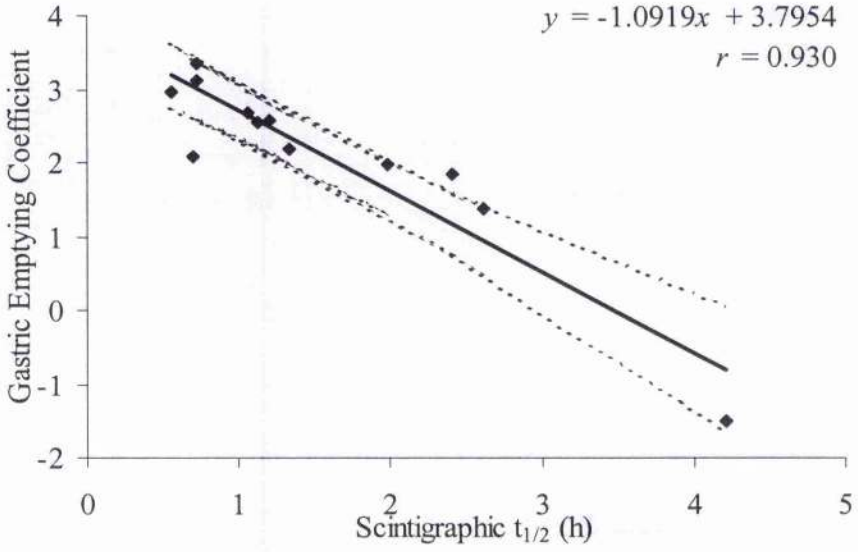


Figure 3.16 Correlation between the gastric emptying coefficient (GEC) derived from the overall shape of the ^{13}C-tracer recovery curve (Maes' formula) after the ^{13}C-OABT and the scintigraphic measurement of $t_{1/2}$ (n = 12). There is a significant negative correlation between the data sets. The best regression line and 95% confidence intervals are plotted: r = 0.930; P < 0.001.

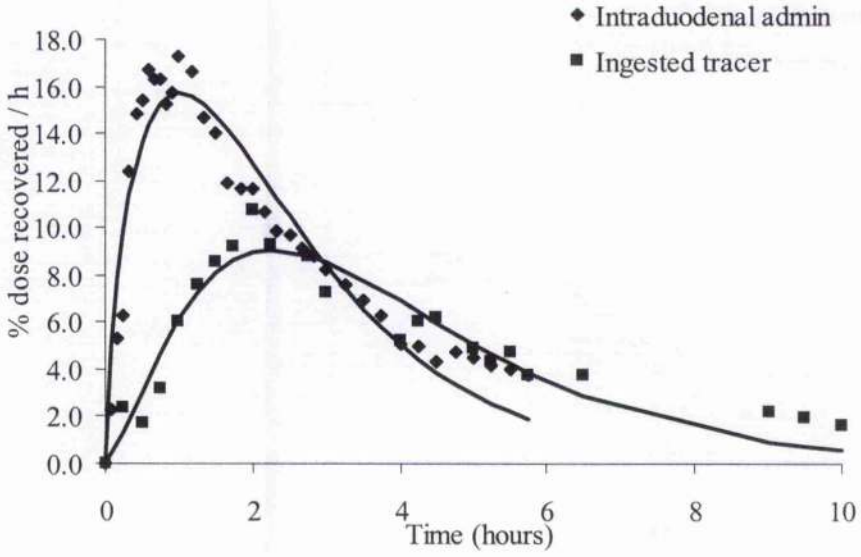


Figure 3.17 Expiratory recovery of $^{13}CO_2$ in subject 1929, following either direct intraduodenal administration of ^{13}C-octanoic acid (♦) or ingestion of the labelled test meal (■). The continuous lines represent the best modelled fit of the data, generated by Maes' formula. Following the ^{13}C-OABT, gastric $t_{1/2}$ = 3.36 h. Following duodenal instillation and recovery (DIR) of the ^{13}C-isotope in this individual, $t_{1/2}$ = 1.97 h.

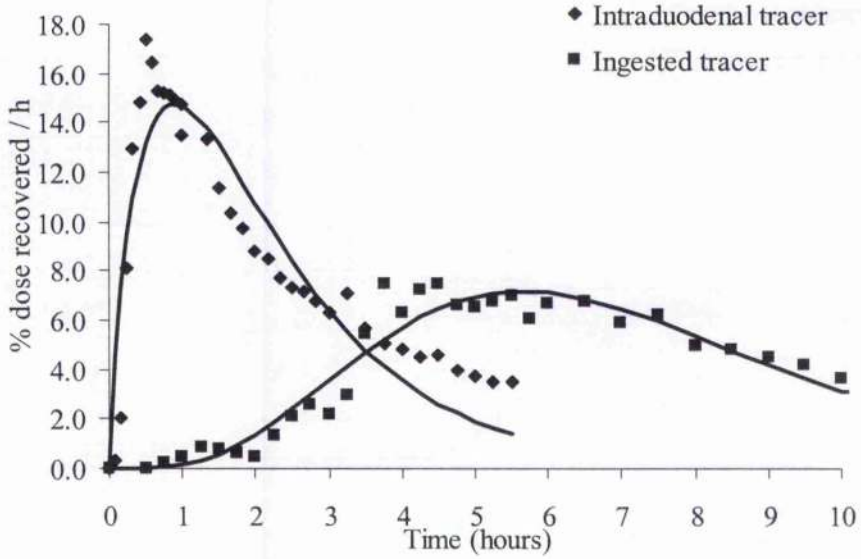


Figure 3.18 Expiratory recovery of $^{13}CO_2$ in subject 1889, following either direct intraduodenal administration of ^{13}C-octanoic acid (♦) or ingestion of the labelled test meal (■). Maes' formula has been used to model the acquired data (continuous lines). Following the ^{13}C-OABT, gastric $t_{1/2}$ = 6.55 h. Following duodenal instillation and recovery (DIR) of the ^{13}C-isotope in this individual, $t_{1/2}$ = 1.76 h.

In the model described by Maes *et al.* (1998), the emptying rate of a labelled solid meal from mouth to pylorus is given by M(t); the rate of postgastric processing (absorption, metabolism and expiration) of the label is given by D(t); and the global process of CO_2 excretion after ingestion of the labelled solid meal is given by T(t). Simplifying, at time t after ingestion of the labelled meal, total $^{13}CO_2$ production is given by equation 3.3:

$$T(t) = \int_0^t D(t - t_0)M(t_0)dt_0 \qquad \textbf{Equation 3.3}$$

where t_0 = zero time (i.e. $^{13}CO_2$ output before test meal ingestion). T(t) is provided by the results of the ^{13}C-OABT and D(t) is gained from DIR data, allowing real-time calculation of M(t) by the given integration process. The function M(t) was deconvoluted for 2 individuals with comparable ^{13}C-OABT and DIR data, and the real-time gastric flow rate curves provided by this function are shown in Figures 3.19 and 3.20. It can be seen that in subject 1929 there was an extremely rapid, early passage of ingesta from the stomach (Fig 3.19), whilst in subject 1889 the process of trituration and gastric emptying of the labelled test meal was slower and more prolonged (Fig 3.20). For completion, the real-time breath test M(t) functions for each subject were also compared to the concurrently measured scintigraphic retention of radioactivity in those individuals. This was achieved by plotting the inverse cumulative M(t) ^{13}C-cumulative recovery for each individual against time, as shown in Figure 3.21. In both cases, there was homology between the scintigraphic and ^{13}C-OABT M(t) retention profiles. Scintigraphic $t_{1/2}$ was also numerically close to the deconvoluted M(t) $t_{1/2}$ for each subject.

3.3.2.6 Comparison of ^{13}C-OABT GE indices derived from breath and blood

In 7 of the subjects, breath and venous blood samples were collected concurrently after ingestion of the ^{13}C-labelled test meal. A previous description of the general comparability of individual equine blood and breath samples for measurement of ^{13}C:^{12}C ratio has been given in section 2.7.2. In this case, a comparison was made of the breath- and blood-derived estimations of gastric $t_{1/2}$ and t_{lag} for each individual. As shown in Figures 3.22 and 3.23, highly significant correlations were found between the different media for calculation of these parameters (r = 0.998 and r = 0.992; $P < 0.001$), which approximated parity. The equation for the best linear regression line and its 95% confidence intervals is shown in each Figure.

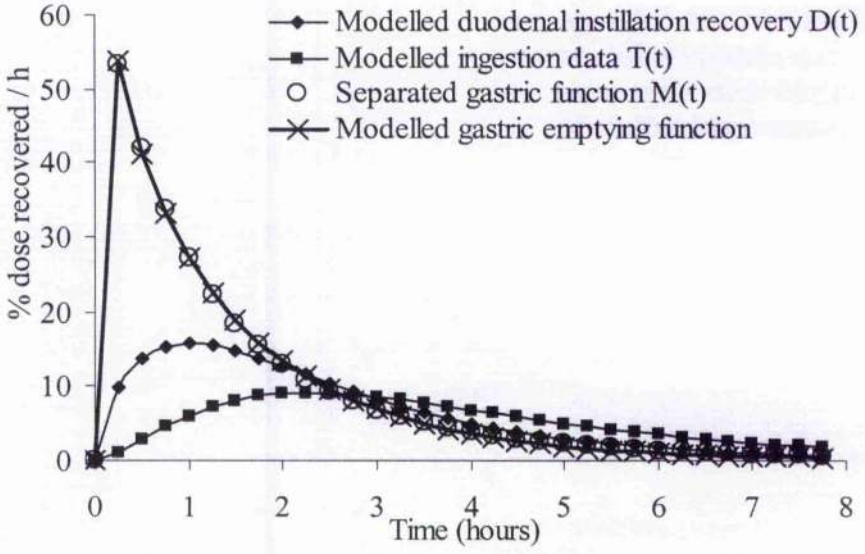


Figure 3.19 Deconvolution of gastric emptying function, M(*t*), in subject 1929. The $t_{1/2}$ values for each function are as follows: duodenal instillation and recovery, D(*t*), $t_{1/2}$ = 1.97 h; ingestion of labelled test meal, T(*t*), $t_{1/2}$ = 3.36 h; separated gastric emptying function, M(*t*), $t_{1/2}$ = 0.88 h. The M(*t*) $t_{1/2}$ is close in numerical value to that obtained from the left lateral scintigraphic study (scintigraphic $t_{1/2}$ = 0.70 h).

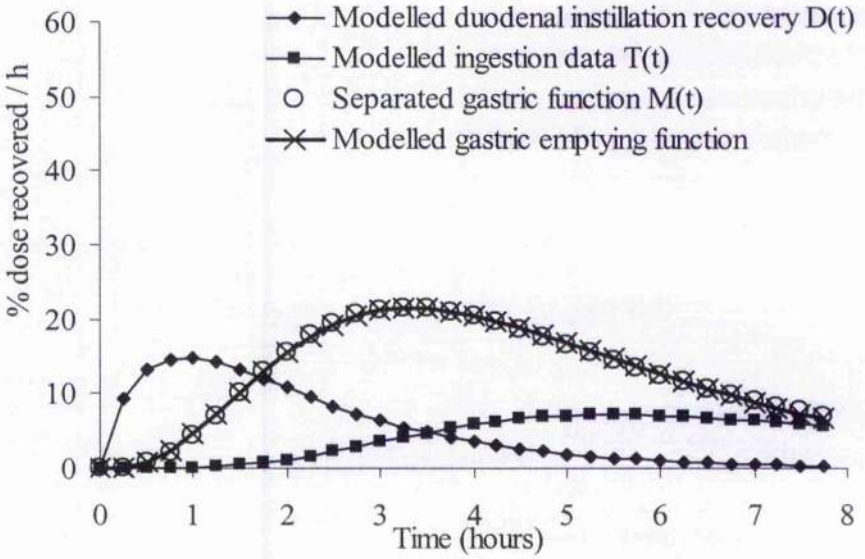
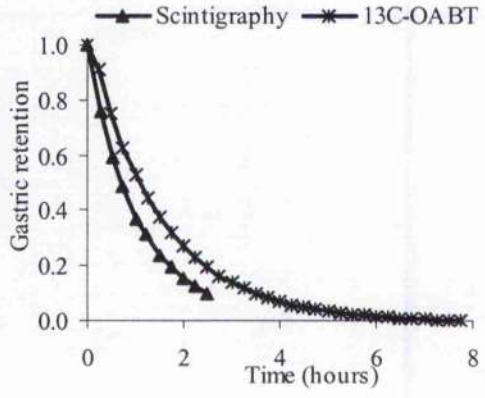


Figure 3.20 Deconvolution of gastric emptying function, M(*t*), in subject 1889. The $t_{1/2}$ values for each function are as follows: duodenal instillation and recovery, D(*t*), $t_{1/2}$ = 1.76 h; ingestion of labelled test meal, T(*t*), $t_{1/2}$ = 6.55 h; separated gastric emptying function, M(*t*), $t_{1/2}$ = 4.37 h. The M(*t*) $t_{1/2}$ is close in numerical value to that obtained from the left lateral scintigraphic study (scintigraphic $t_{1/2}$ = 4.21 h).



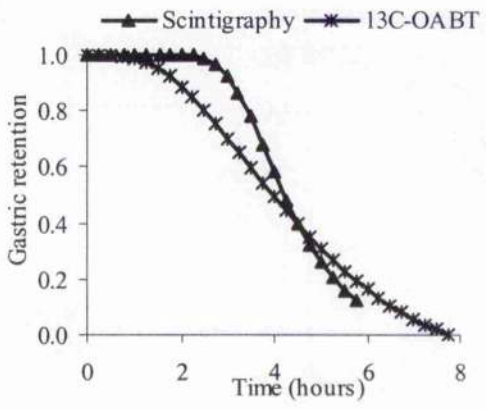
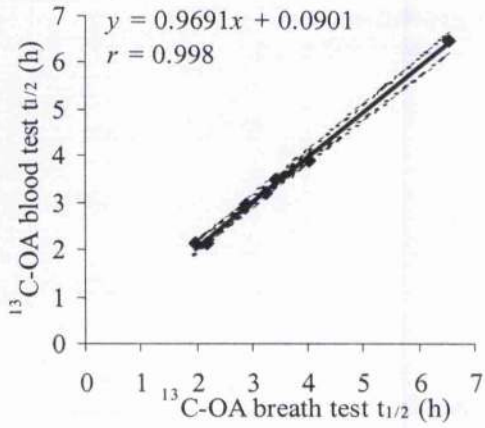


Figure 3.21 Gastric retention of the labelled test meal as determined by either scintigraphy or deconvolution of the ^{13}C-OABT results. The ^{13}C-deconvolution data have been converted to gastric retention by plotting the inverse cumulative ^{13}C-recovery data. The left Figure shows the combined results for subject 1929: scintigraphic $t_{1/2}$ = 0.70h; the separated M(t) function of the ^{13}C-OABT has $t_{1/2}$ of 0.88 h. The results for subject 1889 are presented on the right: scintigraphic $t_{1/2}$ = 4.21 h; $t_{1/2}$ of the deconvoluted ^{13}C-OABT M(t) function = 4.37 h.



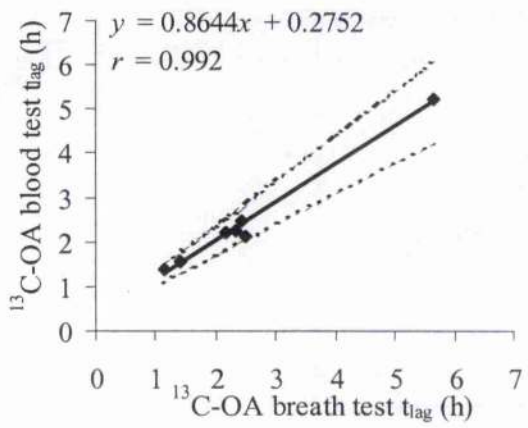


Figure 3.22 (left) Comparison of estimates of gastric $t_{1/2}$ (h) derived from the ^{13}C-OABT using either simultaneously collected breath or blood substrates in 7 healthy individuals. The best linear regression line is shown, and its 95% confidence intervals (dotted lines). There is a highly significant correlation between the two data sets, approximating equivalence (r = 0.998, P < 0.001).

Figure 3.23 (right) The correlation between the same data sets (n = 7) for estimation of t_{lag} (h) is shown, together with the 95% confidence intervals. Once again there is an excellent correlation between the breath and blood media for calculation of the parameter: r = 0.992, P < 0.001.

3.3.2.7 *Effect of fear on equine gastric emptying rate*

Although somewhat anecdotal, the gastric emptying data of 1 individual are worthy of further note. Subject 1925, a 3 y.o. arab mare, became progressively agitated during the course of the study and 2.50 h after test meal ingestion, was walked out of the nuclear medicine room. After 10 min, the subject relaxed and was returned to the scintigraphy room to resume the study. The ^{13}C-OABT and scintigraphic emptying data for this individual are shown in Figures 3.24 and 3.25. Two discrete peaks were seen in $^{13}CO_2$ recovery in this individual. Similarly, the loss of gastric radioactivity was not linear, but exhibited a plateau between 1.50 – 2.50 h, with loss of radioactive ingesta only resuming after this period. It was considered that the fear experienced by the horse during this period may have been responsible for inhibiting the gastric emptying of the labelled ingesta.

3.3.3 Discussion

In this study concurrent radioscintigraphic and ^{13}C-OABT measurement of gastric emptying rate in 12 healthy equine subjects revealed a highly significant positive correlation between the two techniques for the calculation of several parameters of solid phase gastric emptying. The correlation between the gastric half-emptying times derived from the two modalities was linear in nature, with a positive bias in the breath test that increased in size with increasing amplitude of measurement. This mean positive bias was similar in size to the empirical $t_{1/2}$ observed for absorption and metabolism of ^{13}C-octanoic acid after intraduodenal administration. Hence, the rate-limiting step in equine $^{13}CO_2$ production after ingestion of the ^{13}C-labelled test meal appeared to be gastric emptying of the solid phase label into the small intestine.

Deconvolution of the ^{13}C-OABT data (Ghoos 1996; Maes *et al.* 1998; Geypens 2000) allowed production of the real-time gastric flow curves for 2 individuals. The inverse cumulative M(t) function was found to be directly analogous to the scintigraphic gastric retention curves for these individuals, with similar $t_{1/2}$ values, providing further evidence that the ^{13}C-OABT provides an effective indirect measurement of equine gastric emptying rate. Some of the concerns of previous research groups (Choi *et al.* 1997) regarding the variability of octanoic acid absorption and metabolism were therefore addressed by the results of this study.

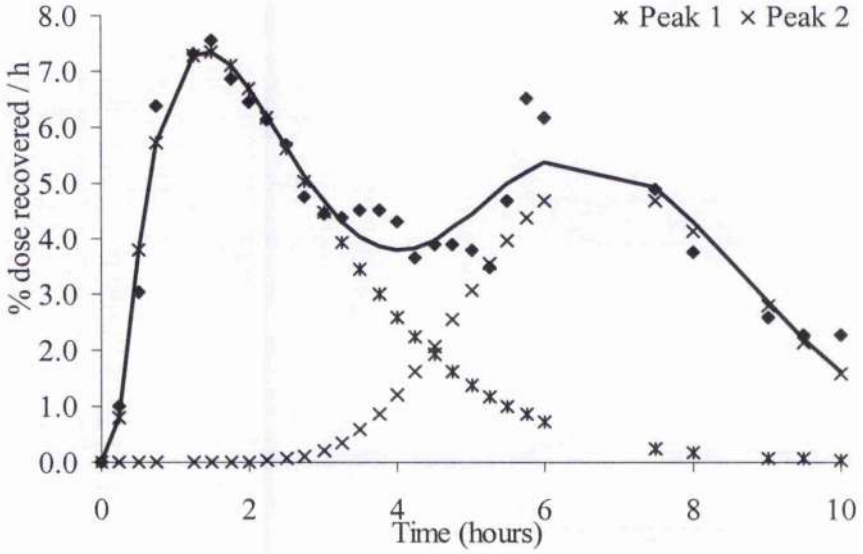


Figure 3.24 ^{13}C-OABT in 3 y.o. arab mare (subject 1925): biphasic expiratory recovery of $^{13}CO_2$ following ingestion of the labelled test meal, possibly caused by stress-induced disruption of gastric emptying.

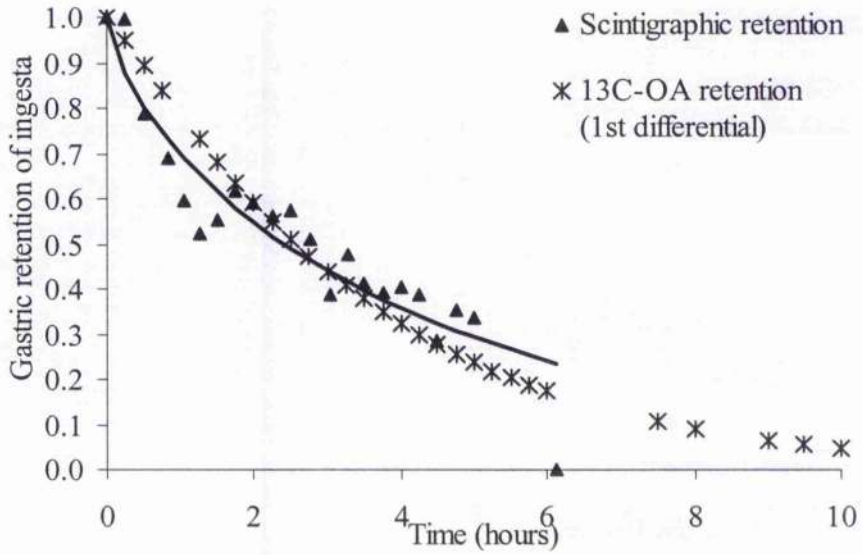


Figure 3.25 Gastric retention of labelled test meal in subject 1925, as determined by both radioscintigraphy and ^{13}C-OABT. A discrete plateau is seen in the loss of radioactive material from the stomach. The gastric retention of ^{13}C-octanoic acid-labelled ingesta has been modelled using the inverse of the first derivative of the cumulative dose recovery curve, and is similar to the modelled scintigraphic retention.

Venous blood was proved to be a suitable alternative medium to breath for ^{13}C:^{12}C analysis, when processed as described. This may enhance the clinical utility of the ^{13}C-OABT in equine medicine should there be reluctance to collect breath for analysis. Plasma and whole blood ^{13}C analysis has been performed previously in dogs, cats and cattle (Metges *et al.* 1990; Cornetta *et al.* 1998; Moeller *et al.* 2001). Similar to this study, Cornetta *et al.* (1998) showed that blood analysis in dogs produced indices that were directly comparable to expiratory breath samples (when used for the ^{13}C-urea breath test).

The pilot ^{13}C-OABT studies performed at Glasgow confirmed that the pattern of expiratory ^{13}C enrichment after ingestion of the developed labelled test was similar in healthy horses to that previously reported in ponies (Wyse 1999) and man (Ghoos *et al.* 1993). However, before undertaking the validation study it was necessary to study the effect of habitual diet on baseline $^{13}CO_2$ production, to determine whether this affected its stability under test conditions after ingestion of the developed test meal. As noted above, endogenous production of $^{13}CO_2$ may be affected by substrate intake/diet (Wolfe *et al.* 1984; Morrison *et al.* 2000), physiological state and metabolic fuel source (Schoeller *et al.* 1984; Wolfe *et al.* 1984) as well as fluctuations in VCO_2 for other reasons (Kalivianakis *et al.* 1997; Amarri *et al.* 1998). As predicted, the group of horses maintained on alfalfa hay (test conditions) had a constant basal production of $^{13}CO_2$ after ingestion of the unlabelled test meal. The selected test meal components of oats and wheat bran have ^{13}C contents approximating those of alfalfa (1.0922 and 1.0930 atom % ^{13}C respectively (Morrison *et al.* 2000) versus 1.0943 atom % ^{13}C (Svejcar *et al.* 1993)). However, the horses maintained on Bermuda grass had a higher mean basal $^{13}CO_2$ output than the alfalfa group, which decreased in the 10-hour period after ingestion of the test meal. As the C_4 plant Bermuda grass has a relatively high natural abundance of ^{13}C (1.1020 atom % ^{13}C; Waller and Lewis 1979), metabolism of the test meal itself is likely to have been responsible for the shift in background ^{13}C-abundance in this group (Schoeller *et al.* 1980). The downward shift in basal ^{13}C output may also have been caused by a shift from glycogenolysis to gluconeogenesis during the test period as the ^{13}C-rich hepatic glycogen stores became depleted, due to the prior period of food deprivation (Tanis *et al.* 1998). In view of this, it is recommended that when performing equine ^{13}C-OABTs a test meal of equivalent ^{13}C:^{12}C ratio to maintenance diet should be used, with general avoidance of foods particularly heavy in ^{13}C. Should this not be practical, then an increased tracer dose of up to 1.5 mg/kg ^{13}C-octanoic acid could be used in the test meal to minimise possible errors in calculation of the gastric emptying indices.

Gastroscintigraphy is widely regarded as the optimum standard for measurement of equine gastric emptying. However, in the few scintigraphic studies reported, liquid phase emptying (Lohmann *et al.* 2000, 2002), or a combination of liquid and solid phase emptying after gastric intubation (Sojka and Cantwell 1988; Neuwirth 1994; Ringger *et al.* 1996) has been performed, and a standard protocol for measurement of solid phase gastric emptying after voluntary ingestion of a standard test meal has not been established. Ringger *et al.* (1996) used geometric mean measurements to calculate scintigraphic half-emptying time, and this is usually the case in small animal (Goggin *et al.* 1998) and human (Moore *et al.* 1988; Siegel *et al.* 1989; Ghoos *et al.* 1993) studies. But, in this present study, imaging of the equine right gastric region was often difficult and considered to be inaccurate, as reported previously by Lohmann *et al.* (2000, 2002). Right lateral gastric images lost detectable radioactivity well in advance of the left gastric region, and were occasionally obscured by overlying loops of radioactive small intestine. In addition, $t_{1/2}$ values calculated from the right gastric images were poorly correlated to those from the left side. Therefore scintigraphic gastric emptying indices calculated from the left region of interest were used preferentially for comparison with ^{13}C-OABT data (Bahr *et al.* 2001).

Equine scintigraphic solid phase gastric $t_{1/2}$ has been reported to vary from mean 0.82 h (Neuwirth 1994) to 1.50 h (Levy and Sojka 1991; Ringger *et al.* 1996). The mean value in this study (1.56 h ± 1.08, range 0.56 - 4.21) was comparable to that of the latter two research groups. However, direct comparison cannot be made due to the different nature of the test meal and conditions. For production of future reference values, the adoption of a standard protocol and test meal is essential. The coefficient of variation reported here for $t_{1/2s}$ (69.23%) is higher than for most human studies, but comparable to that of Levy and Sojka (1991) who gained a CV% of 53.92% in horses using a similar test meal. Interindividual CV%s for $t_{1/2b}$ (40.37%) and GEC (60.48%) were also higher than the first reported equine values derived from ponies (Wyse 1999), and most reported human values (Ghoos *et al.* 1993; Duan *et al.* 1995; Delbende *et al.* 1998). Given the range in age, weight and type of horse used here, the wide variation in gastric $t_{1/2}$ was not unexpected. However, not one of these individual factors was found to correlate significantly with gastric $t_{1/2}$ in this study. This is in contrast to human studies, where both body weight and body surface area have been reported to have an inverse linear relationship with gastric emptying rate (Lavigne *et al.* 1978). Further information is required on basic aspects such as the relation of equine gastric volume itself to body mass before such compounding factors can be considered further.

The apparent effects of psychological stress on equine gastric emptying rate, as noted in subject 1925 of this study, were unexpectedly dramatic. Whilst physical stress has previously been suggested to have an influence on equine GE, psychological factors have generally been considered less important. By contrast, there is evidence for the involvement of psychological factors in the aetiopathogenesis of human gastric ulceration (Lloyd 1993). In a radionuclide case-control gastric emptying study in healthy young adults, Roland *et al.* (1990) found that mild mental stress significantly delayed gastric emptying, caused mainly by prolongation of the lag phase. Their data also suggested that gastric emptying was reactivated once the stress period had ended, as seen in subject 1925 in this study. Experiments in dogs have closely reproduced the above findings; Mistiaen *et al.* (2002) showed that transport to an unfamiliar environment was sufficient to cause a significant delay in scintigraphic gastric emptying in beagle dogs due to decreased antropyloral motor activity and prolongation of the lag phase. Having established a non-invasive means of measuring GE rate in healthy horses, further investigation of the possible effects of psychological factors on GE rate, and their possible involvement in the aetiology of the gastric ulceration syndrome may be justified.

The effects of a variety of physiological stress factors on gastric emptying rate, such as severe thermal strain (Neufer *et al.* 1989) and maximal exercise at above 70% maximal heart rate (Marzio *et al.* 1991) have also been investigated in man, and shown to reduce liquid phase gastric emptying. Given that the prevalence of gastric ulceration has been reported to approach 93% in Thoroughbreds in race training, with the severest lesion scores noted in those that have raced in the previous 2 months (Murray *et al.* 1996), further research on the possible influence of modified gastric emptying rate in the actiopathogenesis of this disorder is now urgently required.

One further interesting phenomenon observed during the scintigraphic studies in 58% (7/12) of the subjects was an occasional increase in the total radioactive counts in the left region of interest at successive time points. This was thought to have been caused by retrogradal peristaltic flow of radioactive material from the small intestine into the stomach, and provides further evidence that reflux of duodenal contents into the stomach occurs regularly in the healthy horse (Kitchen *et al.* 2000). In fact, the scenario of an empty stomach with high acid content and low pH, coupled with mucosal bile salts injury, and perhaps further complicated by reduced gastric motility, has to be considered a strong contender for the aetiopathogenesis of nonglandular gastric ulcer disease of the mature horse (Berschneider *et al.* 1999).

The ^{13}C-octanoic acid breath test has been validated against radioscintigraphy for the measurement of solid phase gastric emptying in humans and is now in regular clinical use for the investigation of upper gastrointestinal tract disorders (Ghoos *et al.* 1993; Veereman-Wauters *et al.* 1996; Choi *et al.* 1998; Delbende *et al.* 1998, 2000; Perri *et al.* 1998; Van Den Driessche *et al.* 1999). In one of the first papers to report its validation for use in humans, Ghoos *et al.* (1993) found a very similar relation between breath test data and gastric scintigraphy as found in this present study. To allow conversion of breath test $t_{1/2b}$ to scintigraphic $t_{1/2s}$, these researchers introduced a 'correction factor' of 1.10 h which was shown to be similar to the observed $t_{1/2}$ for absorption and metabolism of ^{13}C-octanoic acid after intraduodenal administration in eight healthy individuals (mean = 1.03 h). Using the equine data generated here, a mean correction factor of 2.18 h could be used to convert breath test $t_{1/2}$ to scintigraphic $t_{1/2}$ if required. However, establishment of a standard protocol and reference range for equine ^{13}C-OABT gastric indices should allow this diagnostic modality to be used in isolation (see Chapters 5 and 9).

In summary, the data presented in this Chapter revealed a significant correlation between radioscintigraphy and the ^{13}C-OABT for the measurement of solid phase gastric emptying in healthy animals ($P < 0.001$), using both breath and blood media for isotopic analysis. Before routine clinical use of the ^{13}C-OABT in equine medicine, further validation of the test was required to ensure that gastric emptying remained the rate-limiting step in the expiratory excretion of $^{13}CO_2$ in individuals with ileus or significant gastroparesis. The validation of the ^{13}C-OABT for the quantitation of atropine-delayed gastric emptying in adult horses is described in Chapter 4. The results of this present Chapter suggested that the ^{13}C-OABT may have potential application in the investigation of conditions such as gastroduodenal ulceration, post-operative ileus, chronic grass sickness and duodenitis/proximal jejunitis. Because the ^{13}C-OABT is non-invasive and safe, it may be used to perform serial gastric emptying studies in an individual, or to investigate the gastrointestinal effects of specific drugs in a humane manner. As discussed by Lee *et al.* (2000) and Horowitz *et al.* (2002), there is also the possibility that the ^{13}C-OABT may prove useful for screening tests and for large scale epidemiological studies that may lead to enhanced knowledge of (equine) gastric physiology.

Chapter 4 QUANTITATIVE DETECTION OF ATROPINE - DELAYED GASTRIC EMPTYING IN THE HORSE BY THE ^{13}C- OCTANOIC ACID BREATH TEST

4.1 Introduction

Delayed gastric emptying may be involved in the pathogenesis of many important conditions in the horse, such as gastric ulceration, equine dysautonomia, post-operative ileus, gastric impaction and idiopathic recurrent colic, and can be difficult to diagnose clinically. A safe, non-invasive, quantitative test for the measurement of this parameter, that could be used repeatedly in clinical cases, would be of great benefit in equine gastroenterology (Merritt 1997). Previous methods that have been used to assess solid phase GE in the horse have not given direct physiological information, being based on transit measurements of plastic beads at *post mortem* (Adams and MacHarg 1985), or radiographic tracking of radiopaque markers (Baker and Gerring 1994). Gastric radioscintigraphy following intubation with radiolabelled solids (Sojka and Cantwell 1988; Levy and Sojka 1991; Neuwirth 1994; Ringger *et al.* 1996) is considered to be the optimum diagnostic technique, but has the disadvantages of radiation exposure and the requirement for expensive equipment and user expertise. Acetaminophen absorption has been validated in the horse for the measurement of liquid phase emptying, using radioscintigraphy (Lohmann *et al.* 2000). However, solids and liquids empty from the stomach by different mechanisms and rates (Parkman *et al.* 1995), and measurement of solid phase emptying is preferable for clinical purposes.

Having validated the ^{13}C-OABT against scintigraphy for the measurement of solid phase GE in Chapter 3 (Sutton *et al.* 2003), the objective of the work here was to investigate the ability of the test to quantitate equine delayed GE, prior to its clinical application for this purpose. Despite the findings of Choi *et al.* (1997), a number of human studies have found that the test provides gastric function results that are correlated to a predicate method in patients with mild delays in emptying rate or even gastroparesis (Ghoos *et al.* 1993; Duan *et al.* 1995; Veereman-Wauters *et al.* 1996; Ziegler *et al.* 1996; Choi *et al.* 1998; Delbende *et al.* 1998; Van Den Driessche *et al.* 1999; Ritz *et al.* 2001).

An example of the potential diagnostic value of the ^{13}C-OABT for the measurement of solid phase GE in horses presenting with abdominal pain is provided by the following case summary. An 8 y.o. Thoroughbred gelding was presented to the Glasgow University Veterinary School with a history of chronic abdominal pain of 48 h duration. In the previous month, the horse had exhibited weight loss and poor appetite, despite worming

and additional feeding. Symptoms deteriorated after hospitalisation such that an exploratory laparotomy was performed, with the only abnormal finding being massive, doughy distension of the stomach. A primary gastric impaction was diagnosed, and trans-serosal saline injection and digital manipulation were used to reduce the mass.

In order to investigate the aetiology of the gastric impaction, a ^{13}C-OABT was performed 9 days after surgery to measure gastric emptying rate. The test was performed according to the standard protocol outlined in section 2.4.4. The results are shown in Figure 4.1 (i). Gastric emptying rate was significantly delayed, with a prolonged triturative phase (t_{lag} = 6.30 h) and slow half-emptying time ($t_{1/2}$ = 7.03 h). None of the extrinsic factors previously linked to gastric impaction, such as ingestion of fibrous grasses or straw (Owen *et al.* 1987), persimmon seeds or mesquite beans (Honnas and Schumacher 1985; Kellam *et al.* 2000), irregular water supply or poor dentition (Owen *et al.* 1987) were present in this case. It was hypothesised that primary or secondary gastric dysmotility was involved. Although it has been suggested that intrinsic factors such as defective gastric secretion or gastric atony may be implicated in the aetiology of primary impaction (Owen *et al.* 1987) this has not been confirmed to the author's knowledge. The horse was managed conservatively and re-examined after 6 weeks. A ^{13}C-OABT performed at this time had an unusual appearance (Figure 4.1 (ii)), but overall emptying rate had improved (t_{lag} = 1.37 h, $t_{1/2}$ = 4.24 h), allowing further decisions about case management to be made. Further details of this case are provided in Appendix II.

However, before making definitive diagnoses, or conclusions about equine gastric physiology or pharmacological intervention based on the results of the ^{13}C-OABT, it was necessary to prove the validity of the test for solid phase emptying measurement in individuals proven to have a delayed emptying rate. This was required to ensure that the basic premise of the test, namely gastric-emptying being the rate-limiting step in expiratory recovery of the tracer after its ingestion, still held true in individuals with gastric dysmotility. The major objective of this Chapter was to compare the ^{13}C-OABT against the optimum standard of radioscintigraphy in horses with significantly delayed gastric emptying, using a model of atropine-induced gastroparesis. In this way, it was hoped to validate the ^{13}C-OABT for the quantitative diagnosis of equine gastroparesis.

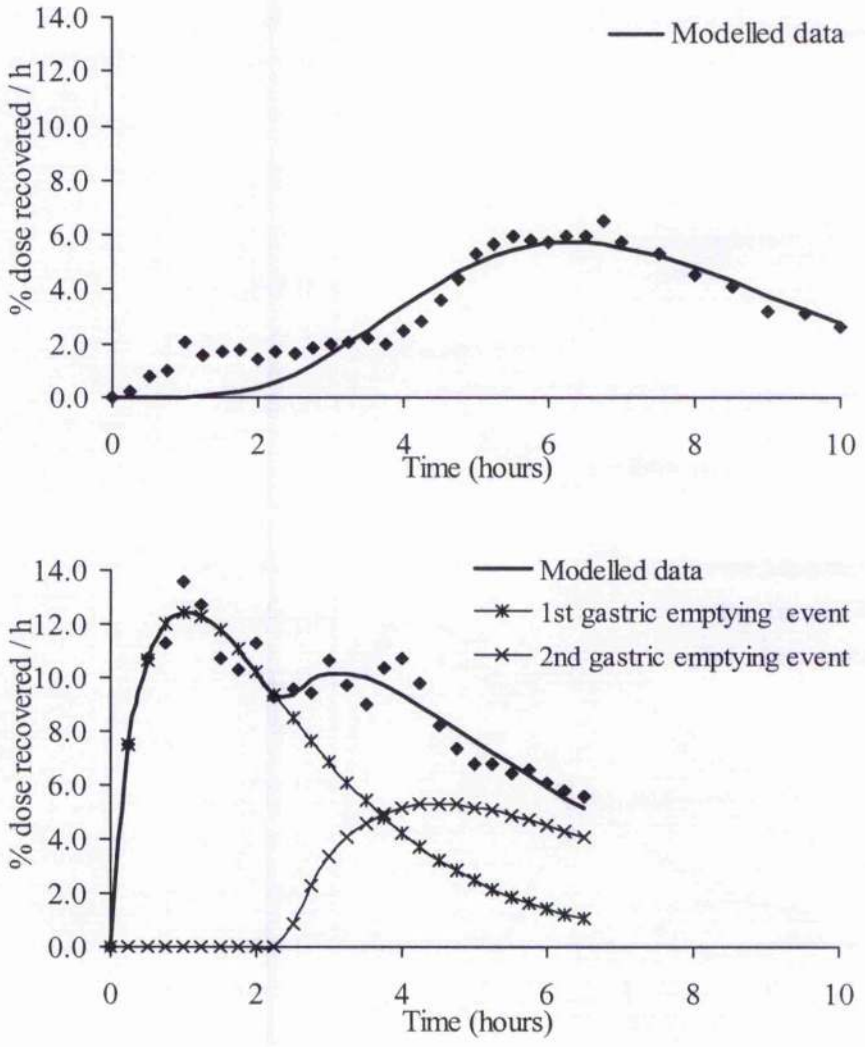


Figure 4.1 (i) Gastric emptying profile generated by the ^{13}C-OABT in an 8 y.o. Thoroughbred gelding (case 138250), 9 days after presentation with, and treatment for, a gastric impaction (top). The % dose recovery of the administered ^{13}C isotope is plotted on the *y*-axis against time. Gastric emptying is delayed: half-emptying time ($t_{1/2}$) = 7.03 h; gastric lag phase (t_{lag}) = 6.30 h; Gastric Emptying Coefficient (GEC) = -3.16.

(ii) ^{13}C-OABT results in the same horse 6 weeks after receiving treatment for the impaction (bottom). The ^{13}C-isotope recovery is best fitted by a dual peak model, possibly due to a second gastric emptying event. The overall rate of gastric emptying is improved compared with (i): $t_{1/2}$ = 4.24 h; t_{lag} = 1.37 h; GEC = 2.67.

4.2 Materials and Methods

4.2.1 Subjects

Eight adult horses (5 Quarter Horses, 3 Thoroughbreds) from the Texas A&M University research herd were used in this study, with a median age of 11.5 years (mean 13.3, range 7 to 25 years) and a median body weight of 520.0 kg (mean 507.4, range 424.5 to 530.0 kg). These animals had no historical or physical evidence of gastrointestinal disease and biochemical and total blood cell parameters lay within the reference range. All subjects were maintained outdoors on a low ^{13}C diet of *ad libitum* alfalfa hay only for at least 2 weeks prior to each experiment, to ensure constant basal metabolic production of $^{13}CO_2$. This management regimen had been shown previously to result in a steady basal output of $^{13}CO_2$ under resting test conditions (see Figure 3.10).

4.2.2 Study design and protocol

Gastric emptying rate of a standard test meal was measured twice in each horse, after randomised treatment with either atropine or saline. The intravenous bolus of saline or atropine was administered via a preplaced jugular catheter, immediately after ingestion of the test meal was completed, and the catheter flushed with heparinised saline. A ^{13}C-OABT was performed on each occasion, together with a concurrent radioscintigraphic study when GE was delayed. Paired tests were separated by a period of at least 7 days. A standard intravenous dose of atropine of 0.035 mg/kg bodyweight was used to induce gastroparesis.

Atropine dosage was determined by a preliminary pilot study, in which the minimum standard dose necessary to produce a significant delay in scintigraphic GE rate, without causing apparent abdominal pain, was investigated. Analysis of reference scintigraphic data collected in a separate experiment from healthy horses in the same population (see Chapter 3) showed that it would be necessary in the present study to extend scintigraphic $t_{1/2}$ to at least 3.20 h (mean + 2.58 x s.d.) in order to delay emptying rate significantly. This cut-off point was selected to be rather high, such that the delay induced in emptying rate was unequivocal. In the reference population of healthy adults, less than 0.50% of individuals would have been expected to have solid phase gastric emptying $t_{1/2}$ above this cut-off point. In the pilot study, a dose of 0.025 mg/kg atropine was insufficient to cause this delay, but the higher dose of 0.035 mg/kg bodyweight was found to be effective.

The study was designed to allow paired comparison of gastric function data after randomised saline or atropine administration, using the ^{13}C-OABT. The design also

allowed correlation of the breath test technique with scintigraphy for the measurement of GE in horses known to have a significant delay in emptying rate. The study was approved by the Texas A&M University Laboratory Animal Care Committee (Animal Use Protocol 2001-35).

4.2.3 Test meal composition

The test meal consisted of 150 g crimped oats, 100 g bran, 200 ml water and two dual-labelled egg yolks. For the combined test, approximately 1 mg/kg ^{13}C-octanoic acid (Octanoic acid-1-^{13}C, Isotec Inc., Miamisburg, OH, USA) and 20 mCi ^{99m}technetium sulphur colloid (CIS-US inc., Bedford, MA, USA) were added to two egg yolks, which were baked in a microwave oven until firm and thoroughly mixed into the test meal. The radiopharmaceutical was omitted when the breath test alone was performed. Food was withdrawn 14 h before the start of the test to ensure that the stomach was empty.

4.2.4 ^{13}C-octanoic acid gastric emptying test

4.2.4.1 Breath sampling protocol

Expiratory air was sampled using a modified Aeromask® (Trudell Medical International, Ontario, Canada) and 250 ml Quintron® breath collection bag (QuinTron Instrument Company, Wisconsin, USA) with a unidirectional valve. This technique was rapid and well tolerated, and has been validated previously as described in sections 2.4.1 and 2.8. Breath samples were stored in duplicate in 10 ml Exetainer® tubes (Labco Ltd., Buckinghamshire, UK), prior to stable isotope analysis. Three basal breath samples were collected 60, 15 and 0 min before test meal ingestion (-60, -15, 0 min), and thereafter at 15 min intervals for 8 h, then 30 min intervals for a further 4 h.

4.2.4.2 Blood sampling protocol

Venous blood samples were taken from a preplaced jugular catheter. An initial 5 ml aliquot of blood was removed from the catheter before withdrawing the 1 ml sample required for analysis, and flushing the catheter. Taking care to avoid air bubbles, the 1 ml of blood was injected into a 10 ml evacuated glass tube (Vacutainer®, Becton Dickinson, NJ, USA), and either acidified immediately by the addition of 2 ml of 6 molar hydrochloric acid, or after a period of storage. After addition of the acid, the sample was vortexed, and the contents then allowed to settle before subsequent mass spectrometric analysis of the resulting headspace gas. This method of processing, and the effect of storage on the quality of sampling results has been described in detail in sections 2.7.1 to 2.7.3

4.2.5 Measurement of sample $^{13}CO_2$ content

A continuous flow IRMS (PDZ Europa Ltd, Cheshire, UK) was used to measure the total CO_2 content and ^{13}C abundance of each sample, by comparison with a calibrated 5% CO_2 in nitrogen standard gas. Poor expiratory samples of below 0.5% CO_2 were rejected for data analysis. The $^{13}CO_2$:$^{12}CO_2$ ratio of each sample was measured relative to the international limestone standard, and expressed as the $\delta^{13}C$ value:

$$\delta_x = ((R_x - R_s)/R_s)*1000 \qquad \textbf{Equation 4.1}$$

where R_s and R_x are the ^{13}C:^{12}C atomic ratio of the standard and sample respectively. Quality control specimens were sampled after each 5 breath samples, with an acceptable s.d. for $\delta^{13}C$ of 0.2 per run. After subtraction of the average ^{13}C-abundance of the three baseline (predose) breath samples, the $\delta^{13}C$ ratio of each sample was converted to absolute units (parts per million excess ^{13}C). Data were then expressed as percentage dose recovery (PDR) of the administered isotope per hour. This latter calculation used the formula for resting adult standardbred and quarter horse VCO_2 described by Gallivan *et al.* (1989), derived from body mass, as given in equation 2.5 of section 2.5.2.

4.2.6 Gastric radioscintigraphy

Each subject was maintained in the nuclear medicine room for the duration of the test and serial left and right gastric scintigraphs were obtained at 15 min intervals, until the radioactive counts per 30 s interval in the region of interest had decreased to less than 10% of that at time zero. The first scintigraph was taken immediately after ingestion of the test meal and intravenous atropine injection had been completed. Using a dedicated nuclear medicine imaging computer (Nuclear Mac, Scientific Imaging, Colorado, USA), regions of interest were hand-drawn and the total counts in the gastric region recorded for each time point. These counts were decay-corrected to time 0, using the equation given previously in Chapter 3 (equation 3.1).

4.2.7 Calculation of gastric emptying indices

4.2.7.1 Radioscintigraphic test data

Counts in the left and right gastric regions of interest were plotted against time as the relative retention of radioactivity after decay correction. The geometric mean data set was also calculated, as the square root of the product of the left and right lateral counts. This measurement, based usually on anterior and posterior counts is used in human gastric emptying studies to correct for tissue attenuation of the radioactive counts (Halkar *et al.*

1999). Siegel's non-linear regression formula (Siegel *et al.* 1988) was then used to fit a modelled curve to the data:

$$y(t) = 1 - 1(1 - e^{-kt})^{\beta} \qquad \textbf{Equation 4.2}$$

where *y(t)* is the fractional meal retention at time *t* (expressed in h); *k* is the rate of exponential decay of the final phase of the curve or GE rate in h^{-1}; and β is a rate constant determined by the extrapolated y-intercept of the initial portion of the curve. As described in section 2.6.3.1, the best-fitting curve to the gastric radiation retention data was generated by minimisation of the root mean square of the residuals. From this fitted curve, the scintigraphic half-emptying time ($t_{1/2s}$) and gastric lag phase duration (t_{lags}) were derived. The formulae used for calculation of these parameters have been given previously in section 2.6.3.1. The left side data were used for standard measurement of scintigraphic emptying (Sutton *et al.* 2002), but the right side values were recorded for interest and the geometric mean values generated if there were obvious errors in use of the left side values alone.

4.2.7.2 ^{13}C-OABT data

Data were plotted as PDR / h against time, and once again, indices of gastric emptying were derived using the best fitting curve modelled using the non-linear regression formula of Ghoos *et al.* (1993):

$$y = at^{b}e^{-ct} \qquad \textbf{Equation 4.3}$$

where *y* is the percentage of the ^{13}C dose recovered in breath per hour; *t* is time in hours; and *a*, *b* and *c* are regression constants. The breath test gastric half-emptying time, $t_{1/2b}$, is equivalent to that time at which the area under the cumulative ^{13}C recovery curve demonstrates recovery of half the administered isotopic dose, and was calculated as given in section 2.6.3.2 (equation 2.13). The duration of the lag phase, t_{lagb}, prior to the onset of maximal gastric emptying rate was also calculated, using equation 2.14, and the gastric emptying coefficient (GEC) was determined as a general index of the shape of the GE curve (see equation 2.15). Further detail about the modelling techniques has been given in sections 2.6.3.1 and 2.6.3.2.

4.2.8 Statistical Analyses

A paired *t*-test was used to determine the effect of randomised atropine versus saline administration on mean breath test $t_{1/2b}$. Where assumption requirements were met, two-

sample *t*-tests were used to compare means in independent groups of observations. A Wilcoxon rank sum test was used for this purpose when there was inequality of variance. Significance was set at $P < 0.05$ in each case.

4.2.8.1 Validation of ^{13}C-OABT versus scintigraphy

The relationships between the GE indices obtained by the two diagnostic modalities were evaluated by Pearson correlation and then linear regression. The relationship between $t_{1/2b}$ and $t_{1/2s}$ was investigated for linearity, normal distribution and variability of the residuals. The S.E. (mean Y given X) and 95% C.I. of the regression line were determined using the formula of Bland (1995). Bland Altman statistics were used to evaluate the mean bias between the two techniques for measurement of gastric $t_{1/2}$ (Bland and Altman 1995; Bland and Altman 1999) and 95% confidence limits were established.

4.2.8.2 Validity of the ^{13}C-OABT for detection of delayed gastric emptying

The concurrent scintigraphic and breath test GE data collected in this study were pooled with comparable data from healthy untreated individuals in the same population, which had been collected and reported previously (see Chapter 3; Sutton *et al.* 2003). The combined population ($n = 21$) showed a normal distribution for $t_{1/2}$ measurement. A cut-off point was determined for scintigraphic $t_{1/2s}$ in the untreated group (Mean + ($t_{0.005}$ x s.d.)) above which $t_{1/2s}$ was described to be 'delayed'. This cut-off point for definition of 'delayed' versus 'normal' gastric emptying was chosen to be relatively high, such that individuals falling above the cut-off (approximating less than 0.05% healthy individuals in the normally distributed population group) had relatively marked delays in emptying rate. The sensitivity and specificity of the breath test for detection of delayed gastric emptying in the entire data set (as directly compared to concurrent scintigraphy) was then investigated. Standard receiver operating characteristic (ROC) curves and two-graph ROC (TG-ROC) curves (Greiner 1995; Greiner *et al.* 2000) were constructed to enable selection of the best ^{13}C-OABT $t_{1/2}$ cut-off time for optimal diagnostic sensitivity and specificity of delayed gastric emptying.

As the normal ('negative') GE set was not normally distributed, a non-parametric minimisation of misclassification plot (MCT) was constructed (Greiner 1995) to assist in selection of the optimum cut-off point. The MCT plot allows the costs of both false-negative and false-positive results to be minimised:

$$MCT = (1 - p)(1 - Sp) + rp(1 - Se) \qquad \textbf{Equation 4.4}$$

where p = prevalence of delayed gastric emptying; Sp = specificity; Se = sensitivity; and r = costs (false-negative result)/(costs (false-positive result)). In this study, false-negative and false-positive costs were weighted equally (r = 1). Using a combination of these techniques, the optimum ^{13}C-OABT $t_{1/2}$ cut-off time, and its confidence values, were determined for detection of delayed GE in the given population.

4.3 Results

4.3.1 Effect of atropine on gastric emptying rate

The effects of randomised intravenous atropine (0.035 mg/kg) versus saline administration on exhaled tracer recovery in eight horses, following ingestion of the ^{13}C-octanoic acid labelled test meal, are presented in Figure 4.2. The combined mean PDR/h is plotted for each time point of both sample sets, together with its standard deviation (*y*-axis error bars). It is seen that the dose of atropine used caused a dramatic prolongation of ^{13}C-OABT solid-phase half-emptying time. The mean (± s.d.) breath test $t_{1/2}$ values for the atropine and saline treatments were 6.76 (± 1.65) and 2.52 (± 0.35) h respectively. The differences between the paired observations were normally distributed and shown by a paired *t*-test to be significant: mean difference = 4.23 (± 1.60) h, n = 8, $P < 0.001$. The calculated 99% confidence interval (C.I.) range for the mean difference excluded zero (2.26 to 6.21 h), confirming that the ^{13}C-OABT was sufficiently sensitive to detect the atropine-induced delay in gastric emptying.

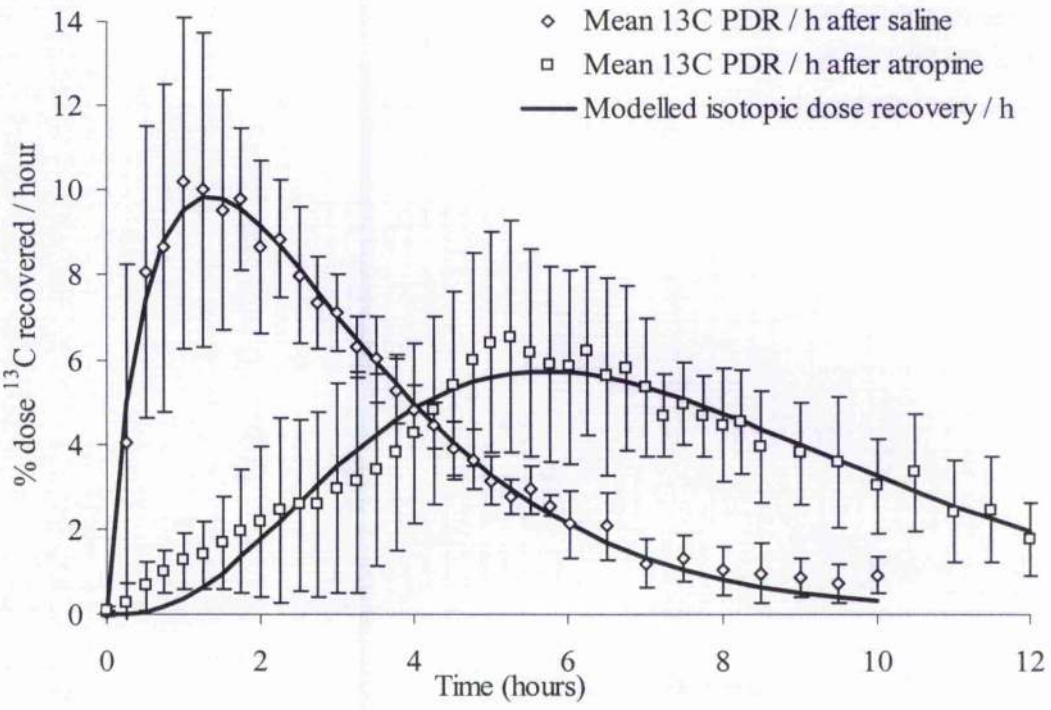


Figure 4.2 Mean percentage dose recovery (PDR / h) ± s.d. of ^{13}C in the breath after ingestion of the ^{13}C-octanoic acid labelled test meal at time 0, followed by immediate saline or atropine injection in 8 horses. The test was performed twice in each horse (n = 16) in randomised order, separated by an interval of at least one week. Mean saline and atropine $t_{1/2}$ values were significantly different (P < 0.001) with no overlap in the 99% C.I. range.

Table 4.1 Gastric emptying indices determined by concurrent radioscintigraphy and ^{13}C-OABT in 8 horses after ingestion of a standard dual-labelled meal, followed by induction of temporary gastroparesis using atropine 0.035 mg/kg.

Modality	*Parameter*	*Mean*	*s.d.*	*Range*	*Sample*	*CV%*
Gastric	$t_{1/2s}$ (h)	4.75	1.60	2.75 - 8.12	n = 8	33.68
radioscintigraphy	t_{lags} (h)	3.64	0.89	2.05 - 4.12	n = 7	21.98
^{13}C-OABT	$t_{1/2b}$ (h)	6.76	1.65	4.01 - 9.48	n = 8	24.41
	t_{lagb} (h)	5.63	1.22	3.17 - 6.91	n = 7	21.67

$t_{1/2s/b}$ = calculated gastric half-emptying time (scintigraphic / breath test); $t_{lags/b}$ = duration of the calculated lag phase (scintigraphic / breath test); CV% = coefficient of inter-individual variation.

Similarly, ^{13}C-OABT measurement of mean t_{lag} duration in the 8 individuals was significantly different between the atropine and saline treatments. The mean (± s.d.) breath test t_{lag} values for the atropine and saline treatments were 6.05 (± 1.65) and 1.51 (± 0.59) h respectively. The differences between the paired observations were also normally distributed and significantly different: mean difference = 4.19 (± 1.12) h, n = 8, $P < 0.001$. The calculated 99% confidence interval (C.I.) range for the mean difference excluded zero (2.49 to 5.89 h), confirming that there was no overlap in the range of emptying values for the two treatment groups.

The scintigraphic $t_{1/2}$ measurement (mean (± s.d.)) after atropine administration in this study was 4.75 (± 1.60) h (n = 8). A non-parametric Wilcoxon rank sum test showed that this value was significantly delayed relative to directly comparable scintigraphic data collected previously from 12 healthy untreated individuals in the same population and as presented in Chapter 3 ($P < 0.01$; Sutton *et al.* 2003). Following atropine treatment, mean (± s.d.) scintigraphic t_{lag} = 3.64 (± 0.89) h (n = 7). This parameter could not be measured from the scintigraphic data in 1 individual. The mean gastric emptying indices as measured by the two diagnostic modalities after atropine treatment are shown in Table 4.1.

Scintigraphic measurement of gastric radiation retention was most reliable from the left lateral view. Examples of 3 series of gastric scintigraphs in subjects with delayed emptying are shown in Figures 4.3 – 4.5. In all subjects, counts were higher in the left gastric region throughout the study, and total loss of radioactive material was first observed on the right side (Figure 4.3). Atropine treatment changed the shape of the gastric outline, resulting in prolonged retention of material in the proximal stomach, often with radioactive material visible in the distal oesophagus and cardia for the first hour (Figures 4.3 – 4.4). Movement of radioactive ingesta from both left to right and right to left was noted, suggesting incoordination of antropyloric contractions and reduced propulsion of ingesta.

4.3.2 Further effects of atropine treatment

Intravenous atropine administration was followed in all individuals by immediate absence or reduction of intestinal borborygmi, tachycardia and pupil dilatation. The tachycardia was used to monitor the effect of the given dose of atropine, with an average return to resting heart rate of approximately 2.5 h. Intestinal borborygmi returned to original frequency and volume approximately 10 h after atropine, and this was similar to the mean time to first defecation. Although there was increased stretching after atropine administration, no overt signs of abdominal discomfort were seen in any individual.

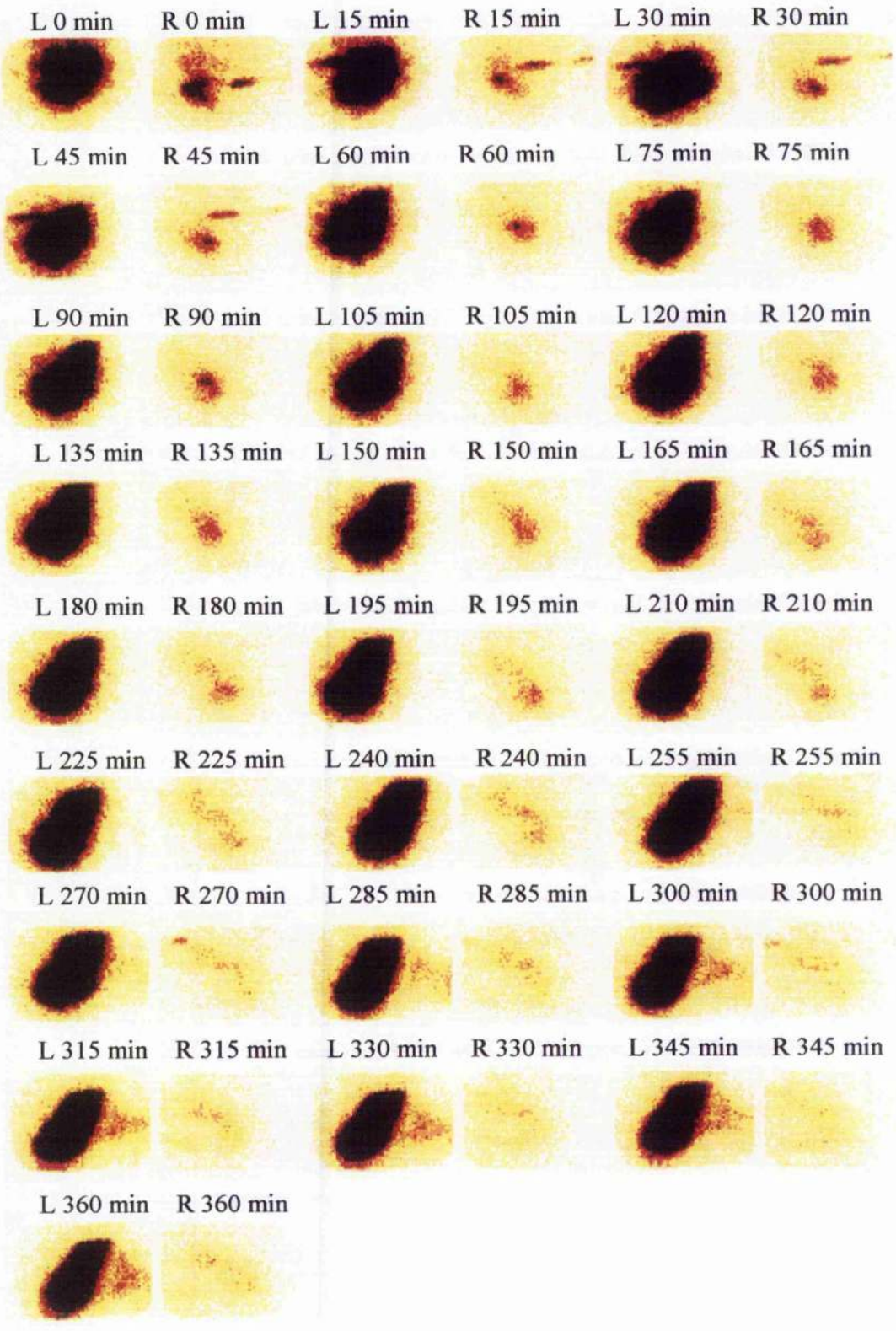


Figure 4.3 Scintigraphic gastric emptying study in subject 1949, after intravenous administration of atropine 0.035 mg/kg at time 0. Radioactive ingesta are visible in the distal oesophagus for the first 45 min after test meal ingestion. The left gastric region regains an elliptical shape from 135 min. Left scintigraphic $t_{1/2}$ = 3.43 h; t_{lag} = 2.97 h. The right gastric region is difficult to discern by 4 h after start of study.

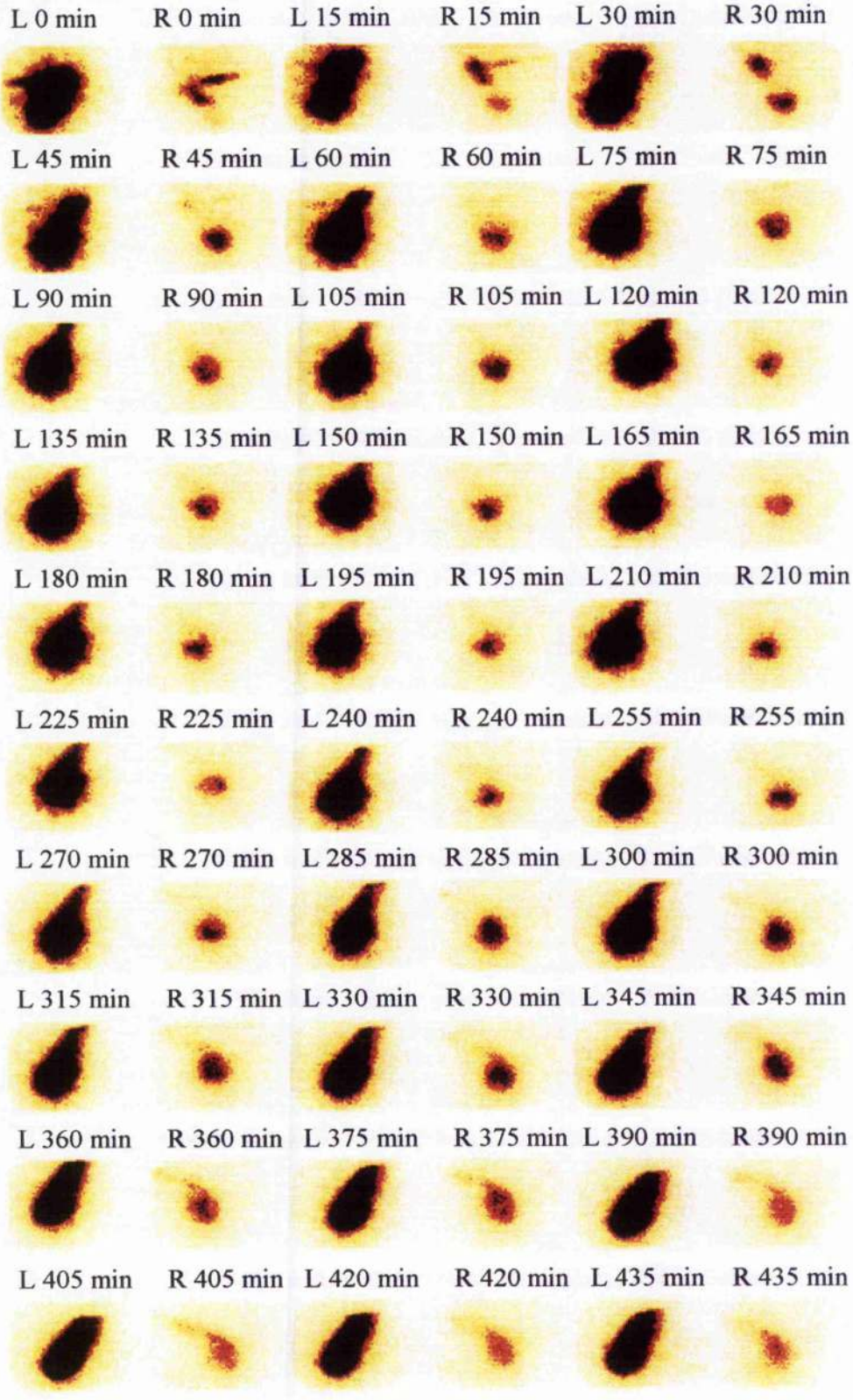


Figure 4.4 Left and right lateral gastric scintigraphic study in subject 1970. Two discrete doses of atropine (0.035 mg/kg i.v.) were given at 0 h and 1.75 h after test meal ingestion. There is clear movement of radioactive ingesta from right to left regions, as well as antropyloric movement (left to right). The *saccus caecus* region (proximal stomach) has prolonged retention of labelled ingesta, although there is some movement of material into the duodenum in the first 30 min. Geometric mean $t_{1/2}$ = 8.12 h; t_{lag} was not calculable.

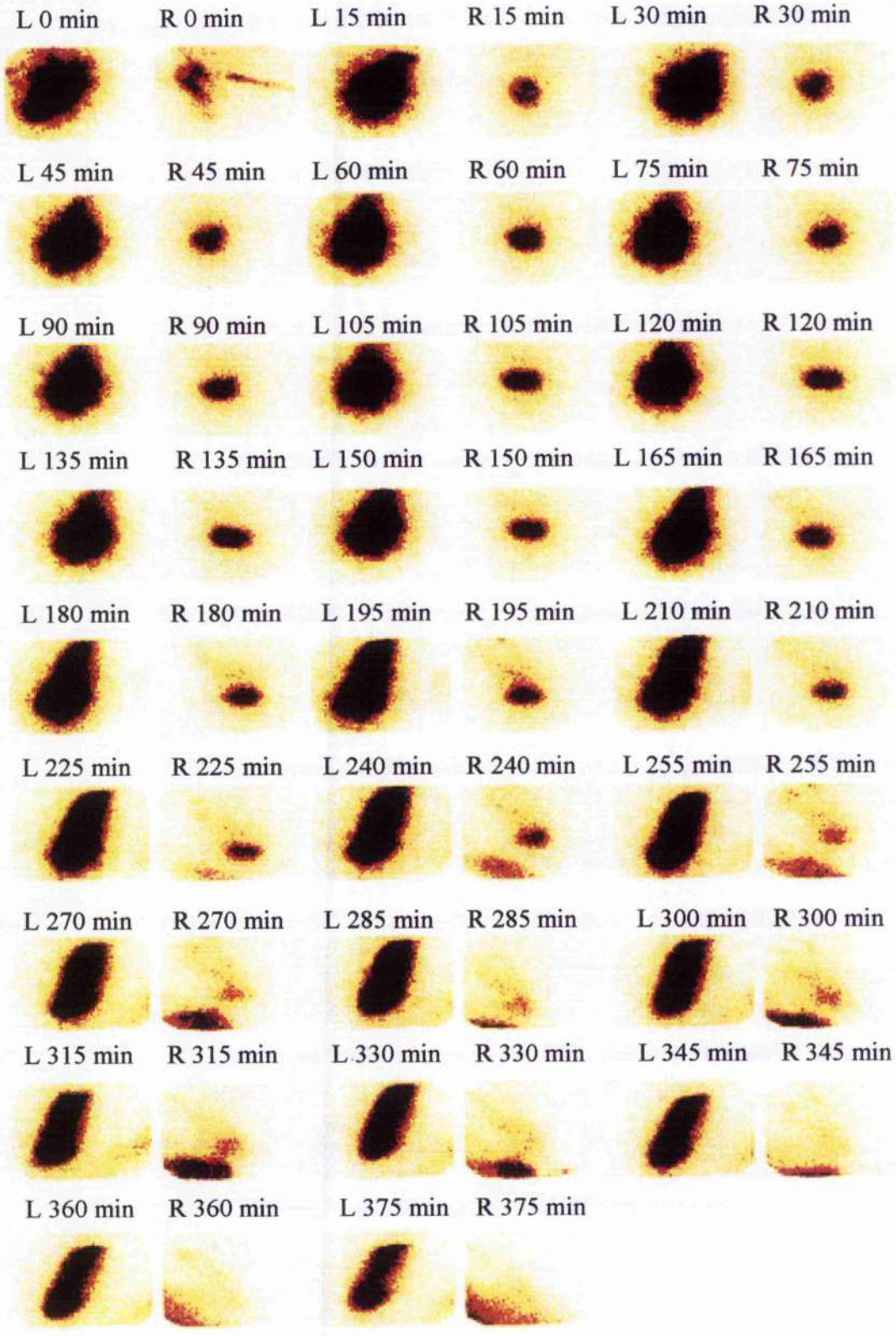


Figure 4.5 Serial left and right gastric scintigraphs in subject 1944 after intravenous administration of atropine (0.035 mg/kg) at time 0. There is prolonged retention of radioactive material in the pyloric antrum region (right side), suggesting reduced antropyloric contractions, following atropine treatment. The left gastric region becomes more elliptical in shape after 3 h as the atropine wears off, and the stomach begins to empty. Scintigraphic left $t_{1/2}$ = 4.51 h; left t_{lag} = 4.12 h.

4.3.3 Correlation between the ^{13}C-OABT and radioscintigraphy for the measurement of delayed gastric emptying

Two typical examples of the results of the combined scintigraphic/^{13}C-OABT study in equine subjects with atropine-induced gastroparesis are shown in Figures 4.6 and 4.7. It can be seen in subjects 1944 and 1970, for example, that the rate of recovery of $^{13}CO_2$ in the breath (^{13}C-OABT) is proportionate to the rate of decrease of gastric radioactivity (scintigraphy). In 6/8 individuals both diagnostic modalities revealed a lag phase before apparent passage of the dual-labelled meal into the small intestine (as presented in Figure 4.6). In these individuals, a constant rate of loss of gastric radioactivity succeeded the initial lag phase, and this was presented in the ^{13}C-OABT as a flattened bell-shaped curve. In the remaining 2/8 individuals, GE proceeded slowly from the start of the test without an obvious discrete scintigraphic lag phase, even after a second dose of atropine (0.07 mg/kg total) was given to these individuals. The combined scintigraphic/^{13}C-OABT results for each of the 8 subjects in the atropine-delayed emptying study are presented in Appendix III. The mean gastric emptying indices in the 8 subjects after atropine administration are presented in Table 4.1. In 7/8 individuals scintigraphic $t_{1/2}$ was calculated using images collected from the left side of the horse. In the remaining individual, it was necessary to use the geometric mean value (based on the square-root of the product of the left and right side counts) due to prolonged activity in the right region of interest; t_{lag} calculations were not included for this horse, due to modelling inaccuracies.

A linear relationship was present between the two techniques for calculation of $t_{1/2}$ (Figure 4.8), with a normal residual distribution, and a Pearson correlation coefficient of 0.867 (95% C.I. = 0.417 – 0.976, $P < 0.01$). The best linear regression line and its 95% upper and lower confidence intervals are shown in Figure 4.8. Although included in Figure 4.8, one outlying value was likely caused by underestimation of scintigraphic emptying, due to the mixing of gastric contents between left and right sides. If this individual is omitted, then the best linear equation for inter-modal $t_{1/2}$ comparison is given by equation 4.5:

$$Y = 1.640 + 1.029x \qquad \textbf{Equation 4.5}$$

with $r = 0.944$ (95% C.I. = 0.663 - 0.992), $n = 7$, $P < 0.001$.

For the 7 subjects in which it was calculable, a positive correlation was also found between the two techniques for measurement of t_{lag} : $r = 0.764$, $P < 0.05$.

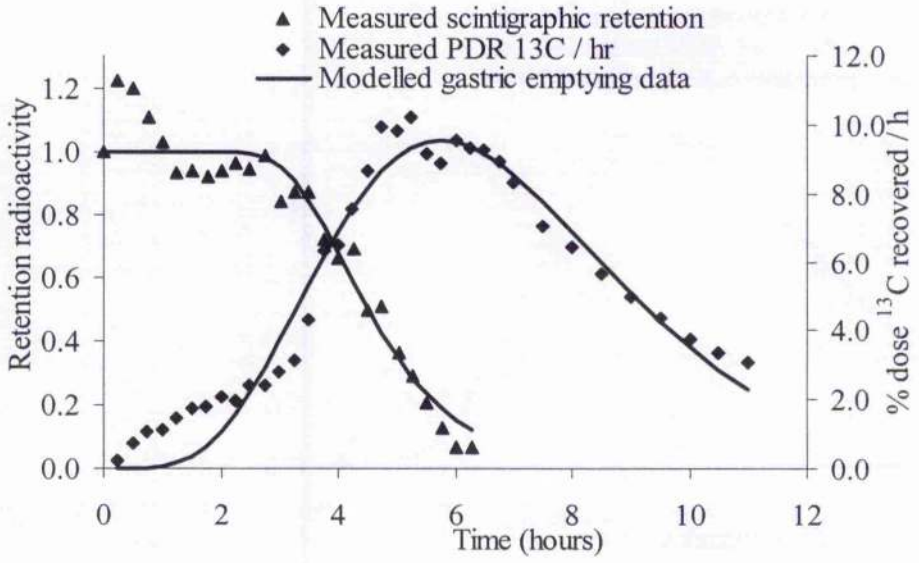


Figure 4.6 Simultaneous scintigraphic and ^{13}C-OABT gastric emptying study in horse 1944, a 12 y.o. QH gelding of body weight 519 kg, after intravenous administration of atropine 0.035 mg/kg bwt. Left scintigraphic $t_{1/2}$ = 4.51; ^{13}C-OABT $t_{1/2}$ = 6.48 h. Scintigraphic and breath test estimates of t_{lag} = 4.12 h and 5.77 h respectively.

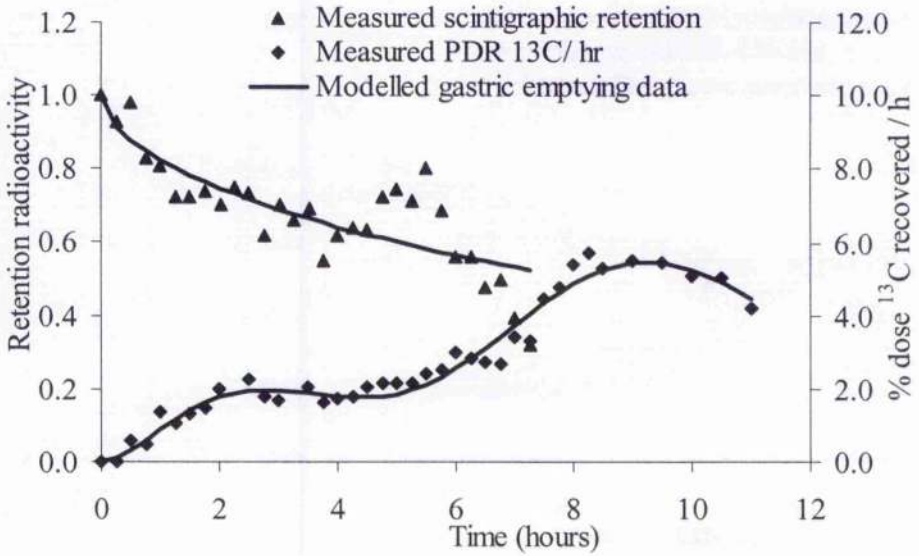


Figure 4.7 Markedly delayed gastric emptying in subject 1970, a 7 y.o. Thoroughbred gelding (body weight 530 kg) as demonstrated both by gastric scintigraphy (left *y*-axis) and ^{13}C-OABT (right *y*-axis). A biphasic expiratory recovery of the ^{13}C-tracer is seen, together with a biphasic loss of gastric radioactivity. Gastric emptying was delayed in this subject by administration of 2 doses of atropine 0.035 mg/kg at times 0 h and 1.75 h after ingestion of the labelled test meal. Geometric mean $t_{1/2s}$ = 8.12 h; $t_{1/2b}$ = 9.48 h. The lag phase duration could not be modelled by the power regression formula in this individual.

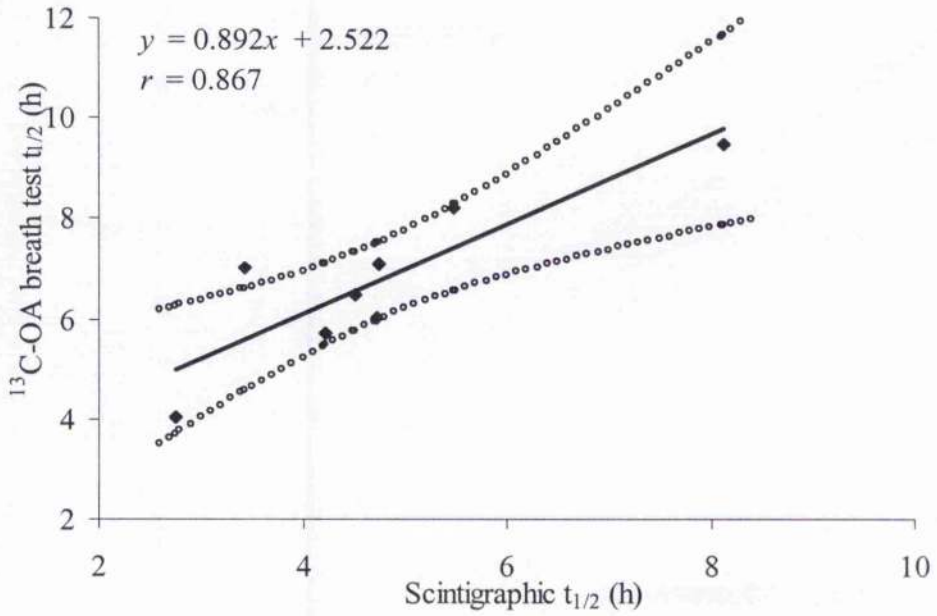


Figure 4.8 Comparison of gastric half-emptying times ($t_{1/2}$) determined by ^{13}C-octanoic acid breath test with those determined by scintigraphy after atropine administration at time 0 in all individuals (n = 8). The best regression line is shown, and the upper and lower 95% confidence limits are plotted. Pearson correlation coefficient = 0.867, P < 0.01.

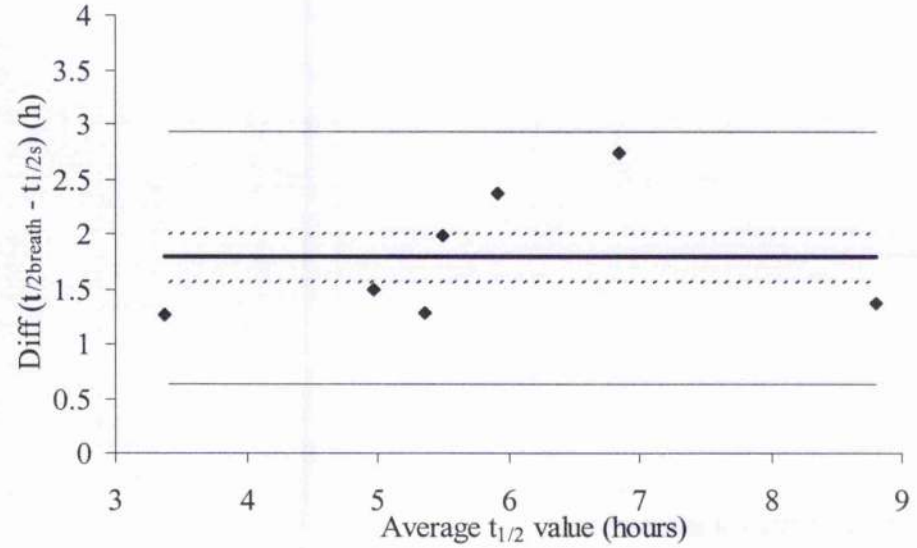


Figure 4.9 Bland Altman plot showing mean difference in estimate of $t_{1/2}$ for the two techniques plotted against average $t_{1/2}$ value. The mean difference (continuous line), 95% confidence intervals and the 95% limits of agreement for the mean bias are plotted. The outlier shown on the linear regression plot has been removed (n = 7). Mean (± s.d.) bias = 1.78 (± 0.59) h. 95% C.I. range for bias = 1.56 – 2.00 h, and 95% limits of agreement = 0.66 – 2.93 h.

Bland Altman statistics were used to investigate the nature of the mean bias between the techniques for measurement of $t_{1/2}$. After removing the single outlier shown in Figure 4.8, the mean difference was plotted against average combined value for $t_{1/2s}$ and $t_{1/2b}$, together with the 95% confidence intervals and 95% limits of agreement (Figure 4.9). The mean bias = 1.78 ± 0.58 h; 95% C.I. for mean bias = 1.56 – 2.00 h; 95% limits of agreement = 0.66 – 2.93 h; n = 7. After confirming normal distribution and homoscedasticity of the data, a two-sample *t*-test showed that the mean difference ($t_{1/2b}$ - $t_{1/2s}$) after atropine treatment was not significantly different in this study to that measured previously in 12 individuals from the same population with normal emptying rate (described in Chapter 3).

4.3.4 Measurement of GE indices from blood-derived ^{13}C:^{12}C ratio values

As described in section 4.2.4.2, blood samples were collected in synchrony with breath samples from each individual after ingestion of the test meal, and also analysed for ^{13}C:^{12}C content. Data from the blood samples were modelled and analysed in the same manner as the corresponding breath samples. Comparison of $t_{1/2}$ values derived from the ^{13}C-OA blood test to those of concurrent scintigraphy (n = 8) is shown in Figure 4.10. The observed correlation was similar to that seen between the ^{13}C-OA breath test and scintigraphy, with the best-fitting linear regression line given by:

$$Y = 1.013x + 2.335 \qquad \textbf{Equation 4.6}$$

with $r = 0.888$ (95% C.I. 0.490 – 0.980; $P < 0.01$).

The mean bias between the ^{13}C-OA blood test and scintigraphy for $t_{1/2}$ estimation is shown in Figure 4.11. Mean (± s.d.) bias = 2.18 (± 0.62) h; 95% C.I. range for inter-modal bias = 1.95 h – 2.41 h, and 95% limits of agreement = 0.97 – 3.39 h (n = 7).

Despite the overall congruity between the blood and breath data sets, some interesting discrepancies were observed in isotope recovery. In 3 of the 8 subjects, the % dose recovery / h of the ^{13}C tracer in both breath and blood was best modelled by a double peaked curve. What was remarkable, was that the magnitude of the first blood peak in these animals was considerably greater than that of the first breath peak in % dose recovery of the ^{13}C tracer / h, even though the second peaks were almost congruent. These findings were consistent on repetition of sample ^{13}C:^{12}C measurement and analysis. Examples of this phenomenon are shown in Figures 4.12 and 4.13. The bold lines in each Figure represent the combination of the individual peaks for each data set, which have finer lines.

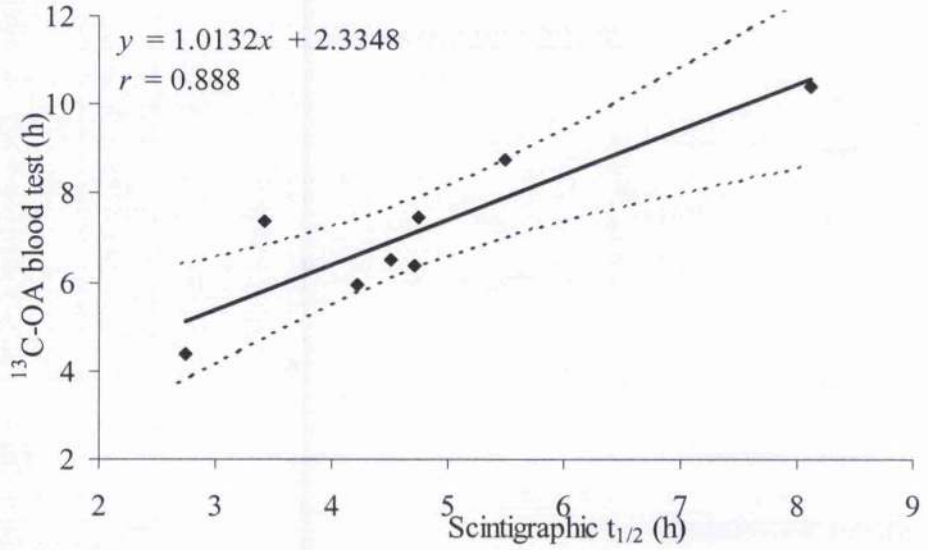


Figure 4.10 Comparison of gastric half-emptying times ($t_{1/2}$) determined by ^{13}C-octanoic acid blood test with those determined by scintigraphy after atropine administration at time 0 in all individuals (n = 8). As in Figure 4.8, the best regression line is shown, and the upper and lower 95% confidence limits are plotted. Pearson correlation coefficient = 0.888, P < 0.01. 95% confidence interval for Pearson correlation coefficient = 0.490 – 0.980.

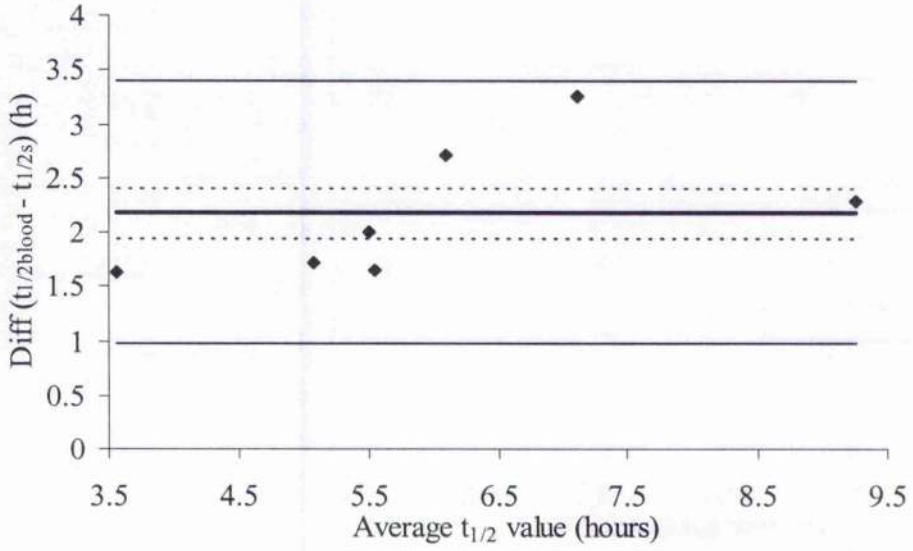


Figure 4.11 Bland Altman plot showing the mean bias in $t_{1/2}$ measurement for the ^{13}C-OA blood test, as compared with gastric scintigraphy. Inter-modal difference is plotted against average $t_{1/2}$ value. The mean difference (continuous line), 95% confidence intervals and the 95% limits of agreement for the mean bias are plotted. The outlier shown on the linear regression plot has been removed (n = 7). Mean (± s.d.) bias = 2.18 (± 0.62) h. 95% C.I. range for bias = 1.95 – 2.41 h, and 95% limits of agreement = 0.97 – 3.39 h.

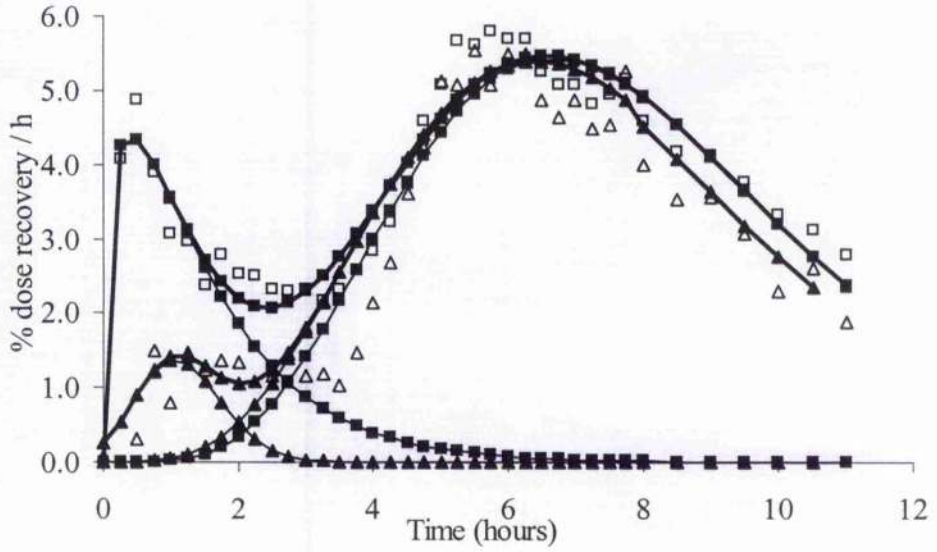


Figure 4.12 % dose recovery/h of ^{13}C tracer in breath (▲) and blood (■) samples collected concurrently from subject 1965, a 25 y.o. Thoroughbred mare, following ingestion of the ^{13}C-octanoic acid-labelled test meal and administration of atropine. The open symbols correspond to the actual data points. The modelled curves for each data set (closed symbols) have been fitted using a dual peak 'best-fit' model, as described in the text. The finer lines represent the individual peaks for each data set, and the bold lines represent the combination of these peaks. Blood peak 1 is of much greater amplitude than breath peak 1 in this horse. The second peak of each data set is almost congruent.

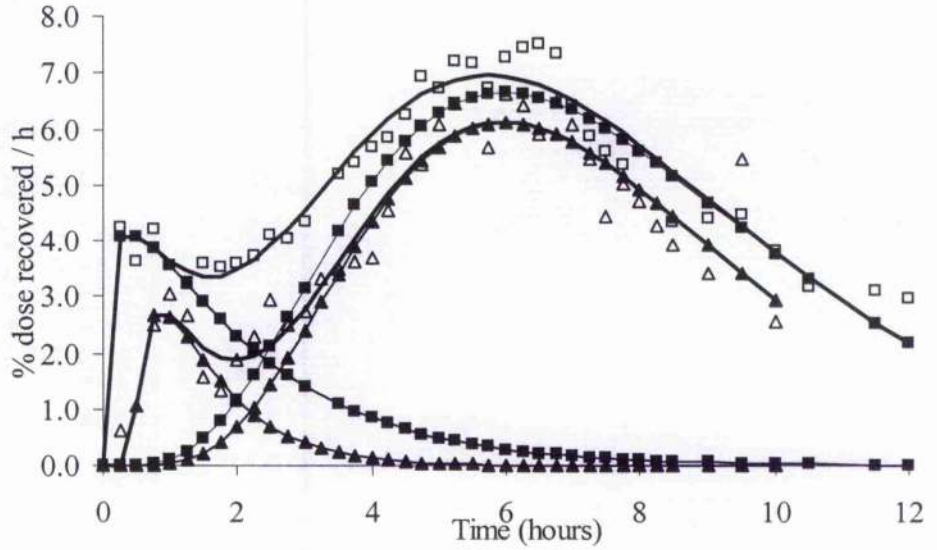


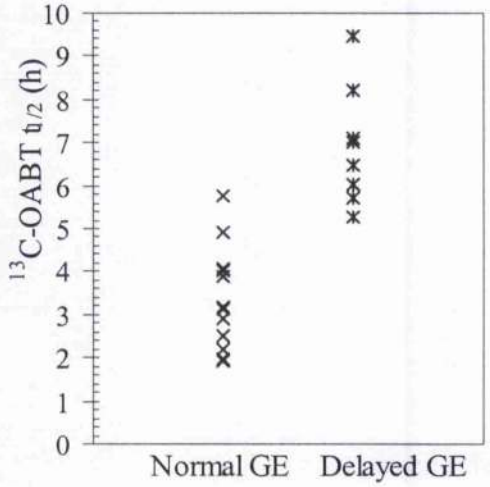
Figure 4.13 Concurrent % dose recovery/h of ^{13}C tracer in breath (▲) and blood (■) samples collected from subject 1937, a 10 y.o. Quarter horse gelding, after ingestion of the ^{13}C-octanoic acid-labelled test meal and administration of atropine at 0.035 mg/kg. The finer lines represent the individual peaks for each data set, and the bold lines represent the combination of these peaks. Again, both data sets are best fitted using a dual peak model, but the first peak is of greater magnitude in the early blood samples.

Although the initial rate of increase in blood ^{13}C:^{12}C ratio exceeded that of breath in 3 individuals, blood dose recovery curves were generally wider and of lower amplitude than the matching breath recovery curves. Hence, mean (± s.d.) gastric $t_{1/2}$ in the 8 horses was 6.76 (± 1.65) and 7.14 (± 1.83) h for breath and blood respectively, with a significant mean difference of 0.39 h (95% C.I. 0.17 – 0.60 h; $P < 0.01$).

4.3.5 Validity of ^{13}C-OABT for measurement of delayed gastric emptying

Based on comparable, normally distributed scintigraphic data from healthy untreated individuals (n = 12) in the same population (Chapter 3; Sutton *et al.* 2002), a cut-off time of 3.151 h was determined below which 99.95% (mean $t_{1/2s}$ + ($t_{0.005}$ x s.d.)) of population $t_{1/2s}$ would be expected to lie. For the purpose of determining sensitivity and specificity values for breath test data in relation to concurrent scintigraphic data, values for $t_{1/2s}$ above/below this cut-off time were described as 'delayed' or 'normal' GE rate. According to this scintigraphic classification of GE in 21 individuals for which combined scintigraphy/^{13}C-OABT data were available, the corresponding breath test $t_{1/2}$ values were grouped as shown in Figure 4.14. The dotplot of the combined distribution of ^{13}C-OABT $t_{1/2}$ values shows distinct separation between the 'normal' and 'delayed' GE individuals, with a small overlap between the ranges. A standard ROC curve for diagnosis of 'delayed' GE by the ^{13}C-OABT was plotted for a selection of $t_{1/2}$ cut-off times (Figure 4.15). The area under the ROC curve was close to 1 (0.899) consistent with an excellent diagnostic accuracy for delayed emptying by the breath test when compared to scintigraphy (Greiner 1995). The actual sensitivity and specificity of a range of $t_{1/2b}$ cut-off values for diagnosis of delayed GE is shown in Figure 4.16. Due to the small combined data set, there is a range of possible values for optimum $t_{1/2b}$ cut-off.

To further define the optimum cut-off for $t_{1/2b}$ selection, a TG-ROC curve was plotted using the technique of Greiner (1995). The TG-ROC plot in Figure 4.17 uses non-parametric techniques to plot the sensitivity (Se) and specificity (Sp) of different ^{13}C-OABT $t_{1/2}$ cut-off points to a level of greater than 95% accuracy. Due to the small data set, there is a range of values about which accuracy falls below 95%, and this is indicated on the chart as the intermediate range (IR). I.R. in Figure 4.17 is relatively narrow, extending for 0.50 h (5.25 – 5.75 h for $t_{1/2}$ cut-off), giving a valid range proportion (VRP) of 0.94. If the object is to detect all individuals with delayed GE, then a $t_{1/2}$ cut-off of < 5.27 h should be selected (Se, Sp = 1.00, 0.90); whereas if the aim is to avoid misclassification of any individuals with normal G.E, it would be necessary to select a cut-off $t_{1/2}$ exceeding 5.72 h (Se, Sp = 0.90, 1.00).



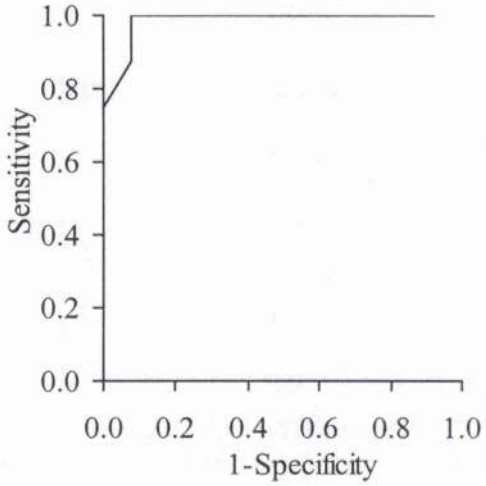


Figure 4.14 (left) Dotplot of the combined distribution of ^{13}C-OABT $t_{1/2}$ values, showing overlap between 'normal' and 'delayed' values when compared to the concurrent scintigraphic value for each individual (n = 21). The optimum scintigraphic cut-off point for 'normal' and 'delayed' gastric emptying has been determined as the healthy population mean value plus ($t_{0.005}$ x s.d.) i.e. that point above which only 0.05% of the normal population would be expected to lie.

Figure 4.15 (right) Standard receiver operating characteristic curve (ROC) for a selection of ^{13}C-OABT $t_{1/2}$ cut-off values for determination of 'normal' and 'delayed' GE as compared to concurrent scintigraphy. Area under the curve = 0.899, indicating an excellent test accuracy when compared to scintigraphy.

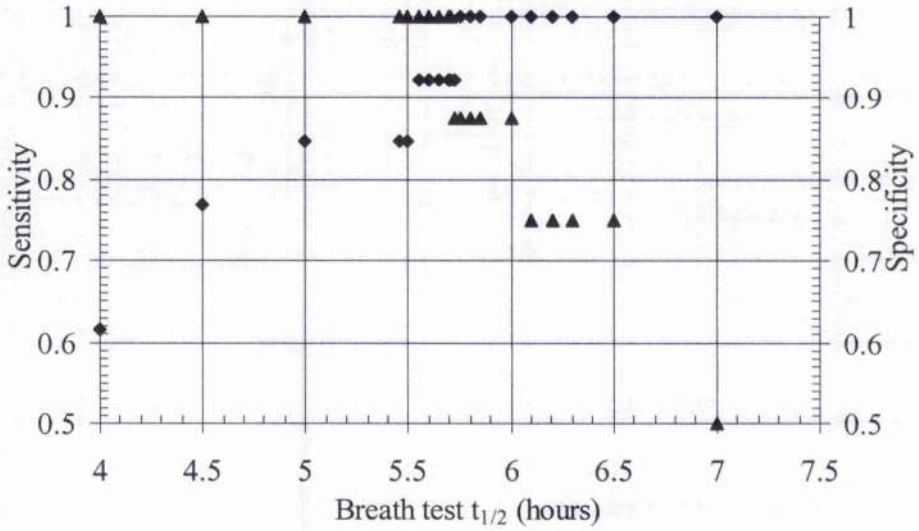


Figure 4.16 Plot of the diagnostic sensitivity (Se) and specificity (Sp) of the actual ^{13}C-OABT $t_{1/2}$ values for determination of equine normal or delayed gastric emptying (as measured by concurrent gastric scintigraphy), as a function of the cut-off value (n = 21).

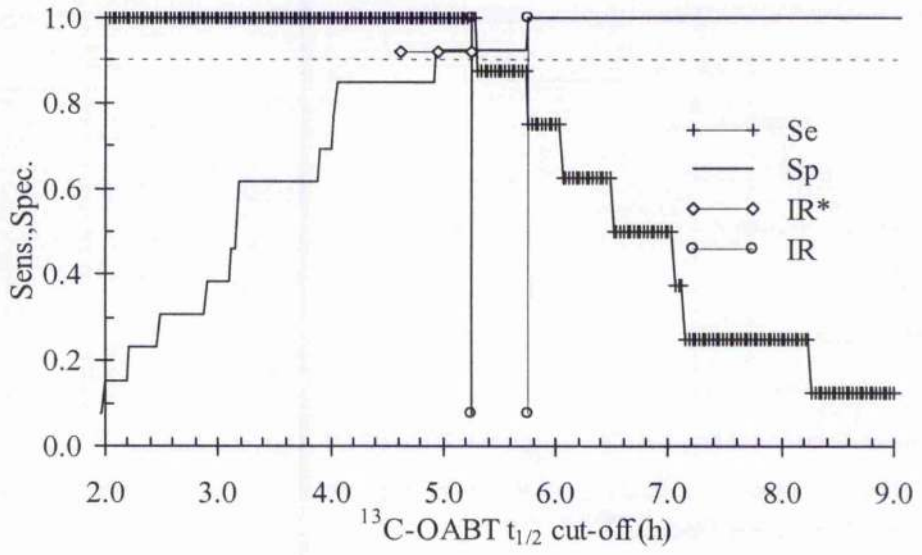


Figure 4.17 Twin graph ROC curve, plotting diagnostic sensitivity (Se) and specificity (Sp) of ^{13}C-OABT $t_{1/2}$ for detection of delayed gastric emptying, as compared with the optimum standard of concurrent gastric radioscintigraphy. An intermediate range (IR) is indicated around cut-off threshold about which the accuracy level (Se, Sp) is less than 95%, according to the model of Greiner (1995). IR extends from 5.246 to 5.748 h, giving a valid range proportion of 0.940. IR* indicates the 95% confidence interval for intersection of the modelled sensitivity and specificity curves.

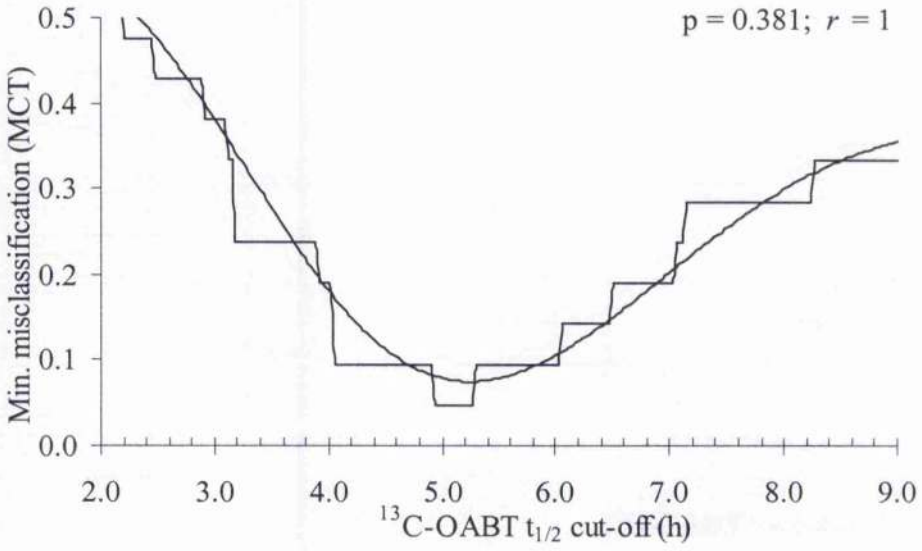


Figure 4.18 Use of the non-parametric misclassification cost term (MCT) to identify that diagnostic cut-off range that minimises misclassification (Greiner *et al.* 2000). The stepped line represents the cost of 'delayed' misclassification/false-negative, versus the cost of 'normal' misclassification or false-positive (curve). With 95% confidence, false-negative costs are minimised in the range for $t_{1/2}$ of 5.00 – 5.27 h; false-positive costs are minimised in the wider range 5.15 – 5.52 h. MCT = (1 – p)(1 – Sp) + *r*p (1 – Se), where p = prevalence and *r* = costs (false-negative result)/costs (false-positive result). In this case, p = 0.381 and *r* = 1.

To further identify the optimum cut-off point, a non-parametric MCT plot was constructed (see Figure 4.18). As described in equation 4.4, the aim of the MCT plot is to minimise the likelihood of both false positive and false negative result classification. In this 95% accuracy plot, it is seen that the costs of false-negative diagnoses of delayed emptying are minimised in the $t_{1/2b}$ range of 5.00 – 5.27 h. The likelihood of misclassifying normal individuals as 'delayed' is minimised in the wider range of 5.15 – 5.52 h. The optimum cut-off for $t_{1/2b}$ may therefore be considered to lie in the narrow range of 5.15 – 5.27 h. This is based on the individuals sampled in this population only (n = 21), in which there was an observed prevalence of 0.38. Values within this range have positive and negative predictive values of at least 0.89 and 1.00 respectively.

4.4 Discussion

4.4.1 General conclusions

In this study, the ^{13}C-OABT was shown to be sufficiently sensitive to detect atropine-induced changes in equine solid phase GE rate. In 8 individuals there was no overlap in the 99% confidence interval range for either mean $t_{1/2}$ or t_{lag} measurement following post-ingestion randomised treatment with atropine or saline. In addition, significant positive correlations were found between the ^{13}C-OABT and radioscintigraphy for the measurement of both gastric $t_{1/2}$ and t_{lag} in a population of horses proven to have significantly delayed gastric emptying (n = 8). It was further shown that either blood or breath media could be used for measurement of equine ^{13}C:^{12}C ratio with very similar accuracy.

Mean inter-modal bias for $t_{1/2}$ calculation was not significantly different in this group of horses with delayed emptying to that measured previously in normal coexisting individuals (Chapter 3; Sutton *et al.* 2003). This suggests that the post-gastric delay period in the processing of ^{13}C-octanoic acid (Ghoos *et al.* 1993) remains constant even in horses with gastroparesis. Validation of this factor is an essential point in the use of the ^{13}C-OABT in individuals with significant delays in emptying rate (Camilleri *et al.* 1998); the combination of results presented here and in Chapter 3 demonstrate that the ^{13}C-OABT is a sensitive diagnostic tool for the measurement of equine delayed solid phase GE, producing quantitative information even when this function is significantly prolonged. Using a cut-off value for $t_{1/2b}$ of 5.15 to 5.27 h, the ^{13}C-OABT was extremely accurate for the diagnosis of delayed GE when compared to scintigraphy (Greiner *et al.* 2000), with excellent sensitivity, specificity and negative and positive predictive values in the combined study population. This would suggest that the ^{13}C-OABT may have diagnostic use in a variety of settings for the detection and quantitation of delayed equine gastric emptying.

4.4.2 Cause of inter-modal difference in $t_{1/2}$ measurement

The mean bias observed between the two diagnostic modalities for calculation of $t_{1/2}$ (1.78 ± 0.58 h) was thought to be due to the post-gastric handling of the ^{13}C-octanoic acid. As described in Chapter 3, the medium chain fatty acid must be absorbed and processed by the liver after the rate-limiting GE event has occurred, before entry of the ^{13}C tracer into the bicarbonate pool and $^{13}CO_2$ expiratory enrichment (Bach and Babayan 1982). Data presented in section 3.3.2.4 of Chapter 3 showed that the mean equine inter-modal bias was close to the measured $t_{1/2}$ value for direct duodenal instillation and recovery of the tracer, as found by Ghoos *et al.* (1993) in man. Furthermore, mathematical deconvolution of the breath test dose recovery data in 2 individuals produced a real-time flow curve for gastric emptying rate, which had a $t_{1/2}$ value directly comparable to that of concurrent gastric scintigraphy. The questions posed by Choi *et al.* (1997) regarding the homogeneity of tracer absorption, metabolism and excretion between different individuals appear to have been answered in part for equidae in these validation studies. It has been shown in the study population that these factors may be assumed to be uniform in healthy adult horses.

The accuracy and reproducibility of the ^{13}C-OABT in people has also been confirmed in several large trials by reference to gastric scintigraphy (Choi *et al.* 1998; Delbende *et al.* 1998, 2000). Most recently, comparison of the gastric emptying functions measured by ^{2}H-octanoate and scintigraphy has indicated that the two methods provide equivalent results (Bluck *et al.* 2002), suggesting the cause of the lag in isotope recovery in the ^{13}C-test is due to recovery from the bicarbonate pool. Inter- and intra-individual repeatability of the ^{13}C-OABT in healthy adult ponies has also been shown to be high (Wyse *et al.* 2001). However, one potential pitfall that still needs to be addressed in the use of the ^{13}C-OABT in both human and equine medicine is whether or not the test remains accurate in the measurement of GE in patients with significant hepatic or pancreatic disease, haemodynamic changes, or even pulmonary disorders that may alter the excretion rate of $^{13}CO_2$ (Camilleri *et al.*1998). The importance of measuring VCO_2 during breath tests, and in maintaining the subject at rest has already been discussed in section 2.4.4. Recent human research has shown that ^{13}C-octanoate metabolism is not affected even by severe hepatic cirrhosis nor by reduction of hepatic blood flow using a transjugular intrahepatic portal shunt (Van de Casteele 2002). However, further research should be performed to assess the validity of the ^{13}C-OABT in subjects with severe metabolic or digestive dysfunction.

4.4.3 Atropine model for equine gastroparesis

In this study, delayed GE was modelled by the administration of atropine (0.035 mg/kg) after meal consumption in healthy horses. This model has been used widely in both equine (Doherty *et al.* 1998; Lohmann *et al.* 2002) and human medicine (Imbimbo *et al.* 1990; Rashid and Bateman 1990), although the specific effects of atropine on equine gastric motility have not been reported. Using an acetaminophen absorption test, Doherty *et al.* (1998) reported that 0.025 mg/kg atropine caused a significant delay in liquid gastric emptying rate in ponies. However, this dose was inadequate to delay solid phase emptying in this study, and dosage was increased to 0.035 mg/kg. This dose delayed GE significantly in 6/8 subjects, but a second treatment (0.035 mg/kg) was required in the two remaining individuals. Hence, a variation was shown to exist in an individual's sensitivity and gastric response to atropine. Signs of abdominal pain were not seen even at the higher combined dose of 0.07 mg/kg. This was in contrast to previous studies in which doses of 0.044 mg/kg atropine (Ducharme and Fubini 1983) and even topical ophthalmic atropine preparations (Williams *et al.* 2000) have been found to cause abdominal pain in ponies.

Atropine administration caused noteworthy changes to the appearance of the scintigraphic gastric emptying profiles. In all individuals, radioactive counts in the gastric region of interest continued to increase for the first 15 min of the study, before reaching plateau. One such example is seen in Figure 4.6. This phenomenon was probably due to rapid disruption of oesophageal motility by the atropine, resulting in delayed orogastric transit of residual radioactive ingesta. The muscularis externa of the caudal third of the equine oesophagus comprises smooth rather than striated muscle fibres (Sisson 1975), and manometric studies have shown that normal peristaltic velocity in this region is reduced, and contraction time increased, when compared to the proximal regions (Stick *et al.* 1982; Clark *et al.* 1987). In reported *in vitro* work, atropine effectively abolished the smooth muscle component of oesophageal contraction in guinea pig, rat and human tissue by postganglionic muscarinic-cholinergic blockade (Kerr *et al.* 1995; Halmai *et al.* 1996; Storr *et al.* 2000), and oesophageal transit is significantly slowed in human patients with parasympathetic nerve dysfunction (Cunningham *et al.* 1991). Hence, the contractility of the terminal smooth muscle segment of the equine oesophagus is likely to have been significantly inhibited by the administration of atropine.

In 3/8 cases, left gastric counts continued to increase for 60 min after meal ingestion. In addition to delayed oesophageal transit in these animals, this appeared to result from movement of radioactive ingesta from the right to left gastric regions, perhaps due to

increased gastric compliance. Lidums *et al.* (2000) showed that atropine administration in humans resulted in increased gastric compliance, with a delay in recovery of postprandial proximal gastric tone, and a consequent decrease in motility. Atropine is also known to reduce the postprandial antral manometric motility index (Parkman *et al.* 1999), and this is the main mechanism by which it delays gastric emptying, as coordinated antral pressure activity is required for the trituration of solid food, and subsequent expulsion of solids and liquids from the stomach (Becker and Kelly 1983; Camilleri *et al.* 1985). The combination of these factors may have resulted in the movement of radioactive ingesta from distal to proximal stomach in the atropinised horses in this study. Maintenance of canine gastric tone is known to require vagal input (Azpiroz and Malageleda 1987), and the scintigraphic findings in this study provide some evidence that cholinergic input is also involved in the control of equine gastric compliance.

4.4.4 Gastric ulceration: possible cause of discrepancies in blood/breath ^{13}C-tracer recovery rate

Isotope recovery in 3/8 individuals in this study was biphasic, with a prominent early peak, which was of significantly greater amplitude in the blood than the breath data sets. This phenomenon requires further investigation, but may offer an unexpected further equine clinical application of the ^{13}C-OA blood test. The most rational explanation for these repeatable findings is perhaps the existence of significant gastric ulcerations in these animals, which allowed the gastric absorption of ^{13}C-OA. Generally, medium chain fatty acids (MCFAs) are rapidly absorbed in the duodenum and transported to the liver by the portal system. Once there, MCFAs are activated on the outer mitochondrial membrane to MCF-acyl CoAs, prior to oxidation in the mitochondrial matrix, with the production of acetyl CoA (Bach and Babayan 1982). After ingestion of ^{13}C-OA, ^{13}C-acetyl CoA is formed, which enters the Krebs cycle, prior to oxidation into $^{13}CO_2$ and subsequent excretion in breath from the bicarbonate pool. Venous drainage from gastric ulcers in the horse may occur via the left parietal gastric vein, which has been noted to enter the posterior vena cava directly, bypassing the liver and returning venous blood directly to the heart (Nickel *et al.* 1981). Since ^{13}C-octanoate cannot be expired as $^{13}CO_2$ until hepatic oxidation has occurred, any tracer absorbed in this way would initially cause a higher ^{13}C:^{12}C ratio in the peripheral blood than expired breath, as noted in this study. However, gastroscopic examination was not performed in the test subjects, and therefore this possibility cannot be substantiated.

Sucrose permeability has recently been investigated for the detection of both human (Sutherland *et al.* 1994) and equine (O'Conor *et al.* 2002) gastric ulceration, and passage of this disaccharide through the gastric mucosa has been demonstrated in subjects with gastric erosions, with its subsequent concentration in the urine. Sutherland *et al.* (1994) reported a sensitivity of 84% for the detection of gastric ulceration by sucrose absorption, and a specificity of 96% for the prediction of abnormal endoscopy. It is possible that the ^{13}C-OA blood test after atropine treatment may prove to be a less cumbersome method for the field diagnosis of equine gastric ulceration. However, validation studies are now required to measure the peripheral circulatory appearance of ^{13}C-OA in individuals with and without gastric ulceration. Given the huge incidence and economic implications of the equine gastric ulceration syndrome (Murray *et al.* 1996), further investigation of this finding is certainly merited. The fact that octanoic acid may be absorbed through the gastric mucosa in the presence of gastric erosions does not appear to complicate the interpretation of ^{13}C-OABT GE data. In the 3 relevant subjects in this study, a discrete early peak in ^{13}C dose recovery was apparent, followed by the large main peak, corresponding to the gastric emptying event.

4.4.5 Conclusions from the ^{13}C-OABT validation studies

The results of the studies presented in Chapters 3 and 4 of this thesis suggest that the ^{13}C-OA breath/blood test is a suitable diagnostic technique for the measurement of solid phase GE in both healthy adult horses and those with atropine-induced gastroparesis. This non-invasive stable isotope test was found to be safe and well tolerated by equidae. As noted in human studies (Perri *et al.* 1998), it also proved to be quantitative, non subjective and simple to perform. Using TG-ROC and MCT techniques, the test was shown to have an excellent sensitivity and specificity for the diagnosis of equine delayed GE when compared to the predicate method of scintigraphy. Based on the small population of horses used in this study, the optimal $t_{1/2b}$ cut-off point for the diagnosis of delayed emptying was concluded to be in the range 5.15 to 5.27 h.

Having been validated against radioscintigraphy for the measurement of delayed GE, it is envisaged that this stable isotope breath test could have wide potential research and clinical applications. Use of the test would be beneficial in cases of recurrent and chronic colic (Hillyer and Mair 1997; Mair and Hillyer 1997), particularly in cases of gastric neoplasia (Mair and Hillyer 1991; McKenzie *et al.* 1997), gastric dilatation (section 4.1; Edwards 1993) or pyloric stenosis (Church *et al.* 1986). In addition, quantitative GE data would be helpful in the diagnosis and management of chronic grass sickness cases (Merritt 1997)

and of any case requiring prokinetic therapy. Further knowledge of the potential pathophysiologcial role of disordered gastric emptying in conditions such as gastroduodenal ulceration in both foals (Becht and Byars 1986) and adults (Berschneider *et al.* 1999) may also be beneficial in management of these common disorders. The ^{13}C-OABT has already been used in a small preliminary study to investigate the effect of different feeding regimens on equine GE (Geor *et al.* 2001). In Chapter 5 of this thesis, it is used to assess the effects of xylazine, detomidine, acepromazine and butorphanol administration on equine GE rate, and to measure whether or not these are dose-dependent.

The subjects described in Chapters 3 and 4 were maintained on alfalfa hay alone to minimise possible error caused by variation in basal $^{13}CO_2$ abundance in breath. This may not always prove practical in equine practice. With hindsight, the signal produced by the quantity of ^{13}C-enriched tracer used was sufficient to overcome the requirement to control the natural ^{13}C dietary intake prior to testing. Algorithms for the reduction of sample collection duration and frequency are presented in Chapter 9, and should make this diagnostic procedure cheaper and still more practical for equine field use. Stored samples also remain stable for at least 60 days (Schoeller *et al.* 1977) and may be safely sent to diagnostic centres for analysis. The number of commercial and institutional analytical centres for stable isotope breath tests is continuing to increase, and dedicated veterinary machines are likely in the near future.

Chapter 5 THE EFFECTS OF XYLAZINE, DETOMIDINE, ACEPROMAZINE AND BUTORPHANOL ON EQUINE SOLID PHASE GASTRIC EMPTYING RATE.

5.1 Introduction

5.1.1 Background to study conception

Although the cardiopulmonary and metabolic effects of many equine sedative, analgesic and preanaesthetic medication protocols have been reported widely, there is little knowledge of their effect *in vivo* on specific regions of the gastrointestinal tract. This may have important implications for their clinical use, particularly when used for the treatment of cases presenting with colic, as inappropriate drug administration could exacerbate the underlying gastrointestinal disturbance. An association between certain anaesthetic regimens and the development of complications following surgery, such as ileus, has also been postulated (Adams *et al.* 1986). Lester *et al.* (1992) found that a variety of induction protocols abolished ileal, caecal and colonic spike burst activity for the duration of general anaesthesia in healthy horses, although myoelectric activity returned to control values within 9 hours of recovery. In a recent colic case-control study, prolonged anaesthesia of above 2.5 hours duration was also associated with an increased risk of post-operative ileus (Roussel *et al.* 2001), although this factor could not be measured in isolation from the increased risk associated with prolonged surgery. Further study is required into the possible involvement of specific pharmacological agents in the development of equine post-operative ileus in both healthy and diseased horses.

5.1.2 Study of pharmacological modulation of equine gastric emptying

5.1.2.1 Technical considerations

The paucity of published information on modulation of equine solid phase gastric emptying rate is due in part to the inherent technical difficulties previously involved in the measurement of this parameter. Principal methods of assessing drug effects on equine gastrointestinal motility *in vivo* have previously included electromyenterography (Roger and Ruckebusch 1987; Merritt *et al.* 1989a), strain gauge transduction (Hunt and Gerring 1986; Clarke *et al.* 1988), duodenal manometry (Merritt *et al.* 1998) and both gastric (Ringger *et al.* 1996) and caecal (Lester *et al.* 1998) radioscintigraphy. When compared to electromechanical techniques, the ^{13}C-OABT offers the distinct advantage of providing a quantitative measurement of the rate of coordinated emptying of labelled ingesta itself, rather than of contractile activity alone. This is particularly important in pharmacological studies, where drugs may alter cellular electrophysiology without affecting propulsive

motility. Previous work has shown that xylazine, for example, increased antroduodenal myoelectrical activity in young cattle, whilst concomitantly reducing duodenal digesta flow rate (Merritt and Ruckebusch 1988). Gastric amplitudes determined by cutaneous electrogastrography have also been shown to bear little correlation to sonographically measured antral mechanical contractions (Pfaffenbach *et al.* 1995). These findings raise questions about the validity of using electromyenterography as the sole technique for assessment of pharmacological intervention on equine gut motility.

5.1.2.2 Statistical considerations

Gastric radioscintigraphy may be the predicate diagnostic modality for accurate assessment of equine solid phase emptying, but the technique does not readily allow large-scale pharmacological studies to be conducted, due to the constraints imposed by time, equipment, and the requirement for a nuclear medicine facility. In order to definitively measure the effect of specific pharmacological agents on gastrointestinal motility, good statistical design considerations point to large study groups (ideally of 60 individuals (Klein 2001)) of both healthy and diseased individuals. Whilst such large study groups are difficult to achieve in equine medicine, use of the ^{13}C-OABT in this study facilitated the efficient conduction of over 64 individual tests of gastric emptying rate in 9 individuals, performed in order to investigate the effect of different sedative/analgesic protocols on this parameter.

5.1.3 Study objectives

The aims of this study were two-fold: (i) to measure the effect of specific commonly used sedative regimens on equine gastric function, using the ^{13}C-OABT; and (ii) to determine whether or not the observed effects of α_2-agonists on this parameter were dose-dependent in nature. To the author's knowledge, the effect of specific sedative agents on equine solid-phase gastric emptying has not previously been determined. Potentially, the results of such a study could have important clinical implications for case selection when these agents are used for purposes of sedation and/or analgesia. The ^{13}C-OABT was used to assess the effects of the individual agents on gastric emptying. As described in Chapters 3 and 4, this stable isotope breath test has been validated against radioscintigraphy for the measurement of solid phase gastric emptying in horses with both normal and delayed gastric emptying (Sutton *et al.* 2002a, 2003). The ^{13}C-OABT is also non-invasive, avoids the use of radioisotopes and is simple to perform, with minimal equipment requirements. The combination of these factors made the ^{13}C-OABT an ideal choice for the pharmacological

studies on solid phase gastric emptying reported here, which involved over 64 individual tests of emptying rate.

5.1.4 Selection of pharmacological agents

The agents routinely used for the sedation and preanaesthetic medication of equidae fall into three main classes: the alpha$_2$ adrenergic agents, the opioids and the phenothiazines. In addition to their use for these purposes, it has been recognised for many years that both the α_2-agonists and opioids may provide good visceral pain relief (Kalpravidh *et al.* 1984; Becht 1986; Lowe *et al.* 1986; Jochle 1989; Jochle *et al.* 1989) and this has lead to their widespread use for the treatment of equine abdominal pain. However, as pointed out by Kohn and Muir in their review article of 1988, it is controversial whether or not α_2 agonists or opiates mediate decreases in gut motility which cause clinically important slowing of ingesta transit, and this requires further investigation.

5.1.4.1 Alpha$_2$-adrenergic agonists

Two commonly used α_2-agonists were chosen for use in this study: xylazine and detomidine. Both of these agents are licensed for equine use in both Britain and the United States. The recommended dose ranges for xylazine are 0.6 – 1.0 mg/kg and 1.1 mg/kg respectively, and those for detomidine are 0.01-0.08 mg/kg and 0.02 – 0.04 mg/kg respectively. Romifidine is the most recent α_2-agonist to be licensed in Britain for equine use (England *et al.* 1992; Browning and Collins 1994) and has been shown by transrectal ultrasonography to reduce both small and large intestinal motility (Freeman and England 2001). However, it was not possible to investigate the effects of romifidine on gastric emptying in this study, as the product is currently not approved in the United States.

The α_2-agonists exert their effects principally via central and peripheral prejunctional inhibitory adrenoceptors within the sympathetic nervous system (Thurmon *et al.* 1996). The analgesic-sedative properties of the compounds result from a central transduction pathway and effector system shared with the opioids. Binding of α_2- or μ-opioid agonists to their receptors results in a cascade of membrane-associated G-protein activation, with eventual outflow of potassium from the postsynaptic neurone and hyperpolarization. This makes the cell unresponsive to further excitation (Thurmon *et al.* 1996). However, xylazine and detomidine also act at peripheral presynaptic α_2-receptors to inhibit release of noradrenaline from sympathetic nerves to the heart in pithed rats (Virtanen and MacDonald 1985). Both compounds also induce vascular smooth-muscle-mediated vasoconstriction,

resulting in regional hypertension, due to action at peripheral post-synaptic α_2 receptors, and possibly α_1 receptors (Virtanen and MacDonald 1985).

There is much evidence in the veterinary literature from electromyenterographic studies that xylazine suppresses motility of the equine caecum and colon (Adams *et al.* 1984; Hunt and Gerring 1986; Roger and Ruckebusch 1987; Clark *et al.* 1988; Rutkowski *et al.* 1991; Sasaki *et al.* 2000) and this is supported by auscultation (Singh *et al.* 1997), caccocolic manometry (Ross *et al.* 1985) and scintigraphic measurement of caecal emptying rate (Lester *et al.* 1998). However, the effect of this compound on intestinal electrical activity is interesting, and may be dose-dependent or differ between subjects or specific regions of the gastrointestinal tract (Hunt and Gerring 1986). The contraction product/min of the left ventral and dorsal colon was increased in response to xylazine (1.0 mg/kg) in some ponies (Hunt and Gerring 1986), whilst Roger and Ruckebusch (1987) reported that the smooth muscle tone of this region was initially enhanced by xylazine (1.2 mg/kg), before a more prolonged inhibition. Very high intra-arterial doses of xylazine were found to increase the amplitude of phasic contractions of isolated equine jejunal loops, in addition to increasing both the vascular resistance and oxygen consumption (Stick *et al.* 1987), although the clinical relevance of this latter study has been questioned due to the dosage regimen used (Hubbell *et al.* 1987).

It has been suggested that the initial stimulatory effects of xylazine on intestinal myoelectrical activity and smooth muscle tone may be due to centrally-mediated presynaptic α_2-adrenoceptor effects (Roger and Ruckebusch 1987), as these are not inhibited by local intra-arterial α_2-antagonists. The inhibitory effect on intestinal motility is likely to be caused by stimulation of presynaptic α_2-receptors in the myenteric plexus (Sellers *et al.* 1985), causing reduced noradrenaline release and a subsequent inhibition of acetylcholine release by parasympathetic postsynaptic neurones (Demol *et al.* 1989). A heterotropic interaction occurs between these transmitter molecules in the myenteric plexus (Rang *et al.* 1999a) with a negative regulation of acetylcholine release by noradrenaline. It has also been shown in the guinea pig that presynaptic α_2-adrenoceptors exist on the vagal cholinergic nerve terminals of the myenteric plexus, similar to those found on the terminals of the sympathetic nerves, and acetylcholine release is also inhibited by direct action of the α_2-agonists at these receptors (Drew 1978).

Few studies have assessed the effects of detomidine on equine gastrointestinal motility, and it has widely been assumed that its effect mirrors that of xylazine. Using a high dose of 0.1 mg/kg detomidine in ponies, Roger and Ruckebusch (1987) found that the compound

produced a biphasic myoelectrical response in the caecum and central colon, with an initial increase in smooth muscle tone, followed by a 3 h inhibition of large intestinal activity. Equine visceral pain studies have suggested that the anti-nociceptive properties of detomidine are dose-dependent (Jochle and Hamm 1986; Lowe *et al.* 1986; Jochle *et al.* 1989; Hamm *et al.* 1995) and superior in depth and duration to equipotent sedative doses of xylazine (Jochle and Hamm 1986; England *et al.* 1992). It has also been demonstrated that detomidine is a more potent α_2-agonist than xylazine (Virtanen and Nyman 1985; Virtanen 1986). In view of these findings, one hypothesis of this pharmacological study was that detomidine would have a significantly greater inhibitory effect on equine gastric emptying rate than equipotent doses of xylazine, due to its higher potency at α_2-receptors.

5.1.4.2 Butorphanol

Butorphanol is a centrally-acting opioid agonist-antagonist with primary agonist and antagonist actions at the κ- and μ-opioid receptors respectively (Branson *et al.* 1995). Experimental analgesic dose-response studies in the horse to superficial and visceral stimuli have shown that the analgesia provided is dose-related, with a dose of 0.2 mg/kg IV appearing optimal and lasting for up to 90 min (Kalpravidh *et al.* 1984a). When this dose was administered IM, the same authors found that visceral analgesia to caecal balloon distention lasted for approximately 4 h, and exceeded that provided by morphine (0.66 mg/kg IM), although was inferior to xylazine (2.2 mg/kg IM) in obtunding visceral pain (Kalpravidh *et al.* 1984b). Further experimental (Muir and Robertson 1985) and clinical (Gingerich *et al.* 1985) trials have reported this dose to produce profound visceral analgesia with good to excellent effect in 90% colic cases. Unfortunately, doses of butorphanol above 0.1 mg/kg (the maximum data sheet recommendation) often cause excessive motor activity, possibly due to increased dopamine release from nigrostriatal system neurones (Kalpravidh *et al.* 1984b) and therefore the analgesic effect of lower doses has been studied. Doses of 0.1 mg/kg butorphanol provide good to excellent visceral analgesia (Becht 1986; Stout and Priest 1986), and most recently a continuous infusion of 13-27 μg/kg bwt/h has also been reported to provide post-operative analgesia (Sellon *et al.* 2001, 2002).

However, further research is required into butorphanol as its effect on equine gastrointestinal motility remains controversial. Myoelectrical and mechanical activity of the jejunum was significantly reduced by treatment with 0.1 mg/kg butorphanol (Sojka *et al.* 1986; Sojka *et al.* 1988), although the same dose was not found to affect the whole gut

transit time of polyethylene glycol in ponies (Roberts and Argenzio 1986). Sellon *et al.* (2001) reported that butorphanol infusion at 27 µg/kg bwt/h produced fewer gastrointestinal effects than a single IV bolus of 0.1 mg/kg, although faecal output during infusion was reduced compared to control animals. In view of the unresolved debate surrounding butorphanol administration, one aim of this study was to quantify the effect of a low dose of this opioid on equine solid phase GE, using the ^{13}C-OABT. Butorphanol is currently most frequently administered at low doses in combination with an α_2-agonist to potentiate the depth of sedation achieved (Clarke and Paton 1988; Taylor *et al.* 1988). Therefore, in this study, it was elected to measure the effect of a standard combined dose of detomidine 0.01 mg/kg/butorphanol 0.02 mg/kg on equine gastric function, using the ^{13}C-OABT, and to compare this to both control values and detomidine 0.01 mg/kg alone.

5.1.4.3 Acepromazine

Acepromazine is a neuroleptic phenothiazine derivative which has affinity for several receptors, acting as an antagonist at dopaminergic 1 and 2 receptors, muscarinic, 5-HT_2 and α-adrenoceptors (Rang *et al.* 1999c). It is used widely in equine medicine for preanaesthetic medication, where its sedative effect is thought to be due to the antagonism of dopamine-mediated synaptic transmission in the CNS (Marroum *et al.* 1994). Drug datasheets recommend a dose of 0.03-0.10 mg/kg bwt acepromazine for this purpose. Including acepromazine in the preanaesthetic medication can prevent the hypertension and bradycardia caused by subsequent α_2-agonist administration (Marntell and Nyman 1996), improve subsequent recovery from anaesthesia (Muir and Mason 1993) and also reduce the required minimum alveolar concentration of halothane (Doherty *et al.* 1997).

However, once again, the effects of acepromazine on equine gastrointestinal motility are poorly known. Opposing extremes, with little evidence in support, have been put forth: Frank (1970) reported its use as having 'revolutionised the treatment of (spasmodic) equine colic', while Adams *et al.* (1986) opined that acepromazine may be useful for the treatment of equine post-operative ileus. Davies and Gerring (1983) discovered that acepromazine at 0.04 mg/kg decreased the electrical activity of pony jejunal Thiry-Vella loops. This finding was associated with decreased resting smooth muscle tone and increased intraluminal fluid transport, rather than inhibited peristalsis, leading to the suggestion that acepromazine may be a useful treatment for impaction colics (Davies and Gerring 1983). In this study, it was elected to use the ^{13}C-OABT to measure the effect of a standard preanaesthetic dose of acepromazine (0.05 mg/kg IM) on equine solid phase GE rate.

In the only previously published study to measure the effect of sedatives on equine gastric function, the recently validated acetaminophen absorption test (Lohmann *et al.* 2002) was used in ponies to assess the action of xylazine (1 mg/kg), butorphanol (0.5 mg/kg) or acepromazine (0.05 mg/kg) on the emptying of 350 ml water administered by nasogastric tube (Doherty *et al.* 1999). Each of these agents delayed the onset of maximal emptying rate as determined by the time to reach peak serum acetaminophen concentration, with most delay caused by xylazine. However, whilst the control mechanisms of liquid emptying are intimately involved with those of solids (Houghton *et al.* 1988), many of the controlling factors differ (Read and Houghton 1989) and the effect of sedative agents on solid phase emptying of a voluntarily ingested meal is likely to bear greater clinical relevance. In this section of the study, the effects of randomised xylazine, detomidine, acepromazine and combined detomidine/butorphanol administration on solid phase emptying were determined, using the ^{13}C-OABT (see Sutton *et al.* 2002b). To the author's knowledge, the effect of these commonly used agents on equine solid phase gastric emptying has not previously been reported.

5.2 Materials and Methods

5.2.1 Study design

The study was divided into two continuous 4-week treatment periods. During each period, the gastric emptying of a standard test meal was measured once weekly in each of 8 horses, using the ^{13}C-OABT. In period 1, ingestion of the test meal was followed by randomised intravenous treatment with saline (2 ml control), xylazine (0.5 mg/kg; Rompun®, Bayer Corporation, Kansas, USA), detomidine (0.01 mg/kg; Dormosedan®, Animal Health, PA, USA) or detomidine / butorphanol (Torbugesic®, Fort Dodge Laboratories Inc., Iowa, USA) combination (0.01 / 0.02 mg/kg). In period 2, test meal consumption was followed by randomised treatment with either saline (2 ml control), xylazine (1.0 mg/kg) or detomidine (0.03 mg/kg) or preceded by 5 min with intramuscular acepromazine (0.05 mg/kg), in line with current sedation protocols.

Treatments were randomised for week and individual, and each horse was its own control. By randomising for both week and individual, it was hoped to eliminate the possible influence of environmental and temporal factors on the study results. One subject had to be removed from the trial at the end of period 1 due to developing a dislike of the test meal, and was replaced. Therefore 7 of the 8 horses used in period 1 were studied in both periods, whilst 2 horses were used only for period 1 or for period 2 (Table 5.1).

Table 5.1 Summary of study design for measurement of effect of specific sedative agents on equine solid phase gastric emptying rate, using the ^{13}C-OABT. Treatments were randomised for both week and individual subject. Total number of tests = 64.

	Time:	Period 1			Time:	Period 2		
Subject	*Week 1*	*Week 2*	*Week 3*	*Week 4*	*Week 1*	*Week 2*	*Week 3*	*Week 4*
1949	Saline	DET 0.01	XYL 0.5	DET/BUT	Saline	XYL 1.0	DET 0.03	ACP 0.05
11 y.o. G	2 ml iv	mg/kg iv	mg/kg iv	0.01/0.02	2 ml iv	mg/kg iv	mg/kg iv	mg/kg im
1936	DET/BUT	Saline	DET 0.01	XYL 0.5	ACP 0.05	Saline	XYL 1.0	DET 0.03
11 y.o. G	0.01/0.02	2 ml iv	mg/kg iv	mg/kg iv	mg/kg iv	2 ml iv	mg/kg iv	mg/kg iv
1938	XYL 0.5	DET/BUT	Saline	DET 0.01	ACP 0.05	DET 0.03	Saline	XYL 1.0
12 y.o. F	mg/kg iv	0.01/0.02	2 ml iv	mg/kg iv	mg/kg iv	mg/kg iv	2 ml iv	mg/kg iv
1940	DET 0.01	XYL 0.5	DET/BUT	Saline	DET 0.03	ACP 0.05	XYL 1.0	Saline
9 y.o. G	mg/kg iv	mg/kg iv	mg/kg iv	2 ml iv	mg/kg iv	mg/kg im	mg/kg iv	2 ml iv
1939	DET 0.01	Saline	XYL 0.5	DET/BUT				
16 y.o. G	mg/kg iv	2 ml iv	mg/kg iv	mg/kg iv				
1947	XYL 0.5	DET 0.01	DET/BUT	Saline	Saline	DET 0.03	ACP 0.05	XYL 1.0
17 y.o. G	mg/kg iv	mg/kg iv	0.01/0.02	2 ml iv	2 ml iv	mg/kg iv	mg/kg im	mg/kg iv
1944	Saline	DET/BUT	DET 0.01	XYL 0.5	DET 0.03	Saline	XYL 1.0	ACP 0.05
12 y.o. G	2 ml iv	0.01/0.02	mg/kg iv	mg/kg iv	mg/kg iv	2 ml iv	mg/kg iv	mg/kg im
1953	Saline	XYL 0.5	DET 0.01	DET/BUT	XYL 1.0	ACP 0.05	DET 0.03	Saline
13 y.o. G	2 ml iv	mg/kg iv	mg/kg iv	mg/kg iv	mg/kg iv	mg/kg im	mg/kg iv	2 ml iv
1937					ACP 0.05	XYL 1.0	DET 0.03	Saline
10 y.o. G					mg/kg im	mg/kg iv	mg/kg iv	2 ml iv

XYL = xylazine; DET = detomidine; ACP = acepromazine; DET/BUT = detomidine/ butorphanol given in combination; iv = intravenous; im = intramuscular; G = gelding; F = female. Gastric emptying tests and treatments were staggered over the appropriate week of both periods 1 and 2. A minimum of 6 days was allowed for each subject between tests. Subject 1939 was replaced by subject 1937 in period 2, due to acquired intolerance of the test meal.

In total, 64 completed breath tests were performed in this section of the study in order to determine the relative effects of the pharmacological agents on solid phase GE rate. In addition to allowing measurement of the relative effect of different sedative protocols on gastric function, the study was designed to investigate possible dose-dependent inhibition of GE with minimal loss of statistical power, by comparison of results between the two periods. Each test was performed in the same place each week, outdoors in the horses' enclosures, so that minimal stress or disruption was caused to the horses' daily routine. During the breath collection procedure each horse was loosely restrained by a lead rope. The study was approved by the Texas A&M University Laboratory Animal Care Committee (Animal Use Protocol 2001-60). A summary of the study design is presented in Table 5.1.

5.2.2 Subjects

Nine healthy adult horses (6 Quarter Horses and 3 Thoroughbreds) of median age 12.0 y (mean 12.3 y, range 9 to 17 y) and weight 518.5 kg (mean 497.5, range 422.7 to 530.7 kg) were used in the study. The 8 geldings and 1 mare in this group comprised part of the Texas A&M research herd. In order to be acceptable to study criteria, each subject was required to be in good body condition, free from clinical evidence of systemic disease, and without historical record of gastrointestinal disease. Each horse also had total blood cell counts and biochemical parameters within the reference range as detailed in section 2.1.1. From 2 weeks before and throughout the study, the horses were maintained outdoors on a low ^{13}C diet of *ad libitum* alfalfa hay, to ensure a constant basal metabolic production of $^{13}CO_2$. Previous work, detailed in Chapter 3, had shown that this diet resulted in a constant basal output of $^{13}CO_2$:$^{12}CO_2$ over the 12 h study period, allowing subsequent dietary enrichment to be used to determine GE rate.

5.2.3 ^{13}C-octanoic acid breath test protocol

The ^{13}C-OABT was performed as has been detailed previously (Sutton *et al.* 2002a, 2003). Food was withheld for 12-14 h to ensure an empty stomach, before voluntary ingestion of the labelled test meal (150 g crimped oats, 100 g bran, 200 ml water) containing 1 mg/kg octanoic acid-1-^{13}C, of minimum 99 atom % ^{13}C (Isotec Inc., Ohio, USA) in 2 baked egg yolks. Two expiratory breath samples were collected prior to test meal ingestion, and thereafter samples were collected in duplicate at 15-min intervals for 6 h, then at 30-min intervals for 3 h, using a modified Aeromask® and 250 ml Quintron® collection bag as described in section 2.4.1. Breath samples were stored in 10 ml Exetainer® tubes until analysed. Food and exercise were prohibited for the duration of the test to minimise basal fluctuations in VCO_2 and $^{13}CO_2$ production, but there was free access to water. The individual treatments were administered intravenously immediately after consumption of the test meal, by injection into the left jugular vein, except in the case of acepromazine which was administered into the left neck muscle 5 min before test meal ingestion.

5.2.4 Expiratory breath $^{13}CO_2$ measurement

The total CO_2 content and ^{13}C:^{12}C ratio of each sample was analysed by continuous flow isotope ratio mass spectrometry (PDZ Europa ABCA) using a calibrant 5% CO_2 in nitrogen standard gas. This was performed as detailed in section 2.5.2 and in Sutton *et al.* (2002a). The analytical accuracy of the generated $\delta^{13}C$ value was maintained below an acceptable s.d. of 0.2 per run for quality control $\delta^{13}C$ values. After subtraction of the average ^{13}C-abundance of the baseline (predose) breath samples, the $\delta^{13}C$ ratio of each

sample was converted to S.I. units (parts per million excess ^{13}C). Data were then expressed as percentage dose recovery (PDR) of the administered isotope per hour using equation 2.5, described in section 2.5.2.

5.2.5 Calculation of gastric emptying indices

The equine solid phase gastric emptying data generated in this study were modelled using the power regression formula developed by Ghoos *et al.* (1993) for human gastric emptying data, as described in section 2.6 of this thesis:

$$y(t) = at^b e^{-ct}$$ where $y(t)$ is the % $^{13}CO_2$ excretion in breath per h; t is time (h) and a, b and c are regression constants. Curve fitting and calculation of constants was performed by non-linear least squares regression analysis using the Microsoft Excel Solver function (section 2.6.4). From the modelled curve, 3 parameters of gastric emptying were calculated for each test, as described in sections 2.6.2 and 2.6.3: (i) the gastric half-emptying time, $t_{1/2}$ (h), equivalent to that time point under the cumulative dose recovery curve at which half of the total ^{13}C cumulative dose recovery has occurred (corresponding to movement of half of the test meal from the stomach to the small intestine); (ii) the gastric lag phase, t_{lag} (h), equivalent to the inflexion point of the cumulative ^{13}C dose recovery curve (and approximating the duration of trituration); and (iii) the gastric emptying coefficient (GEC), a universal index of gastric emptying rate, introduced by Ghoos *et al.* (1993) to reflect the shape of the ^{13}C-OABT tracer recovery curve. Fuller definitions and the specific formulae used to generate these parameters are given in sections 2.6.2 and 2.6.3.

5.2.6 Statistical analysis

A general linear model ANOVA and Tukey pairwise 95% simultaneous confidence interval two-tailed tests were used to determine the effect of the different sedatives on gastric emptying parameters within each period of treatment, relative to the saline control. This analysis was performed using Minitab for Windows, release 13 (Minitab Inc., State College, PA, USA). For inter-period comparisons, paired data sets were available from 7 horses. Paired *t*-tests were used to determine whether or not there was a significant difference between the control (saline) GE results in periods 1 and 2. Due to the small sample size, it was also elected to investigate this using the more conservative non-parametric Wilcoxon signed rank test. In order to examine the effect of dosage of xylazine/detomidine on GE rate, the mean differences in $t_{1/2}$ between saline and xylazine 0.5/1.0 mg/kg and between saline and detomidine 0.01/0.03 mg/kg were calculated for

each treatment period. This served to control for both temporal and environmental factors that may have exerted differential influence on the results between periods. The significance of inter-period differences (i.e. dose-related differences) in these data sets was then examined using Wilcoxon signed rank tests.

5.3 Results

Of the 64 solid phase gastric emptying tests performed, 61 were successful. Data from 3 tests in period 2 were not included in the analysis due to incomplete ingestion of the test meal (n = 2) or problems with quality control of sample analysis by mass spectrometry (n = 1). Some horses became less keen to consume the test meal as the 8-week trial period progressed, perhaps due to increased sensitivity to the taste of the tracer. One horse (no.1939) was removed from the study for this reason, and replaced by horse no. 1937. However, in the remaining individuals, this potential problem was obviated by refrigeration of the ^{13}C-octanoic acid-labelled egg substrate, prior to addition to the test meal. Palatability was enhanced by this procedure, and the average meal consumption time thereafter remained approximately 5 min. The indices of gastric emptying calculated for each specific test, according to individual, week and treatment, are shown in Table 5.2.

5.3.1 Period 1 results

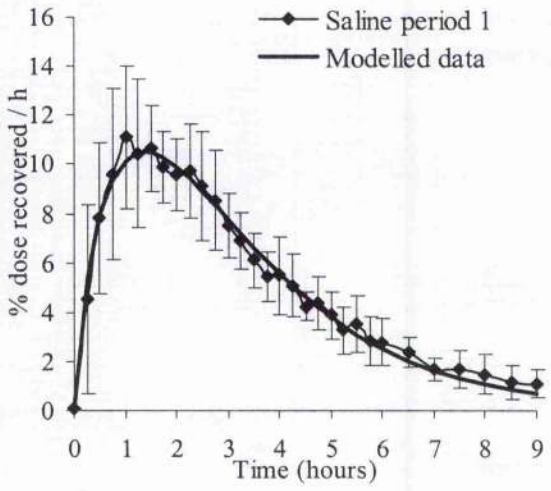
The mean % dose recovery per hour of the ^{13}C tracer in the breath of the 8 horses was calculated for every time point of each treatment and modelled using the given formula of Ghoos *et al.* (1993). These mean (± s.d.) values, together with the modelled dose recovery curves are shown in Figures 5.1 – 5.4. It can be seen that the shape of the modelled curves varies for each treatment. Although most of the curves describe a characteristic skewed bell-shaped distribution, xylazine 0.5 mg/kg (XYL 0.5) administration tended to result in a rapid early peak for isotope recovery with subsequent exponential decay. As shown in Figure 5.2, the rate of initial recovery of the isotope was underestimated by the modelling function, resulting in slight over-estimation of t_{lag} duration after this treatment.

For ease of comparison, the modelled mean values for each treatment in period 1 are also shown plotted together in Figure 5.9. Compared to the saline control, XYL 0.5 had little effect on the mean time of maximal emptying rate (t_{lag}), but did reduce the duration of the lag phase in certain individuals. In contrast, it can be seen that t_{lag} was prolonged by detomidine 0.01 mg/kg (DET 0.01) and delayed further still by the detomidine / butorphanol (DET/BUT) combination.

Table 5.2 Summary of the gastric emptying parameters generated for each individual, according to treatment, period and week of the trial. Treatments were randomised according to individual and week.

	Time:	Period 1			Time:	Period 2		
Subject	*Week 1*	*Week 2*	*Week 3*	*Week 4*	*Week 1*	*Week 2*	*Week 3*	*Week 4*
1949	Saline	DET 0.01	XYL 0.5	DET/BUT	Saline	XYL 1.0	DET 0.03	ACP 0.05
$t_{1/2}$ (h)	2.079	3.186	2.526	3.858	2.354	4.049	6.405	3.378
t_{lag} (h)	1.119	2.761	1.910	3.470	1.430	3.722	5.993	2.705
1936	DET/BUT	Saline	DET 0.01	XYL 0.5	ACP 0.05	Saline	XYL 1.0	DET 0.03
$t_{1/2}$ (h)	3.172	2.283	3.068	2.688	2.887	*	3.352	6.173
t_{lag} (h)	2.752	1.179	2.743	1.937	1.272	*	3.046	5.697
1938	XYL 0.5	DET/BUT	Saline	DET 0.01	ACP 0.05	DET 0.03	Saline	XYL 1.0
$t_{1/2}$ (h)	2.179	3.188	2.580	3.516	3.280	5.095	2.719	4.211
t_{lag} (h)	1.469	2.522	1.670	3.050	2.046	4.797	2.013	3.551
1940	DET 0.01	XYL 0.5	DET/BUT	Saline	DET 0.03	ACP 0.05	XYL 1.0	Saline
$t_{1/2}$ (h)	2.553	2.636	2.861	2.855	4.321	2.906	3.678	2.683
t_{lag} (h)	2.018	1.804	2.482	1.846	4.008	1.805	3.239	1.850
1939	DET 0.01	Saline	XYL 0.5	DET/BUT				
$t_{1/2}$ (h)	2.512	3.289	3.415	3.870				
t_{lag} (h)	1.573	2.151	2.109	3.518				
1947	XYL 0.5	DET 0.01	DET/BUT	Saline	Saline	DET 0.03	ACP 0.05	XYL 1.0
$t_{1/2}$ (h)	2.387	3.169	4.447	2.823	4.364	5.509	*	3.590
t_{lag} (h)	1.681	2.494	4.048	2.122	2.108	5.118	*	3.245
1944	Saline	DET/BUT	DET 0.01	XYL 0.5	DET 0.03	Saline	XYL 1.0	ACP 0.05
$t_{1/2}$ (h)	2.190	2.532	3.246	2.195	4.241	*	3.022	2.750
t_{lag} (h)	0.404	2.012	2.728	1.267	3.932	*	2.136	1.787
1953	Saline	XYL 0.5	DET 0.01	DET/BUT	XYL 1.0	ACP 0.05	DET 0.03	Saline
$t_{1/2}$ (h)	2.361	2.214	2.897	3.560	4.414	4.286	5.346	3.946
t_{lag} (h)	1.174	1.762	2.593	3.279	4.002	3.069	5.096	2.716
1937					ACP 0.05	XYL 1.0	DET 0.03	Saline
$t_{1/2}$ (h)					3.217	3.434	5.121	2.787
t_{lag} (h)					1.337	2.733	4.330	1.367

XYL = xylazine; DET = detomidine; ACP = acepromazine; DET/BUT = detomidine/butorphanol given in combination; $t_{1/2}$ = gastric half-emptying time; t_{lag} = duration of gastric lag phase. A minimum of 6 days was allowed for each subject between tests. Subject 1939 was replaced by subject 1937 in period 2, due to acquired intolerance of the test meal. *results not included due to incomplete ingestion of test meal (n = 2) or failure of mass spectrometer analytical quality control system (n = 1).



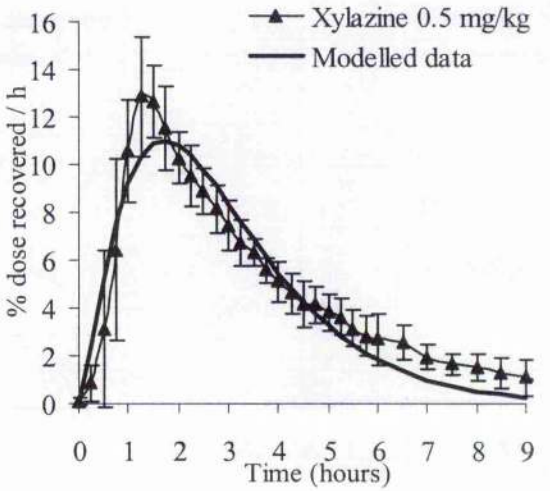
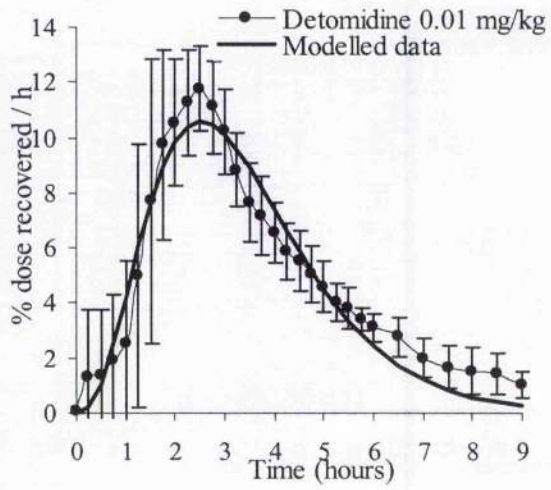


Figure 5.1 (left) Mean % dose recovery of the ^{13}C label in the breath of 8 horses after a ^{13}C-OABT, followed by intravenous injection of saline (control) in period 1. The standard deviation is shown at each point, together with the best fit curve produced by the modelling function.

Figure 5.2 (right) Mean % dose recovery of ^{13}C in the breath of the same 8 horses, following injection of xylazine 0.5 mg/kg immediately after test meal ingestion. The actual data produce a curve that is more exponential in shape than the best fit curve produced by the modelling function.



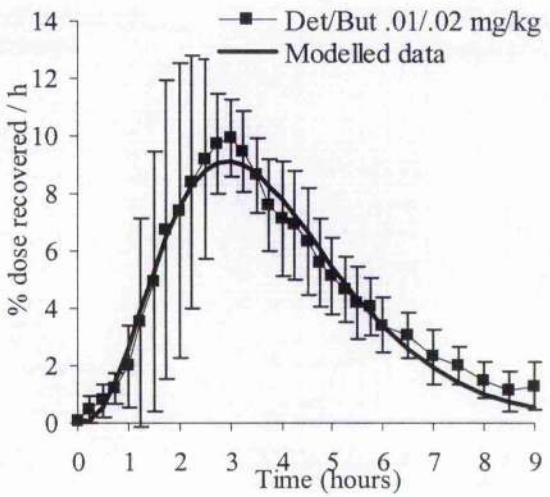
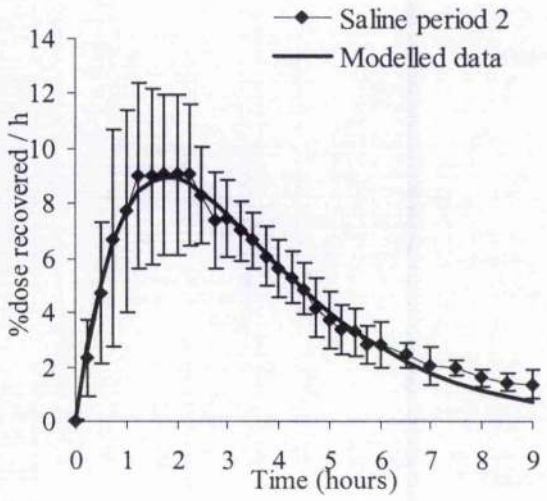


Figure 5.3 (left) The effect of intravenous detomidine administration (0.01 mg/kg) on % dose recovery of the ^{13}C tracer after ingestion of the standard labelled test meal (n = 8).

Figure 5.4 (right) Test meal ingestion followed by immediate intravenous treatment with detomidine/butorphanol combination (0.01/0.02 mg/kg) in 8 individuals. The mean (± s.d.) and modelled data points are plotted.



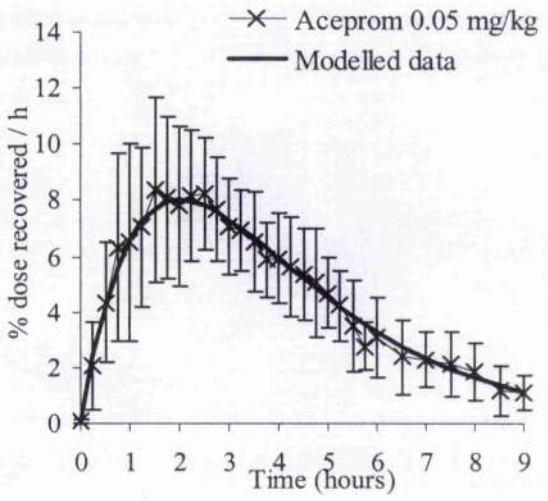
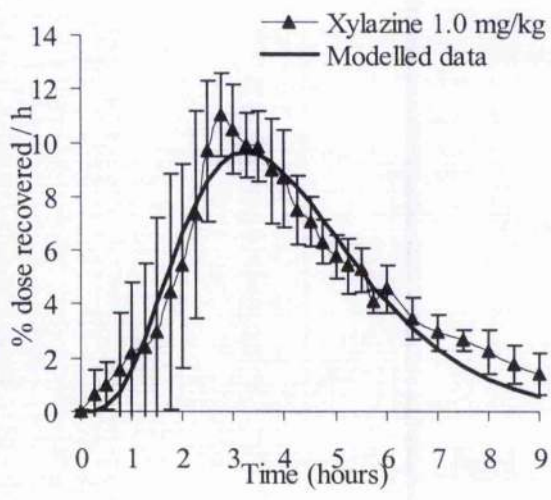


Figure 5.5 (left) Mean (± s.d.) and modelled % dose recovery of the ^{13}C label in the breath of 6 horses after a ^{13}C-OABT, followed by intravenous injection of saline (control) in period 2.

Figure 5.6 (right) Mean ^{13}C-dose recovery in the test subjects in period 2 (n = 7), following intramuscular injection of acepromazine 0.05 mg/kg 5 min before ingestion of the labelled test meal.



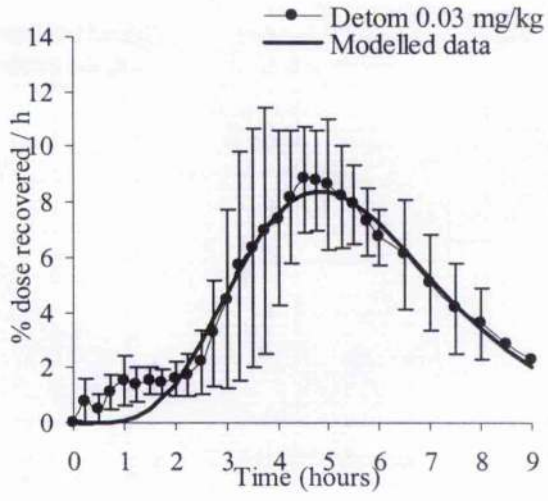
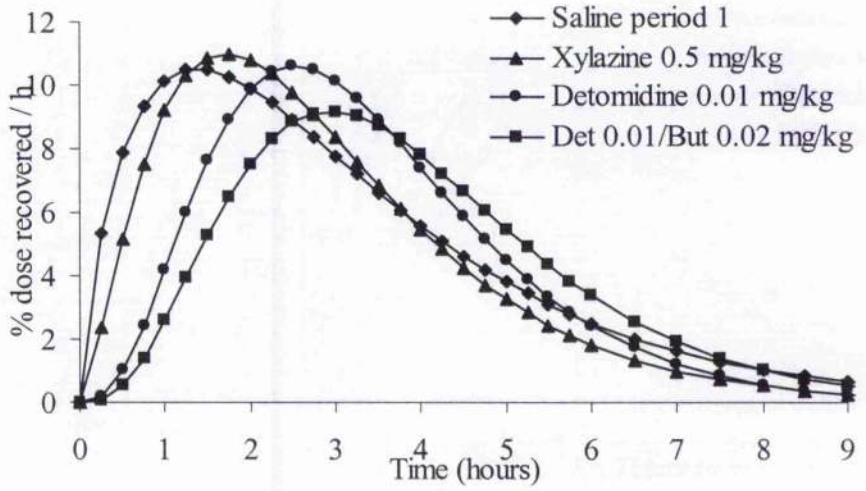


Figure 5.7 (left) Mean (± s.d.) and modelled % dose recovery of the ^{13}C label in the breath of 8 horses in period 2, when test meal ingestion was followed immediately by intravenous injection of xylazine 1.0 mg/kg.

Figure 5.8 (right) Mean (± s.d.) and modelled % ^{13}C dose recovery in 8 horses in period 2. Test meal ingestion was followed immediately by intravenous injection with detomidine 0.03 mg/kg.



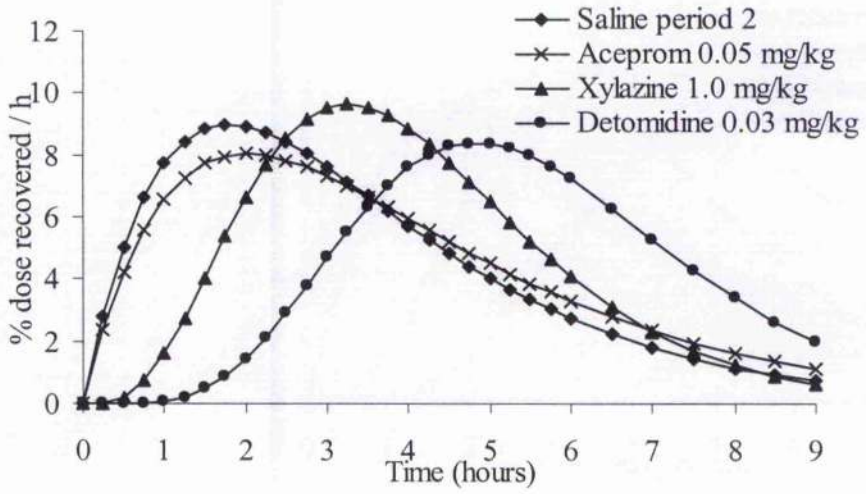


Figure 5.9 (top) Modelled mean % dose recovery curves of the ^{13}C tracer in the breath of 8 horses after a ^{13}C-OABT, followed by immediate treatment with saline, xylazine (0.5 mg/kg), detomidine (0.01 mg/kg) or detomidine/butorphanol (0.01/0.02 mg/kg) combination in random order, at weekly intervals.

Figure 5.10 (bottom) Modelled mean % dose recovery curves of the ^{13}C tracer in period 2. Test meal ingestion accompanied by randomised treatment with either saline, acepromazine (0.05 mg/kg), xylazine (1.0 mg/kg) or detomidine (0.03 mg/kg). Seven of the 8 horses used in period 2 were also used in period 1 (Fig 5.9). A total of 32 breath tests were performed in each 4-week period.

The mean (± s.d.) values for $t_{1/2}$, t_{lag} and GEC in the 8 horses after treatment with the saline control or the different sedative agents are shown in Table 5.3. Mean gastric $t_{1/2}$ after saline was 2.58 h, which was within the range previously reported in healthy horses using this technique (see Chapter 3; Sutton *et al.* 2003). The effect of each treatment on $t_{1/2}$ was consistent, with low coefficients of variation (CV%; (s.d./mean)*100). The inter-individual CV%s for saline, XYL 0.5, DET 0.01 and DET/BUT for $t_{1/2}$, t_{lag} and GEC are shown in Table 5.4.

Table 5.3 Effects of saline, xylazine (0.5 mg/kg), detomidine (0.01 mg/kg) and detomidine/ butorphanol (0.01/0.02 mg/kg) on mean gastric emptying indices in 8 horses (Period 1). Tests were performed in random order at weekly intervals. Matching superscript letters denote a significant difference between treatments (P < 0.05).

Treatment (mg/kg)	N	Mean (sd) $t_{1/2}$ (h)	Mean (sd) t_{lag} (h)	Mean (sd) GEC
Saline	8	^{a}2.58 (0.46)	^{c,d}1.24 (0.63)	^{g}2.81 (0.40)
Xylazine 0.5	8	^{b}2.53 (0.41)	^{e,f}1.74 (0.27)	^{h}3.13 (0.39)
Detomidine 0.01	8	3.02 (0.35)	^{c,e}2.50 (0.48)	2.58 (0.49)
Det. 0.01/But. 0.02	8	^{a,b}3.44 (0.62)	^{d,f}3.01 (0.68)	^{g,h}1.62 (1.31)

Specific P-values for superscript letters: a = 0.0082; b = 0.0018; c, d, f < 0.0001; e = 0.0023; g = 0.0414; h = 0.0031.

Table 5.4 Inter-individual coefficients of variation (CV%) for ^{13}C-OABT-derived gastric emptying indices following the different treatments administered in period 1.

Treatment (mg/kg)	N	$t_{1/2}$ CV%	t_{lag} CV%	GEC CV%
Saline	8	17.83	50.81	14.23
Xylazine 0.5	8	16.21	15.52	12.46
Detomidine 0.01	8	11.59	19.20	18.99
Det. 0.01/But. 0.02	8	18.02	22.59	80.86

DET 0.01 delayed gastric $t_{1/2}$ with respect to both saline (P = 0.244) and XYL 0.5 (P = 0.076) although this was not significant. However, the addition of the low dose of butorphanol (0.02 mg/kg) to DET 0.01 resulted in a further mean reduction in gastric emptying rate, and DET/BUT produced a significant delay in $t_{1/2}$ compared to both saline (P = 0.0082) and XYL 0.5 (P = 0.0018). Following XYL 0.5, $t_{1/2}$ was marginally faster than that following the saline control treatment.

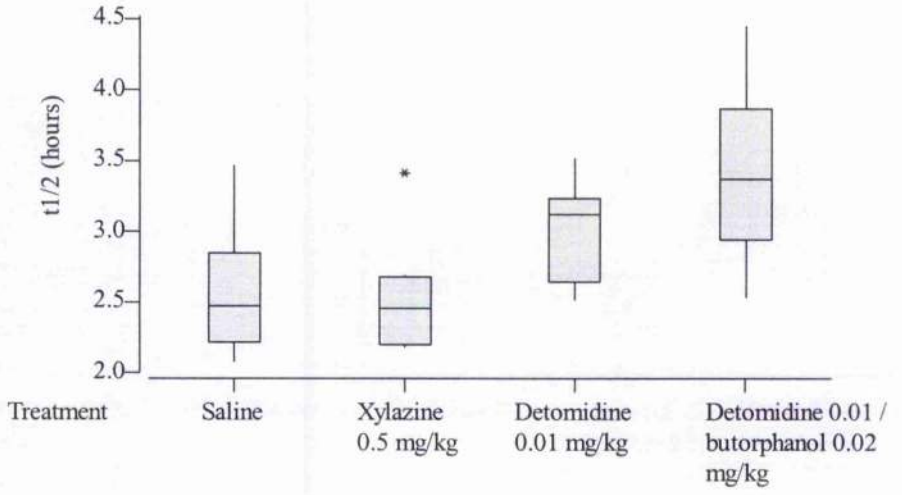


Figure 5.11 Boxplots demonstrating the effect of different treatments on gastric half-emptying time ($t_{1/2}$) of a standard test meal as measured by the ^{13}C-OABT. Each treatment was given to 8 horses in randomised order over a 4-week period (period 1). Total number of tests = 32. *denotes an outlying value.

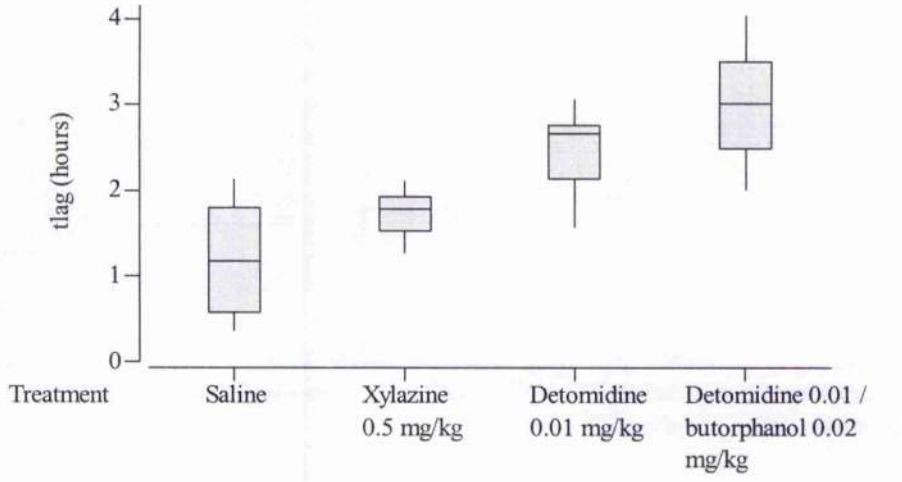


Figure 5.12 Boxplots showing the effect of period 1 treatments on gastric lag phase (t_{lag}) in 8 horses as measured by the ^{13}C-OABT. The total range, inter-quartile range (shaded box) and median values (horizontal bar) are represented by each symbol. Total number of tests = 32.

When considering t_{lag}, both DET 0.01 and DET/BUT delayed this parameter with respect to both saline (Ps < 0.0001) and XYL 0.5 (P = 0.0023; P < 0.0001). Boxplots have been used to summarise the effect of the period 1 treatment protocols on both $t_{1/2}$ (Figure 5.11) and t_{lag} (Figure 5.12). Mean GEC mapped the changes in the initial rate of isotope recovery caused by the different treatments, with DET/BUT causing a significant change relative to both saline (P = 0.0414) and XYL 0.5 (P = 0.0031).

5.3.2 Period 2 results

The modelled curves for mean % dose recovery per hour of the ^{13}C tracer in the 8 horses after the treatments administered in this period are shown in Figures 5.5-5.8. It can be seen that the dose recovery curves following both saline and acepromazine 0.05 mg/kg (ACP 0.05) administration were similar, while recovery of the ^{13}C isotope (and hence gastric emptying) was delayed following xylazine 1.0 mg/kg (XYL 1.0) and dramatically so, following detomidine 0.03 mg/kg (DET 0.03). The mean values for $t_{1/2}$, t_{lag} and GEC for the 8 horses after the treatments administered in this period are shown in Table 5.5. The inter-individual coefficients of variation for these parameters after period 2 treatments were relatively small, and are presented in Table 5.6.

Table 5.5 Effects of saline, acepromazine (0.5 mg/kg), xylazine (1.0 mg/kg) and detomidine (0.03 mg/kg) on mean gastric emptying indices in 8 horses (Period 2). Tests were performed in random order at weekly intervals. Matching superscript letters denote a significant difference between treatments (P < 0.05).

Treatment (mg/kg)	N	Mean (sd) $t_{1/2}$ (h)	Mean (sd) t_{lag} (h)	Mean (sd) GEC
Saline	6	^{a}3.14 (0.81)	^{d,e}1.91 (0.50)	^{i}2.70 (0.43)
Acepromazine 0.05	7	^{c}3.24 (0.52)	^{f,g}2.00 (0.67)	^{j}2.39 (0.43)
Xylazine 1.0	8	^{b}3.71 (0.48)	^{d,g,h}3.19 (0.61)	^{k}1.35 (1.07)
Detomidine 0.03	8	^{a,b,c}5.28 (0.77)	^{e,f,h}4.87 (0.75)	^{i,j,k}-3.01(2.33)

Specific P-values for superscript letters: a = 0.0001; b = 0.0007; c = 0.0005; d = 0.0004; g = 0.0031; e, f, h, i, j, k = < 0.0001.

Table 5.6 Inter-individual coefficients of variation (CV%) for ^{13}C-OABT-derived gastric emptying indices following the different treatments administered in period 2.

Treatment (mg/kg)	N	$t_{1/2}$ CV%	t_{lag} CV%	GEC CV%
Saline	6	25.80	26.18	15.93
Acepromazine 0.05	7	16.05	33.50	17.99
Xylazine 1.0	8	12.94	19.12	79.26
Detomidine 0.03	8	14.58	15.40	129.18

ACP 0.05 did not have a significant effect on any parameter when compared to the saline control treatment in this period. In marked contrast, DET 0.03 delayed both $t_{1/2}$ and t_{lag} with respect to saline, ACP 0.05 and XYL 1.0 (in each case, $P < 0.001$). Mean $t_{1/2}$ after XYL 1.0 was not significantly different to that following saline or ACP 0.05 treatment. However, XYL 1.0 did delay t_{lag} relative to both saline ($P = 0.0004$) and ACP 0.05 ($P = 0.0027$). As expected (since the derivation of GEC is numerically related to both $t_{1/2}$ and t_{lag}), DET 0.03 also changed GEC with respect to saline, ACP 0.05 and XYL 1.0 ($P < 0.0001$). Boxplots are presented to highlight the effect of treatments on $t_{1/2}$ (Figure 5.12) and t_{lag} (Figure 5.13) in this period. The boxplots represent in each case the range, inter-quartile range (shaded box) and median value (horizontal bar) of the gastric index plotted on the *y*-axis.

5.3.3 Inter-period comparison of results

5.3.3.1 Temporal effects on gastric emptying control values

There was a tendency in period 2 for gastric $t_{1/2}$ after saline to exceed that of period 1, and this was seen in 4 of the available 5 paired control data sets. However, a paired *t*-test showed that this was not significant ($0.05 < P < 0.1$). When all the data (paired and unpaired) were combined, mean $t_{1/2}$ and mean t_{lag} after saline remained greater in period 2 than period 1, but neither difference was significant. Two control data sets from period 2 could not be used, due in one case to the subject consuming less than half of the test meal, and, in the second, to a quality control fault on the mass spectrometer. Unfortunately, due to the nature of the study design, further control data sets could not be acquired without the risk of introducing new error factors. With a larger number of control (saline) data sets, it is possible that a statistically significant reduction in the rate of gastric emptying during the progression of the study may have been noted. However, the data presented here do not provide statistical proof for this suggestion.

5.3.3.2 Dose-related effects of α_2-adrenergic agonists on gastric emptying

In order to determine the effect of α_2-agonist dose on GE, the mean difference in $t_{1/2}$ between saline and XYL 0.5/XYL 1.0, and saline and DET 0.01/DET 0.03 was calculated for each treatment period. This procedure served to control for the possible temporal effects on GE in the study animals outlined in section 5.3.3.1. Due to the relatively small size of these data sets, the significance of inter-period differences in the mean (α_2 agonist $t_{1/2}$ – saline $t_{1/2}$) values was examined using conservative non-parametric Wilcoxon signed rank tests, as criteria for *t*-tests were not satisfied.

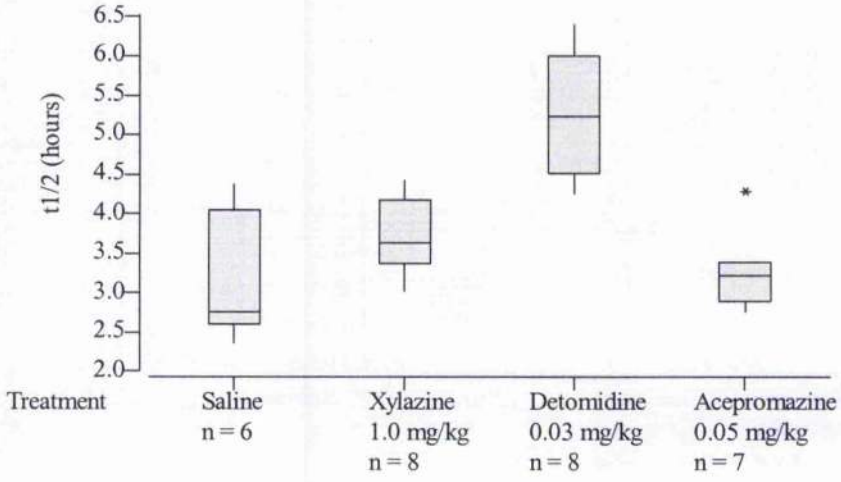


Figure 5.13 Boxplots demonstrating the effect of period 2 treatments on gastric $t_{1/2}$, as measured by the ^{13}C-OABT. Four randomised treatments were given to each of 8 individuals at weekly intervals. Data from 3/32 tests were not suitable for inclusion in the study. *denotes outlying value.

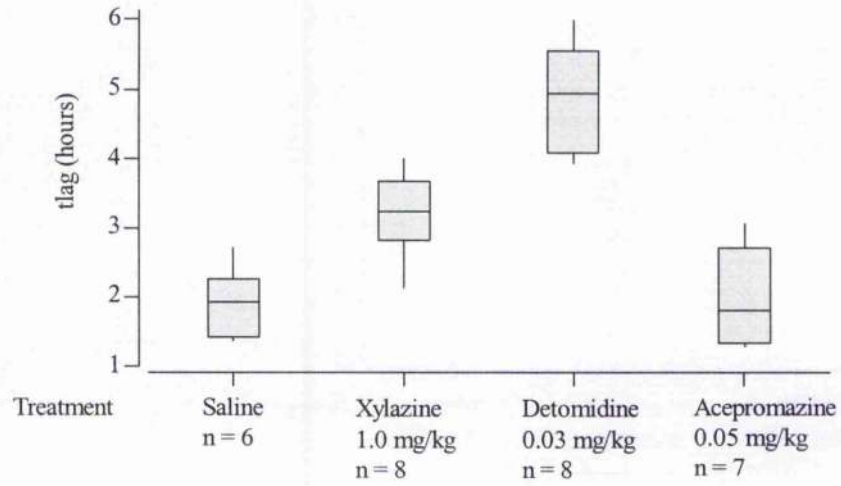


Figure 5.14 Boxplots highlighting the range, inter-quartile range, and median values for t_{lag} in the 8 individuals in period 2, following administration of the specific sedative agents after ingestion of the test meal. Treatments were randomised for both week and individual.

Although there was a trend towards a slowing of GE with increased xylazine dose, neither mean $t_{1/2}$ nor mean t_{lag} following XYL 1.0 were significantly different compared to XYL 0.5. Therefore xylazine was not shown to have a significant dose-dependent effect on equine GE rate. However, the delays in mean $t_{1/2}$ and mean t_{lag} caused by DET 0.03 were significantly greater than those caused by DET 0.01 ($P < 0.05$). Hence, detomidine was shown to produce a significant dose-dependent slowing of equine solid phase GE rate.

5.4 Discussion

5.4.1 Summary of study results

The results of this study suggest that doses of xylazine (0.5 mg/kg) and detomidine (0.01 mg/kg) with apparent equipotent sedative effect (England *et al.* 1992) differ in their effect on solid phase GE rate in healthy horses. Interestingly, XYL 0.5 tended to promote early initial GE, without affecting $t_{1/2}$, whilst DET 0.01 delayed both $t_{1/2}$ and t_{lag}. When the common clinical combination of detomidine / butorphanol (0.01/0.02 mg/kg) was used (Clarke and Paton 1988; Taylor *et al.* 1988), gastric $t_{1/2}$ and t_{lag} were delayed significantly. In addition, detomidine produced a marked dose-dependent slowing of GE rate, which was not seen with xylazine, although gastric emptying was slowed with XYL 1.0. Based on the results presented, DET 0.03 is likely to have a clinically significant effect on equine solid phase GE rate. By contrast, acepromazine used at a standard premedicant intramuscular dose of 0.05 mg/kg (Marntell and Nyman 1996) had negligible effect on equine gastric transit time.

The effect of sedative agents on equine solid phase GE has not, to the author's knowledge, been previously investigated. In the United States, where the study was performed, xylazine and detomidine are both licensed for equine analgesia and sedation at intravenous dosages of 1.1 mg/kg and 0.02 or 0.04 mg/kg respectively, and the marked difference on GE observed between the agents within these dose ranges was greater than expected. The effect of lower doses of the agents on GE was investigated in period 1, as these are the doses frequently used in clinical practice for sedation (Muir and Mason 1993; Bueno *et al.* 1999) and/or treatment of abdominal pain (Clarke and Paton 1988; Hamm *et al.* 1995). Once again, there was a marked difference in effect between XYL 0.5 and DET 0.01, which was augmented by the addition of butorphanol 0.02 mg/kg to the detomidine.

5.4.2 Specific drug effects

5.4.2.1 Alpha$_2$-adrenergic agonists

The differential effects of xylazine and detomidine on equine solid phase GE must be interpreted with reference to the existing pharmacological studies of their actions. Xylazine and detomidine cause sedation by depression of the locus coeruleus neurons in the pons of the lower brainstem (England and Clarke 1996). Their antinociceptive action is mediated by stimulation of presynaptic α_2-adrenoceptors on pain modulating noradrenergic neurones in the brainstem, causing a reduction in the release of noradrenaline (Virtanen 1986). However, these agents also affect post-synaptic CNS α_2 receptors (Virtanen and MacDonald 1985) and at higher concentrations are partial agonists at α_1 receptors (Virtanen and Nyman 1985), resulting in reduced pain responses at the spinal level.

In vitro experiments have shown consistently that detomidine has a greater potency at α_2 receptors than xylazine (Virtanen and Nyman 1985), and this is matched by its superior visceral analgesia in both clinical cases (Jochle *et al.* 1989) and experimental models (Lowe *et al.* 1986) of equine colic. Using a caecal balloon induced model of colic, Lowe *et al.* (1986) found that detomidine at 0.02 mg/kg provided good equine visceral analgesia for about 40 min, which was of similar depth, but greater duration to xylazine 1.1 mg/kg. Similar results have been reported for response in clinical case series of equine colic (Jochle and Hamm 1986; Jochle 1989). The lower doses of xylazine (Jochle *et al.* 1989) and detomidine (Lowe *et al.* 1986) used in period 1 of this present study both provide lesser, dose-dependent visceral analgesia. However, in light of the results presented in section 5.3.3.2 of this study, it should be remembered that augmenting detomidine dosage above 0.02 mg/kg principally increases duration rather than depth of analgesia (Jochle *et al.* 1989; Hamm *et al.* 1995).

The inhibition of gastric emptying seen in this study following DET 0.01/0.03 and XYL 1.0 administration is likely to have resulted from peripheral presynaptic stimulation of inhibitory α_2 receptors, causing decreased release of noradrenaline and consequent reduction in acetylcholine release from the enteric plexi (Gerring and Hunt 1986; Rang *et al.* 1999). As mentioned in section 5.1.4.1, it has also been shown in other species that presynaptic α_2-adrenoceptors exist on the vagal cholinergic nerve terminals of the myenteric plexus, similar to those found on the terminals of the sympathetic nerves (Drew 1978). Although this has not been documented in the horse specifically, α_2 agonist administration may inhibit acetylcholine release directly via action at such receptors. Central noradrenergic inhibition is also likely to have affected gastric motility (Virtanen

1986). The greater inhibitory effect of detomidine, compared with xylazine, on equine GE is perhaps not surprising in view of the pharmacological and clinical studies reported above, and is likely to result from its increased potency at α_2 receptors. The significant dose dependent effect of detomidine, and the lesser dose-related effect of xylazine, may be mediated by action at α_1 receptors at higher doses, or at a specific α_2 subtype (Rang *et al.* 1999). Further GE studies in the presence of selective antagonists would be required to investigate this hypothesis further. In the dog, it has also been demonstrated that both xylazine and medetomidine decrease gastrin release after ingestion (Nakamura *et al.* 1997). Altered gastrin release may be a further potential mechanism in the horse leading to reduced gastric motility after α_2 agonist administration, but this has not been proven.

An interesting and unexpected phenomenon in this study was the slight shortening of the gastric lag phase observed after XYL 0.5 administration. A similar phenomenon has been observed in human liquid phase emptying after pre-treatment with clonidine, a related α_2-agonist (Asai *et al.* 1997). It has been suggested that this may be due to inhibition of pyloric motor activity by action at presynaptic α_2 receptors (Merritt and Ruckebusch 1988; Allescher *et al.* 1989), allowing early entry of chyme into the duodenum. The α_1 agonist, phenylephrine, has also been shown to have this effect on canine pyloric tissue (Allescher *et al.* 1989). The effects of both xylazine and clonidine on upper gastrointestinal contraction in other species are conflicting. Xylazine has been reported to delay small intestinal transit in mice, without affecting gastric emptying (Hsu 1982), but prolonged both gastric residence and small intestinal transit of barium in dogs (Hsu and McNeel 1983). Clonidine delayed small intestinal transit but not gastric emptying in the rat (Ruwart *et al.* 1980), but did not delay either of these parameters in humans (Baxter *et al.* 1987). It seems that both species specificity and dose-related effects are likely to be in operation. At the higher dose (1.0 mg/kg) of xylazine used in this study, the inhibition of pyloric motility seen at lower doses is likely to have been outweighed by antral hypomotility, resulting in the overall reduction observed in emptying rate.

5.4.2.2 Butorphanol

In this study, butorphanol acted synergistically with detomidine to further delay gastric emptying. Butorphanol is a morphinan derivative that produces sedation and analgesia via partial antagonism and partial agonism at μ and κ opioid receptors respectively. In isolation, minimum doses of 0.1 mg/kg are usually required for effective equine visceral analgesia (Kalpravidh *et al.* 1984; Muir and Robertson 1985; Becht 1986; Stout and Priest 1986). However, the addition of low doses of butorphanol to α_2 agonists has been reported

to enhance sedation and analgesia while minimising cardiopulmonary depression, as lower doses of the α_2 agonists may be used (Taylor *et al.* 1988; Thurmon *et al.* 1996). Whilst its inhibitory effects at higher doses (0.1 mg/kg) on equine jejunal and large intestinal motility have been documented (Sojka *et al.* 1986), the additive effect of butorphanol 0.02 mg/kg on GE was greater than expected. Using electromyenterography, Merritt *et al.* (1989b) reported that butorphanol 0.05 mg/kg caused only a 'resetting' of the activity of the gastric antrum and duodenum, with minimal disruption to periodicity. The results of this study may therefore suggest genuine synergistic inhibition of butorphanol and detomidine on equine GE.

Studies in other species have suggested that there is vagal enkephalinergic neural control of the pylorus. Feline immunohistochemical studies have revealed a rich enkephalinergic innervation of the pylorus, with vagal neuronal enkephalin-like immunoreactivity in both the circular smooth muscle layer and the myenteric plexus (Edin *et al.* 1980). Intra-arterial injection of enkephalin in this species elicited a pyloric contraction and gastric relaxation, which could be inhibited by naloxone (Edin *et al.* 1980). Similarly, infusion of beta-endorphin in humans increased pyloric phasic pressure activity, and caused an overall reduction in antral phasic pressure activity (Camilleri *et al.* 1986). Increased basal pyloric pressure has been shown to correlate to closure of the pyloric sphincter, and cessation of antral flow into the duodenum (Tougas *et al.* 1992). These findings suggest that the inhibition of equine GE caused by butorphanol administration in this study may have resulted from direct stimulation of pyloric contraction in addition to antral hypomotility.

5.4.2.3 Acepromazine

Acepromazine is a potent neuroleptanalgesic (Thurmon *et al.* 1996). It is most frequently administered intramuscularly prior to anaesthesia to potentiate the effects of anaesthetic agents (Becht 1986). Acepromazine also inhibits α_2 agonist-induced bradycardia (Marntell and Nyman 1996) in addition to decreasing the required minimum alveolar concentration of inhalation agents (Doherty *et al.* 1997). Indeed, epidemiological evidence suggests that acepromazine administration prior to general anaesthesia may reduce the incidence of anaesthetic-related fatalities (Johnston *et al.* 1995). Previous equine work has suggested that acepromazine may decrease the electrical activity of jejunal Thiry-Vella loops (Davies and Gerring 1983) and also delay liquid phase emptying (Doherty *et al.* 1999). However, in accordance with the results of canine studies (Zontine 1973; Voges *et al.* 1995), and the equine myoelectrical study on the effects of acepromazine on ileal, caecal and colonic

activity (Lester *et al.* 1992), the results outlined here suggest that premedication doses of acepromazine have insignificant effect on equine solid phase GE rate.

5.4.3 Effects of specific sedatives on different regions of the equine gastrointestinal tract

The results of this study document both the comparative and dose-related effects of specific sedative agents on equine solid phase gastric emptying, using a new diagnostic technique (Wyse *et al.* 2001; Sutton *et al.* 2002a, 2003). However, although GE is a rate-determining step in small bowel transit, extrapolations should not be made to the entire gastrointestinal tract as regional differences exist, particularly in response to specific autonomic transmitters (Hunt and Gerring 1986). In general terms, the results of electromechanical studies have suggested that the left dorsal colon, left ventral colon and caecum have greater sensitivity than the small intestine (most studies have assessed jejunal response) to equipotent doses of the α_2 agonists (Adams *et al.* 1984; Sasaki *et al.* 2000), with detomidine causing the most potent inhibition of activity (Lowe *et al.* 1986). It is relevant to note that whilst xylazine 0.5 mg/kg has been reported to have negligible (Merritt *et al.* 1989a) or temporary (Merritt *et al.* 1998) inhibitory effect on equine duodenal motility, the same dose has also been reported to reduce the spike burst duration of the ileum, right ventral colon, small colon and to decrease the caecal emptying rate of radiolabelled markers (Lester *et al.* 1998). Xylazine may also produce a dose dependent inhibition of both equine caecal mechanical activity (Hunt and Gerring 1986; Singh *et al.* 1997) and blood flow (Clarke *et al.* 1988; Rutkowski *et al.* 1991). Hence, it is reiterated that the results of this study specifically document the effect of these sedative agents on equine gastric motility alone.

5.4.4 Longitudinal temporal effects on gastric emptying rate

Although not of statistical significance, there was a trend in period 2 of the study for the subjects to have relatively slower GE after control saline injection than seen in period 1. Had the study involved a greater number of subjects, or been of greater duration, it is possible that this difference may have been significant. This finding in itself is worthy of further investigation. Colic is cited as the most common medical disease problem of adult horses (Traub-Dargatz *et al.* 1991), and in prospective and retrospective epidemiological investigations of risk factors, several managemental changes have been implicated. These include a change in hay feeding (Tinker *et al.* 1997; Hudson *et al.* 2001) or other recent dietary change (Cohen *et al.* 1995; Hudson *et al.* 2001), decreased exposure to pasture (Hudson *et al.* 2001) and a recent change in the level of daily activity (Cohen *et al.* 1995).

All of these factors were in fact present in the horses used in this study, although the dietary change from coastal grass pasture to *ad libitum* alfalfa hay was effected at least 2 weeks prior to the start of the study. There is some evidence from a single, poorly designed study that sudden management change from pasture to stabling with concentrate feed resulted in significantly increased gastrointestinal motility in 6 horses (Nicholls and Freeman 2002), followed by a subsequent reduction in large intestinal motility. Unfortunately, Nicholls and Freeman (2002) employed no case-control, and gastric emptying was not assessed. However, long-term changes in gastrointestinal motility associated with managemental change may help to explain the colic risk factors identified by epidemiological studies. Further investigation of the effect of specific managemental changes on equine GE, using the ^{13}C-OABT, may have potential value in determining the aetiology of equine colic.

In retrospect, gastroscopy of each subject before the start of the study and again, at its conclusion would have been helpful in determining whether or not development of gastric ulceration was linked to the changes observed in control GE rates in periods 1 and 2. Alfalfa hay has been reported to buffer gastric acid, and to protect the squamous mucosa from ulcer development (Nadeau *et al.* 2000), but the possibility of ulcer development during the course of the study cannot be disregarded.

5.4.5 Possible limitations of the study

The underlying principle of the ^{13}C-OABT is that gastric emptying is the major rate-determining step in the recovery of the ^{13}C-label in the breath following its ingestion, and that the rate of post-gastric handling of the isotope remains constant. It is recognised in this study that factors other than gastric emptying rate may possibly have affected the rate of expiratory recovery of the isotope.

5.4.5.1 Rate of absorption from the small intestine

If any of the administered drugs increased the rate of absorption of liquids from the small intestine, then the rate of hepatic delivery of the ^{13}C-octanoic acid via the portal vein may have been enhanced. In isolated rabbit ileum, alpha$_2$-agonists decreased the short-circuit current, and enhanced both ion and fluid transport (Chang *et al.* 1982). However, the medium-chain fatty acids are absorbed from the small intestine as rapidly as glucose (Bach and Babayan 1982), before travelling to the liver bound to serum albumin. Changes in the rate of small intestinal fluid absorption are unlikely to have had significant effect on the overall rate of post-gastric metabolism of the tracer.

5.4.5.2 *Effect of arterial blood pressure on gastric emptying rate*

After producing an initial hypertension due to increased peripheral vascular resistance (Hubbell *et al.* 1999), both xylazine (Stick *et al.* 1987; Singh *et al.* 1997) and detomidine (Muir and Mason 1993; England and Clarke 1996) then result in decreased mean arterial blood pressure. This induced hypotension may either have influenced gastric emptying directly, or have decreased metabolism of the ^{13}C-tracer by reducing hepatic blood flow. Though blood pressure effects may have had a role, XYL 0.5 did not delay the expiratory recovery of the isotope to any extent, whilst undoubtedly reducing arterial blood pressure (Bucno *et al.* 1999). In Muir and Mason's study (1993) of the cardiorespiratory effects of xylazine 0.5/1.0 mg/kg, detomidine 0.01 mg/kg and acepromazine 0.1 mg/kg, acepromazine was found to cause the greatest initial fall in systemic arterial blood pressure, followed in order by xylazine 0.5 mg/kg, xylazine 1.0 mg/kg and detomidine 0.01 mg/kg. Acepromazine induces hypotension by α_1-adrenoceptor blockade (Hubbell *et al.* 1999) and at the dose used in this study would have resulted in a significant fall in arterial pressure (Ballard *et al.* 1982), but its administration did not result in altered $^{13}CO_2$ recovery when compared with the control values.

It therefore appears unlikely that changes induced in arterial blood pressure or hepatic blood flow during this study were responsible for significant alterations in the rate of hepatic metabolism of the ^{13}C-octanoic acid. The effects of hypotension alone are also unlikely to have accounted for the changes observed in gastric emptying rate following drug administration. Administration of calcium channel blockers in humans at doses sufficient to reduce arterial blood pressure did not result in any slowing of either liquid or solid phase gastric emptying rate (Traube *et al.* 1985; Santander *et al.* 1988; Krevsky *et al.* 1992).

5.4.5.3 *Hepatic enzyme function*

Several factors affect hepatic enzyme activity and it is possible that elimination of the alpha$_2$-agonists may have influenced metabolism of the ^{13}C-octanoic acid, thereby reducing the rate of appearance of $^{13}CO_2$ in the breath. Oxidation of ^{13}C-octanoic acid relies on the action of hepatic mitochondrial matrix enzymes. After acylation on the outer mitochondrial membrane, the acyl CoA enters the β-oxidation pathway, a series of oxidation, hydration, oxidation and thiolysis reactions, ending in the formation of acetyl CoA and acyl (n-2) CoA (Bach and Babayan 1982). The alpha$_2$-agonists are substituted imidazoles, and have been shown to inhibit reactions mediated by cytochrome-P450 enzymes, such as adrenal steroidogenesis (Maze *et al.* 1991) and ketamine demethylation

(Kharasch *et al.* 1992), by direct binding to P-450 haem iron via the substituted imidazole moiety (Rodrigues and Roberts 1997). Compounds requiring hepatic β-oxidation, such as tianeptine (Fromenty *et al.* 1989) and sodium benzoate (Kalbag and Palekar 1988) have been shown to cause a significant reduction in octanoic acid metabolism *in vivo*. Given the shared pathways, this may not be surprising, but compounds requiring cytochrome P450 enzymic activity for their degradation, such as ibuprofen (Frencaux *et al.* 1990), have also been shown to inhibit octanoic acid β-oxidation. Clonidine, a similar α_2-agonist, has been shown to reduce the hepatic clearance of both sulfobromophthalein (Ben-Zvi and Hurwitz 1985) and bupivacaine (Bruguerolle *et al.* 1995), but data on the specific interactions between xylazine/detomidine and MCFA oxidation have not been found.

In order to establish the significance of any interaction between the α_2-agonists and equine ^{13}C-octanoic acid metabolism *in vivo*, it would be necessary to determine $^{13}CO_2$ recovery rate after intra-duodenal administration of the labelled MCFA (as described in Chapter 3) in several subjects, before and after drug administration. Alternatively, *in vitro* hepatic preparations could be used in the first instance. This has yet to be performed in human studies, despite the use of the ^{13}C-OABT to measure the effect of anaesthetic agents such as propofol on gastric emptying rate (Chassard *et al.* 2002). Steps were taken to minimise any influence of hepatic metabolism on the study results by accepting only subjects with hepatocellular and bile acid parameters within the reference range. Studies in humans have shown that the rate of hepatic oxidation of octanoic acid is not affected even by marked cirrhosis, nor by reduction of hepatic blood flow using a transjugular intrahepatic portal shunt (Van de Casteele 2002). There is also the possibility of extra-hepatic, possibly renal, oxidative metabolism of the octanoic acid (Bach and Babayan 1982; Van de Casteele 2002). It is concluded that more investigation of this possible interaction would be desirable, but that this factor alone is unlikely to have accounted for more than a small part of the variation in test results observed in this study.

5.4.6 Clinical implications of the study results

It is of considerable clinical interest that detomidine at doses of 0.03 mg/kg or greater is likely to have detrimental effect on GE rate of solids. This effect may be more marked if given in combination with even low doses of butorphanol. Furthermore, in conditions such as endotoxaemia, where there is enhanced sympathetic neurotransmitter release (Bades and Moore 1993) or post-operative ileus, which is associated with reflex adrenergic stimulation (Glise *et al.* 1980; Gerring and Hunt 1986), the inhibitory effect of α_2 agonists on equine GE may be further enhanced. Yohimbine, a specific α_2 antagonist, has been shown to have

beneficial effect on small intestinal motility in a model of equine post-operative ileus (Gerring and Hunt 1986). Hence, in the clinical setting, high doses of detomidine should perhaps be avoided in cases likely to have delayed gastric emptying. In cases presenting with nasogastric reflux or gastric impaction, low doses of xylazine would be preferentially recommended to facilitate diagnostic procedures. Finally, it can also be concluded from this section of the study that the preanaesthetic administration of acepromazine to colic cases presenting without cardiovascular compromise would be unlikely to inhibit gastric emptying rate. Given the beneficial properties of acepromazine in equine anaesthetic protocols (Johnston *et al.* 1995), this finding may be of value when selecting the most appropriate anaesthetic regimens for equine exploratory laparotomy.

Chapter 6 INVESTIGATION OF EQUINE BICARBONATE POOL KINETICS AND LIQUID PHASE GASTRIC EMPTYING RATE

6.1 Introduction

6.1.1 Measurement of liquid phase emptying rate

The ^{13}C-Bicarbonate breath test (^{13}C-BBT) has been used to investigate liquid phase gastric emptying rate. The results of all $^{13}CO_2$ breath tests are ultimately dependent on the rate of recovery of labelled CO_2 from the body bicarbonate pool. As discussed in section 3.3.2.5, the real-time differences in estimates of solid phase $t_{1/2}$ derived from the ^{13}C-OABT and gastric scintigraphy for example are largely attributable to the delay caused by post-gastric handling of the isotope. Pallikarakis *et al.* (1991) demonstrated that the delay in peak $^{13}CO_2$ production after exogenous ^{13}C-substrate oxidation was directly linked to the influence of the body CO_2 stores. Bicarbonate pool kinetics are responsible for the rate of expiration of $^{13}CO_2$ after its production by the liver, and have previously been found to fit a 3-compartmental exponential model (Irving *et al.* 1983; Sphiris and Pallikarakis 1995; Van Hall 1999), corresponding to one central and two peripheral pools, as shown in Figure 6.1. This remains true at rest and during exercise (Barstow *et al.* 1990). The precise physiological identity of these three pools has not been established. However, the central pool is likely to represent vascular and interstitial bicarbonate, whilst the fast pool comprises metabolically active tissue of the visceral organs and the slow peripheral pool is contained primarily in resting skeletal muscle (Irving *et al.* 1983). During breath tests performed at rest, tracer entry and $^{13}CO_2$ loss is most likely to occur from the central pool (Van Hall 1999), whilst in all such tests (other than bicarbonate) $^{13}CO_2$ is likely to originate from the tissue of the fast pool, such as liver.

Pharmacokinetics of the equine bicarbonate pool have been little investigated, and the first aim of the work presented in this Chapter was to establish the pattern of $^{13}CO_2$ recovery after a simple sodium ^{13}C-bicarbonate breath test (^{13}C-BBT). It was hypothesised that this would be multi-phasic, due to kinetic differences in $^{13}CO_2$ exchange and recovery from the different pools. Terminal $^{13}CO_2$ recovery from the ^{13}C-OABT is frequently phasic in horses (as discussed in Chapter 3, see Figure 3.11 for example) and the cause of this phenomenon required further investigation. In addition, ^{13}C-Stable isotope tests have been used to measure whole body substrate oxidation rates (see Wolfe 1992c), based on expiratory $^{13}CO_2$ output following ingestion, but need to be corrected by a 'bicarbonate correction factor' due to body $^{13}CO_2$ retention (Hoerr *et al.* 1989; Wolfe 1992c).

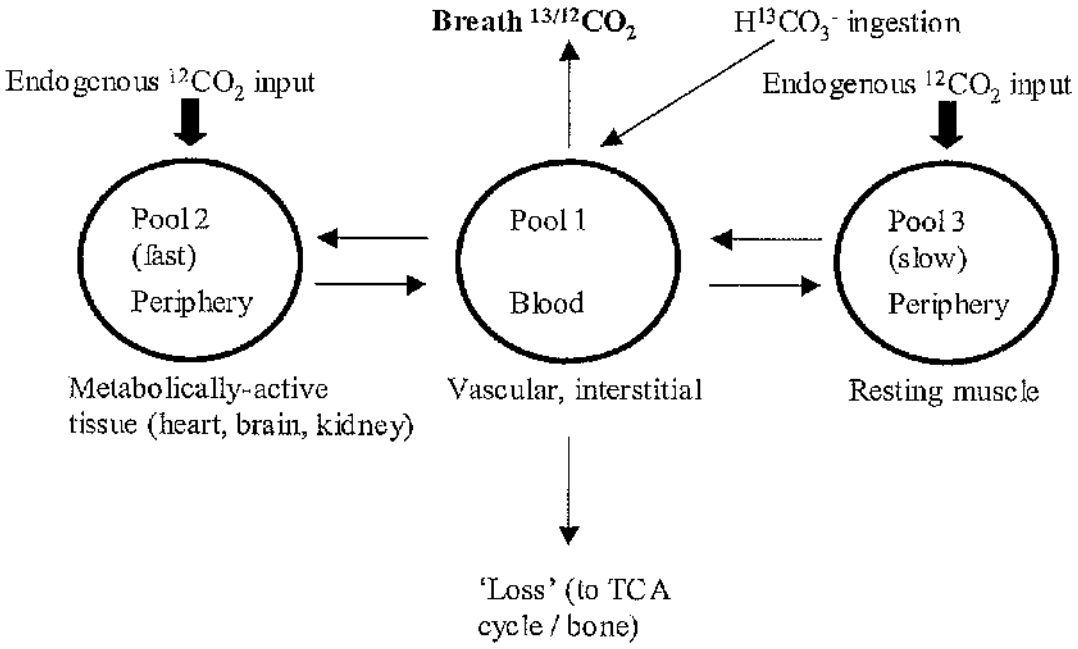


Figure 6.1 Three-compartment model for the mammalian bicarbonate pool, adapted from Van Hall 1999.

To aid future metabolic work in horses, a further aim was to measure the cumulative dose recovery of orally administered ^{13}C-bicarbonate, to determine if such a correction factor was necessary for equine oxidation studies. To avoid the potential error induced by rapid loss of $^{13}CO_2$ in the first few breaths after ingestion, prior to mixing with the entire bicarbonate pool (Wolfe 1992c), breath samples were collected at 5 min intervals for the first 1 h period in this study.

Bjorkman *et al.* (1991) investigated use of the ^{13}C-BBT in 15 gastric patients, and compared results with those of simultaneous scintigraphic measurement of solid and liquid phase gastric emptying rates. These investigators reported no correlation between the results of the breath test and scintigraphy for either measurement in man. However, the method used to calculate emptying rate from the ^{13}C-BBT in this study was poor; cumulative dose recovery of the isotope at 40 min was used to compare against scintigraphic t_{lag} and $t_{1/2}$, rather than the more specific corresponding measurements, such that the conclusions drawn from the study may be invalid.

6.1.1.1 Gastric emptying physiology

Solid, liquid and oil phases of gastric emptying are generally regarded to proceed at different rates (Maes *et al.* 1994). Low-nutrient liquids leave the stomach more quickly than solids (Collins *et al.* 1983), which must be triturated before passage through the pylorus with any residual liquid (Read and Houghton 1989). Liquids also redistribute much faster than solids within the stomach (Mistiaen *et al.* 2000) and emptying is usually exponential, with solid material remaining in the fundus until approximately 80% of the liquid has emptied (Houghton *et al.* 1988a). Whilst the GE rate of digestible solids is dependent on antral motor activity (Becker and Kelly 1983; Marciani *et al.* 2001), the emptying of liquids is more dependent on contraction of the fundus (Parkman *et al.* 1995). After initial receptive relaxation in response to the entry of fluids, progressive distention results in fundic contractions which increase the movement of fluid into the antrum and duodenum (Chaudhuri and Fink 1991). Both proximal and complete gastric vagotomy may increase the rate of liquid emptying, by removing the initial receptive relaxation, whilst inhibiting the emptying of solids (Wilbur and Kelly 1973).

However, it has been shown more recently that intragastric pressure alone is not the only factor regulating gastric liquid emptying rate (Paraskevopoulos *et al.* 1988). After fluid ingestion, initial pyloric pressure waves are gradually replaced by propagated antroduodenal contractions (Houghton *et al.* 1988b), and the role of fundic contractions may be to prime the antral pump to enhance its efficiency (Read and Houghton 1989). Hyperosmotic fluids or solutions containing fat empty more slowly in proportion to nutrient content (Hunt and Stubbs 1975; Collins *et al.* 1983; Collins *et al.* 1991; Gonzalez *et al.* 2000), mediated by small intestinal receptors, and produced by a combination of fundic relaxation, suppression of antral motility, increased pyloric resistance and a change in duodenal motor activity to favour mixing rather than propulsion (Paraskevopoulos *et al.* 1988; Read and Houghton 1989). Sosa Leon *et al.* (1997) reported that hyperosmotic saline and glucose solution emptied at the same rate as isotonic saline in Standardbred horses, but this study was fundamentally flawed by the concurrent medication of each individual with xylazine, which may enhance early gastric emptying rate, as discussed in Chapter 5 of this thesis.

A vital consideration when interpreting the results of liquid phase studies is whether or not the results are representative of solid phase emptying, and applicable to the clinical investigation and diagnosis of gastroparesis. During the early rapid emptying of liquid that occurs during the lag phase for solids, Camilleri *et al.* (1985) have shown that there is no

relation between overall liquid emptying and antral phasic pressure activity. This factor was the main reason for the predominance of work presented in this thesis towards the development of a non-invasive test for the measurement of solid (rather than liquid) phase GE in horses. GE of solids may be delayed in the presence of normal or accelerated liquid emptying rate (Mistiaen *et al.* 1998, 2001). However, a relationship has been noted between antral motility and liquid emptying after the initial lag period for solids has passed (Camilleri *et al.* 1985). Siegel *et al.* (1989) proposed that the slope of the terminal liquid emptying curve should be used for evaluation of liquid emptying, rather than a $t_{1/2}$ value. Using this approach in 37 patients with symptoms of gastroparesis, they reported a sensitivity of 96% and specificity of 100% for the diagnosis of delayed emptying of solids based on liquid phase slope, even though overall estimates of $t_{1/2}$ for the two phases were not correlated. The physiological theories of gastric emptying support the approach of Siegel *et al.* (1989), since solids empty with the terminal liquid component (Houghton *et al.* 1988a) as mentioned above.

6.1.1.2 *Value of liquid emptying measurement*

Although measurement of solid phase emptying may be a more robust method of diagnosing gastroparesis (Duan *et al.* 1995), information about the passage of liquid has clinical value. In their special report on gastrointestinal motility measurement, Camilleri *et al.* (1998) suggested that measurement of liquid GE may be preferable in patients with suspected gastric dumping syndrome, and that dual phase measurement is optimal in certain patients with symptoms that may be secondary to either rapid emptying of liquids or slow emptying of solids. In addition, information regarding liquid emptying physiology is of importance in equine medicine to determine, for example, the optimum time after exercise to allow a horse to drink, to maximise rehydration and minimise the risk of colic; the optimum management of post-operative ileus and gastric reflux, and the optimum feed composition, volume and regimen for neonates requiring milk replacement feeding.

6.1.1.3 *Previous methods of equine liquid emptying measurement*

The three major methods that have been reported for measurement of equine liquid emptying rate are dye dilution techniques (Baker and Gerring 1994a; Sosa Leon *et al.* 1997), acetaminophen absorption (Doherty *et al.* 1998, 1999b; Lohmann *et al.* 2000, 2002) and scintigraphy using ^{111}In – diethylenetriaminepentaacetic acid (DTPA) in water (Neuwirth 1994; Neuwirth *et al.* 1995; Lohmann *et al.* 2000). More experimental techniques have included impedance epigastrography (Baker and Gerring 1994b), or have required surgical creation of a gastric fistula (Healy *et al.* 1993) or implantation of a gastric

cannula (Campbell-Thompson and Merritt 1990). Of these, the latter two techniques are invasive, and not suited to the clinical investigation of GE rate.

In the dye dilution technique, a known concentration and volume of phenol red solution is administered by nasogastric tube. Measurement of progressive reduction in gastric volume is made by repeated aspiration of gastric contents, coupled with instillation of further boluses of phenol red of specific concentration. The phenol red concentration ratio of instilled to aspirated fluid is used to calculate the residual gastric volume. The technique requires frequent mixing and aspiration of gastric contents, and has large error margins caused by incomplete mixing of the dye with the gastric contents, and binding of the dye to particulate matter (Baker and Gerring 1994a). In common with radioscintigraphy and acetaminophen absorption tests, the procedure also necessitates nasogastric intubation. This may not be a good physiological model for the integrated responses involved in deglutition and gastric emptying. Both receptive and adaptive gastric relaxation appear to be present in the horse (Lorenzo-Figueras *et al.* 2002). The former is initiated by stimulation of pharyngeal mechanoreceptors during swallowing (Abrahamsson and Jansson 1973); vagal efferent preganglionic cholinergic fibres synapse with inhibitory intramural neurones, which exert their effect via non-adrenergic non-cholinergic transmission (Curro and Preziosi 1998). This mechanism may not be replicated by gastric intubation of fluids.

The measurement of serum acetaminophen concentration after ingestion of a labelled liquid was established as a valid means of measuring liquid phase gastric emptying rate in 1973 (Heading *et al.* 1973). Gastric absorption of acetaminophen is insignificant, and in the absence of pathology, absorption occurs in the proximal small intestine (Clements *et al.* 1978) after emptying from the stomach, which is the rate-limiting step (Heading *et al.* 1973). No correlation was found between gastric scintigraphy and acetaminophen for the measurement of semi-solid meal emptying, and the method is useful for estimation of liquid emptying rate only (Petring *et al.* 1986). The technique has been validated in horses against radioscintigraphy for the measurement of both normal (Lohmann *et al.* 2000) and delayed (Lohmann *et al.* 2002) gastric emptying rate, and has been used to look at the effect of phenylbutazone, cisapride, metoclopramide and yohimbine on endotoxin-mediated delayed GE (Valk *et al.* 1998a; Valk *et al.* 1998b; Doherty *et al.* 1999a; Meisler *et al.* 2000). Although time of maximal serum concentration (T_{max}) was correlated to scintigraphic $t_{1/2}$ in horses with delayed emptying, not all variables of acetaminophen absorption are consistently correlated to scintigraphy (Lohmann *et al.* 2002). Occasional horses may fail to absorb acetaminophen sufficiently to permit accurate estimation of

gastric emptying rate, due to possible differences in intestinal permeability, intestinal blood flow or perhaps enterohepatic circulation of acetaminophen (Lohmann *et al.* 2000, 2002). The use of the test in horses with abnormal absorptive function, such as in chronic inflammatory bowel disease has also to be assessed.

In impedance epigastrography, transcutaneous electrodes are used to measure gastric emptying by detection of the reduction in electrical impedance in the epigastric region as a low conductivity meal moves from the stomach into the small intestine. This technique has been validated in man against dye dilution (McClelland and Sutton 1985) and radioscintigraphy (Sutton *et al.* 1985) for the measurement of GE rate. Its use has also been reported in foals (Baker and Gerring 1994b) although this technique would not be practical in adult horses, due to the need to immobilise the patient in a sling to reduce electrical interference.

Finally, of the reported techniques, scintigraphic assessment of the emptying rate of ^{111}In-DTPA containing solution is considered to the optimal standard. However, the radioactive test solution requires nasogastric intubation as discussed above, and presents a not inconsiderable hazard to personnel. The requirement of a gamma camera also precludes use of this technique in most diagnostic settings.

6.1.1.4 Stable isotope measurement of equine liquid phase emptying rate

The ^{13}C-BBT may be unreliable for the measurement of liquid phase GE in man (Bjorkman *et al.* 1991) as discussed in section 6.1.1, but is investigated here for possible evaluation of this parameter in horses, and to yield information regarding equine bicarbonate pool pharmacokinetics. In addition, the ^{13}C-acetate breath test (^{13}C-acetate BT) is investigated in this Chapter for measurement of equine liquid phase GE, and the results compared with those of the ^{13}C-BBT and solid phase measurements derived from the ^{13}C-OABT. The ^{13}C-acetate BT has been validated in both human patients and healthy controls for gastric liquid emptying using either liquid or semi-solid meals, with a sensitivity and specificity of approximately 80% for detection of delayed liquid GE when compared with scintigraphy (Braden *et al.* 1995). Similar to the ^{13}C-OABT, a constant delay factor was noted between scintigraphic and ^{13}C-acetate BT liquid phase $t_{1/2}$ measurements, thought to correspond to the kinetics of tracer absorption and oxidation (Braden *et al.* 1995). Acetate absorption occurs by non-mediated diffusion in the small intestine (Watson *et al.* 1991) but must then undergo oxidation before entry of CO_2 to the bicarbonate pool. The ^{13}C-acetate BT also has been validated against scintigraphy for neonatal use (Gatti *et al.* 2000), and

against the double-dilution technique for the measurement of GE during exercise (Van Nieuwenhoven, M.A. *et al.* 1999). In addition to its use in exercise physiology studies (Mudambo *et al.* 1997), the test has proved useful for establishing the effects of cisapride on GE of liquids (Duan *et al.* 1995).

6.2 Materials and Methods

6.2.1 ^{13}C-Bicarbonate breath test

6.2.1.1 Study Animals

^{13}C-bicarbonate breath tests were performed in 4 animals of mixed type (2 Thoroughbreds and 2 native ponies), ranging from 6 to 14 y of age. Two tests were performed in each individual at 1-week intervals. As with the ^{13}C-OABT, each subject was maintained on a diet of ryegrass seed hay only during the testing period, designed to be low and constant in ^{13}C content. Before each test, food was withheld for at least 12 h to ensure an empty stomach, and the subjects were maintained at rest during each procedure so as to minimise fluctuations in basal VCO_2 and $^{13}CO_2$ production.

6.2.1.2 ^{13}C-bicarbonate breath test protocol

Use of the ^{13}C-bicarbonate breath test has not previously been reported in the horse. A dose of 0.25 mg/kg sodium ^{13}C-bicarbonate ($NaH^{13}CO_3$, Isotec Inc., Ohio, USA) was dissolved in water, and administered orally by dosing syringe in 250 ml water. This quantity of tracer (minimum 99% chemical purity, 99 atom %^{13}C) was found in pilot studies to result in sufficient enrichment of breath ^{13}C:^{12}C ratio. The method of administration was chosen carefully to minimise disruption to the normal process of deglutition. Recent evidence has suggested that the passage of a nasogastric tube may increase the rate of gastric emptying (Lammers and Cohen, *unpublished*). Expiratory breath samples were collected and stored in duplicate before tracer ingestion for measurement of basal $^{13}CO_2$ output (using the methods described in section 2.4.1). After dosing with the dissolved tracer solution, duplicate expiratory samples were collected at 5 min intervals for 1 h, then 10 min intervals for 1 h, followed by 15 min intervals for a further 4 h. Subjects were not allowed access to food or water until the end of the collection period. The test protocol was approved by the University of Glasgow Ethics and Welfare committee.

6.2.1.3 Measurement of ^{13}C:^{12}C ratio

The ^{13}C:^{12}C ratio of each breath sample was determined by CF-IRMS (AP2003 mass spectrometer, Analytical Precision Products Ltd., Northwich, Cheshire) and expressed

relative to the international PDB standard (δ_{PDB}). As described in more detail in section 2.5.2, this ratio was converted to ppm excess ^{13}C, before plotting the expiratory percentage dose recovery (PDR) of the administered isotope against time. Expiratory samples containing less than 0.5% CO_2 were rejected for analysis to minimise possible inaccuracies. Estimation of resting VCO_2 measurement for the 2 ponies was based on the formula of Orr *et al.* (1975), whilst that of the 2 horses was calculated from the formula of Gallivan *et al.* (1989), as detailed in section 2.5.2.

6.2.1.4 Modelling of PDR data and determination of gastric emptying indices

One of the main aims of the ^{13}C-bicarbonate breath tests was to investigate the nature of $^{13}CO_2$ recovery from the equine bicarbonate pool to determine whether discrete steps were identifiable in this process. Terminal $^{13}CO_2$ recovery in many of the ^{13}C-OABT tests appeared to be phasic in nature. Possible causes of this phenomenon were considered to be distinct gastric emptying events, or rate-limiting steps during the exchange of $^{13}CO_2$ with, and excretion from, the rest of the bicarbonate pool. ^{13}C-isotope recovery data were therefore fitted using the multi-peak modification of Macs' formula (Maes *et al.* 1994) as described by Morrison *et al.* (1998):

$$y(t) = a_1(t-d_1)^{b1}e^{-c1(t-d_1)} + a_2(t-d_2)^{b2}e^{-c2(t-d_2)} + \ldots\ldots + a_n(t-d_n)^{bn}e^{-cn(t-d_n)} \qquad \textbf{Equation 6.1}$$

where *y(t)* is the % $^{13}CO_2$ excretion in breath per h; *t* is time (h) and *a*, *b* and *c* are constants. Although this is a mathematical, rather than physiological, approach to multi-compartment curve-fitting, it has proved successful with complex data sets. Its mathematical derivation has been described in more detail in section 2.6.5. To provide liquid phase gastric emptying indices comparable to those previously described in this thesis for measurement of solid phase emptying, a simple single peak model was also used to fit the data, with minimisation of the root mean square term achieved by the Solver function of Excel® (Microsoft Corporation, Redmond, USA) as detailed in section 2.6.4. The terms $t_{1/2}$, t_{lag} and GEC were then derived from the fitted models as described in sections 2.6.3.1 – 2.6.3.2 (Equations 2.13 – 2.15).

6.2.2 Sodium acetate (1-^{13}C) breath tests

6.2.2.1 Study Animals

Sodium 1-^{13}C-acetate breath tests were performed in 6 individuals on at least one occasion. These comprised 3 healthy adult ponies (subjects 101, 103 and 108) of mean (± s.d.) age 12.0 (± 2.0) y and 3 healthy Thoroughbred horses (subjects 102, 104 and 107) of mean age

8.67 (± 3.06) y. Comparable ^{13}C-bicarbonate data were available for 4 of these individuals. Once again, maintenance diet for these individuals was designed to be of low, constant $^{13}C:^{12}C$ ratio, and food was withheld for 12 h before administration of the Stable isotope tracer.

6.2.2.2 *Sodium ^{13}C-acetate breath test protocol*

^{13}C-acetate breath test data have not previously been reported in the horse. In initial pilot studies, a dose of 0.65 mg/kg sodium 1-^{13}C-acetate ($NaCH_3{}^{13}CO_2$, Isotec Inc., Ohio, USA) of minimum 99% chemical purity and 99 atom %^{13}C was found to result in sufficient enrichment of breath $^{13}C:^{12}C$ ratio. This quantity of tracer was dissolved in water, and then administered orally to each subject in a total of 250 ml water by dosing syringe. Expiratory breath samples were collected as for the ^{13}C-bicarbonate breath test: -60 and 0 min (pre-tracer samples), and then at 5 min intervals for the first 1 h, followed by 10 min intervals for the second h, and then 15 min intervals for a further 4 h. No access to food or water was allowed during each test. In those subjects in which repeat tests were performed, a minimum interval of 6 days elapsed between tests.

6.2.2.3 *Calculation of liquid phase GE indices and statistical analysis*

Measurement of the $^{13}C:^{12}C$ ratio of the expiratory samples, and modelling of the derived % dose recovery data was performed as described in sections 6.2.1.3 and 6.2.1.4 respectively. In those individuals in whom both tests had been performed, paired *t*-tests were used to compare the GE indices generated.

6.3 Results

6.3.1 *^{13}C-bicarbonate breath test*

The % dose recovery curves (PDR/h) of the administered tracer in the 4 test subjects are shown in Figures 6.2 to 6.5. In each subject it is seen that isotopic recovery is not monoexponential in nature, having more than 1 phase. Almost without exception, the recovery of isotope in the breath was best fitted by 3, or even 4 exponential non-linear regression curves. This indicates that in the equid subjects there were at least 3 discrete and detectable rate-limiting stages in the recovery of $^{13}CO_2$ from the breath after ingestion of solution containing $NaH^{13}CO_3$. The overall cumulative recovery of the tracer in the breath at 6 h after ingestion was exceptionally high: a mean (± s.d.) recovery of 93.37 (± 14.61) % was achieved, and in 3/8 tests there was near complete recovery of the tracer in this period without obvious loss due to eructation as has been observed in human studies.

The liquid phase GE indices generated by the ^{13}C-bicarbonate breath test are shown in Table 6.1. The mean cumulative half-dose recovery time was markedly quicker than that seen for solid phase GE with the ^{13}C-OABT (see Chapter 5, Table 5.3). Mean (± s.d.) estimates for liquid phase $t_{1/2}$ and t_{lag} were 1.13 (± 0.35) h and 0.19 (± 0.08) h respectively.

Table 6.1 Liquid phase gastric emptying parameters derived from the ^{13}C-bicarbonate breath test, performed twice in each of 4 healthy individuals. The inter- and intra-individual coefficients of variation (CV%) are shown.

Parameter	*Mean ± s.d.*	*Range*	*Inter-individual* CV%	*Intra-individual* CV% (Mean ± s.d.)
$t_{1/2}$ (h)	1.13 ± 0.35	0.69 - 1.91	30.92	20.48 ± 16.48
t_{lag} (h)	0.19 ± 0.08	0.09 - 0.30	38.60	43.36 ± 28.89
GEC	4.23 ± 0.37	3.44 - 4.58	8.81	5.90 ± 6.03

$t_{1/2}$ = gastric half-emptying time; t_{lag} = gastric lag phase; GEC = gastric emptying coefficient

Table 6.2 Liquid phase gastric emptying parameters derived from the ^{13}C-acetate breath test, performed at least once in 3 healthy ponies and 3 healthy horses (n = 6). The inter- and intra-individual coefficients of variation (CV%) are shown. The mean gastric emptying indices for the ^{13}C-acetate breath test are greater than those of the ^{13}C-bicarbonate test.

Parameter	*Mean ± s.d.*	*Range*	*Inter-individual* CV%	*Intra-individual* CV%
$t_{1/2}$ (h)	1.72 ± 0.21	1.41 - 2.00	12.35	8.83
t_{lag} (h)	0.70 ± 0.30	0.42 - 1.29	41.75	34.87
GEC	3.22 ± 0.31	2.95 - 3.88	9.55	4.90

$t_{1/2}$ = gastric half-emptying time; t_{lag} = gastric lag phase; GEC = gastric emptying coefficient

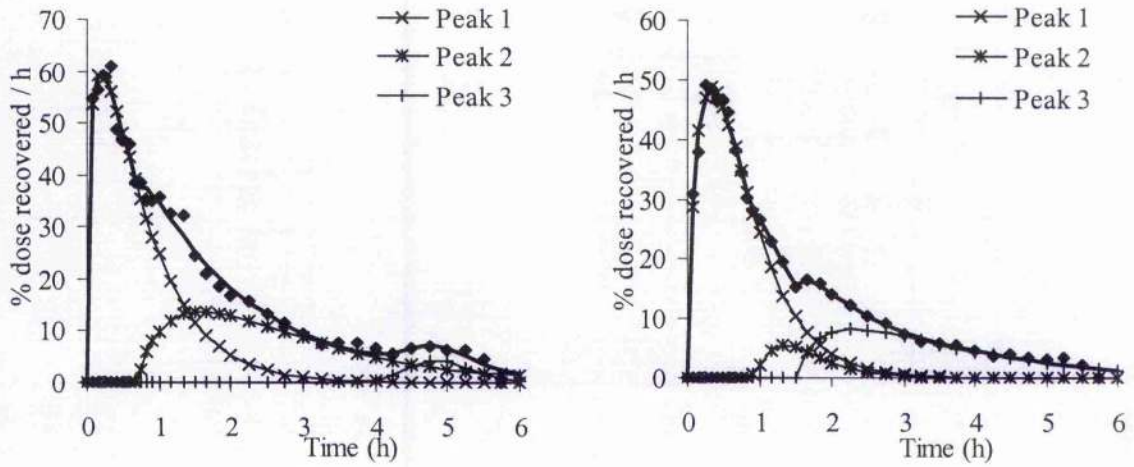


Figure 6.2 Liquid phase gastric emptying rate: ^{13}C-bicarbonate breath tests in subject 108, a 14 y.o. Welsh mountain pony mare. Two tests were performed with a 1-week interval. The expiratory recovery of $^{13}CO_2$ was best modelled in each case by a 3 peak exponential model. In week 1 (left), $t_{1/2}/t_{lag}$ were 1.201/0.091 h, and in week 2 (right) the same parameters were 0.977/0.245 h respectively.

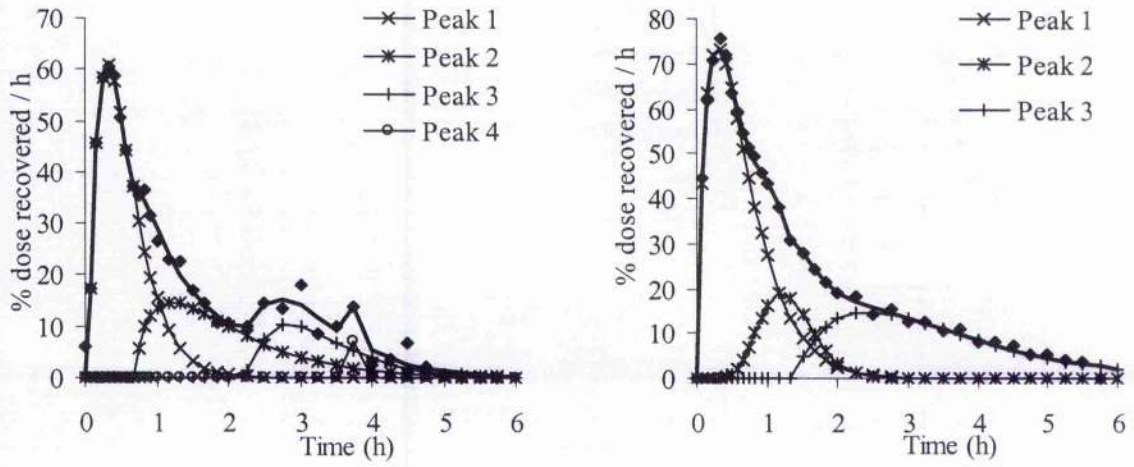


Figure 6.3 ^{13}C-bicarbonate breath tests in subject 101, a 10 y.o. Welsh mountain pony stallion. The pattern of ^{13}C recovery in the breath is clearly multi-phasic in nature, and was best fitted by 4-(left) and 3-(right) peak models. The gastric emptying parameters were very similar in each case: $t_{1/2}/t_{lag}$ = 1.056/0.181 h (left) and 1.067/0.197 h (right).

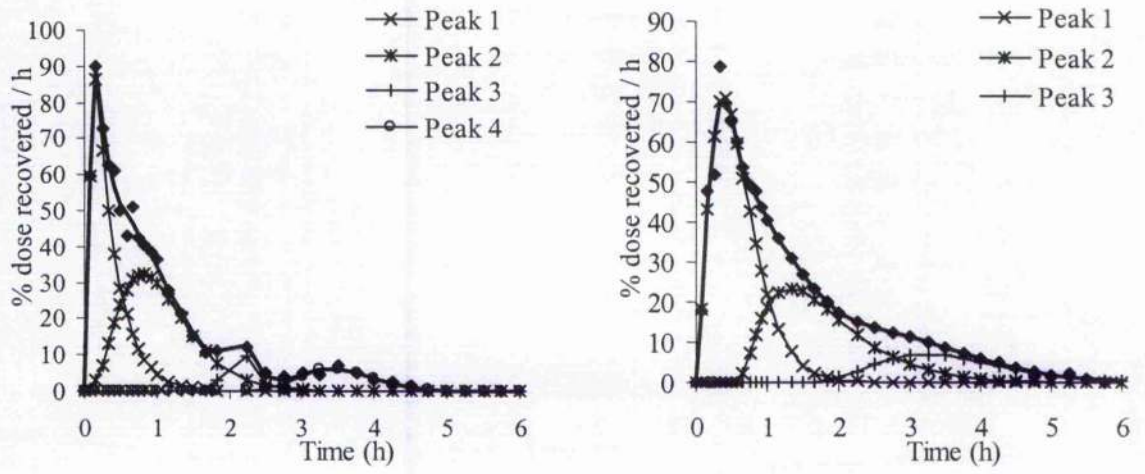


Figure 6.4 ^{13}C-bicarbonate study in subject 102, a 12 y.o. Thoroughbred mare. Again, the recovery of isotope is clearly multi-phasic in each case, best fitted by 4-peak (left) and 3-peak (right) models. The liquid phase gastric emptying parameters in each case were: $t_{1/2}/t_{lag}$ = 0.691/0.107 h (left) and 1.030/0.302 h (right).

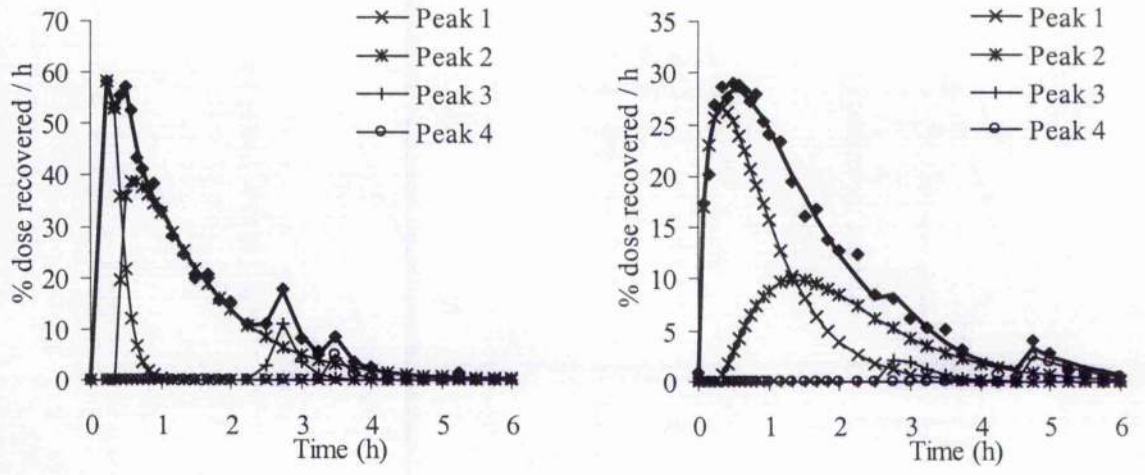


Figure 6.5 Investigation of bicarbonate pool kinetics and liquid phase gastric emptying rate in subject 107, a 6 y.o. Thoroughbred mare. Gastric emptying was slower in the second week (right) than in the first: $t_{1/2}/t_{lag}$ = 1.088/0.271 h and 1.910/0.163 h respectively. Once again, the expiratory recovery of $^{13}CO_2$ was best fitted by multi-peak models.

The ^{13}C-bicarbonate test was also relatively repeatable, with a mean inter-individual CV% for $t_{1/2}$ of 30.92%, and a mean (± s.d.) intra-individual CV% of 20.48 (± 16.48) %. The variability of the observations for mean liquid phase $t_{1/2}$ described here and those for solid phase $t_{1/2}$ described in Table 5.3 were homoscedastic, allowing a two-sample *t*-test to be performed. The mean difference in $t_{1/2}$ between the populations was significantly different ($P < 0.01$). A non-parametric Wilcoxon signed rank test was used to establish the difference between mean duration of the lag phase for the solid and liquid phase emptying test in the two different populations. Mean liquid phase t_{lag} was of significantly shorter duration than that of solid phase emptying as measured by the ^{13}C-OABT in the population described in Chapter 5 ($P < 0.01$).

6.3.2 ^{13}C-acetate breath test results

Examples of the ^{13}C-acetate liquid phase PDR/h curves in each of the 6 test subjects are shown in Figures 6.6 (ponies) and 6.7 (horses). Although isotopic recovery in each case is biphasic, a third rate-limiting step in $^{13}CO_2$ recovery is less apparent, save for in a single subject (101). The mean liquid phase GE indices as measured by this test are given in Table 6.2. Mean (± s.d.) estimates for $t_{1/2}$ and t_{lag} were 1.72 (± 0.21) h and 0.70 (± 0.30) h respectively. Inter-individual CV% for $t_{1/2}$ was very low (12.25%) as was intra-individual CV%, although this was based on a small number of individuals.

Both tests were performed in randomised order in 4 individuals, and a paired *t*-test used to examine the intermodal differences in parameter estimation. Mean $t_{1/2}$ ($n = 4$) derived from the ^{13}C-acetate test was significantly greater than that from the ^{13}C-bicarbonate test ($P < 0.02$) as was mean t_{lag} estimation ($P < 0.01$). The mean (± s.d.) difference in $t_{1/2}$ estimation between the two tests was 0.71 (± 0.29) h. This delay factor may have been attributable to the time taken for oxidative metabolism of the ^{13}C-acetate tracer. Cumulative dose recovery of the ^{13}C-acetate label at 6 h was significantly lower than that of ^{13}C-bicarbonate in this study at 42.81 ± 8.35%, versus 93.37 ± 14.61%. Although cumulative dose recovery is dependent on VCO_2 estimation, this term remained constant between the two tests.

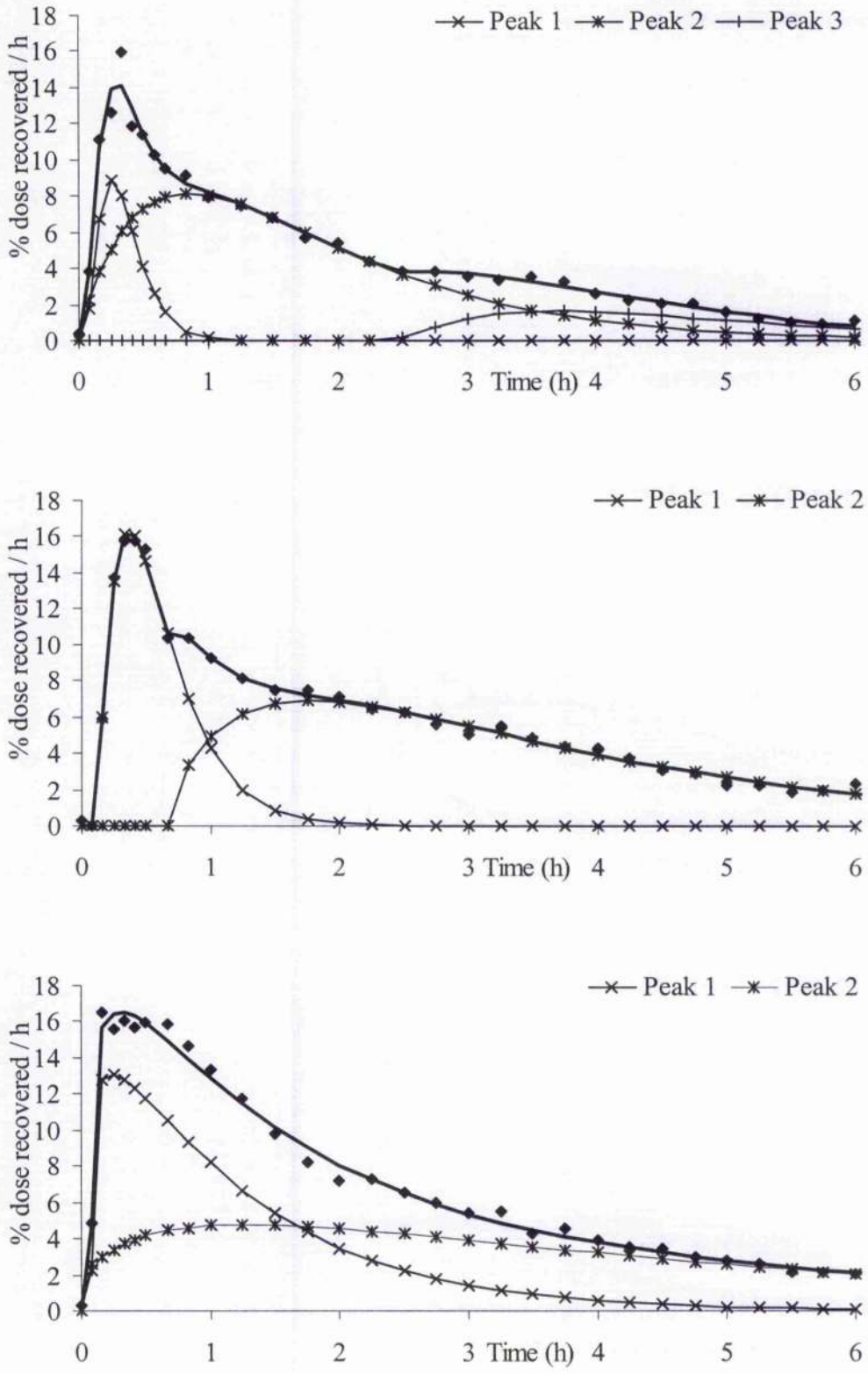


Figure 6.6 ^{13}C-acetate liquid phase gastric emptying results in pony subjects 101 (top), 103 (middle) and 108 (bottom). The estimated values for $t_{1/2}/t_{lag}$ in each case are: 1.41 and 0.42 h; 1.75 and 0.67 h; 1.56 and 0.52 h respectively. The pattern of expiratory $^{13}CO_2$ recovery is generally biphasic in nature with a less pronounced third phase than in the ^{13}C-bicarbonate tests.

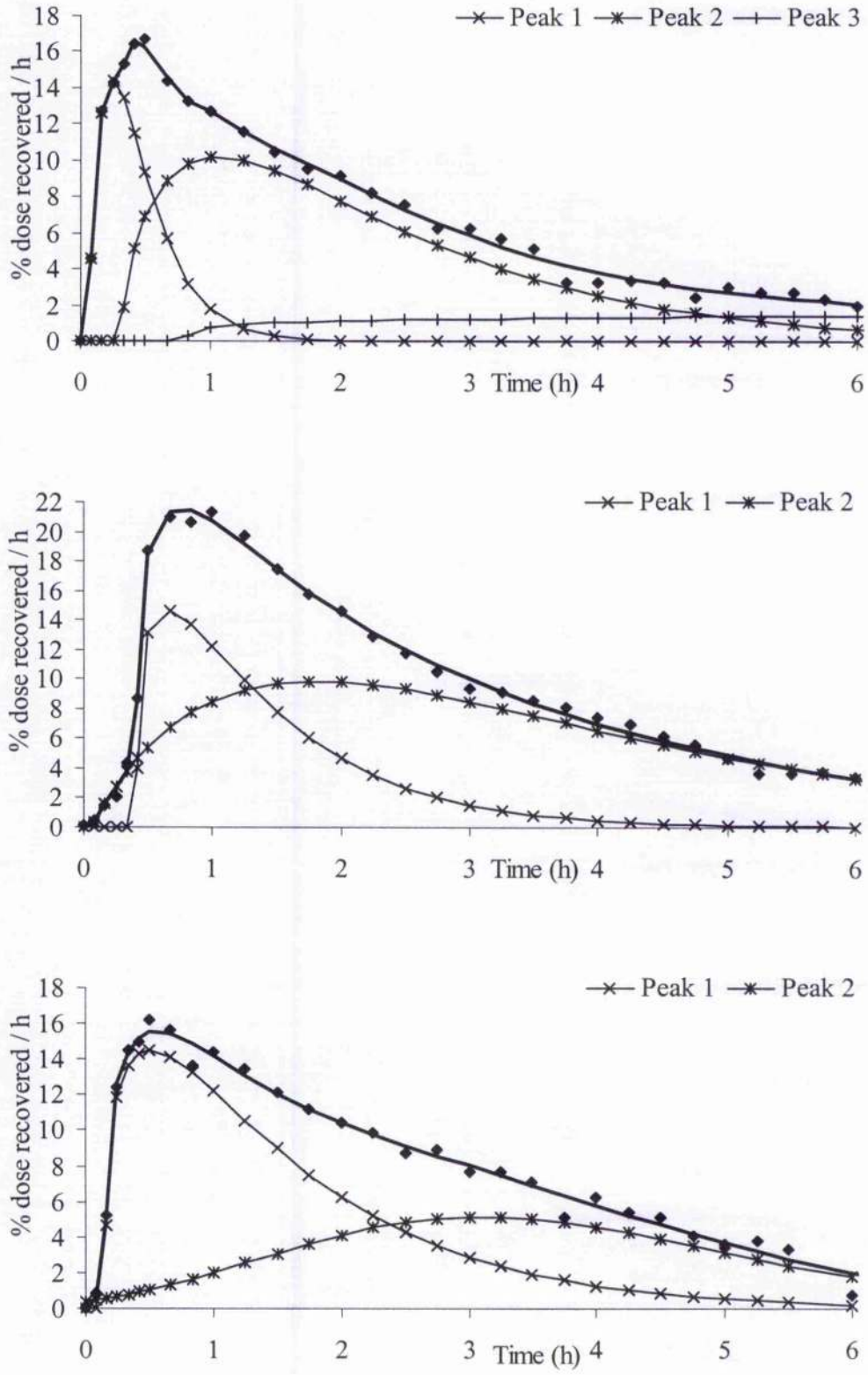


Figure 6.7 **^{13}C-acetate liquid phase gastric emptying results in horse subjects 104 (top), 102 (middle) and 107 (bottom). The estimated values for $t_{1/2}/t_{lag}$ in each case are: 1.66 and 0.61 h; 2.10 and 1.29 h; 2.00 and 0.94 h respectively. Again, the pattern of expiratory $^{13}CO_2$ recovery is markedly biphasic, with a less apparent third phase than in the ^{13}C-bicarbonate tests. A summary of the gastric emptying indices derived in the test subjects from the ^{13}C-acetate test is given in Table 6.2.**

6.4 Discussion

6.4.1 *Equine bicarbonate pool kinetics*

The results of the ^{13}C-BBTs in each of the 4 subjects in this study suggest that the pattern of $^{13}CO_2$ recovery from the equine bicarbonate pool is multiphasic in nature, with more than one rate-liming step, as reported in man (Irving *et al.* 1983; Wolfe 1992c; Van Hall 1999). The rate and pattern of recovery of $^{13}CO_2$ from the bicarbonate pool was also repeatable both within and between individuals ($t_{1/2}$ CV% = 20.5 and 30.9% respectively). These are important findings as bicarbonate pool kinetics have the same final influence on all equine Stable isotope tests, regardless of the compound used (Pallikarakis *et al.* 1991), and are largely responsible for the decay of any breath test curve. The frequent phasic appearance of the terminal section of the ^{13}C-OABT gastric emptying curves, as described in section 3.2.3.2 (Figures 3.3 and 3.4) is therefore likely to have been attributable to rate-limiting stages in $^{13}CO_2$ recovery from the bicarbonate pool rather than multiple discrete gastric emptying events. This is consistent with the three-compartment model for the bicarbonate pool (Irving *et al.* 1985; Van Hall 1999) as given in Figure 6.1, with different rate constants for the exchange of $^{13}CO_2$ between the large, central vascular pool of bicarbonate and the smaller, slow peripheral and fast visceral pools.

Although there are rate-limiting stages in the recovery of the given tracer from the equine bicarbonate pool, these are thought to remain constant at rest, such that in the ^{13}C-OABT, for example, gastric emptying remains the major rate-limiting factor in $^{13}CO_2$ recovery. However, the compartmental dynamics of CO_2 transport and storage are very sensitive to changes in metabolic rate induced by exercise (Barstow *et al.* 1990; Leese *et al.* 1994) with an increased ^{13}C-bicarbonate washout caused by an increase in size of the central bicarbonate pool. Leese *et al.* (1994) also showed that elimination of CO_2 from labelled bicarbonate was lower during acidosis, due to a decreased CO_2 pool size. These findings further underscore the importance of minimising changes in metabolic rate during Stable isotope tests (Kalivianakis *et al.* 1997).

Since the terminal $^{13}CO_2$ recovery curve may be determined by bicarbonate pool kinetics rather than the specific physiologic parameter of interest, the results suggest that there may be more appropriate means of calculating GE rate than the traditional parameters of $t_{1/2}$ and t_{lag} (Ghoos *et al.* 1993) which are based on the overall rate of cumulative dose recovery. An alternative approach in GE studies may be to use the time to peak appearance of $^{13}CO_2$ as a parameter for gastric emptying, as suggested by van Nieuwenhoven *et al.* (1999).

Schommartz *et al.* (1998) proposed measurement of the latency phase (t_{lat}) and the ascension time (t_{asc}). Using the first derivative of Siegel's formula (equations 2.7 and 2.9; Siegel *et al.* 1988), these researchers were able to determine the rate constants directly from the experimental data. The initial delay in the cumulative exhalation curve was termed the 'latency' phase, and obtained as the point of intersection of the tangent at the inflection point of the curve and the *x*-axis:

$$t_{lat} = t_{lag} - (1-1/\beta)/\kappa \qquad \textbf{Equation 6.2}$$

The ascension time was defined as that time interval between the latency phase and $t_{1/2}$:

$$t_{asc} = t_{1/2} - t_{lag} - (1-1/\beta)/\kappa \qquad \textbf{Equation 6.3}$$

Although these parameters have not yet gained wide acceptance, they are less dependent on the terminal portion of the ^{13}C dose recovery, and do not exhibit the high level of correlation that exists between t_{lag} and $t_{1/2}$ terms (Schommartz *et al.* 1998; Bluck *et al.* 2002), such that they might offer enhanced diagnostic value.

6.4.2 *Equine bicarbonate dose recovery*

A very high cumulative dose recovery of the bicarbonate tracer, approaching 100%, was achieved in each of the subjects in this study. This suggests that there is very little non-respiratory loss of ingested bicarbonate in the horse at rest. The quoted values for labelled bicarbonate recovery at rest in human studies approximate 60 – 80% (Irving *et al.* 1985; Hoerr *et al.* 1989; Barstow *et al.* 1990; Meineke *et al.* 1993; Leese *et al.* 1994), with variability attributable to methodological differences, such as use of a bolus dose or constant infusion, and recent nutritional status (Leijssen and Elia 1996). The high expiratory recovery of $^{13}CO_2$ from the equine bicarbonate pool suggests that in future oxidative metabolism studies, use of a 'bicarbonate correction factor', incorporated in human studies to account for non-respiratory CO_2 consumption (Van Hall 1999), should not be necessary in this species. However, these observations need to be repeated in a study in which CO_2 output has been measured rather than estimated.

6.4.3 *^{13}C-Acetate breath test results*

The estimated values for $t_{1/2}$ and t_{lag} were significantly greater for the ^{13}C-acetate breath tests than those from the corresponding individuals using the ^{13}C-BBT. This difference is likely to have been caused by the time taken for absorption and oxidative metabolism of the acetate label, prior to entry of $^{13}CO_2$ to the bicarbonate pool. The mean (± s.d.)

difference in $t_{1/2}$ estimation between the two tests in this study was 0.71 (± 0.29) h. Although the ^{13}C-acetate BT has been validated against scintigraphy in both adult (Braden *et al.* 1995) and paediatric (Gatti *et al.* 2000) human populations for the measurement of GE, it has also been noted that the absorption and/or oxidation rate of acetate is enhanced at exercise (van Nieuwenhoven *et al.* 1999). During exercise, ^{13}C-acetate absorption may be altered by increased splanchnic blood flow, and oxidation increased due to increased metabolism in the liver and/or working muscles (van Nieuwenhoven *et al.* 1999). Therefore the test should always be performed under standard conditions to enable comparison of results to be made, and studies using this test alone to measure gastric emptying rate before and during exercise should be interpreted with caution (e.g. Mudambo *et al.* 1997). However, using the term 'time to peak (TTP)' $^{13}CO_2$ output, van Nieuwenhoven *et al.* (1999) validated the ^{13}C-acetate BT against the double dilution test during exercise and found a good correlation between $t_{1/2}$ and TTP. There is thus potential for the use of this test to measure the effect of exercise on equine GE rate, but further validation would first be required against a standard method such as scintigraphy, both at rest and during exercise.

Cumulative dose recovery of the ^{13}C-acetate label at 6 h was significantly lower than that of ^{13}C-bicarbonate in this study (42.81 ± 8.35%, versus 93.37 ± 14.61% respectively). Acetate is converted to acetyl-CoA after absorption and oxidation, and thereafter may enter the tricarboxylic acid (TCA) cycle where its carbon skeleton can undergo exchange with that of glutamic and aspartic acid, or function as a substrate for lipogenesis or ketogenesis. Lipogenesis is likely to be reduced under the test conditions of overnight food deprivation, but ketogenesis may be increased, further reducing substrate recovery (Van Hall 1999). The fraction of acetate recovered in this study is similar to the 49% recovery of $^{13}CO_2$ from [1-^{13}C] acetate reported in man (Trimmer *et al.* 2001). Trimmer *et al.* (2001) also found that [1-^{13}C] acetate recovery in resting adults was markedly lower than ^{13}C-bicarbonate recovery (78%), and attributed this to CO_2 fixation and incorporation in alternative metabolite pools. Oxidation of acetate also differs in obese and lean subjects, and is enhanced at exercise (Horowitz and Klein 2000; Borghouts *et al.* 2001) due to a relative increase in TCA turnover compared to TCA exchange reactions (Trimmer *et al.* 2001).

6.4.4 Duration of equine liquid emptying phases

6.4.4.1 Duration of t_{lag}

The results of the ^{13}C-BBTs presented here suggest that there is very little gastric lag in horses before low nutrient liquids start to empty from the stomach after ingestion. This is

in accordance with the results of human studies (Gonzalez *et al.* 2000). The duration of this lag phase has not previously been determined in equine liquid phase studies (Neuwirth 1994; Neuwirth *et al.* 1995; Doherty *et al.* 1998; Lohmann *et al.* 2000). Of even greater clinical interest than the emptying of water, would be the effect of increasing caloric and/or electrolyte content on the rate of equine liquid phase GE. In human studies, the ingestion of nutrient liquids has been shown to reduce GE rate in proportion to the energy density of the meal, via interaction of specific nutrients with small intestinal receptors. These receptors have been shown to include osmoreceptors, lipid receptors, probable glucose receptors and receptors for the amino acids tryptophan and phenylalanine (Read and Houghton 1989), and emptying is delayed via alteration of all components of the emptying process. Similar experiments on the effects of specific nutrients on equine liquid phase GE have yet to be performed satisfactorily, and would potentially be useful for optimising oral rehydration therapy regimens. As mentioned in section 6.1.2.1, Sosa Leon *et al.* (1997) reported that electrolyte concentration did not produce a significant effect on equine liquid GE rate, but this study was flawed by the prior medication of each subject with xylazine. Either the ^{13}C-acetate breath test or ^{13}C-BBT would potentially be suitable for the further investigation of the physiology of liquid phase emptying in this species.

6.4.4.2 Duration of liquid phase $t_{1/2}$

Estimates of $t_{1/2}$ derived from both the ^{13}C-acetate BT (1.72 ± 0.21 h) and the ^{13}C-BBT (1.13 ± 0.35 h) were significantly shorter than those for solid phase $t_{1/2}$ derived from the ^{13}C-OABT (3.79 ± 1.53 h; see Table 3.1). This is consistent with the generally accepted theories of solid-liquid discrimination, whereby the majority of liquid leaves the stomach, before antral grinding of the solids into particles sufficiently small to exit through the pylorus with the residual liquid (Read and Houghton 1989). Measurement of solid phase t_{lag} by the ^{13}C-OABT (2.32 ± 1.14 h; see Table 3.1) was also significantly greater than that of either of the liquid phase studies, reflecting the period of trituration required before the movement of solids through the pylorus.

6.4.5 Validation of Stable isotope tests for liquid phase emptying

This Chapter contains the first reported use of either the ^{13}C-acetate BT or the ^{13}C-BBT in the horse, and although appearing potentially useful, both tests require validation before further clinical use. The optimum standard for validation would be to compare the generated gastric parameters with the results of a simultaneous scintigraphic study, using ^{99m}Tc-DTPA (see Lohmann *et al.* 2000). Although the ^{13}C-BBT has not been validated for human use (Bjorkman *et al.* 1991), the test may be useful for equine studies, having

excellent substrate recovery with minimal inter-individual variation, and lesser dependence on metabolic rate. Both stable isotope tests are relatively cheap and easy to perform, and have the advantages of requiring neither a radiation source, nor a nasogastric tube. Although the acetaminophen absorption test has been validated against radioscintigraphy for equine use (Lohmann *et al.* 2000; 2002), occasional individuals may exhibit aberrant absorption profiles. Moreover, drug interaction may significantly alter acetaminophen metabolism, and large individual differences in first-pass metabolism and elimination may complicate estimation of GE (Medhus *et al.* 1999), although algorithms have been developed to improve accuracy (Medhus *et al.* 2001). Unequal distribution of acetaminophen in body fluids during periods of rapid changes in serum concentration may also confound emptying indices derived from its serum levels. In contrast, both the ^{13}C-BBT and ^{13}C-acetate BT have been shown to be independent of concentration-related pharmacokinetic effects (Van Hall 1999).

In comparison with other reported techniques for measurement of equine liquid phase GE such as impedance epigastrography (Baker and Gerring 1994b), and double dilution techniques (Baker and Gerring 1994a), the stable isotope tests also have greater ease of application, and are likely to offer superior accuracy with sufficient sensitivity to detect subtle changes in emptying rate.

6.4.6 *Future applications of the ^{13}C-BBT/^{13}C-acetate BT in equine medicine*

Further studies must be performed in horses with both normal and delayed gastric motility to correlate the findings of the stable isotope techniques with a standard method such as gastric scintigraphy. In light of the findings described in Chapters 3 and 4 of this thesis, it seems likely that one or both of these tests will prove suitable for the measurement of equine liquid phase GE. As so little is known regarding control of equine GE rate, either the ^{13}C-acetate BT or ^{13}C-BBT could then be used to investigate the role of factors such as electrolyte content/osmolality/volume of test liquids on equine gastric motility. Read and Houghton (1989) have also reported that measurement of GE following ingestion of both a non-nutrient liquid meal and a nutrient meal may provide the most useful diagnostic information for identification of abnormal gastric function syndromes. Important stable isotope studies in human neonates have also investigated the relative effects of milk formula replacements and expressed breast milk on neonatal gastric function (Van den Driessche *et al.* 1999). Similar studies in neonatal foals may be useful to further investigate the aetiology of neonatal gastroduodenal ulceration syndrome.

Chapter 7 VALIDATION OF THE LACTOSE ^{13}C-UREIDE BREATH TEST FOR THE MEASUREMENT OF EQUINE OROCAECAL TRANSIT TIME

7.1 General Introduction

7.1.1 Measurement of equine orocaecal transit time

The development of a non-invasive test for the routine measurement of orocaecal transit rate would represent a major diagnostic advance in the field of equine gastroenterology. Although tests for solid and liquid phase gastric emptying rate have been described and validated in the previous Chapters of this thesis, there are a number of equine ailments that specifically affect small intestinal transit. Gastric emptying may well be normal in these cases, even if orocaecal transit is significantly prolonged. Such conditions may include post-operative ileus (Gerring and Hunt 1986), ileal impaction (Hanson *et al.* 1996; Proudman *et al.* 1998; Little and Blikslager 2002), idiopathic or secondary ileal muscular hypertrophy (Chaffin *et al.* 1992), ileo-ileal or ileocaecal intussusception (Ford *et al.* 1990) and chronic grass sickness (Milne *et al.* 1996). Disrupted small intestinal motility has also been noted in conditions such as tapeworm (*Anoplocephala perfoliata*) infestation (Proudman and Holdstock 2000); nonstrangulating infarction of the small intestine caused by the vascular migratory stages and/or intra-luminal larval antigen of *Strongylus vulgaris* (Berry *et al.* 1986); intra-abdominal neoplasia or inflammation, and may be involved in the aetiology of spasmodic colic. In addition to facilitating the diagnostic investigation of such cases, a non-invasive modality for OCTT measurement would permit determination of the effects of specific drugs, such as potential prokinetic agents and commonly used sedative and anaesthetic agents, on small intestinal motility.

Tests that have been reported for the measurement of equine OCTT include the hydrogen breath test (Bracher and Steiger 1995; Murphy and Love 1995; Murphy 1997; Murphy *et al.* 1998) and the sulphasalazine/sulphapyridine (SLZ/SP) test (McGreevy and Nicol 1998), as described in detail in Chapter one. Due to the lack of an established standard for the detection of OCTT in equidae, neither the H_2BT nor the SLZ/SP test has been validated for specific assessment of this parameter, and their efficacy is dependent on the presence of the requisite microflora in the desired gastrointestinal locus. In human medicine, the most accurate modality for detection of the arrival of ingesta in the caecum is gastroenterocolonic scintigraphy (Camilleri *et al.* 1998) and this is used as the reference technique for validation of new methodology. However, scintigraphic examination of the equine abdomen is not sufficiently specific for determination of OCTT due to obscuring of

the caecal base by loops of radioactive small intestine, necessitating development of an alternative technique.

A possible limitation of both the H_2BT and the SLZ/SP test for equine use is that, although the caecum and colon are the primary sites for microbial digestion, substantial populations of bacteria have been demonstrated in the small intestine of healthy horses on a grass diet (Mackie and Wilkins 1988). Mackie and Wilkins (1988) reported that bacterial counts increased along the length of the equine small intestine. This may lead to elevations of expiratory H_2 or cleavage of the SLZ azo-bond prior to entry of ingesta into the caecum, with consequent underestimation of OCTT.

Murphy *et al.* (1998) defined OCTT as that time at which breath H_2 concentration first exceeded the (mean + 2 s.d.) basal output level, following ingestion of the test meal. Using a meal of 1 kg oats in 7 ponies, Murphy *et al.* (1998) reported a range (mean ± s.d.) of 60 to 300 min (218 ± 87 min) for OCTT estimation. A wheat flour test meal in the same individuals produced a narrower range for OCTT of 150 to 270 min (184 ± 50 min). This latter value approximates the estimate of 135 ± 57 min (60 to 300 min) for OCTT produced by McGreevy *et al.* (2001) using the SLZ/SP test in 4 normal horses. It seems likely that individual subjects in both of these studies exhibited small intestinal fermentation of the chosen substrates, resulting in underestimation of OCTT. Evidence for this comes from the same study of Murphy *et al.* (1998) in which one subject with an apparent OCTT of 60 min after oat consumption also produced a 30 min peak in expiratory H_2 output after the ingestion of glucose. Early monosaccharide fermentation in both human and canine studies is most consistent with small intestinal bacterial overgrowth (Taylor *et al.* 1981; O'Connor *et al.* 1987; Rutgers *et al.* 1995). An early transient H_2 peak has also been reported after xylose ingestion in a horse with suspected pyloric outflow obstruction due to apparent gastric microbial activity (Murphy *et al.* 1994).

Further limitations of the H_2BT, though not reported for the SLZ/SP test, include the presence of individual animals that do not produce a H_2 signal in response to the arrival of specific carbohydrates in the large intestine (Bracher and Baker 1994; Murphy 1997; Bracher and Steiger 1998). For example, lactulose is commonly used as the test substrate for the H_2BT in human medicine, but ingestion of lactulose resulted in detectable increases in expiratory H_2 output in just 2 of 7 pony subjects investigated by Murphy *et al.* (1998). Sasaki *et al.* (1999) also reported that expiratory H_2 was not detected in 13/52 horses immediately before and after feeding a combination of hay, grass and concentrates, although methane was detectable in the breath of these individuals. The latter group

hypothesised that variable H_2 output in certain horses was due to a relatively higher ratio of methanogenic bacteria in the hindgut of these individuals, which utilise H_2 for CH_4 production. This theory has also been proposed in normal human populations (Cloarec *et al.* 1990) as detailed in the introductory Chapter. Sulphate-reducing bacteria (Miller and Wolin 1983) and acetate-forming organisms (Lajoie *et al.* 1988) have also been noted to reduce colonic H_2 output. As low H_2 output may be confused with prolongation of OCTT if test duration is not sufficient, the presence of H_2 'non producers' may reduce the specificity of the test for diagnostic purposes.

Most of the extant reports regarding equine small intestinal motility and physiology have been based upon electrophysiological and strain gauge techniques, which require prior implantation of silver electrodes on the serosal surface of the intestine. From such studies, the slow wave frequencies of the duodenum, jejunum and ileum have been reported to approximate 12 to 14/min, decreasing aborad (Davies and Gerring 1983; Adams *et al.* 1984; Merritt *et al.* 1989; Ross *et al.* 1990), with a migrating myoelectrical complex (MMC) periodicity of around 125 to 153 min (Merritt *et al.* 1989; Lester *et al.* 1998a). Unlike carnivores, there is minimal disruption to the equine MMC during *ad libitum* feeding. After initial gastric emptying of ingesta, there is a period of duodenal repetitive spiking activity (RSA) which is coincident with suppression of antral spike activity, and propagates along the small intestine (Merritt *et al.* 1989a). Ileal contractions have a unique pattern compared to the rest of the small intestine. Periodic spike bursts known as migrating action potential complexes (MAPCs) occur in the ileum, mostly during phase II of the MMC. These MAPCs occur at a frequency of 2 to 5/h, and are frequently followed by a high amplitude caecal then colonic spike burst (Ross *et al.*1990; Lester *et al.* 1998). Ileal MAPCs occur at greater frequency during the fed state, and are thought to be partly responsible for propagation of ingesta from the ileum into the right ventral colon, via the caecocolic orifice (Lester *et al.* 1998a).

Although myoelectric studies have yielded much valuable information of relevance to the subject matter of this thesis, such an approach is not suitable for the investigation of OCTT in a clinical setting. Furthermore, intestinal electrical activity is informative only about contractility rather than the movement of ingesta, for which coordinated propulsion is required. Use of labelled ingesta is also necessary for physiological studies in which it is aimed to determine the effect of nutrient composition itself on small intestinal motility. Such studies are yet to be performed with any complexity in the horse, and are of vital importance for the optimisation of feeding regimens. The major aim of this Chapter of the

thesis was therefore to develop a new non-invasive test to allow measurement of the rate of movement of ingesta along the equine small intestine.

7.1.2 Use of the lactose ^{13}C-ureide breath test for measurement of OCTT

Geypens *et al.* (1999) reported that the LUBT was closely correlated to GECS for the measurement of OCTT (r = 0.94, n = 22, P = 0.0001) and concluded that it was a valid alternative for determination of this parameter. The principal of the LUBT has been detailed in Chapter one. The test substrate consists of a lactose (glucose-galactose) molecule bound to a ^{13}C-urea molecule, and crystallises as the dihydrate, with a molecular mass of 421 mass units (see Figure 7.1):

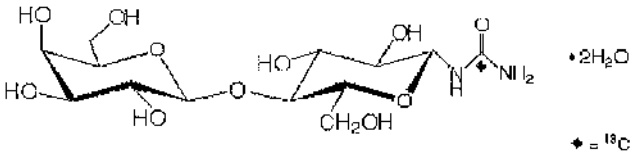


Figure 7.1 Chemical structure of β-lactosyl ^{13}C-ureide dihydrate molecule used for the determination of orocaecal transit time.

As the glucose-urea bond cannot be cleaved by mammalian brush border enzymes (Ruemmele *et al.* 1997), hydrolysis of the molecule and subsequent liberation of $^{13}CO_2$ after urea degradation is under the control of microbial enzymes. In the same manner as the ^{13}C-OABT, the labelled $^{13}CO_2$ moiety enters the bicarbonate pool after absorption from the large intestine and is subsequently exchanged, resulting in an increased expiratory $^{13}CO_2$:$^{12}CO_2$ ratio. Sensitivity and specificity of this test for OCTT estimation is dependent on: (i) inter- and intra-individual variations in microbial hydrolytic rate and capability; (ii) distribution of the glucoseureide-hydrolysing microflora in the intestinal tract; and (iii) nature of expiratory recovery of $^{13}CO_2$ from the body bicarbonate pool. The characteristics of human faecal (Mohr *et al.* 1999; Morrison 2000) and ruminant (Merry *et al.* 1982b, 1982c) microbial glycosylureide metabolism are relatively well known. Previous exposure to unlabelled substrate (induction) has been shown to increase the subsequent rate of hydrolysis of the ^{13}C-compound in both populations. This phenomenon results either from selected bacterial proliferation or enzymic induction, and clarifies the caecal arrival time of labelled ingesta by augmenting the expiratory $^{13}CO_2$ signal. An induced LUBT protocol has recently been developed for clinical practice, which has helped to confirm the presence of delayed OCTT in paediatric patients with Crohn's disease and with functional gastrointestinal symptoms (Van den Driessche 2001).

Although potentially suitable for equine use, the LUBT has not previously been reported in this species. Due to its dependence on microbial substrate fermentation, the test may be limited by the same factors that affect both the H_2BT and the SLZ/SP test. However, unlike the basal production of H_2 (Murphy 1997), basal output of $^{13}CO_2$ remains relatively constant in horses (see Chapter 3) such that the LUBT should not experience this particular complication of the H_2BT.

7.1.3 Aims of the study

The major aim of this project was to develop a LUBT for equine use, and to attempt its validation by comparison with existing methods. In Part I, a preliminary protocol for the equine LUBT was established. Estimation of equine OCTT by this test was compared with the results of a simultaneous H_2BT in 8 healthy subjects. The effects on OCTT estimation of previous subject exposure to lactose ^{12}C-ureide (unlabelled) were also determined. Based on the results of Part I, it was aimed to further validate the LUBT for equine OCTT estimation by *in vitro* microbial fermentation techniques. The glucoseureide hydrolytic capability of microflora collected from specific regions of the gastrointestinal tract *post mortem* was investigated. The effect of previous exposure to unlabelled substrate on microbial enzymic function was also measured. The *in vitro* studies of the validation process, and refinement of the test protocol, are described in Part II of this Chapter.

PART I

7.2 Comparison of the lactose ^{13}C-ureide breath test with the hydrogen breath test for the measurement of orocaecal transit time

7.2.1 Materials and methods

7.2.1.1 Subjects

Pilot breath studies were performed in 8 healthy adult individuals. These comprised 4 native ponies of mean age 14.0 ± 4.3 y (range 10 – 20 y) and 4 Thoroughbreds of mean age 8.3 ± 3.3 y (range 6 13 y). The subjects had no known history or physical evidence of gastrointestinal disease, and were rejected if biochemical or total blood cell parameters were found to lie outside the reference range. All subjects were maintained on a daily ration of 30 g ryegrass seed hay per kg bodyweight to minimise fluctuations in basal $^{13}CO_2$ output, and treated with an Ivermectin anthelmintic (Merial Animal Health Ltd, Essex, UK) at 8- to 10-weekly intervals during the study. Exercise consisted of daily turnout into an indoor school.

7.2.1.2 Test meal composition

The aim was to develop a palatable, rapidly consumed test meal that resulted in emission of a significant expiratory H_2 signal after ingestion, and that was suitable for labelling with lactose ^{13}C-ureide. Variation from the ^{13}C-OABT test meal was minimised in order to facilitate potential combination of the stable isotope tests in a single test meal at a future time (see Chapter 8). Murphy (1997) reported the emission of a detectable H_2 signal in 7/7 native ponies after the ingestion of either oats or a pelleted high fibre ration. Hay consumption was also reported to increase expiratory H_2 production in the same study animals (Murphy 1997). A number of test meals were investigated accordingly, with the optimum 3 shown in Table 7.1:

Table 7.1 Trial test meals used for the detection of equine orocaecal transit time by simultaneous measurement of first significant increase in exhaled H_2 output, and expiratory recovery of 3% administered dose of lactose ^{13}C-ureide.

Test Meal 1	Test Meal 2	Test Meal 3
400 g crimped oats	400 g chopped hay	400 g high fibre pellets
100 g wheat bran	150 g crimped oats	150 g crimped oats
200 ml water	100 g wheat bran	100 g wheat bran
1 baked, chopped egg white, containing ^{13}C-LU	200 ml water	200 ml water
	1 baked, chopped egg white, containing ^{13}C-LU	1 baked, chopped egg white, containing ^{13}C-LU

In addition to inducing a detectable H_2 signal after ingestion, the test meals were selected to be of similar ^{13}C content to the habitual ryegrass hay diet. Basal expiratory ^{13}C:^{12}C ratio after ingestion of these test meals had been shown in pilot studies to remain stable (see sections 2.2.2 and 3.3.2.1). To maintain the lactose ^{13}C-ureide in the solid phase of gastric emptying, it was mixed with an egg white, which was microwaved until firm, homogenised and mixed thoroughly with the test meal.

7.2.1.3 Study design

Expiratory H_2 production was measured in at least 5 individuals after ingestion of test meals 1, 2 and 3. Individuals were maintained on a constant diet between tests, which were performed at a minimum of 7 d intervals. Hydrogen output was monitored at 30 min intervals for 24 to 36 h after ingestion of the test meal. In order to measure repeatability of

the H_2 signal after meal ingestion, test meal 3 was given to 4 subjects (101, 102, 104 and 108) on 3 occasions.

Measurement of OCTT by the two tests was compared in 2 individuals (101 and 108), using both a non-induced LUBT and an induced LUBT. Finally, using a prior induction protocol for the LUBT, estimation of OCTT by the H_2BT and induced LUBT was compared in a further 5 individuals.

7.2.1.4 Lactose ^{13}C-ureide breath test

Pilot studies showed that administration of a dose of 3.0 mg/kg β-lactosyl ^{13}C-ureide dihydrate (Bell College of Technology, Glasgow) was sufficient to produce a repeatable increase in the expiratory ^{13}C:^{12}C ratio. Food was withheld for 14 h prior to each test to ensure an empty stomach and two expiratory samples were collected for basal ^{13}C:^{12}C analysis before voluntary ingestion of the labelled test meal. Breath collection was performed using a modified equine Aeromask® (Trudell Medical International, Ontario, Canada) as detailed in section 2.4.1, and sample ^{13}C:^{12}C ratio was measured by CF-IRMS (see section 2.5.1). Samples were collected in duplicate at 30 min intervals for up to 36 h after test meal ingestion. For the preliminary induction protocol, 5 g lactose ^{12}C-ureide was added to the diet 12 – 16 h before the start of the test, as recommended by Morrison (2000). Non-induced LUBT protocols were performed before induced LUBTs in each subject, as the duration of any 'induction' effect was not known.

7.2.1.5 Hydrogen breath test

7.2.1.5.1 Breath collection

Exhaled breath was collected using the same technique employed for the Stable isotope breath tests (section 2.4.1) but care was taken to collect end expiratory tidal air, as this has been reported to contain the highest concentrations of hydrogen (Murphy *et al.* 1998).

7.2.1.5.2 Measurement of H_2 abundance

Sample H_2 concentration (parts per million) was measured using an Exhaled Hydrogen Monitor (GMI Medical Ltd, Renfrew, Scotland) as shown in Figure 7.2, according to the method of Corbett *et al.* (1981). This instrument measures H_2 concentration using an electrochemical detector, which consists of a metallised membrane electrode held at constant potential relative to a reference electrode. When H_2 diffuses through the metallised membrane it yields electrons to the electrode, generating an electric current in proportion to the partial pressure of H_2 in the sample. This current is converted to a digital

reading of hydrogen partial pressure, which is determined by initial calibration of the instrument with a reference gas of 95 parts per million (ppm) H_2 (GMI Medical Ltd, Renfrew, Scotland).

Figure 7.2 Exhaled hydrogen monitor showing calibration with a 95 ppm H_2 reference gas.

The unit is able to detect changes of 1 ppm H_2, and is reproducible and linear over the range 1 to 250 ppm. Samples of end expiratory air were injected slowly into the inlet port in 20 ml aliquots, and the maximal value reached was recorded, before flushing the unit with the same volume of room air. For each time point, samples were analysed in duplicate and the mean value determined. As the monitor is heat sensitive, it was maintained in a thermostat-regulated room at constant temperature.

7.2.1.6 Calculation of orocaecal transit time

For the H_2BT, OCTT was determined as that time at which there first was a sustained increase of at least 5 ppm in expiratory H_2 output. This cut-off has been shown to produce the best correlation to OCTT measurement in humans when compared to both barium meal studies (Hirakawa *et al.* 1988) and GECS (Sciarretta *et al.* 1994; Miller *et al.* 1997). Although the cusum technique (see section 9.2.3.3) has been used previously for estimation of OCTT using the LUBT (Geypens 2000; Morrison 2000), it is adversely affected by fluctuations in the basal output of $^{13}CO_2$ and so in this study, OCTT was defined as that point at which 3% of the total expired cumulative dose recovery (PDR) of the administered isotope had occurred. This technique was thought to be more robust and although subjective, the 3% value was considered to represent the first significant recovery of hydrolysed tracer. Isotope PDR data were modelled by non linear regression as described in section 2.5.2, and fitted by the least squares method to a delayed Maes' model (Equation 7.1). Specifically, estimated OCTT (3% PDR time) was calculated by a

Gammainv function using Microsoft Excel (Microsoft Corporation, Redmond, USA) software as given in Equation 7.2:

$$y(t) = a(t-d_1)^b e^{-c(t-d_1)} \quad \textbf{Equation 7.1}$$

$$OCTT = Gammainv\ (0.03;\ b + 1;\ 1/c) \quad \textbf{Equation 7.2}$$

where $y(t)$ is the % $^{13}CO_2$ excretion in breath per h; t is time (h); a, b and c are rate constants and d is the delay factor. As for the ^{13}C-OABT, the related parameters $t_{1/2}$ (see Equation 2.13) and t_{lag} (Equation 2.14) were also calculated for the LUBT. These parameters were considered to indicate the time to recovery of 50% of the total cumulative dose recovered, and that time at which recovery of the ^{13}C label was maximal in rate.

7.2.1.7 Statistical evaluation of results

In those individuals in which dual H_2/LUBT tests were performed successfully, the relationship between the OCTT estimates by the two modalities was examined by linear regression, and calculation of Pearson correlation coefficient. The coefficient of variation was generated for both inter-and intra-individual variations in OCTT of test meal 3 as detected by the H_2BT. Paired *t*-tests were used to assess the effects of the induction process on the measurement of OCTT by the LUBT.

7.2.2 Results

7.2.2.1 H_2 production

H_2 production was found to be dependent on test meal composition, and varied both between and within different individuals. In addition to fluctuations in the time of peak H_2 signal, its magnitude varied markedly among subjects. Basal expiratory H_2 output at the start of the test was also variable, hindering interpretation when elevated. The probability of detecting a clear increase in expiratory H_2 output in the test subjects was maximised by ingestion of test meal 3 (see Table 7.2). Only 2 of 5 subjects produced a detectable H_2 signal after ingestion of both meals 1 and 2. By contrast, in 15 tests performed in 7 subjects with the third test meal, 11 (73%) resulted in a detectable increase in H_2 output thought to correspond to OCTT. This included responses from 2 ponies (101 and 103) that had emitted only negligible H_2 after ingestion of test meals 1 and 2. One subject (103) failed to product a detectable increase in H_2 output after ingestion of any test meal, and 2 further subjects (101 and 102) demonstrated the tendency to maintain a low constant H_2 output after meal consumption.

Table 7.2 Summary of expiratory H_2 production in 8 equine subjects after ingestion of test meals 1, 2 or 3.

Test meal	SUBJECTS							
	101	102	103	104	105	106	107	108
Test meal 1	0	-	0	-	0	-	1	1
Test meal 2	0	0	-	1	-	-	1	0
Test meal 3	1,1,0	1,0,0	0	1,1,1	1	1	-	1,1,1

where 1 = detectable peak in H_2 output, exceeding previous point by at least 5 ppm H_2, and 0 = poor magnitude of H_2 signal with no detectable peak after meal ingestion.

The intra-individual variability of the increase in expiratory H_2 output after ingestion of test meal 3 is demonstrated in Figures 7.3 and 7.4. In subjects 101 and 102, increased H_2 output was not a constant factor after test meal consumption, such that calculation of OCTT was not always possible. Basal H_2 production also varied, as seen in subject 104, which hindered interpretation. A summary of the estimates of OCTT derived from the H_2BT in the 4 subjects after ingestion of test meal 3 is shown in Table 7.3.

Table 7.3 Orocaecal transit time in 4 equine subjects as measured by the hydrogen breath test, after ingestion of test meal 3.

SUBJECT	OROCAECAL TRANSIT (h)			Mean (h)	s.d. (h)	CV%
	Week 1	Week 2	Week 3			
101	10.50	1.50	-	6.00	6.36	106.07
102	5.00	-	-	5.00	-	-
104	3.50	4.00	3.50	3.67	0.29	7.87
108	2.75	4.00	5.50	4.08	1.38	33.72

7.2.2.2 Results of the LUBT

In contrast to the H_2BT, ingestion of the lactose ^{13}C-ureide labelled test meal was followed in each case by the production of an increase in expiratory ^{13}C:^{12}C ratio, which allowed estimation of OCTT to be made. The pattern of increase in ^{13}C:^{12}C ratio was best modelled by a 2- or 3-peak Maes' model (Maes *et al.* 1994), as shown in Figures 7.5 – 7.8, and cumulative dose recovery of the isotope remained relatively constant with a range of 29 – 47%. Prefeeding of 5 g lactose ^{12}C-ureide on the day prior to ingestion of the lactose ^{13}C-ureide test meal resulted in a steeper, more discrete peak in $^{13}CO_2$ output, and an apparent shortening of OCTT. The effects of this induction process on substrate hydrolysis rate and OCTT measurement in subjects 101 and 108 are demonstrated in Figures 7.5 – 7.8.

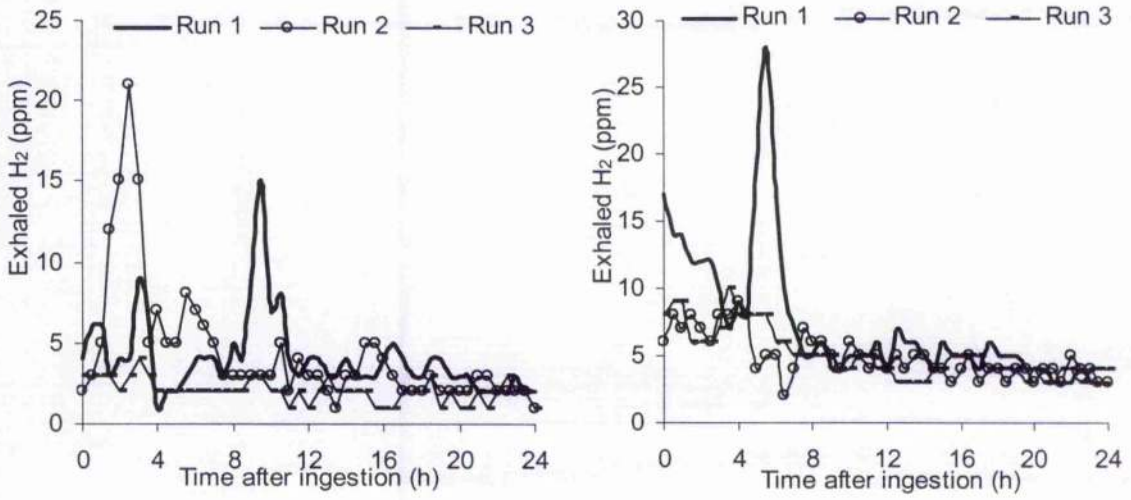


Figure 7.3 Hydrogen breath test results for subjects 101 (left), a 10 y.o. native pony stallion, and subject 102 (right), a 13 y.o. TB female, after ingestion of test meal 3 on 3 separate occasions. There is marked intra-individual variability in both magnitude and timing of H_2 signal after ingestion of the test meal. Of the 6 tests, only 3 produced an H_2 signal sufficient for OCTT estimation.

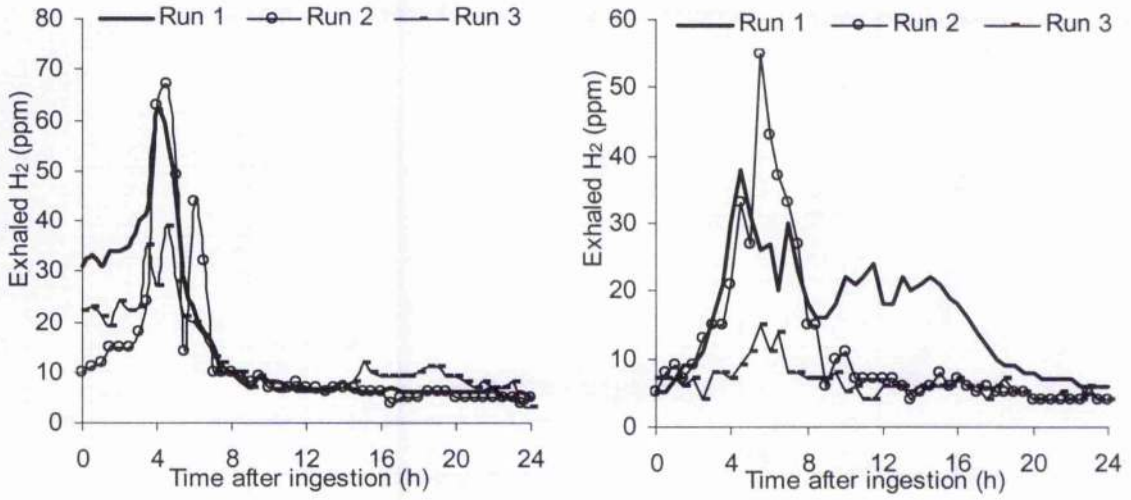


Figure 7.4 Hydrogen breath test results for subjects 104 (left), an 8 y.o. TB gelding, and subject 108 (right), a 14 y.o. native pony female, after ingestion of test meal 3 on 3 separate occasions. Detectable H_2 signals were produced on each occasion in both individuals, but interpretation was complicated in 104 by high basal H_2 output. Peak H_2 production occurred at a similar time after meal ingestion in both 104 and 108 on each occasion.

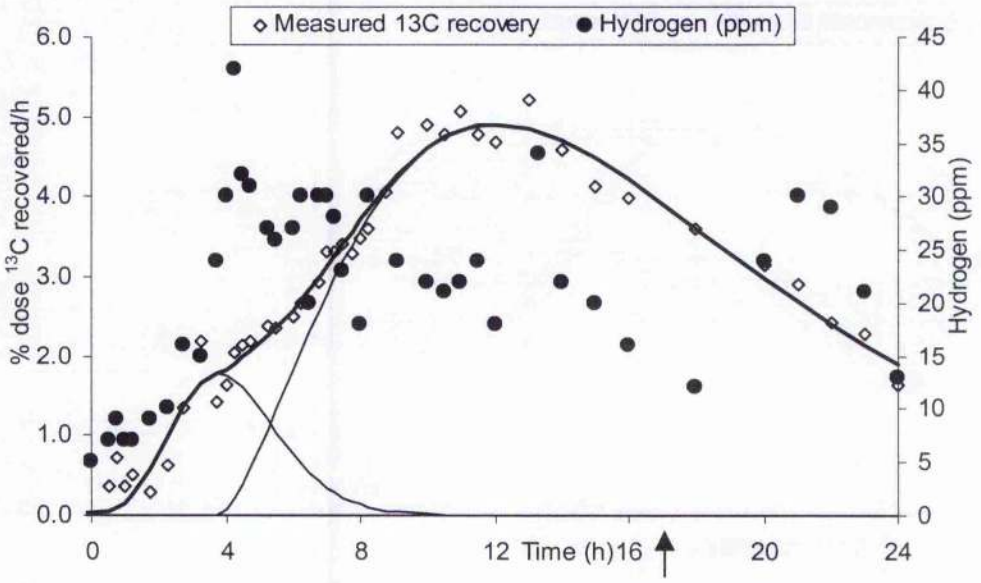


Figure 7.5 Combined lactose ^{13}C-ureide breath test (non-induced) and H_2 breath test in subject 108, after ingestion of test meal 2 at time 0 h. The expiratory recovery of $^{13}CO_2$ was best fitted by a dual peak model (solid line). The first significant increase (> 5 ppm) in expiratory H_2 output occurred at 2.75 h. Estimation of the OCTT (time to 3% cumulative dose recovery) by the LUBT = 4.34 h. Arrow denotes feeding of second meal.

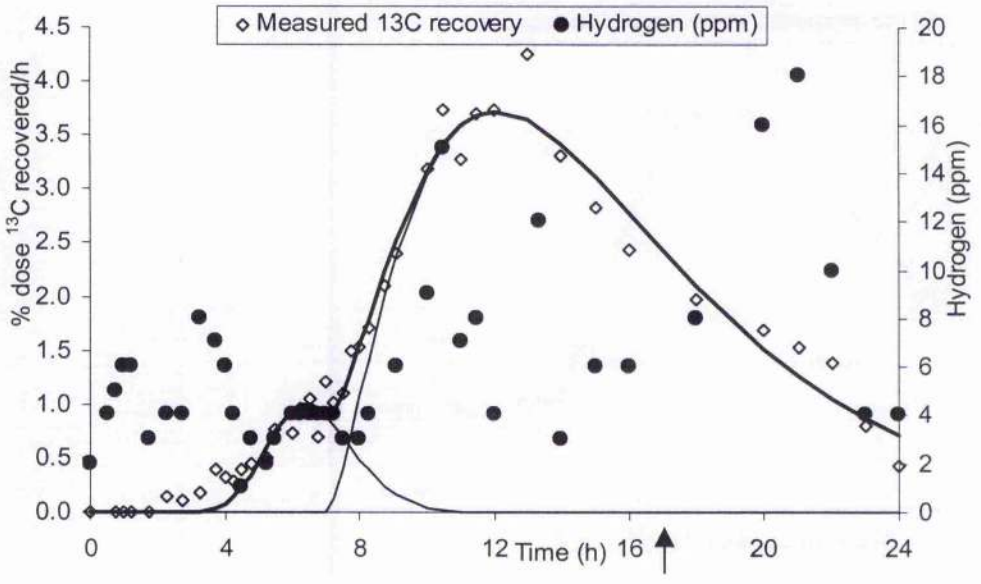


Figure 7.6 Combined lactose ^{13}C-ureide breath test (non-induced) and H_2 breath test in subject 101, after ingestion of test meal 2 at time 0 h. As in Figure 7.5, expiratory $^{13}CO_2$ recovery was best fitted by a dual peak model (solid line). The first significant increase (> 5 ppm) in expiratory H_2 output occurred at 3.15 h. Estimation of the OCTT by the LUBT = 6.72 h. Arrow denotes feeding of second meal.

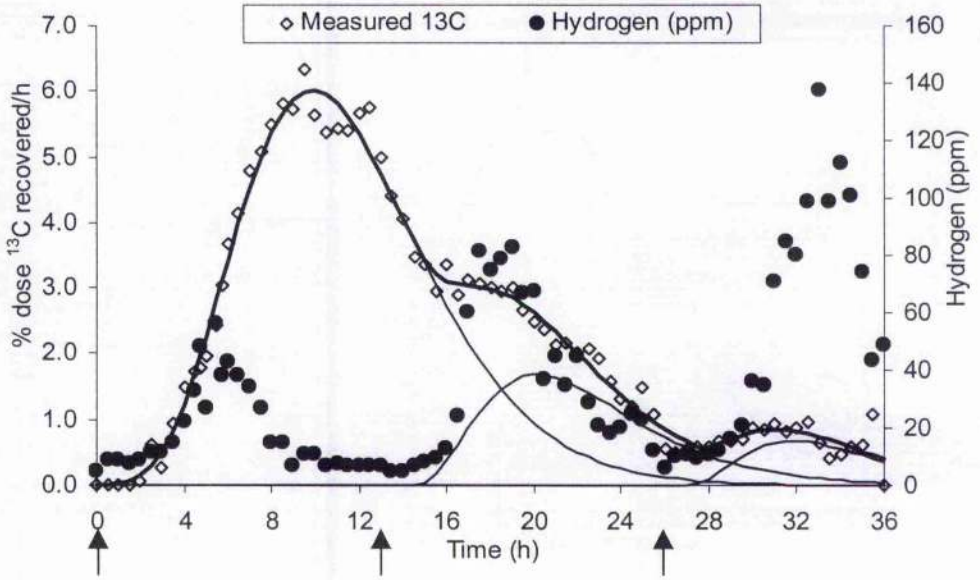


Figure 7.7 Combined LUBT and H_2BT in subject 108 after ingestion of test meal 3 at time 0 h. Unlabelled lactose ureide (5 g) was added to the diet 24 h prior to the test. Two further meals of soaked high fibre cubes (1.6 kg) were fed at 13 h and 26 h, as denoted by the arrows. Three discrete peaks in H_2 production occurred at constant intervals after feeding. Recovery of $^{13}CO_2$ was triphasic in appearance. OCTT estimation by LUBT and H_2BT = 4.49 h and 4.00 h respectively.

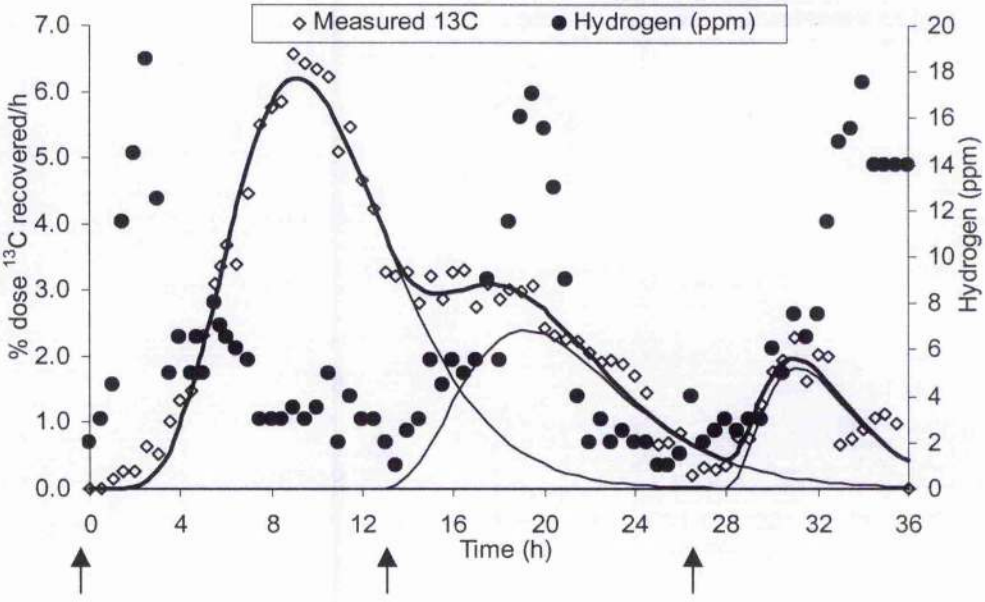


Figure 7.8 Combined induced LUBT and H_2BT in subject 101 after ingestion of test meal 3 at time 0 h. Two further meals of soaked high fibre cubes (1.6 kg) were fed at 13 h and 26 h, as denoted by the arrows. A discrete H_2 peak is seen at a constant interval after ingestion of each meal. Recovery of $^{13}CO_2$ was also triphasic in appearance, but with a larger primary peak. Apparent OCTT estimation by LUBT and H_2BT = 4.69 h and 1.50 h respectively.

The induction process also increased the overall rate of isotope hydrolysis, with significant reductions in both caecal t_{lag} and $t_{1/2}$ ($P < 0.05$) in paired induced/non-induced tests. The characteristics of this mechanism are investigated further *in vitro* in part II of this Chapter, and further measured *in vivo* in Chapter 8.

7.2.2.3 *Comparison of the induced LUBT and H_2BT for OCTT measurement*

Due to the variation in H_2 production, direct comparison between the results of simultaneous H_2BTs and LUBTs for the measurement of OCTT was possible in just 4 individuals. These data are presented in Table 7.4. For accuracy of comparison, data from both tests were modelled using Maes' formula (see section 7.2.1.6) so that estimates of OCTT, caecal t_{lag} and caecal $t_{1/2}$ could be compared directly.

Table 7.4 Comparison of the induced LUBT and H_2BT in 4 individuals for the measurement of orocaecal transit time and caecal fermentation rate.

Subject	*Induced LUBT (h)*			*Hydrogen BT (h)*			*Diff (LUBT - H_2BT) (h)*		
	OCTT	t_{lag}	$t_{1/2}$	OCTT	t_{lag}	$t_{1/2}$	**OCTT**	**t_{lag}**	**$t_{1/2}$**
101	4.69	9.16	10.04	1.31	2.3	2.45	**3.38**	**6.86**	**7.59**
102	4.86	7.12	8.21	3.81	5.69	5.86	**1.05**	**1.43**	**2.35**
104	3.67	5.00	6.26	2.42	3.95	4.14	**1.25**	**1.05**	**2.12**
108	4.49	9.86	10.94	3.58	5.6	5.83	**0.91**	**4.26**	**5.11**
Mean	**4.43**	**7.79**	**8.86**	**2.78**	**4.39**	**4.57**	**1.65**	**3.40**	**4.29**
S.d.	**0.53**	**2.19**	**2.07**	**1.15**	**1.60**	**1.63**	**1.16**	**2.72**	**2.58**

OCTT = orocaecal transit time; t_{lag} = time of maximal rate of hydrolysis; $t_{1/2}$ = time at which 50% of cumulative dose recovery of isotope has occurred.

In 3 of 4 subjects, the LUBT estimate for OCTT was approximately 1 h greater than that of the H_2BT. In the remaining subject (101), the difference was much greater, due to a very early H_2 peak, possibly reflecting gastric or small intestinal fermentation. Consequently, the correlation between the two modalities for OCTT estimation was not significant in this sample group. The mean difference in t_{lag} and $t_{1/2}$ measurements was much more variable between the tests, but generally, expiratory H_2 recovery was steeper and declined more quickly than that of $^{13}CO_2$.

The effect of subsequent feeds on the production of both marker gases was assessed as shown in Figures 7.7 and 7.8. After ingestion of the test meal at time 0, subsequent meals resulted in detectable further peaks in both $^{13}CO_2$ and H_2, occurring at constant intervals after feeding. These peaks were greater in magnitude for H_2 output.

7.2.3 Discussion – Part I

In the experiments described in Part I of this study, the H_2BT was shown to have a number of limitations as a possible test of equine OCTT. Of 25 H_2BTs performed in 8 subjects, only 15 yielded useful information due to the variability of H_2 production. The addition of high fibre pellets to the test meal ration resulted in a greater probability of subsequent H_2 output as noted previously (Murphy and Love 1995; Murphy 1997), but 1 subject still failed to produce a detectable H_2 signal, and this signal was inconsistent in 2 further individuals. These results showed the H_2BT to be less reliable than previously reported for the measurement of equine OCTT, and insufficiently robust for validation of the new LUBT technique.

The presence of low or non H_2 producers has been noted in human studies (Levitt and Donaldson 1970; Bond and Levitt 1974; Pressman *et al.* 1987; Cloarec *et al.* 1990; Corazza *et al.* 1993; Hammer *et al.* 1996) and variously attributed to an acidic colonic microclimate (Perman *et al.* 1981) or the prevalence of H_2-consuming microbes (Miller and Wolin 1983; Gibson *et al.* 1988; Lajoie *et al.* 1988). The absence of basal H_2 output in horses has been noted previously, and Sasaki *et al.* (1999) hypothesised this to be caused by a relative increase in intestinal methanogenic bacteria. However, the intra-individual variability of this phenomenon has not previously been noted. It may reflect either rapid changes in the equine gastrointestinal ecosystem or changes in gut motility such that altered faecal H_2 tension permits increased H_2 consumption and decreased absorption (Strocchi and Levitt 1992; Hammer 1993).

The observed variability in equine basal H_2 output is also likely to reduce the sensitivity of the test for OCTT measurement (Flatz *et al.* 1984), but could be improved by a more prolonged initial period of food deprivation (Kotler *et al.* 1982; Perman *et al.* 1984; Romagnuolo *et al.* 2002). Stimulation of the gastrocolic reflex during test meal ingestion may itself stimulate further movement and fermentation of previously consumed complex carbohydrates, thus increasing bacterial H_2 production. This is the putative mechanism for the multiple H_2 peaks shown in Figures 7.7 and 7.8. Phasic movement of the test meal into the caecum may also have caused the smaller peaks noted in $^{13}CO_2$ recovery. Two factors are thought to contribute to the inter-modal difference noted in OCTT estimation: different production rates of H_2 and $^{13}CO_2$ by the specific caecal bacterial populations, and the time taken for equilibration of $^{13}CO_2$ in the bicarbonate pool before expiration (see 6.1.1).

Preliminary results with the LUBT indicated that it could be an effective means of monitoring equine OCTT. Ingestion of lactose ^{13}C-ureide was followed in all individuals by a discrete peak in expiratory $^{13}CO_2$ output as described in man (Heine *et al.* 1994). The clarity of this peak was enhanced by prior administration of lactose ^{12}C-ureide as noted in both ruminants (Merry *et al.* 1982b) and humans (Wutzke *et al.* 1997b; Geypens *et al.* 1999) due to possible induction of bacterial enzymic activity. In contrast to the H_2BT, intra-individual variation in production of the marker gas (H_2/$^{13}CO_2$) was relatively small, and no anomalous early peaks were seen with the LUBT. This may reflect greater specificity of the LUBT for caecal microbial action than seen with the H_2BT. Whilst most intestinal microbes produce H_2, only *Clostridium innocuum* has been proven to hydrolyse the glucose ^{13}C-ureide bond (Mohr *et al.* 1999).

Due to the promising early data with the equine LUBT, and the problems outlined with the equine H_2BT, an alternative means of validating the LUBT for equine OCTT measurement was required. It was elected to investigate the locus of specific glucoseureide-cleaving intestinal microbes using *in vitro* fermentation techniques, and to further elucidate the nature of the induction process by this means. The results of the *in vitro* validation studies are detailed in Part II of this Chapter.

PART II

7.3 *In vitro* investigation of the fermentation of glycosyl^{13}C-ureides by equine gastrointestinal microbes

7.3.1 Study objectives

Although the LUBT has been validated against scintigraphy for the measurement of human OCTT (Geypens *et al.* 1999) and has increasing clinical uptake, relatively little is known of the kinetics of microbial glycosylureide fermentation. *In vivo* studies of large intestinal microbial fermentation are difficult to perform due to the inaccessibility of this section of the bowel, and must by their nature be invasive. By contrast, models based on the incubation of stool or gut contents collected *post mortem* circumvent several of these problems. *In vitro* reports are restricted to glycosylureide metabolism by ruminal microbes (Merry *et al.* 1982b) or human faecal microbes (Morrison 2000), and the ubiquity or specific intestinal locus of the requisite microbes has not been investigated in any species. Having established the ability of equine microbes to ferment lactose ^{13}C-ureide *in vivo* (see 7.2), the major aim of the *in vitro* project was to determine the location and ubiquity of those organisms in the gastrointestinal tract. In order to validate the LUBT for the

measurement of OCTT, it was necessary to demonstrate that the microbes of interest were restricted to the caecum and large intestine, and that this was a consistent finding.

As the kinetics of equine lactose ^{13}C-ureide fermentation were not known, secondary aims of these studies were: (i) to investigate the existence of additional rate-limiting steps in the fermentation pathway of L^{13}C-U that, if present, would limit the efficacy of the test. The rate of metabolism of a number of related ^{13}C-substrates was measured *in vitro*, and the effect of substrate competition on bacterial enzymic function was studied; (ii) to quantify the 'induction' of glucoseureide hydrolase activity, stimulated by the prior exposure of the gastrointestinal microbes to lactose ^{12}C-ureide; (iii) to produce an optimum protocol for clinical application of the LUBT, based on the results of the *in vitro* induction studies. It was planned to develop the final protocol for *in vivo* use from bacterial incubations of both fresh faecal samples and intestinal contents collected anaerobically at *post mortem* examination.

7.3.2 Materials and Methods

7.3.2.1 Fermentation techniques

Several different techniques have been reported for *in vitro* substrate fermentation studies and major factors that affect outcome include the inoculum size, type of media, maintenance of anaerobic conditions, choice of buffer, rate of mixing and sampling protocol (Khin-Maung *et al.* 1992; Edwards *et al.* 1996; Parrett and Edwards 1997). Due to the difficulties in predictable availability of fresh equine gastrointestinal material, initial fermentation techniques were developed using fresh faecal samples as the inoculum.

7.3.2.1.1 Faecal incubation

Faecal specimens were collected from the rectum of adult horses and ponies (subjects 101 – 108) maintained on a ryegrass hay diet, and which had not received medication within the previous 2-month period. Samples were placed in an airtight container and processed within 30 min of collection. A 400 g litre^{-1} faecal slurry was formed using anaerobic Sorensen's phosphate buffer at pH 7.0 (Table 7.5), and homogenised for 2 min using a liquidiser. The buffer was boiled vigorously for 10 min immediately before use and cooled under a stream of oxygen-free nitrogen (BOC gases, Guildford, Surrey) to ensure anaerobic conditions. As described by Edwards *et al.* (1996), the slurry was then filtered through nylon meshing to remove coarse particulate matter, before transferring 40 ml aliquots to 120 ml glass serum bottles. The bottles were closed with gas tight crimp lids and teflon seals (Figure 7.9).

Table 7.5 Composition of Sorensen's phosphate buffer for pH range 5.4 – 8.0.

Required pH of final solution	$^1/_{15}$ M KH_2PO_4 ml/100ml solution	$^1/_{15}$ M $Na_2HPO_4.2H_2O$ ml/100ml solution
5.4	96.9	3.1
5.6	95.0	5.0
5.8	92.0	8.0
6.0	88.0	12.0
6.2	81.5	18.5
6.4	73.8	26.2
6.6	64.0	36.0
6.8	50.0	50.0
7.0	39.0	61.0
7.2	28.0	72.0
7.4	20.0	80.0
7.6	13.0	87.0
7.8	8.5	91.5
8.0	5.5	94.5

Chemicals supplied by Sigma-Aldrich Company Ltd., Dorset, England.

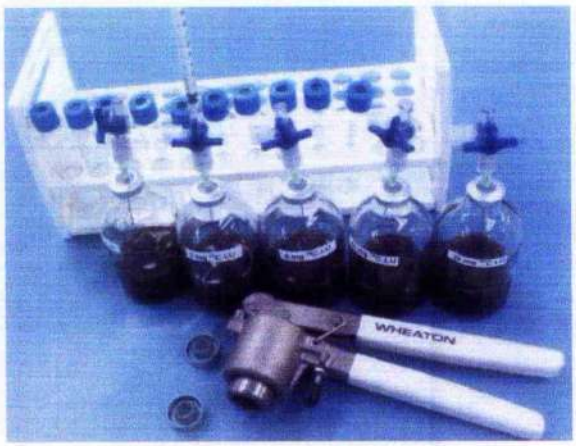


Figure 7.9 Anaerobic faecal incubation chambers showing collection of headspace gas via luer lock and syringe.

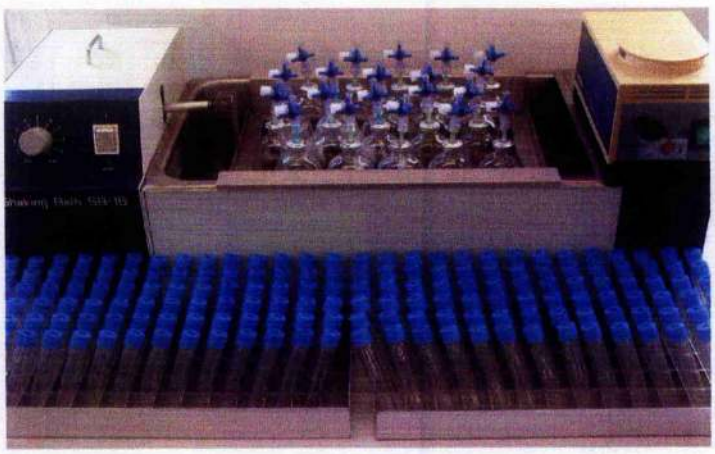


Figure 7.10 Incubation of anaerobic chambers at 38^{0}C. Headspace gas samples stored in Exetainers previously flushed with oxygen-free nitrogen.

After sealing, each chamber was fitted with a needle and luer lock tap system, and flushed with oxygen free nitrogen (OFN) for 60 s to create an anaerobic environment. With the tap system closed, the chambers were then placed in a shaking water bath at 38^0C and 50 revolutions/min to mimic the action of large intestinal fermentation. In order to sample the headspace gas, 1 ml aliquots were sequentially withdrawn with a syringe and second luer lock and transferred to an Exetainer® (Labco Ltd., Buckinghamshire, UK) which had previously been filled with OFN (volume 11.75 ml). Initial bacterial fermentation activity was assessed by measurement of H_2 (ppm) in the headspace gas using the Exhaled Hydrogen Monitor. At a fixed period for each experiment, ^{13}C-lactose ureide solution was added to the incubation and ^{13}C-LU degradation rate was assessed by measuring the rate of appearance of $^{13}CO_2$ in the headspace gas. A range of 2-10 mg ^{13}C-LU (or equimolar ^{13}C-glucoseureide) was used per chamber, and this was scaled from an *in vivo* dose of 3.0 mg/kg bwt (see 7.2.1.4). The effects of specific substrate enrichment on anaerobic bacterial activity were investigated. Substrates included glucose, lactose and galactose (Sigma-Aldrich Company Ltd.), $^{12}/^{13}$C-urea (Euriso-top, Saint-Aubin Cedex, France), and blood culture and cooked meat anaerobic media (Oxoid, Basingstoke, UK).

7.3.2.1.2 Specific intestinal incubations

Samples of luminal content and intestinal mucosa were collected from the stomach, duodenum, mid-jejunum, ileum (60 cm orad of ileocaecal orifice), caecal apex and caecal base of adult horses as soon as possible after euthanasia. The materials were collected from horses euthanased for reasons not relating directly to the intestinal tract, or from abattoir specimens. Medications that may have been received by the subjects were noted where possible, together with most recent diet. Samples were stored in airtight containers flushed with OFN and processed as quickly as possible. Luminal contents in the small intestine were often scarce, and therefore samples were homogenised together with the superficial mucosa in a liquidiser. Specimen pH was measured with a handheld digital pH meter (Sigma-Aldrich Company Ltd.) and a 90% w/v ingesta slurry created using the appropriate mixture of preboiled Sorensen's buffer (Table 7.5). As for the faecal incubations, these samples were filtered to remove coarse debris and then separated into 40 ml aliquots in 120 ml anaerobic chambers.

7.3.2.2 Measurement of ^{13}C-substrate fermentation rate

The rate of ^{13}C-substrate fermentation was analysed indirectly by measuring the rate of appearance of $^{13}CO_2$ in the headspace gas. Basal samples were collected for measurement of ^{13}C:^{12}C ratio before addition of the labelled substrate, and then at 30-60 min intervals for

up to 48 h. The 1 ml samples of headspace gas were added to OFN-flushed Exetainers before automated analysis of $\delta^{13}C$ value by continuous flow IRMS (Analytical Precision Products Ltd., Northwich, Cheshire) as described in section 2.5.1.

7.3.2.3 *Calculation of ^{13}C-compound fermentation rate*

The sample $\delta^{13}C$ values generated by the IRMS were converted to ppm excess ^{13}C as described in equations 2.2 and 2.3. Recovery rates of the labelled substrate were plotted either as nmol excess ^{13}C or % dose recovery of the isotope, against time on the *x*-axis. In order to convert ppm excess ^{13}C to PDR of the isotope it was necessary to account for the dilution in the sampling process, as shown in Equations 7.3 – 7.4:

$$\text{nmol excess } ^{13}C = \frac{\text{ppm excess } ^{13}C \text{ x Exetainer volume x } \%CO_2 \text{ x volume headspace gas}}{\text{ml } CO_2 / \text{mmol x 100 x gas aliquot (ml)}} \qquad \textbf{Equation 7.3}$$

$$\%\ ^{13}C \text{ dose recovery} = \frac{\text{nmol excess } ^{13}C \text{ recovered x 100\%}}{\text{nmol substrate added}} \qquad \textbf{Equation 7.4}$$

In addition to the initial rate of fermentation, the cumulative % hydrolysis of substrate at infinite time was predicted for each specific incubation chamber and test condition.

7.3.2.4 *Statistical comparison of fermentation rates*

The rate of appearance of $^{13}CO_2$ in the headspace gas was described in most cases by a sigmoid curve as expected for a closed bacterial fermentation system. Since the concentration of $^{13}CO_2$ was representative of the cumulative dose recovery of the isotope at any time in the closed fermentation system, its recovery was modelled using Siegel's formula, as given in equation 7.5:

$$y(t) = m(1 - e^{-kt})^{\beta} \qquad \textbf{Equation 7.5}$$

where *y(t)* is the cumulative % ^{13}C excretion in breath at time *t*, and *m* is the total cumulative ^{13}C recovery when time is infinite. Mathematical curve-fitting was performed as previously given in section 2.6.4. When required, the difference in fermentation rates between specific systems was tested for statistical significance, based on the time to hydrolysis of % substrate as given by the modelled function. For example, the time to 3% hydrolysis was given by equation 7.6:

$$\text{Time to 3\% dose recovery} = -1/k * \ln(1 - 0.03^{1/\beta}) \qquad \textbf{Equation 7.6}$$

7.3.3 Fermentation of lactose ^{13}C-ureide by equine faecal flora

7.3.3.1 Effect of induction period on glucoseureide hydrolase activity

7.3.3.1.1 Aims

Having observed the effect of prior treatment with lactose ^{12}C-ureide *in vivo* on subsequent ^{13}C-substrate hydrolysis rate (section 7.2.2.2), it was aimed to further quantify this process *in vitro*. By using a variety of induction protocols, it was hoped to optimise both the relative ratio of ^{12}C:^{13}C substrates required, and to determine the optimum 'pre-dosing' time for addition of the lactose ^{12}C-ureide to the fermentation chambers. It was considered that maximisation of lactose ^{13}C-ureide hydrolysis rate *in vivo* would increase the reliability of OCTT estimation.

7.3.3.1.2 Study design

Initial pilot studies were required to determine the optimum concentration and preparation of the faecal slurry. Fermentation techniques reported for human faecal bacteria have used slurries varying from 5% w/v (Christian *et al.* unpubl.) to 33% w/v (Edwards *et al.* 1996; Parrett and Edwards 1997; Heavey *et al.* 2003). However, little fermentation activity was present in this study at these concentrations and a minimum slurry concentration of 40% w/v was required. In most reported preparation methods, all particulate matter is filtered from the slurry after homogenisation. However, in the equine faecal samples, this resulted in poor fermentative activity. Accordingly, the technique was modified to include more prolonged homogenisation in a liquidiser (2 – 3 min) prior to sieving through coarse nylon to remove the largest particles only. Removal of all particulate matter has been reported to reduce the concentration of cellulolytic bacteria in the slurry (Merry *et al.* 1982b) and may also have reduced target organisms in the pilot studies.

Faecal samples were collected from 6 individuals and prepared as given in section 7.3.2.1.1. Seven fermentation chambers were prepared for each individual, and incubated under anaerobic conditions in the shaking water bath at 38^{0}C. These were subjected to the given test protocol: Lactose ^{12}C-ureide (50 mg) was added to 4 chambers at set interval (T-19 h, T-14 h, T-10 h and T 0 h) before the addition of 10 mg of ^{13}C-LU at zero time (T 0 h). Three control chambers were used for each individual: these received no induction dose of ^{12}C-LU, and were treated with either 10/20 mg ^{13}C-LU or equimolar ^{13}C-urea (0.11 mg) at T 0 h. The study of Maczulak *et al.* (1985) showed that at least 18% of equine caecal bacterial isolates were able to use urea for growth. The rate of $^{13}CO_2$ production from ^{13}C-urea was therefore used as a marker for suitable fermentative conditions in these

experiments. Basal 1 ml aliquots of headspace gas were collected from each chamber prior to addition of the ^{13}C-LU and thereafter at 30 min intervals for 6 h. These were stored and analysed as given in 7.3.2.2 – 7.3.2.4, and results plotted as nmol excess ^{13}C against time.

7.3.3.1.3 Results

The effect of pre-treatment with ^{12}C-LU ('induction') at a dose ratio of 5:1 ^{12}C-LU:^{13}C-LU was very similar in the systems from each individual. Two examples are given in Figures 7.11 and 7.12. Induction at T-19 h resulted in a significant increase in the initial rate of ^{13}C-LU hydrolysis, when compared with the non-induced, T-14 h and T-10 h samples. The time to reach 1% PDR was significantly shorter in the T-19 h samples in all individuals (paired *t*-tests; $P < 0.01$). The initial rate of isotope recovery was greater in the T-14 h samples than the T-10 h samples, but this was not significantly different. The cumulative dose recovery of the ^{13}C-LU was also increased significantly by the earlier addition of ^{12}C-LU to the incubation systems at T-19 h. In the control samples, ^{13}C-urea metabolism proceeded rapidly without a lag phase. Urea metabolism was thus considered unlikely to form a significant rate-determining stage in the faecal degradation of ^{13}C-LU by equine gut microbes. Very little ^{13}C-LU fermentative activity was recorded in the chambers that were not induced before addition of the isotope.

7.3.3.2 Nature of induction process

7.3.3.2.1 Aims

Although prior ingestion of ^{12}C-LU has been described to 'induce' increased bacterial degradation of ^{13}C-LU (Heine *et al.* 1995; Wutzke *et al.* 1997; Mohr *et al.* 1999), this would suggest an upregulation of specific protein translation and transcription, which has not been demonstrated for lactose ureide. The induction of *E.Coli* β-galactosidase production by lactose is perhaps the most studied example. In the presence of lactose, a repressor protein is inactivated, allowing rapid transcription of enzymic protein from the operon genes (Stryer 1995). Bacterial enzymic induction is a very rapid process, which facilitates utilisation of specific carbohydrate when available. Further examples include the induction of *Eubacterium* sp. 7α-dehydroxylase activity by cholic acid, both *in vitro* (White *et al.* 1980) and *in vivo* (Thomas *et al.* 2001). This results in a marked increase in bacterial bile acid conversion rate in the presence of cholic acid. However, rather than enzymic induction, Morrison (2000) suggested that bacterial adaptation or selective multiplication was responsible for the increase in lactose ureide degradation after previous exposure. By studying the exposure of the target bacteria to different substrates and protocols, it was aimed here to further elucidate the mechanism of this process.

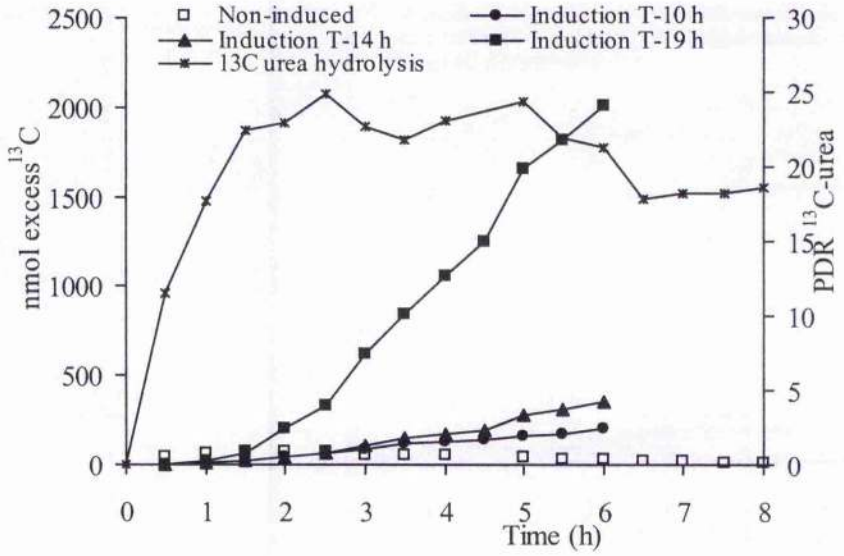


Figure 7.11 Rate of lactose ^{13}C-ureide digestion by faecal anaerobe preparations from subject 108, demonstrating effect of pre-treatment with lactose ^{12}C-ureide (50 mg) at different intervals prior to addition of lactose ^{13}C-ureide (10 mg) at time zero (0 h). The rate of ^{13}C-urea hydrolysis is plotted for comparison.

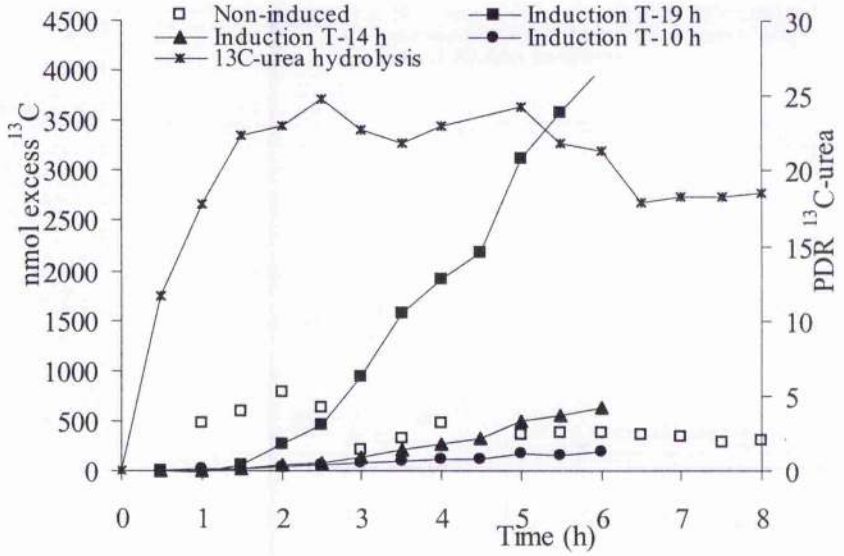


Figure 7.12 Rate of lactose ^{13}C-ureide digestion by faecal anaerobe preparations from subject 106. An induction dose ratio of 5 parts ^{12}C-LU: 1 part ^{13}C-LU was used, with addition of the ^{12}C- substrate at set intervals before that of the heavier isotope.

7.3.3.2.2 Study design

Faecal samples were collected from 3 adult ponies and processed within 30 minutes to form 40% w/v slurries, as described in section 7.3.3.1.2. For each individual, 18 anaerobic fermentation chambers were created, each containing 40 ml of slurry. After sealing, these were flushed with OFN for 60 s and treated according to the protocol given in Table 7.6:

Table 7.6 Study design for assessment of specific substrates on subsequent fermentation rate of lactose ^{13}C-ureide by equine faecal anaerobes.

Fermentation chamber	DURATION OF INDUCTION PERIOD -18 h	-14 h	-10 h - 8 h -2 h	0 h
A-18 h	10 mg ^{12}C-LU			2 mg ^{13}C-LU
B-18 h	20 mg ^{12}C-LU			2 mg ^{13}C-LU
C-18 h	4.28 mg galac			2 mg ^{13}C-LU
A-14 h		10 mg ^{12}C-LU		2 mg ^{13}C-LU
B-14 h		20 mg ^{12}C-LU		2 mg ^{13}C-LU
C-14 h		4.28 mg galac		2 mg ^{13}C-LU
" "		" "	" " " "	" "
A 0 h				10 mg ^{12}C-LU + ^{13}C-LU
B 0 h				20 mg ^{12}C-LU + ^{13}C-LU
C 0 h				4.28 mg galac + ^{13}C-LU

galac = galactose; $^{12/13}$C-LU = $^{12/13}$C-lactose ureide; ^{13}C-isotope was added to all fermentation chambers at time 0 h.

Three chambers were treated with either 10/20 mg ^{12}C-LU or equimolar galactose solution, at each of 18, 14, 10, 8, 2 and 0 h before the addition of 2 mg ^{13}C-LU. Prior to the addition of the ^{13}C-isotope, 1 ml samples of headspace gas were collected for basal ^{13}C:^{12}C measurement, and thereafter at 30 - 60 min intervals for 14 h, with further sampling at 21 and 26 h. Once again, samples were stored and analysed as given in 7.3.2.2 – 7.3.2.4, and results plotted as nmol excess ^{13}C against time. Ruemmele *et al.* (1997) showed that LU may be cleaved by small intestinal β-galactosidase, resulting in the arrival of glucoseureide and galactose in the large intestine. Therefore a comparison was made here between the effects of adding ^{12}C-LU or equimolar galactose to the slurries. In so doing, it was hoped to determine whether the increase observed in subsequent ^{13}C-LU fermentation rate was simply due to energy provision in the form of galactose, or due to more specific properties of the LU molecule. Earlier pilot experiments had shown that prior addition of lactose to the fermentation chambers did not have significant effects on subsequent LU fermentation rate. As it was possible that the faecal preparations contained insufficient lactase to allow cleavage of this disaccharide, the experiments were repeated with galactose.

7.3.3.2.3 Results

The rate of production of $^{13}CO_2$ in the headspace gas was not enhanced significantly in any individual by the earlier addition of galactose. Nor was the effect of galactose altered by the time point at which it was added to the fermentation chamber. By contrast, the addition of equimolar ^{12}C-LU at T-18 h caused a significant decrease in the time to 1% dose recovery of ^{13}C-LU in all individuals (paired *t*-test, $P < 0.01$) when compared to the effect of galactose. Although the changes were not always significant, ^{13}C-LU fermentation rate was positively correlated to the duration of the interval between addition of the ^{12}C-LU primer and the ^{13}C-LU marker. Examples from one subject are given in Figures 7.13 and 7.14. If the interval between addition of the primer and of the substrate was less than 10 h, there was little effect on ^{13}C-LU fermentation rate. Although time period was very important, the ratio of primer:substrate was less important in this experiment, with no significant different noted between the ratios of 5:1 and 10:1 for ^{12}C-LU:^{13}C-LU.

7.3.4 Fermentation of lactose ^{13}C-ureide by specific intestinal anaerobes

7.3.4.1 Ubiquity of equine bacteria with glucoseureide hydrolase activity

7.3.4.1.1 Aims

In order to validate the ^{13}C-LUBT for measurement of equine OCTT, it was necessary to establish whether organisms capable of cleaving the glycosylureide bond were restricted to the large intestine, or were more widespread. Having developed efficacious faecal fermentation systems, these techniques were applied to equine intestinal contents to determine this. The initial aims were twofold: (i) to determine whether caecal anaerobes cleaved ^{13}C-LU, and if so, whether 'priming' with ^{12}C-LU or different substrates had the same effect as noted in the faecal systems; (ii) to determine whether or not there was significant small intestinal fermentation under the conditions required for maximal caecal fermentation.

7.3.4.1.2 Study design

Intestinal mucosal and luminal contents were collected from 3 adult horses as soon as possible after death. These horses were euthanased on humane grounds for reasons not relating to the gastrointestinal tract. In each case, samples were collected from the stomach, duodenum, mid-jejunum, ileum and caecal body, and anaerobic conditions established as quickly as possible. As described in section 7.3.2.1.2, 90% w/v intestinal slurries were created, using the appropriate pH of preboiled Sorensen's buffer. When luminal contents were scarce, superficial mucosa was included in the homogenisation process.

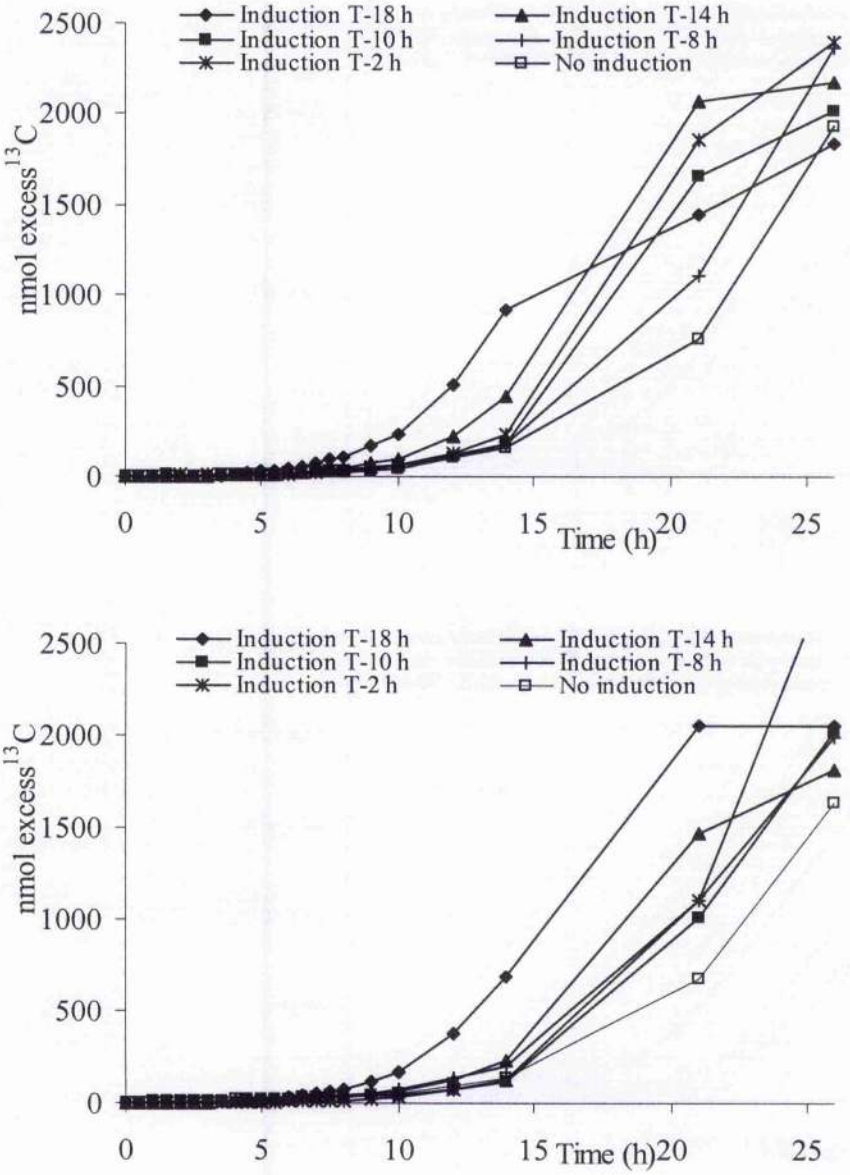


Figure 7.13 (top) Effect of induction duration on subsequent rate of ^{13}C-lactose ureide digestion by equine faecal anaerobes. Lactose ^{12}C-ureide (10 mg) or equimolar galactose solution ('No induction') was added to the chambers 2, 8, 10, 14 or 18 h prior to the addition of lactose ^{13}C-ureide (2 mg).

Figure 7.14 (bottom) Effect of inducing bacterial activity with a primer (^{12}C-Lactose ureide) to substrate (^{13}C-Lactose ureide) ratio of 10:1, rather than 5:1. The rate of appearance of $^{13}CO_2$ in the headspace gas is shown, following addition of ^{13}C-LU to the system at zero time.

Four 40 ml chambers were created for each intestinal locus, sealed and flushed with OFN prior to incubation at 38^0C in a shaking water bath. These chambers were primed with 10 mg ^{12}C-LU at either 14, 12 or 8 h prior to the addition of 2 mg ^{13}C-LU at time 0 h. The remaining chamber did not have a primer dose added. Basal samples of headspace gas were collected for ^{13}C:^{12}C analysis at T-1 h, and thereafter 1 ml samples were collected hourly for 10 h, and stored in OFN-flushed Exetainers. Continuous flow IRMS was used to measure isotopic enrichment and analysis was performed as detailed in sections 7.3.2.2 and 7.3.2.3. The mean cumulative dose recovery of the isotope at 10 h was compared for the different intestinal loci under the same induction conditions, in addition to the time taken for 1% dose recovery.

7.3.4.1.3 Results

A summary of the dose recovery data from the different intestinal regions is given in Figure 7.15. The caecal slurries from each individual were found to ferment ^{13}C-LU. The rate of $^{13}CO_2$ production and the cumulative dose recovery at 10 h were both increased by priming with ^{12}C-LU. Mean (s.d.) time to 1% dose recovery for the caecal non-induced and induced samples was 4.70 (1.58) h and 0.55 (0.22) h respectively ($P < 0.001$). Mean (s.d.) dose recovery at 10 h in the induced caecal samples was 39.88 (11.34) % compared with 16.44 (15.72) in the non-induced fermentation chambers ($P < 0.1$). In contrast to the caecal samples, there was no fermentation in the gastric samples and very little fermentative activity in the duodenal/jejunal or ileal samples. Priming with ^{12}C-LU did not produce a significant difference in the subsequent fermentation rate of ^{13}C-LU in any of the small intestinal samples. In the duodenal/jejunal samples, dose recovery was frequently below 1%; mean (s.d.) dose recovery at 10 h in the induced chambers was 0.51 (0.46) %, which was significantly different to the caecal samples ($P < 0.001$). In the T-14 h induced ileal samples, mean (s.d.) time to 1% PDR was 3.73 (1.26) h, with a mean cumulative dose recovery of just 4.78 (1.12) %; both of these values were significantly different to the induced caecal samples ($P< 0.001$).

Similarly to the faecal samples, there was a positive correlation in the caecal samples between the duration of the induction period and subsequent ^{13}C-LU fermentation rate. However, the difference in time to 1% PDR for the T-14 h, T-12 h and T-8 h was not significant.

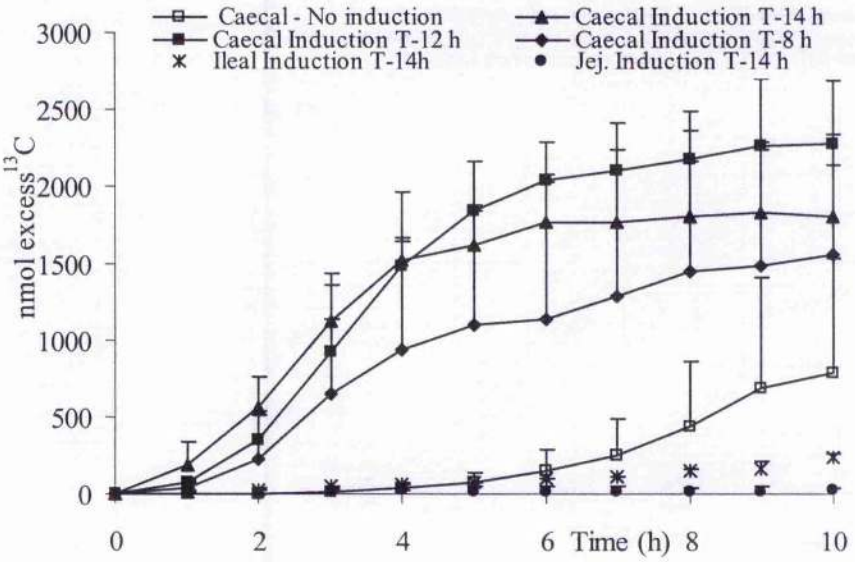


Figure 7.15 Mean rate of fermentation (n = 3) of lactose ^{13}C-ureide by equine jejunal, ileal and caecal anaerobes, as determined by appearance of $^{13}CO_2$ in the chamber headspace gas. The standard deviation (bar) is shown for each mean value. The maximum values gained for jejunal and ileal ^{13}C-LU fermentation are shown.

7.3.4.2 Characteristics of caecal induction process

7.3.4.2.1 Aims

Having established some of the properties of the 'induction' process in the faecal systems, it was aimed to determine whether the results of 7.3.3.2 would be replicated by caecal anaerobe fermentation. Specifically, the prior addition of ^{12}C-LU was compared with that of equimolar quantities of glucose, galactose or lactose. By addition of specific anaerobe culture media to the intestinal broths, it was aimed to increase the total dose recovery of the ^{13}C-LU.

7.3.4.2.2 Study design

Caecal contents were collected from an adult horse as soon as possible *post mortem*, and 90% w/v slurries created with preboiled/OFN-cooled Sorensen's buffer. Anaerobic fermentation chambers were set up as detailed in section 7.3.2.1.2. For those chambers undergoing enrichment with culture media, a 2:1 ratio was used for slurry:media solution. To each chamber, 10 mg ^{13}C-LU was added at time 0 h, and this alone was added to the control chambers. To the remainder, one of the following substrates was added at T-24 h: ^{12}C-LU (40 mg/9.5 x 10^{-5} mol); glucose (1.9 x 10^{-4} mol); galactose (1.9 x 10^{-4} mol) or lactose (9.5 x 10^{-5} mol), or the chamber was supplemented with blood culture medium or

cooked meat broth. All chambers were duplicated, to enable mean measurements to be calculated for each point. Aliquots of headspace gas were taken for ^{13}C:^{12}C analysis at T-1 h and thereafter at 30-60 min intervals for 12 h, with further end samples collected at 23 and 25 h. Sample isotopic enrichment and analysis was performed as detailed in sections 7.3.2.2 and 7.3.2.3.

7.3.4.2.3 Results

The mean rate of recovery of $^{13}CO_2$ in the different fermentation conditions is summarised in Figure 7.16. Once again, priming with ^{12}C-LU at T-24 h resulted in a marked increase in the subsequent rate of ^{13}C-LU fermentation, with a significant reduction in time to 1% PDR when compared with the non-induced sample ($P < 0.001$). The effect of ^{12}C-LU was significantly greater than equimolar quantities of lactose, galactose or glucose. In fact, the addition of galactose or lactose to the caecal slurry at T-24 h did not cause an appreciable increase in digestion rate of ^{13}C-LU at time 0 h when compared with the control chambers. Addition of glucose at T-24 h did increase ^{13}C-LU fermentation rate from 8 h onward, suggesting that glucose provided a required energy source for this procedure, which was more readily available to the microbes than either lactose or galactose. Neither of the specific anaerobe culture media resulted in improved microbial digestion of the ^{13}C-isotope, such that these media are unlikely to be helpful in future studies.

The mean (± s.d.) cumulative dose recovery of the ^{13}C-LU at 25 h was significantly increased by the addition of both lactose (52.20 ± 1.83 h) and glucose (44.94 ± 0.23 h) when compared with the ^{12}C-LU induced (32.15 ± 0.93 h) and non-induced (25.60 ± 0.42 h) samples. Thus, although initial ^{13}C-LU digestion rate was slower in these systems, it was more sustained, resulting in a higher end PDR.

7.3.4.3 Intracaecal distribution of lactose ureide-cleaving organisms

7.3.4.3.1 Aims

The objective of this experiment was to determine whether equine glucoseureide –cleaving organisms were more abundant in one section of the caecum than another, and whether they were adherent to the mucosa or free in the lumen. This was a preliminary study, to facilitate possible isolation, culture and speciation of the target organisms in the future.

7.3.4.3.2 Study design

Caecal aliquots were collected immediately *post mortem* from a stab incision made in the caecal apex or base, or were scraped from the mucosa of the caecal body with a spatula.

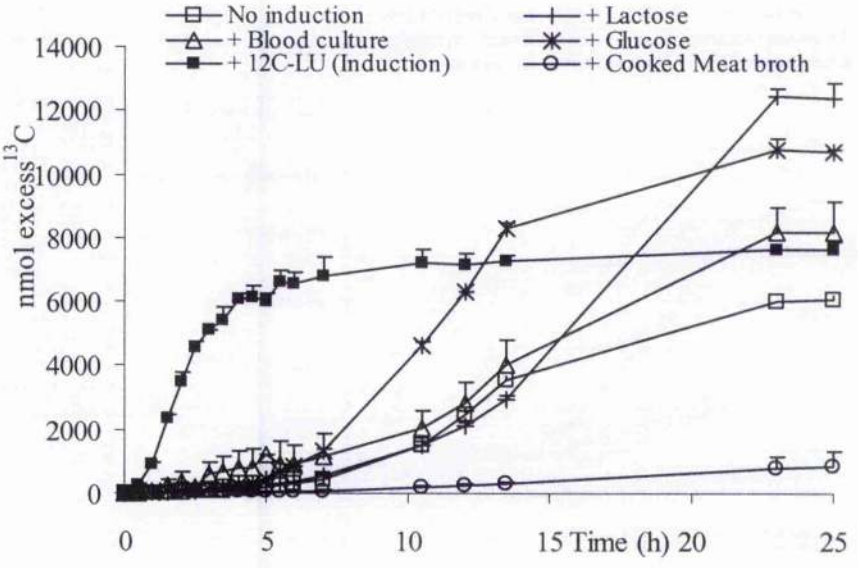


Figure 7.16 Effect of specific substrates and culture media on the rate of digestion of lactose ^{13}C-ureide by equine caecal anaerobes. The mean values (n = 2) and standard deviation are shown at each point. For comparison, the carbohydrate substrate doses were chosen to provide the systems with equivalent moles of free glucose. A ratio of 4:1 ^{12}C-LU:^{13}C-LU was used for the induction process.

Faecal samples were also collected from the small colon. For each locus, 90% w/v slurries were created and split into 40 ml aliquots in 120 ml fermentation chambers under anaerobic conditions. Five chambers were established for each site: 2 controls, and 3 'primed' chambers, to which 10, 20 or 40 mg ^{12}C-LU was added at T-12 h. At T 0 h, 10 mg ^{13}C-LU was added to all 5 chambers. Headspace gas was sampled at T-1 h and thereafter at 1 h intervals for 10 h. The ^{13}C:^{12}C ratio was measured, and fermentation rate calculated, as described in sections 7.3.2.2 and 7.3.2.3.

7.3.4.3.3 Results

In the subject of this study, the faecal microbes showed the most fermentative activity, with a significantly greater cumulative dose recovery at 10 h than any of the caecal samples ($P < 0.001$). The time to 1% dose recovery was also shortest in the faecal chambers. Of the caecal samples, 1% dose recovery was reached quickest in the apical chambers, but this was not significantly different to either the base or mucosal samples. The rate of $^{13}CO_2$ production in the different chambers is summarised in Figures 7.17 and 7.18. Of particular note in this study was the effect of the different 'priming' doses of ^{12}C-LU: maximal rate of digestion was achieved with a 1:1 ratio of ^{12}C-LU:^{13}C-LU. This may have reflected over-saturation of the target enzyme with unlabelled LU at the higher induction doses, causing corresponding reduction in ^{13}C-LU digestion.

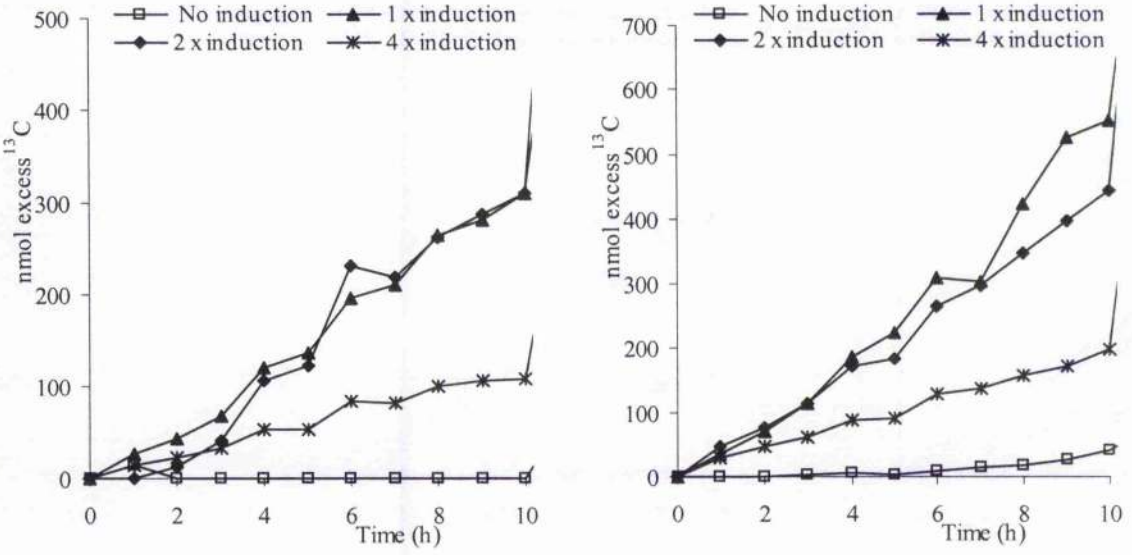


Figure 7.17 Effect of primer (lactose ^{12}C-ureide) to substrate (lactose ^{13}C-ureide) dose ratio on anaerobe digestion rate. The left chart maps fermentation by equine caecal base organisms; digestion by caecal apex anaerobes is given on the right for comparison.

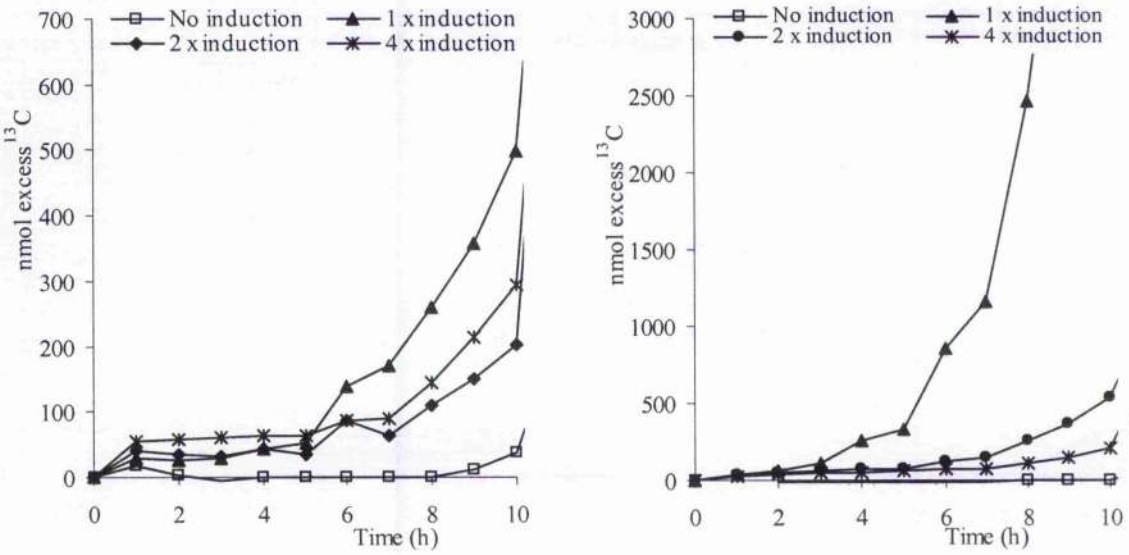


Figure 7.18 Effect of primer (lactose ^{12}C-ureide) to substrate (lactose ^{13}C-ureide) dose ratio on anaerobe digestion rate. The left chart maps fermentation by equine caecal mucosal organisms; digestion by faecal anaerobes from the same individual is given on the right for comparison.

7.3.4.4 *Inter-individual variation in locus of glucoseureide-cleaving microbes*

7.3.4.4.1 Aims

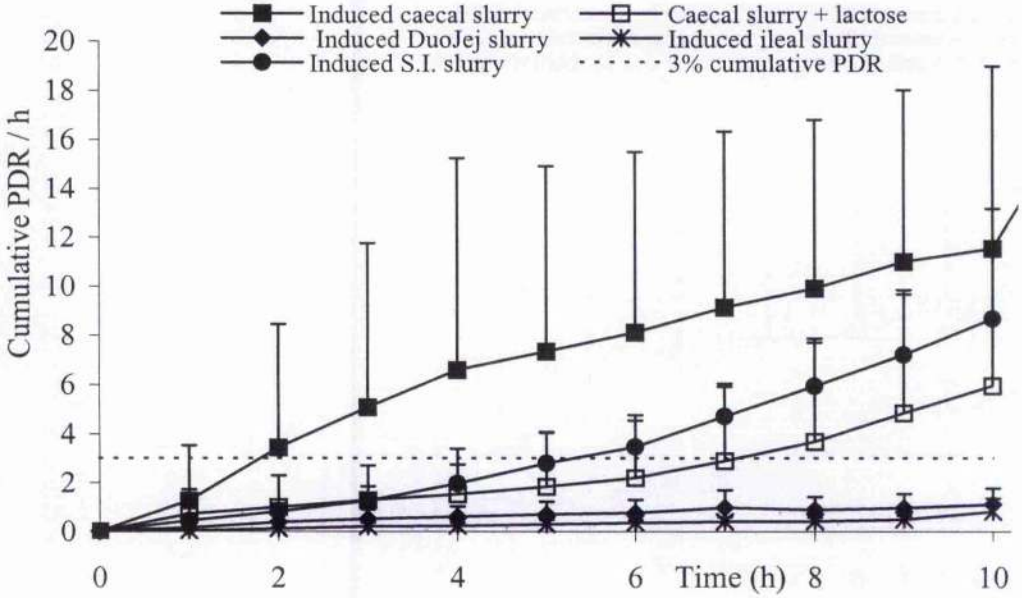
Prior to using the LUBT for clinical measurement of equine OCTT, it was necessary to establish whether or not there was marked inter-individual variation in LU fermentation capacity, or whether there was variation in the location of the organisms responsible for this function. Due to the dependence of microbial fermentation on experimental conditions, it was aimed to sample a range of horses, and to perform simultaneous incubations on their intestinal contents to ensure identical experimental conditions. The objective was to incubate contents from the duodenum/jejunum, ileum and caecum of 10 horses, and subject each to the final developed induction protocol. The difference between the mean rates of fermentation at the different loci was then to be measured and statistical comparison performed.

7.3.4.4.2 Study design

Intestinal samples were collected from 10 adult abattoir specimens as soon as possible after death. Stab incisions were made in the mid-jejunum, ileum (60 cm orad of the ileocaecal valve) and caecal apex. Intestinal contents were then collected into air-tight containers and flushed with OFN. Ingesta from the duodenum and jejunum were combined. In 4/10 individuals, there were very limited ingesta in the bowel, such that combined samples were collected from the entire small intestine. Samples were stored in airtight containers at room temperature until processing as described in section 7.3.2.1.2. The pH of each sample was measured, before homogenising with the appropriate pH of preboiled Sorensen's buffer to create a 90% w/v slurry. Four incubations were performed per individual: caecal controls (equimolar lactose/no induction), and duodenal/jejunal, ileal and caecal samples, all induced at T-15 h with ^{12}C-LU at a ratio of 10:1 for primer to ^{13}C-substrate. Chambers were maintained in anaerobic conditions in a shaking water bath at 38°C throughout the induction and collection process. Samples of headspace gas were collected at T-1 h for basal ^{13}C:^{12}C measurement, and thereafter at 1 h intervals for 10 h. As before, the rate of ^{13}C-LU fermentation was measured and calculated for each system as detailed in sections 7.3.2.2 and 7.3.2.3.

7.3.4.4.3 Results

For each fermentation chamber, the indirect rate of ^{13}C-LU digestion was determined as PDR in the headspace gas against time. The mean (± s.d.) rate of fermentation (n = 10) was calculated for each intestinal locus and this was plotted against time (Figure 7.19).



PDR = % dose recovery; duojej = duodenal/jejunal; S.I. = small intestine

Figure 7.19 Mean rate of lactose ^{13}C-ureide fermentation by anaerobes from the small and large intestinal tract of 10 adult horses. Each sample was induced with a 10:1 ratio of unlabelled substrate at 15 h prior to addition of the ^{13}C-isotope. The vertical bars represent 1 s.d. The time to reach 3% cumulative dose recovery of the isotope in the headspace gas is shown (dotted line).

Table 7.7 Numerical summary of the rate of digestion of lactose ^{13}C-ureide by intestinal microbes in 10 adult horses. These data are presented graphically in Figure 7.20.

Equine Intestinal Locus	Sample Size	Time to 1% PDR Mean +/- s.d. (h)	Time to 3% PDR Mean +/- s.d. (h)	Cum PDR at 10 h Mean +/- s.d. (h)
Duodenum/jejunum	6	[a]7.48 +/- 3.95	[e]18.58 +/- 7.40	[i]1.11 +/- 0.66
Ileum	6	[b,c,d]10.76 +/- 1.74	[f,g,h]15.42 +/- 4.52	[j]0.81 +/- 0.55
Combined small intestine	4	[c]2.59 +/- 0.80	[f]5.51 +/- 1.22	8.67 +/- 2.78
Caecal apex (Not induced)	10	[d]4.53 +/- 3.32	[g]9.18 +/- 3.71	5.95 +/- 7.22
Caecal apex (Induced)	10	[a,b]1.81 +/- 1.42	[e,h]3.65 +/- 2.38	[i,j]11.53 +/- 7.44

Matching superscript letters denote significant difference between pairs ($P < 0.05$)

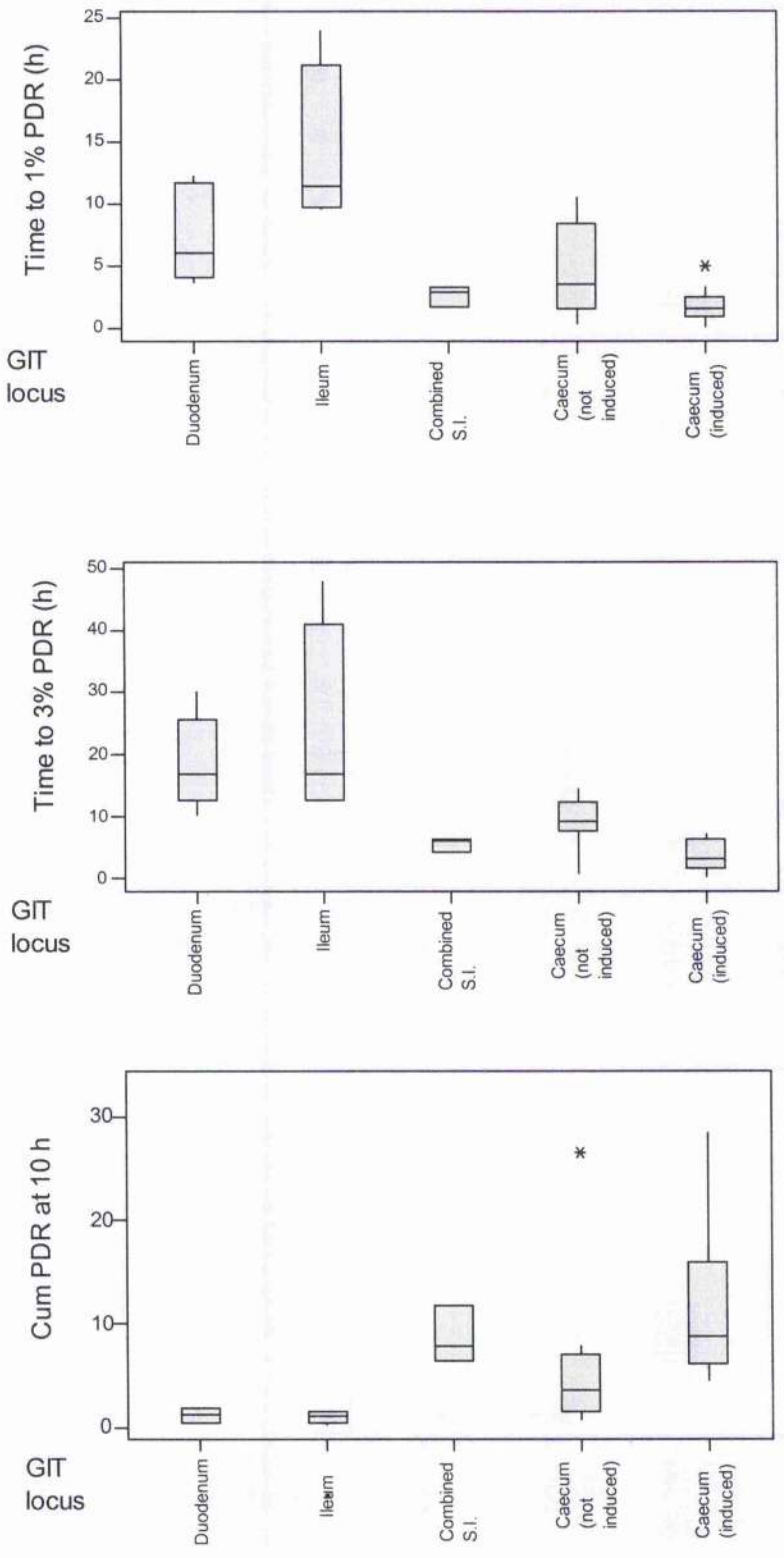


Figure 7.20 Boxplots providing summary of the fermentation rates of lactose ^{13}C-ureide by anaerobes from different intestinal locations in 10 adult horses: time to reach 1 % PDR (top); time to each 3% PDR (middle) and the cumulative dose recovery at 10 h are shown. Each incubation chamber was 'primed' by the addition of unlabelled LU at T-15 h.

Each box represents the median (horizontal line) and interquartile range of values. *denotes an outlying value

To quantify the differences observed in ^{13}C-LU fermentation rate, the times to reach 1% and 3% PDR were calculated for each chamber, and the cumulative isotopic dose recovery at 10 h noted. The mean values for each of these parameters was compared between the intestinal loci by a general linear model ANOVA, using Tukey pairwise comparisons with 95% confidence intervals (Minitab Inc., State College, PA, USA). These values are presented numerically in Table 7.7 and are also given in boxplot form in Figure 7.20. The mean rate of ^{13}C-LU fermentation as measured by time to both 1% PDR and 3% PDR was significantly shorter in the induced caecal apex samples than in the duodenal/jejunal and ileal samples ($P < 0.05$), and cumulative dose recovery at 10 h was also significantly higher in this group ($P < 0.05$). In fact, in the duodenal/jejunal and ileal samples, there was negligible fermentation of ^{13}C-LU, with mean cumulative PDRs of 1.11 ± 0.66% and 0.81 ± 0.55% respectively at 10 h. These statistical differences are summarised in Table 7.7. Mean pH increased along the length of the small intestine, and then decreased in the caecum: mean pH measurements for duodenum/jejunum, ileum and caecum were 6.96, 7.36 and 6.65 respectively.

In the combined small intestinal samples, 2 individuals had fermentative activity, causing appreciable ^{13}C-LU digestion as shown. In this sampled population, there was therefore significant small intestinal fermentation in 2/10 individuals. However, in each of these individuals the induced caecal samples showed either an equivalent or greater rate of ^{13}C-LU fermentation. Both of these individuals were noted to have particularly empty bowels at *post mortem* in comparison with the other subjects.

7.3.5 *Discussion* – in vitro *digestion of lactose ureide by equine anaerobes*

7.3.5.1 *Interpretation of study results*

In this study, anaerobic fermentation chambers were established, permitting indirect measurement of equine microbial lactose ^{13}C-ureide digestion by detection of the rate of $^{13}CO_2$ appearance in the headspace gas. The initial experiments demonstrated that equine faecal flora were capable of cleaving the glycosylureide bond, and that this capacity was enhanced significantly by prior exposure to LU. The duration of LU exposure was critical, with a minimum period of 14 h required before a significant increase appeared in the subsequent rate of ^{13}C-LU digestion. A positive correlation was also noted between the duration of the 'induction' period, and rate of $^{13}CO_2$ production. This was not attributable to changes in the rate of ^{13}C-urea hydrolysis, as this occurred rapidly without a lag phase. Nor was it due to the provision of energy in the form of LU, as equimolar quantities of glucose, galactose and lactose were shown to have very little effect on subsequent ^{13}C-LU

digestion. It was therefore concluded that prior exposure to LU caused either specific induction of a bacterial glucoseureide hydrolase, or resulted in selective multiplication of those organisms capable of its digestion.

The specific intestinal incubations also yielded useful information. The caecum was demonstrated *in vitro* to contain significant ^{13}C-LU fermentative activity, and was the most orad section of the intestinal tract to so do. As for the faecal samples, caecal digestion rate of ^{13}C-LU was increased in each individual by prior addition of LU to the fermentation. Once again, a minimum 'priming' period of 14 h was required to cause significant enhancement of ^{13}C-LU digestion, and the duration of this period was positively correlated to subsequent rate. The required ^{12}C-LU primer:^{13}C-LU substrate ratio was a less important factor in fermentative activity, but excessive quantities of primer resulted in reduced substrate digestion, possibly due to enzyme saturation. The optimum ratio *in vitro* appeared to be between 2 and 5:1 for ^{12}C-LU:^{13}C-LU ratio. The 'priming' effect was not replicated by glucose, galactose or lactose, and was concluded to result from specific properties of the LU molecule.

The specificity of the equine caecum for LU fermentation was excellent, with maximal digestion occurring in the apex. In samples from 15 individuals, small intestinal ^{13}C-LU fermentative activity was negligible in 13/15 (86.67%), whilst marked caecal digestion was present in 15/15. In the 2 individuals with small intestinal microbial capability, ^{13}C-LU digestion rate and cumulative dose recovery were lower or equivalent to those in the caecal chambers. In relative terms, small intestinal microbial digestion of ^{13}C-LU may also be of less significance *in vivo* as the transit of material through this intestinal segment is more rapid than that of the caecum (Argenzio *et al.* 1974). In classic experiments, Argenzio *et al.* (1974) reported that 2 h after its intragastric administration, 68 ± 6% of a soluble marker was recovered in caecal contents. This was compared to a caecal liquid transit time of approximately 5 h, with most prolonged transit of liquid and particulate markers in the ventral and dorsal large colon (Argenzio 1975). In addition, although equine caecal volume is often based on anatomical capacity (Nickel *et al.* 1979) rather than physiology and overestimated (e.g. Dabarciner and White 1997), its volume does generally exceed that of the small intestine (Argenzio *et al.* 1974). On the basis of the *in vitro* fermentations, it is therefore concluded that the ^{13}C-LUBT is likely to be a valid test for the measurement of equine OCTT, with arrival of ingesta in the caecum marked by an increased ^{13}C:^{12}C expiratory ratio. The data suggest that a priming dose of ^{12}C-LU is essential to maximise caecal ^{13}C-LU fermentative activity and aid measurement of OCTT.

7.3.5.2 *Microbial cleavage of lactose ureide*

Of the 174 different strains of faecal microbes obtained from healthy adults, Mohr *et al.* (1999) identified just one organism capable of degrading lactose ureide: *Clostridium innocuum*, a normal intestinal microbe. This clostridial species has no urease activity, such that hydrolysis of the ^{13}C-urea moiety is dependent on further microbial action. Attempts to isolate those bacterial species responsible for LU digestion from rumen contents with high *in vitro* LU-degradative activity were not successful (Merry *et al.* 1982b) and certainly the existence of *C. innocuum* has not been established in the horse. Mohr *et al.* acknowledged that further organisms may have LU hydrolase activity, but fortunately for the purposes of the equine LUBT, the property does appear to be exclusive. The equine stomach, duodenum, jejunum and ileum all have significant bacterial flora, averaging 27, 2, 30 and 40 x 10^6 culturable bacteria/g of lumen content respectively (Mackie and Wilkins 1988), but exhibited minimal LU fermentation in this study. Bacteria capable of lactate production and utilisation have been isolated from the stomach and proximal small intestine, but the greatest proportion of small bowel organisms are proteolytic (Kern *et al.* 1973; Mackie and Wilkins 1988). Estimates of equine caecal bacterial counts range from 5 – 50 x 10^8/g contents, of which up to 20% are proteolytic and 1% cellulolytic (Kern *et al.* 1973; Julliand *et al.* 1999). The caecum has the greatest population of cellulolytic organisms in the horse (Kern *et al.* 1974). Gram positive rods comprise 8.6% of the caecal population (Kern *et al.* 1973) but speciation of these has not been performed. Culture, isolation, and identification of caecal microbes, followed by thin layer chromatographic study of substrate digestion would be required to detect those species responsible for equine LU fermentation.

7.3.5.3 *Bacterial adaptation to LU digestion*

In this study naïve equine bacterial preparations exhibited little fermentative capability for LU. This has also been reported in steers and sheep given GU or LU for the first time (Merry *et al.* 1982c). In ruminants, the first significant increases in rumen digestion of GU occurred 9 d after daily addition of this substrate to the diet; the adaptation period for LU was shorter at 5 d, but fermentation of both compounds increased with more prolonged exposure (Merry *et al.* 1982c). Once adapted, cleavage of the sugar-urea bond in LU was completed in 2-4 h. Interestingly, Merry *et al.* (1982b,c) also noted that 'de-adaptation' occurred at 7 days after substrate withdrawal. Bacterial enzymes can be induced within a few hours of specific substrate exposure (e.g. lactose) and likewise lost after substrate removal (Stryer 1995b). In the ruminant studies, given the extended period required for adaptation, proliferation of a selected minority bacterial population seems to be the best explanation. However, in this equine study significant adaptation occurred within 14 h,

suggesting that true substrate induction of glucoseureide hydrolase activity may have occurred in the caecal populations. Although ureides do form a class of nitrogen compounds in plant metabolism, glucoseureide is not likely to be a natural constituent of the equine diet, such that the significance of this induction mechanism is not clear. As LU digestion rate continued to increase with more prolonged induction periods, selective bacterial division may have been present in addition to substrate induction. Molecular studies would be required to further investigate a possible induction mechanism.

7.3.5.4 Caecoileal reflux

A possible cause of the distal small intestinal ^{13}C-LU fermentation noted in 2 individuals is caecoileal reflux, which may be a normal occurrence in the horse. Roger *et al.* (1990) noted frequent dips in terminal equine ileal pH, caused by entry of acidic caecal contents containing short chain fatty acids. These were immediately counteracted by propagated phasic contractions of the terminal ileum, as noted in healthy humans (Coffin *et al.* 1997). Intraluminal manometry of the ileocaecal junction has indicated the presence of a high pressure zone over a distance of 5 cm, in which phasic pressure waves occur following caecal distention or acidification (Roger *et al.* 1990, 1995), effectively hindering the reflux of caecal content into the ileum. However, the mechanisms of caecoileal reflux are not fully known in the horse. Using a porcine model, Cuche and Malbert (1998) detected retrogradal propagated ileal contractions which preceded pH dips, and suggested that these facilitated caecal reflux. Prolonged retention of caecal reflux in the ileum could potentially confound interpretation of the ^{13}C-LUBT in the horse. However, from initial results of this study, it would appear that caecal reflux must be cleared rapidly and efficiently from the ileum. No significant difference in small intestinal pH was found here between individuals with and without ^{13}C-LU fermentative capability.

It is worth noting that caecoileal reflux does not occur during propulsive caecocolic contractions. Longitudinal propulsive contractions occur approximately every 4 minutes in the caecum, and move ingesta from the apex into the caecal base, through which it enters the cupola. The expanded cupola is then separated from the caudal base by a constriction of the medial and lateral folds and floor, which occurs cranial to the ileal papilla (Dyce *et al.* 1976; Sellers *et al.* 1982a,b), preventing reflux. This is followed by contraction of the isolated cupola, with emptying of contents through the opened caecocolic ostium into the right ventral colon. Caecal propulsive activity is stimulated by a pacemaker in the vicinity of the caecal apex (Ross *et al.* 1986, 1989) and also by migrating action potential complexes (MAPCs) that originate in the ileum (Lester *et al.* 1998a).

7.3.5.5 *Limitations of the study*

There are limitations to *in vitro* models of large intestinal fermentation that must be acknowledged. Most importantly, the removal of faeces or intestinal contents from the body inevitably changes bacterial composition and metabolic activity. Inadequate speed of preparation may have reduced isotopic dose recovery in some experiments, due to the death of anaerobes. A significant difference between human and equine faeces is that the latter has an anaerobe:aerobe ratio of 1:1, compared with at least 1000:1 in humans (Garrett *et al.* 2002; Guarner and Malagelada 2003). This factor may explain the high concentration of faecal slurries required in these studies for adequate fermentation, and the difficulty experienced in developing the faecal incubation technique as intestinal anaerobes are readily killed on exposure to air (Weese *et al.* 2000).

As fermentation products are not absorbed in an incubation chamber they accumulate and can be bacteriostatic (Christian *et al.* unpubl.). Bacterial growth patterns may also be abnormal *in vitro*, since rapid bacterial division may be slowed by exhaustion of a substrate unrelated to the one of specific interest (Merry *et al.* 1982b). Although each incubation chamber was buffered, it is also difficult to regulate pH in such systems, which in itself can have an important effect on individual bacterial metabolism (Edwards *et al.* 1985). As a final technical limitation, the build up of pressure was difficult to regulate in the simple incubation systems used here. This does not happen *in vivo* due to specific intestinal gas clearance mechanisms, and may have reduced viability of the target organisms. However, the methods described allowed rapid assimilation of informative data that would have been impossible *in vivo* without extensive surgical preparation of equine subjects.

7.3.5.6 *Clinical Considerations*

Before clinical application of the LUBT, certain further factors must be considered. Dietary composition is known to influence equine intestinal microbial numbers, and potentially could affect LU fermentation rate. Kern *et al.* (1973) found significant increases in caecal bacterial concentrations when ponies consumed diets consisting of 25% oats, rather than an all-forage timothy hay diet (7.0 vs. 4.8 x 10^9/g caecal ingesta). Goodson *et al.* (1988) produced similar results: concentrate feeding increased numbers of total viable caecal anaerobes, with a specific increase in lactate-utilising bacteria after 3 to 7 days. Concentrate diets may promote bacterial multiplication by increasing available energy (Goodson *et al.* 1988; Moore and Dehority 1993). Different feeding and management systems have also been shown specifically to affect the faecal CFU of

Clostridium perfringens/g equine faeces (Wierup and DiPietro 1981). Dietary variation may therefore alter cumulative dose recovery of ^{13}C-LU after ingestion. However, if OCTT is measured as the time to recovery of 3% of the end cumulative ^{13}C-LU dose, it is unlikely to be significantly affected by dietary composition.

Significant intestinal disease may potentially be a more serious obstacle to reliable use of the LUBT for equine OCTT measurement. For example, horses with acute grass sickness had a 10-fold increase in ileal Gram positive rods compared to controls, comprising up to 21 clostridial species (Garrett *et al.* 2002). Faecal clostridial counts were also increased in horses with both acute and chronic grass sickness (Garrett *et al.* 2002). An overgrowth of certain species, such as *C. perfringens*, may also be involved in the aetiology of postsurgical diarrhoea (Wierup and DiPietro 1981). In fact, it may be speculated that any condition resulting in intestinal stasis may lead to an increase in intestinal anaerobes with possible clostridial overgrowth of the small intestine. In small intestinal ileus, it is also possible that the normal mechanisms preventing caecoileal reflux may not be operational. Therefore, further *in vitro* investigation of LU digestion may be required using intestinal contents collected from cases of gastrointestinal disease.

The specific effects of antibiotic administration on LUBT results have yet to be established in any species (Geypens 2000). Clostridial organisms are generally sensitive to antibiotics such as penicillin G, metronidazole, tetracycline and modern penicillin derivatives with specific Gram positive activity. As all of these agents may be used in equine medicine, further *in vitro* studies of their effects on LU digestion may be necessary. Oral administration of oxytetracycline to horses was followed by large increases in faecal counts of coliforms, *Bacteroides* and *Streptococcus* species, and the appearance of *Clostridium perfringens* type A (White and Prior 1982). However, oral trimethoprim did not have a significant effect on faecal flora (White and Prior 1982), and nor did amikacin in either intravenous or oral form (Horspool *et al.* 1994). In this study, intestinal contents were incubated from at least 2 individuals that had received penicillin G treatment immediately before death, and the pattern of ^{13}C-LU digestion was found to be normal. Therefore any antibiotic-related effects on the outcome of the LUBT may be minimal, but should perhaps be tested *in vitro* for each class of antibiotic.

Finally, the efficacy of the LUBT in neonates would depend on colonisation of the hindgut by the requisite microbes. Van den Driessche (2001) has shown in human neonates that the ability to digest LU develops quicker in bottle-fed than breast-fed infants, and is only reliably present from 8 months onward. The LUBT is therefore unsuitable for

measurement of OCTT in neonates. Although young foals exhibit coprophagy, particularly during the first two months of life (Crowell-Davis and Houpt 1985), gastrointestinal microbes with glucoseureide hydrolase activity may not be present before weaning. The time of appearance of this microbial property should be ascertained prior to clinical use of the LUBT for OCTT estimation in this age group.

7.4 General discussion

The results of Part I of this study provided further evidence that the H_2BT is a relatively poor test for the measurement of equine OCTT. Inter- and intra-individual variation in H_2 production was considerable, and a high proportion of tests did not yield useful information due to the absence of an adequate H_2 output. By contrast, ingestion of lactose ^{13}C-ureide was followed in all cases by a detectable increase in expiratory $^{13}CO_2$, the magnitude of which was increased by prior ingestion of ^{12}C-LU. The potential value of the ^{13}C-LUBT for equine OCTT measurement was further confirmed in Part II of this Chapter. Using *in vitro* techniques, the ability to digest LU was shown to be a select property among equine intestinal microbes, with the requisite organisms being chiefly resident in the caecum. The effect of prior exposure to LU noted in Part I was also substantiated by the *in vitro* findings in Part II: naïve microbial populations had poor fermentative capability, whilst previous exposure to LU of at least 14 h duration augmented the rate and extent of digestion. This priming effect was found to be attributable to specific properties of the glucoseureide molecule rather than to energy provision alone, and both enzymic induction and bacterial adaptation may have roles in this process.

The initial cleavage of the lactose ureide moiety into galactose and glucose ureide by β-galactosidase did not appear to be a rate-limiting factor *in vitro*. In fact, further pilot experiments revealed that equine caecal microbes generated $^{13}CO_2$ at a similar rate following addition of either lactose ^{13}C-ureide or glucose ^{13}C-ureide to *in vitro* incubations. These data support the conclusion made by Argenzio (1975) that lactase deficiency in horses is rare. Roberts (1975) suggested that lactase declined in horses after the age of 4 months. However, Dyer *et al.* (2002) have more recently shown by molecular characterisation that lactase expression remains in adults, with greatest production in the jejunum. This suggests that glucose ^{13}C-ureide and lactose ^{13}C-ureide could be interchangeable as markers for orocaecal transit in the horse.

These results confirm that under normal conditions significant LU digestion is restricted to microbial action in the large intestine. For the first time in any species, the LUBT is more

specifically confirmed here to be a potentially suitable test for the measurement of equine OCTT. This is also the first time that a non-invasive test has been formally validated for the estimation of OCTT in this species. Using the induction and sampling protocols developed herein, it was elected to perform further LUBTs in healthy individuals in order to gain more information about small intestinal transit times in the horse. These results are presented in Chapter 8.

Chapter 8 In vivo Application of the Lactose ^{13}C-Ureide Breath Test for Equine Orocaecal Transit Time Measurement

8.1 General Introduction

Having investigated the characteristics of lactose ^{13}C-ureide fermentation by equine gastrointestinal microbes *in vitro*, the first objective of this Chapter was to ascertain whether or not the measured patterns of digestion and $^{13}CO_2$ liberation were repeatable *in vivo*. Using the *in vitro* techniques detailed in Chapter 7 it was demonstrated that equine glucoseureide hydrolase activity was inducible, and restricted to the large intestine, with sporadic terminal small intestinal activity. It was also shown that an exposure period to lactose ureide of at least 14 h was required to produce a significant increase in subsequent bacterial fermentative activity. The ratio of induction (lactose ^{12}C-ureide) to substrate (lactose ^{13}C-ureide) dose was also found to influence subsequent microbial activity, with an optimum ratio ranging between 2 and 5:1. These factors were therefore incorporated into the production of a clinical protocol for measurement of equine OCTT using the LUBT. In addition it was planned to further determine the effect of the induction process on subsequent rate of $^{13}CO_2$ production *in vivo*. In this way it was aimed to generate summary data for the mean orocaecal transit time of a standard labelled test meal in healthy adult horses.

For the second part of this study on non-invasive evaluation of equine small intestinal transit, it was aimed to develop a combined test for both gastric emptying rate and orocaecal transit. By combining ^{13}C-octanoic acid and lactose ^{13}C-ureide in a single standardised test meal and measuring the subsequent rate of recovery of $^{13}CO_2$, it has proved possible in children to measure GE, small intestinal half-transit time and OCTT from a single procedure (Van den Driessche *et al.* 1997). The combined test has proved useful for differentiating disorders of GE from those of small intestinal motility in children with Crohn's disease, in which condition the former is usually normal and the latter often abnormal (Van den Driessche 2001). The combined Stable isotope test has also proved a useful non-invasive diagnostic tool in children with severe functional gastrointestinal symptoms caused by a variety of other conditions (Van den Driessche 2001). Compared to the variety of whole gut transit scintigraphic (WGTS) techniques reported (Read *et al.* 1986; Madsen and Jensen 1989; Camilleri *et al.* 1991; Bonapace *et al.* 2000), the combined ^{13}C-OABT/^{13}C-LUBT has the advantages of being easier to perform, without the requirement for radiopharmaceuticals, and potentially may yield a similar quality of information.

Very little is known of the relationship between gastric emptying and small intestinal transit rate in the horse due to the difficulty in assessing these parameters. As mentioned in Chapter 1, technical imaging difficulties currently prohibit scintigraphic assessment of small intestinal transit in this species. In human patients with gastrointestinal motility disorders Camilleri *et al.* (1986) used manometry to prove that some had primary small intestinal dysmotility while others had antral hypomotility. This group also demonstrated that gastric stasis in gut dysmotilities could occur because of impaired antral peristalsis due to antral hypomotility or increased resistance to flow into the small bowel due to intestinal dysmotility. In healthy humans it is generally accepted that small bowel transit time is independent of GE rate (Read *et al.* 1980, 1984a, 1986), whilst SBTT and colonic filling rate are closely correlated (Read *et al.* 1984a). Similarly, Read *et al.* (1984a) did not find any correlation in healthy individuals between GE and rate of colonic filling. However, patients with irritable bowel syndrome with diarrhoea tended towards short SBTT and early colonic filling, whereas those with constipation tended towards long SBTT and delayed colonic filling (Read *et al.* 1986). Improved knowledge of these interactions in horses would improve physiological understanding of the gastrointestinal tract. In addition, it would be advantageous clinically to be able to distinguish gastric from small intestinal dysmotility to allow a more rational approach to therapy.

Measurement of small intestinal half-transit time using the combined test may also provide more relevant information than simple measurement of OCTT. This is because arrival of the head of a meal in the caecum ('OCTT') may not be representative of the transit of the bulk of ingesta as some particles will move faster than others, producing a spectrum of transit times (Read *et al.* 1986). Small bowel transit time (SBTT) has been derived in a variety of ways, either by subtraction of gastric emptying $t_{10\%}/t_{50\%}$ from colonic filling $t_{10\%}/t_{50\%}$ (Malagelada *et al.* 1984) or deconvolution of gastric emptying and caecal filling functions (Madsen and Jensen 1989; Camilleri *et al.* 1998). Both of these techniques would potentially be possible for the horse using the double isotope test (as explained in 3.3.2.5). Calculation of equine SBTT in this way may then be of diagnostic value in conditions such as post-operative ileus, equine dysautonomia, obstructive colic, gastroduodenal ulceration syndrome, chronic weight loss and wind sucking. Recent work has suggested that small intestinal dysmotility may be involved in the aetiology of wind sucking behaviour (McGreevy *et al.* 2001) and this important topic merits further research.

A non-invasive test for equine SBTT would also facilitate improved understanding of the physiological control mechanisms for gastrointestinal motility in this species. In human subjects, for example, elegant experiments have demonstrated that the ileum can regulate

small intestinal motility in the presence of undigested nutrients. Ileal infusion of either protein or lipid delayed the transit of a solid meal through both the stomach and small intestine of healthy adults, with inhibition of jejunal motor activity (Read *et al.* 1984b). Infusion of nutrients into the jejunum or colon did not produce this effect on SBTT (Read *et al.* 1984b). This 'ileal brake' mechanism is thought to be mediated via intestinal lipid receptors for medium chain triglycerides, oleic acid and triolein that stimulate the release of peptides such as Peptide YY, with consequent inhibition of jejunal motility, delayed GE and satiety (Welch *et al.* 1985; Spiller *et al.* 1988). It has also been demonstrated in man that nutrient stimulation of polymodal mucosal receptors in the orad small intestine has a remarkable retardation effect on GE. Nutrient-rich chyme results in relaxation of the gastric fundus, redistribution of food from the distal to proximal regions of the stomach, and suppression of antral motility. This is accompanied by increased pyloric contractility and tone (Heddle *et al.* 1988; Fraser *et al.* 1992), and also a modulation of duodenal motor activity to mixing rather than propulsive patterns (Houghton *et al.* 1988; Read and Houghton 1989). Whilst these mechanisms may also be found in the horse, such knowledge is in its infancy in equine gastroenterology, and easy measurement of SBTT may facilitate generation of such vital physiological information.

In this study, the clinical protocols and results are thus presented for two novel, non-invasive tests of equine gastrointestinal function: the lactose ^{13}C-ureide breath test, for the measurement of OCTT, and the combined ^{13}C-OABT/^{13}C-LUBT for the measurement of gastric emptying rate, small bowel half-transit time and OCTT. Summary data are presented for the estimation of transit parameters in healthy horses, as measured using these new diagnostic modalities.

8.2 Application of the lactose ^{13}C-ureide breath test for measurement of orocaecal transit time in healthy horses

8.2.1 Aims

The aims of this study were to apply the modified ^{13}C-LUBT *in vivo*, using a modified protocol based on the results of the *in vitro* studies. By applying the test to healthy horses it was aimed to produce an initial reference range for OCTT as measured by this technique, and to examine its inter- and intra-individual variability. In addition, the effect of the induction procedure *in vivo* was measured by comparing the results of non- induced tests with those of the developed optimum induced protocol in each individual.

8.2.2 Materials and methods

8.2.2.1 Subjects

Induced and non-induced ^{13}C-LUBT's were each performed on 2 occasions in 3 healthy adult individuals with no known history or clinical evidence of gastrointestinal disease. Each subject was in good body condition and, as previously, was maintained on a constant diet of ryegrass seed hay (30 g per kg bodyweight) throughout the study to minimise fluctuations in basal $^{13}CO_2$ output. As in all of the experiments described, the subjects were free from significant clinicopathological abnormalities and were treated at 8- to 10-weekly intervals with an ivermectin anthelmintic. Daily routine was kept as regular as possible in order to minimise physiological fluctuations in gastrointestinal motility. The 3 subjects were pony 105, a 20 y.o. native pony mare with body weight of 440 kg; 106, a 10 y.o crossbred mare with body mass 650 kg; and 108, a 14 y.o. welsh mountain pony mare of body weight 335 kg.

8.2.2.2 Test meal composition

As the aim of the latter part of this study was to develop a combined Stable isotope test for GE, SBTT and OCTT measurement, it was elected to use the same test meal for these studies as developed for the ^{13}C-OABT. The criteria for selecting this test meal have been detailed in sections 2.2.2 and 3.2.2.2. Briefly, the test meal comprised 150 g crimped oats, 100 g wheat bran and 200 ml water. The ^{13}C-LU powder (3.0 mg/kg bwt β-lactosyl ^{13}C-ureide dihydrate, Bell College of Technology, Glasgow) was homogenised with 1 egg white, which was then cooked in a microwave for approximately 60 s, before being chopped and thoroughly mixed into the test meal.

8.2.2.3 Study design

Two induced and 2 non-induced ^{13}C-LUBTs were performed in each individual in randomised order. These tests were performed at a minimum interval of 7 days, and 14 days were allowed for microbial deadaptation to occur before performing a non-induced test. Subjects were maintained on a constant exercise and feeding regimen during the test period. The study was designed to allow paired comparisons to be made of the effect of the induction process *in vivo*.

8.2.2.4 Test protocols

8.2.2.4.1 Induced ^{13}C-LUBT

Each subject was given 15 mg/kg β-lactosyl ^{12}C-ureide dihydrate (Bell College of Technology, Glasgow) in a small feed of oats and bran at 15 and 19 h before administration of the labelled substrate. Assuming an average OCTT of 3-4 h, these time intervals were chosen to ensure arrival of at least 1 bolus of ^{12}C-LU in the caecum by 14 h before the start of the test. The *in vitro* work presented in Chapter 7 revealed that an exposure period of at least 14 h was required in order for intestinal microbes to show a significant increase in subsequent ^{13}C-LU fermentative activity, with an optimum ratio of 2-5:1 for primer: tracer dose. Food was then withheld for at least 14 h before the start of the test to ensure an empty stomach. Two breath samples were collected for basal $^{13}CO_2$ measurement before ingestion of the test meal at time 0 h. The described test meal, incorporating a tracer dose of 3.0 mg/kg β-lactosyl ^{13}C-ureide dihydrate (Bell College of Technology, Glasgow) was then voluntarily ingested.

Expiratory breath samples were collected in duplicate at 15 min intervals for 6 h, followed by 30 min intervals for 6 h, then 60 min intervals for a further 2 h. The breath collection technique has been described earlier in section 2.4.1.

8.2.2.4.2 Non-induced ^{13}C-LUBT

This procedure was performed exactly as for the induced ^{13}C-LUBT but without the administration of priming doses of ^{12}C-LU on the previous day.

8.2.2.5 Measurement of Orocaecal transit time

The ^{13}C:^{12}C ratio of each expiratory sample was measured by CF-IRMS as given in section 2.5.1, and expressed as $\delta^{13}C_{PDB}$ value. This was then converted to % dose recovery of the isotope per hour as described in section 2.5.2, using the specific formula stated in Equation 2.5. Orocaecal transit time was taken to be that time at which 3% of the total cumulative dose recovery of isotope had occurred. In addition caecal t_{lag}, the time at which caecal filling rate was maximal, was calculated and also caecal $t_{1/2}$, the approximate time at which half of the administered isotopic dose had reached the caecum. These parameters were calculated from a modelled curve fitted to the PDR data using non-linear regression, based on the formula described by Maes (see Equation 7.1). The techniques used to curve-fit the data have been presented in section 7.2.1.6, and generation of OCTT was performed as given in Equation 7.2.

8.2.2.6 *Statistical evaluation of results*

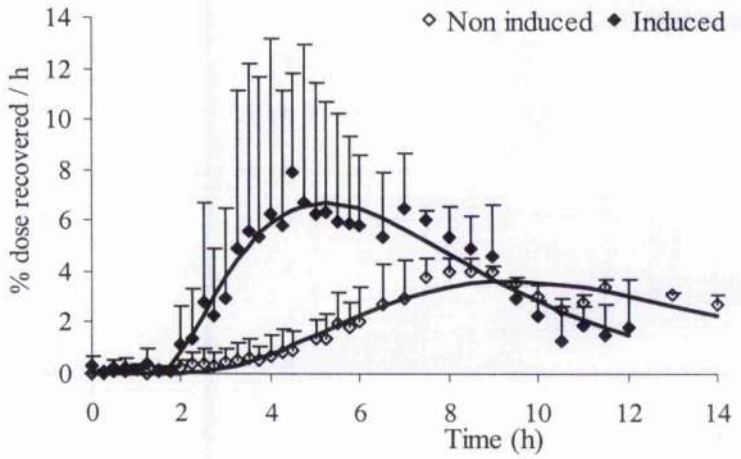
The mean differences in parameter estimation for each individual, between the non-induced and the induced test protocols, were compared using paired *t*-tests and also presented graphically. The overall differences between the groups of non-induced and induced data were also compared by 2-sample *t*-test.

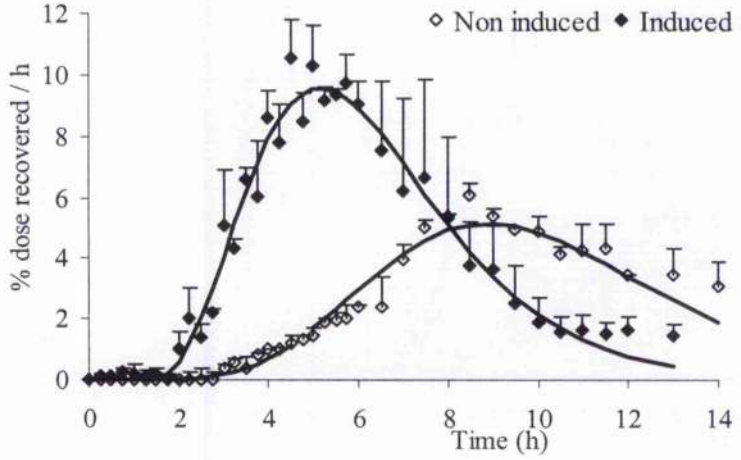
8.2.3 ^{13}C-LUBT results in healthy horses

All individuals produced a discernible peak in expiratory $^{13}CO_2$ output after ingestion of lactose ^{13}C-ureide. The mean expiratory isotopic dose recovery curves for each individual are presented in Figures 8.1-8.3. The mean (+ s.d.) data for each test protocol are presented on the same chart for comparison, together with the best-fit curves as modelled using Maes' formula. It can be seen for each individual that the induction process resulted in an increased rate of recovery of $^{13}CO_2$ in the breath. Furthermore, prior exposure to ^{12}C-LU resulted in an increased cumulative dose recovery of ^{13}C-LU. Mean (± s.d.) cumulative dose recoveries for the induced and non-induced test protocols were 50.66 (± 13.52) and 34.82 (± 4.74) % respectively ($n = 6$, $t = 2.708$, $P < 0.05$). The increased rate and cumulative dose recovery seen with the induced test protocol resulted in a more distinct initial deviation of expiratory $^{13}CO_2$ from basal output, when compared with the non-induced tests. However, initial isotope recovery in the non-induced tests could still be fitted without problem by a single peak Maes' model.

The tail of the isotope recovery curves was in many cases multiphasic and best fitted by a 3-peak Maes' model as demonstrated in Figure 8.4. This phenomenon was most likely caused by rate-limiting steps in the recovery of $^{13}CO_2$ from the body bicarbonate pool as discussed in section 6.4.1. Equine ^{13}C-bicarbonate dose recovery curves also showed this pattern (see Figures 6.2-6.5), and the body bicarbonate pool is usually described by a three-compartment model (Irving *et al.* 1985; Van Hall 1999) with separate rate constants for the exchange of $^{13}CO_2$ between the large, central vascular pool of bicarbonate and the smaller, slow/fast peripheral pools. However, the main peak in $^{13}CO_2$ recovery was distinct in all cases, and modelling of the data was not compromised by this factor.

In one single induced ^{13}C-LUBT in subject 106, a separate early peak was seen in $^{13}CO_2$ recovery, such that a biphasic model was required to fit the data. This is shown graphically in Figure 8.5. The smaller peak in $^{13}CO_2$ output occurred discretely and returned almost to baseline prior to the main $^{13}CO_2$ dose recovery.





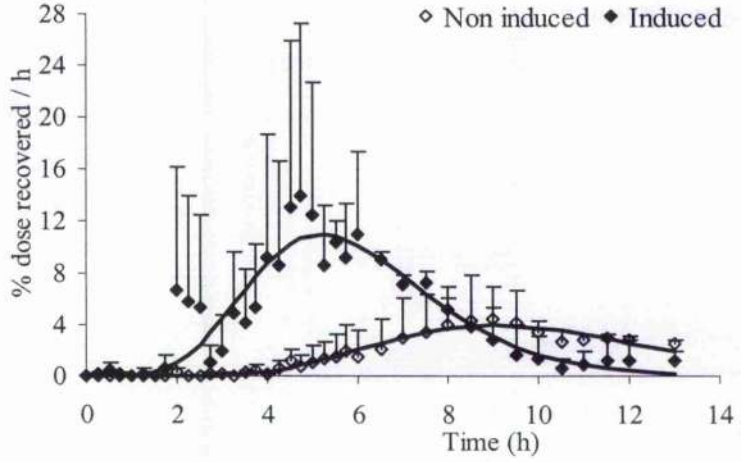


Figure 8.1 (Top) Comparison of mean expiratory dose recovery of lactose ^{13}C-ureide in subject 105, using either an induced or non induced protocol for the lactose ureide breath test. Each test was performed on 2 occasions at a minimum of 7 days' interval. The mean values and standard deviation are given at each point.

Figure 8.2 (Middle) Mean recovery of ^{13}C-LU in subject 108, using a non induced or induced protocol for the lactose ureide breath test.

Figure 8.3 (Bottom) Measurement of OCTT in subject 106, with comparison of mean non induced and induced test protocols for the LUBT (n = 2).

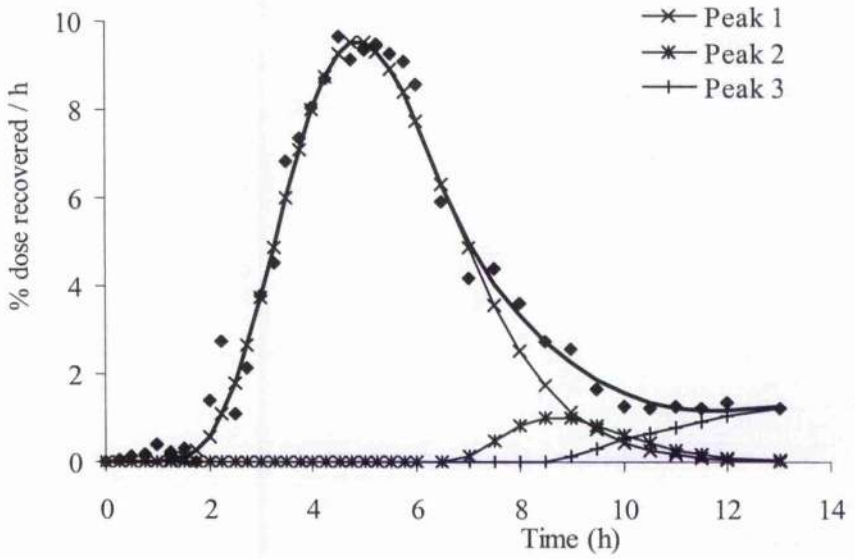
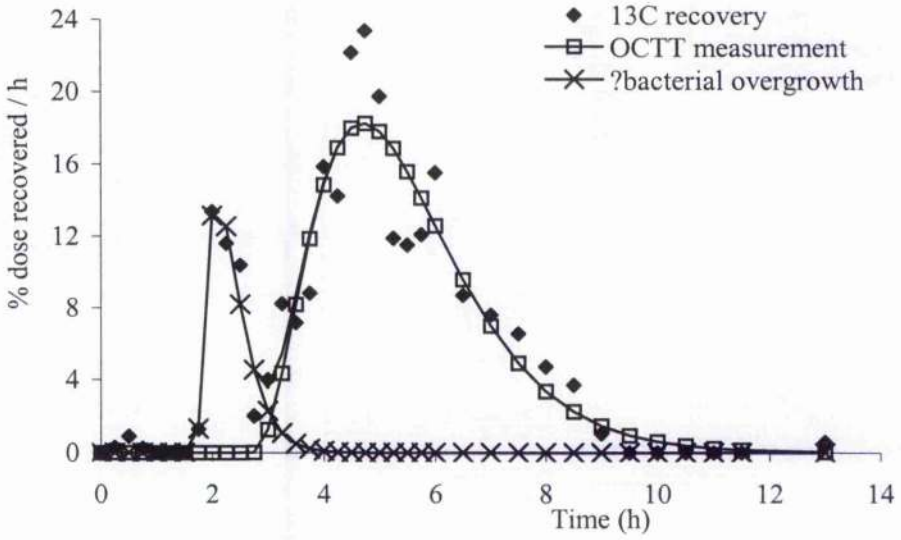


Figure 8.4 Induced lactose ureide breath test in subject 108. The expiratory dose recovery of the ^{13}C-isotope is plotted against time, and modelled using a 3-peak Maes' model. As shown in Chapter 6 with the ^{13}C-bicarbonate breath test, the final phasic stages in the recovery of the isotope are thought to correspond to rate-limiting steps in the recovery of $^{13}CO_2$ from the bicarbonate pool.



OCTT = orocaecal transit time

Figure 8.5 Induced lactose ureide breath test results in subject 106, showing expiratory isotopic dose recovery plotted against time. In this example there is an early peak in isotope recovery, possibly caused by small intestinal fermentation of the ^{13}C-substrate. The main peak is consistent with caecal fermentation secondary to arrival of the substrate in the large intestine.

The most likely cause of the early ^{13}C-LU fermentation was considered to be small intestinal glucoseureide hydrolase activity, either due to small intestinal bacterial overgrowth or alternative changes in bacterial flora. The mean group and individual indices for orocaecal transit for the different test protocols are presented in Tables 8.2 and 8.3 respectively. The mean group ($n = 6$) measurements for OCTT, caecal t_{lag} and caecal $t_{1/2}$ were all significantly lower for the induced test protocol than for the non-induced tests ($P < 0.05$), although the group coefficients of variation for each of these parameters were similar. Using the final induced ^{13}C-LUBT protocol, the mean (± s.d.) values for OCTT, caecal t_{lag} and caecal $t_{1/2}$ were 3.24 (± 0.65) h, 5.62 (± 1.22) h and 6.31 (± 1.21) h respectively. This value for OCTT was similar to that previously noted by McGreevy and Nicol (1998) using the unvalidated SLZ/SP test in 6 adult horses (2.83 ± 0.40 h).

The intra-individual variation in OCTT measurements varied between individuals. Using the induced ^{13}C-LUBT, CV% for OCTT ranged from just 1.19% for subject 108 to 33.31% for subject 105. Because of the relatively large CV% within individuals for most of the parameters, significant differences between induced and non-induced protocol results were only found in subject 108, which had the lowest level of weekly variation in gut transit rate. However, mean induced protocol measurements were shorter than the comparative non-induced ones for each individual.

As expected, a positive correlation was found between the parameters OCTT and t_{lag}, and between OCTT and $t_{1/2}$ for both the induced and non-induced test protocols. The relationship between these indices is shown in Figures 8.6 – 8.8. However, the positive correlation between OCTT/t_{lag} and OCTT/$t_{1/2}$ was relatively weak for the induced test protocols, and it can be seen that certain individuals had specific values falling far outside linearity.

8.2.4 Preliminary discussion

The induction protocol used in this study, as developed from the *in vitro* techniques described in Chapter 7, appeared to optimise interpretation of the ^{13}C-LUBT in all subjects in which it was used. Prior ingestion of 15 mg/kg ^{12}C-LU at 19 and 15 h before ingestion of the ^{13}C-LU test meal, resulted in a significantly increased rate of caecal fermentation of the isotope, and greater cumulative dose recovery. The induced ^{13}C-LUBT dose recovery curves were straightforward to model, and allowed calculation of OCTT, caecal t_{lag} and caecal $t_{1/2}$ in each case. The induced ^{13}C-LUBT therefore would appear to be a successful, easy to perform test for the non-invasive measurement of equine small intestinal transit.

Table 8.1 Intestinal transit parameters derived from 3 adult horses using the lactose ^{13}C-ureide breath test, giving comparison between mean results gained with the induced and non induced test protocols. Each protocol was performed twice in each individual in randomised order.

Test/Parameter	OC transit time (h)			Caecal t_{lag} (h)			Caecal $t_{1/2}$ (h)		
	Mean	s.d.	CV%	Mean	s.d.	CV%	Mean	s.d.	CV%
Induced lactose ^{13}C-ureide BT	3.24*	0.65	20.08	5.62*	1.22	21.63	6.31*	1.21	19.12
WITHOUT induction	5.12*	1.01	19.70	9.42*	1.37	14.58	10.43*	1.59	15.28

OCTT = orocaecal transit time; caecal t_{lag} = time from ingestion to maximal caecal filling rate; caecal $t_{1/2}$ = time until expiratory recovery of $^1/_2$ of total isotopic dose recovered; CV% = coefficient of variation; * denotes significant difference ($P < 0.05$).

Table 8.2 Lactose ^{13}C-ureide breath test results for 3 adult horses, showing intra-individual coefficients of variation for both induced and non induced test protocols. Each test was performed twice in each individual.

Subject/Measure	OC transit time (h)			Caecal t_{lag} (h)			Caecal $t_{1/2}$ (h)		
	Mean	s.d.	CV%	Mean	s.d.	CV%	Mean	s.d.	CV%
Subject 105									
Induced	3.23	1.08	33.31	6.08	2.23	36.74	6.89	2.06	29.85
Non induced	4.88	1.55	31.70	9.26	1.18	12.75	10.85	1.72	15.82
Subject 106									
Induced	3.70	0.38	10.35	5.57	1.20	21.48	6.16	1.28	20.75
Non induced	5.73	1.22	21.20	9.97	2.65	26.55	10.60	2.94	27.71
Subject 108									
Induced	2.80*	0.03	1.19	5.20**	0.44	8.38	5.86*	0.57	9.69
Non induced	4.73*	0.21	4.47	9.04**	0.32	3.52	9.86*	0.30	3.04

*denotes $P < 0.05$; ** denotes $P < 0.02$

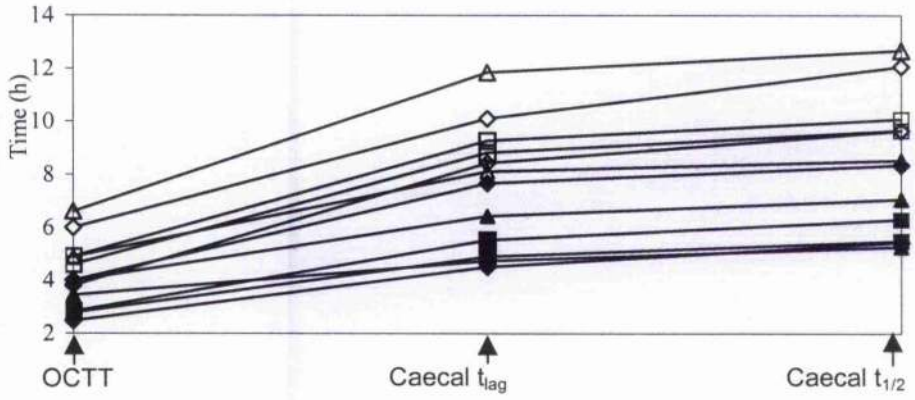


Figure 8.6 Summary diagram showing relationship between OCTT, caecal t_{lag} and caecal $t_{1/2}$ in 3 healthy adult horses. Open symbols represent values gained from non-induced ^{13}C-LUBT, and closed symbols those from induced test protocol. Matching symbols denote same individual.

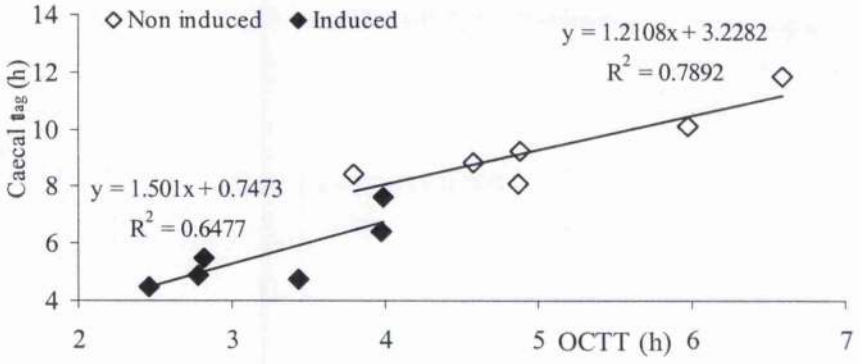


Figure 8.7 Correlation between OCTT and caecal t_{lag} measurement for 12 ^{13}C-LUBTs in 3 individuals. The difference between the non-induced and induced test protocols is shown. The lines are the lines of 'best-fit' as determined by simple linear regression.

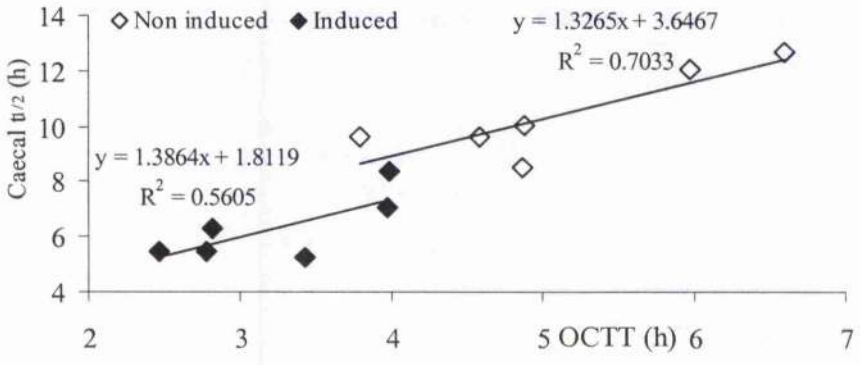


Figure 8.8 Correlation between OCTT and caecal $t_{1/2}$ measurement for the same 12 ^{13}C-LU breath tests. The difference between the non-induced and induced test protocols is shown. A weak positive correlation is seen for the induced test protocol.

The ^{13}C-LUBT has several advantages to the H_2BT for the measurement of equine OCTT. It is reproducible, not affected by dietary fluctuations, and appears to result in a detectable signal in all individuals upon entry of labelled ingesta into the caecum. Unlike the SLZ/SP test for OCTT measurement (McGreevy and Nicol 1998), the ^{13}C-LUBT has been demonstrated to be relatively specific for the fermentative activity of caecal flora (see Chapter 7) and does not result in the generation of pharmacologically active compounds. The effect of sulphasalazine on the equine colon has not been fully investigated.

Prior exposure to ^{12}C-LU seems to be a vital part of the test protocol for the ^{13}C-LUBT, as reported previously in humans, resulting in a greater and steeper generation of $^{13}CO_2$, and more reliable estimation of OCTT (Wutzke *et al.* 1997a,b; Van den Driessche 2001). The one disadvantage of the induction procedure is that it necessitates a 24 h preparation period before the test can be utilised. This may be a drawback if using the test for clinical diagnostics, although the majority of clinical cases to which the test is applicable will be chronic rather than acute in nature. A further possible disadvantage of the ^{13}C-LUBT for equine use was demonstrated in Figure 8.5. In the presence of bowel stasis or small intestinal bacterial overgrowth, small intestinal fermentation of the isotope may occur. Since luminal contents are of much lower volume in the small intestine than the caecum (Dart *et al.* 1999), it is anticipated that any small intestinal digestion would be distinguishable from caecal activity. As shown in Figure 8.5, the early peak in ^{13}C-LU was transient, of significantly lower magnitude than the main peak, and $^{13}CO_2$ excretion also returned to baseline prior to the start of caecal fermentative activity.

The fact that OCTT did not always exhibit a linear relationship with caecal t_{lag} and caecal $t_{1/2}$ was of interest in this study, as this implies that caecal filling was not linear in nature in these individuals. As discussed in section 8.1, an 'ileal brake' mechanism has been demonstrated to exist in man (Spiller *et al.* 1988) and also the dog (Pappas *et al.* 1986), cat (Read and Houghton 1989), pig (Treacy *et al.* 1990) and mouse (Brown *et al.* 1987) whereby ileal lipid receptors can regulate the rate of small intestinal transit via the release of Peptide YY from the ileocolonic mucosa. As yet, the specific lipid receptors that regulate this mechanism have not been identified in any species, and the existence of this mechanism has not been demonstrated in the horse. Based on the variability in equine caecal filling rate demonstrated here, it is possible that an ileal brake mechanism may also act to regulate small intestinal motility in this species. To investigate this effect further, the induced ^{13}C-LUBT could be used to determine the effect of ileal lipid infusion on subsequent OCTT.

A further possibility for the fluctuation in caecal filling rate seen in these experiments could be alterations in the pulsatile frequency of gastric emptying in response to digestion of the test meal. A variety of specific proximal small intestinal receptors has also been described for acid, glucose and amino acids, in addition to polymodal receptors, that have negative feedback effect on gastric emptying rate in the presence of excessive nutrients (Read and Houghton 1989). Hunt and Stubbs (1975) have suggested that the net effect of all of these receptor mechanisms is to maintain homoeostatic delivery of calories to the duodenum, despite the wide variety of meal composition. Duodenal distension has also been shown to result in inhibition of antro-pyloric pressure waves, and stimulation of isolated pyloric pressure waves, both of which act to reduce gastric emptying (Treacy *et al.* 1996). Pulsatile flow during antro-pyloric pressure waves accounted for emptying of more than half the total gastric content in pigs (Treacy *et al.* 1990). If this is assumed to be true in the horse, it is logical that caecal inflow of ingesta may also be pulsatile in nature.

In order to investigate the relationship between gastric emptying and caecal inflow of ingesta, it would be necessary to measure both these parameters simultaneously. This would also allow generation of small bowel half transit times, as has previously been ascertained by whole gut transit scintigraphy (Read *et al.* 1986; Madsen and Jensen 1989; Camilleri *et al.* 1991; Bonapace *et al.* 2000). In the second part of this study, a combined Stable isotope breath test was developed and tested in 4 healthy animals: the combined ^{13}C-OABT/induced ^{13}C-LUBT test, for the measurement of solid phase gastric emptying, small bowel half transit time (SBTT) and OCTT.

8.3 Development and application of the combined stable isotope breath test for the measurement of equine gastric emptying rate, SBTT and OCTT.

8.3.1 Aims

The objective of this study was to combine the acquired data from the ^{13}C-OABT and the induced ^{13}C-LUBT to form a single, dual isotope test, which could be used to gain further information about small intestinal motility, and the relationship between gastric emptying and orocaecal transit time in the horse. The initial objective was to determine the optimum dose ratio of each tracer, so as to facilitate separate modelling of the gastric emptying and orocaecal transit functions. The further aim of pilot studies was to determine whether modification of the existing test meal was required, to assist with temporal spatiation of these functions. In health, overall small bowel transit times of solids and liquids are reported to be similar (Malagelada *et al.* 1984; Graff *et al.* 2001) and in certain conditions, such as chronic intestinal pseudo-obstruction, transit of solids and liquids may be affected

to the same degree (Read *et al.* 1980). Meal physical composition may therefore have little effect on small intestinal transit, but specific nutrient content may relatively prolong SBTT, thus separating out GE and caecal arrival functions.

Having developed an optimum combined test meal and protocol, it was aimed to study both the variability of intra-individual SBTT and also to examine the relationship between solid phase GE rate, SBTT and OCTT in the horse. The physiological relationship between these parameters has not previously been established in this species. A specific test for equine small intestinal motility would also provide an optimum non-invasive means of testing specific prokinetic drugs.

8.3.2 Materials and Methods

8.3.2.1 Subjects

In addition to subjects 105 (20 y.o. pony mare, bwt 440 kg), 106 (10 y.o crossbred mare, bwt 650 kg) and 108 (14 y.o. welsh mountain pony mare, bwt 335 kg) used in section 8.2, subject 102 was used in this study: a 13 y.o. Thoroughbred mare of body weight 516 kg. The criteria for subject eligibility were as reported in section 8.2.2.1, as were feed intake and daily management.

8.3.2.2 Test meal composition

As the same test meal had been validated separately for the measurement of gastric emptying rate and for orocaecal transit time, this meal was also used for the dual isotope breath test. Once again, this comprised 150 g crimped oats, 100 g wheat bran and 200 ml water (see also sections 2.2 and 3.2.2.2). 1mg/kg ^{13}C-octanoic acid (Octanoic acid-1-^{13}C, Isotec Inc., Miamisburg, OH, USA) was added to egg yolk (1 yolk per 250 mg tracer), and blended before baking in a microwave until firm. The ^{13}C-lactose ureide powder (3.0 mg/kg bwt β-lactosyl ^{13}C-ureide dihydrate, Bell College of Technology, Glasgow) was homogenised with 1 egg white, which was then cooked in a microwave for approximately 60 s. Both of these isotope-labelled substrates were then finely chopped and cooled before thorough mixing with the test meal.

8.3.2.3 Study design

A combined ^{13}C-OABT/^{13}C-LUBT was performed in each of the 4 individuals on 4 occasions at approximately weekly intervals. Cost restrictions prohibited further application of the test, and it was considered most useful to monitor intra-individual variations in intestinal motility, rather than to attempt to establish a reference range. As

with all the stable isotope breath tests, the horses were stabled throughout the test period, and attempts made to minimise external stimuli so as to avoid marked fluctuations in VCO_2 and metabolic rate. Variations in basal $^{13}CO_2$ production during the test period were minimised by feeding a maintenance diet of ryegrass hay only. Previous work had shown that ingestion of the test meal itself did not cause a significant change in expiratory ^{13}C:^{12}C ratio (see 3.3.2.1).

8.3.2.4 Test protocol

At 15 and 19 h before ingestion of the dual labelled meal, each horse was given 15 mg/kg ^{12}C-lactose ureide (Bell College of Technology, Glasgow) in a small test meal as a microbial priming dose. Food was then withdrawn 14 h before the start of the test to ensure an empty stomach. Duplicate breath samples were collected before ingestion of the test meal for basal ^{13}C:^{12}C measurement, and thereafter at 15 min intervals for 6 h, then 30 min intervals for 6 h, and finally at 60 min intervals for a further 2 h. Subjects had access to water throughout, but were only allowed a small quantity of hay 10 h after the start of the test.

8.3.2.5 Calculation of indices of small intestinal transit

Expiratory breath sample ^{13}C:^{12}C ratio was measured by CF-IRMS as given in section 2.5.1, and expressed as $\delta^{13}C_{PDB}$ value. These values were then converted to % dose recovery of the isotope per hour as described in section 2.5.2, using the specific formula given in Equation 2.5. A multi-peak Maes' formula was used to model the dose recovery data, incorporating 'd' or 'delay' terms:

$$y(t) = a_1(t\text{-}d_1)^{b1}e^{-c1(t-d1)} + a_2(t\text{-}d_2)^{b2}e^{-c2(t-d2)} + a_n(t\text{-}d_n)^{bn}e^{-cn(t-dn)} \qquad \textbf{Equation 8.1}$$

where *a*, *b* and *c* are rate constants. The derivation of this equation has been detailed in section 2.6.4. The Solver function of an Excel 9.0 spreadsheet was used to fit a modelled curve to the ^{13}C recovery data by non-linear regression. In 15/16 cases, a dual peak model was used. In the remaining dual test, a 4-peak model was required due to apparent discrete gastric emptying events, and subsequent arrival of discrete boluses of ^{13}C-LU at the caecum.

The gastric emptying and caecal transit parameters were derived from the first and second modelled peaks respectively. The gastric parameters $t_{1/2}$ and t_{lag} were generated as

described in section 2.6.3. In addition, the parameter $t_{10\%}$, corresponding to the time of 10% gastric emptying, was generated as shown in Equation 8.2:

$$t_{10\%} = \text{Gammainv.}(0.1;\ b + 1;\ 1/c) \qquad \textbf{Equation 8.2}$$

where b and c are rate constants. Orocaecal transit time, caecal t_{lag} and caecal $t_{1/2}$ were calculated as given in section 7.2.1.6. Caecal $t_{10\%}$ was also determined by application of equation 8.2 to the second modelled peak of ^{13}C recovery.

Four main approaches have been suggested to measure small bowel transit data: (i) deconvolution of gastric emptying and colonic filling curves (Malagelada *et al.* 1984; Van den Driessche 2001), which requires complex mathematics; (ii) subtraction of gastric $t_{1/2}$ from caecal $t_{1/2}$ (Read *et al.* 1980, 1984a), which can approximate the deconvolution process (Read *et al.*1986) but may be inaccurate as gastric emptying is linear, and caecal filling is more likely to consist of irregular bolus movements of ingesta (Camilleri *et al.* 1989); (iii) subtraction of gastric $t_{10\%}$ from caecal $t_{10\%}$ (Camilleri *et al.* 1998; Bennink *et al.* 1999), which primarily assesses transit of the leading column of chyme; and (iv) 10% caecal filling time of a liquid meal (Maurer and Krevsky 1995), which assumes almost instantaneous gastric emptying of this component of the meal, making this unsuitable for cases of marked gastroparesis. As small bowel transit (SBT) has not been reported in the horse, methods (ii) and (iii) were used here to calculate SBT in terms of both 10% and 50% transit times:

Small bowel $t_{1/2}$ time (h) = Caecal $t_{1/2}$ (h) – Gastric $t_{1/2}$ (h) **Equation 8.3**

Small bowel $t_{10\%}$ time (h) = Caecal $t_{10\%}$ (h) – Gastric $t_{10\%}$ (h) **Equation 8.4**

8.3.2.6 Statistical evaluation of results

The intra-individual coefficients of variation were assessed for each of the individual transit parameters and compared. The relationship between the different gastric and small intestinal transit parameters was also assessed by simple linear regression and Pearson's coefficient of correlation calculated to determine statistical significance.

8.3.3 Results

The expiratory ^{13}C recovery curves for the dual isotope tests were plotted for each individual and are shown in Figures 8.9 – 8.12. It can be seen that isotopic recovery was clearly biphasic in 15/16 tests, such that the gastric emptying and caecal filling components of ^{13}C recovery were clearly separable, and amenable to fitting with the dual peak model.

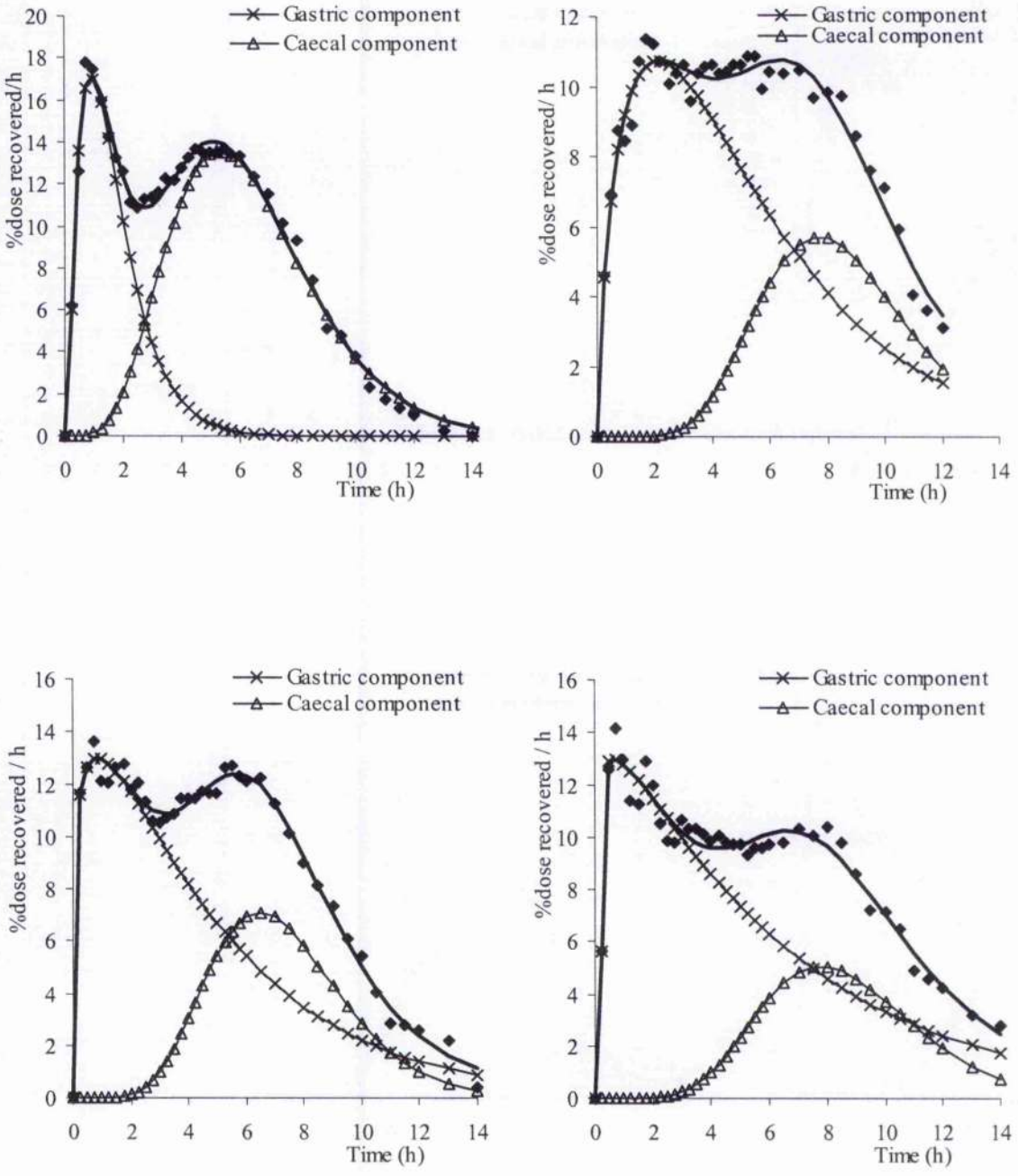


Figure 8.9 Combined ^{13}C-OABT/^{13}C-LUBT results in subject 102. Test results from weeks 1 to 4 are shown clockwise from top left. All results were modelled successfully using a dual peak Maes' model. The modelled gastric and caecal components of isotope recovery are shown, plus combined expiratory recovery of $^{13}CO_2$ (thick line). Solid diamonds mark the actual isotope recovery. Individual parameter values are given in Table 8.3.

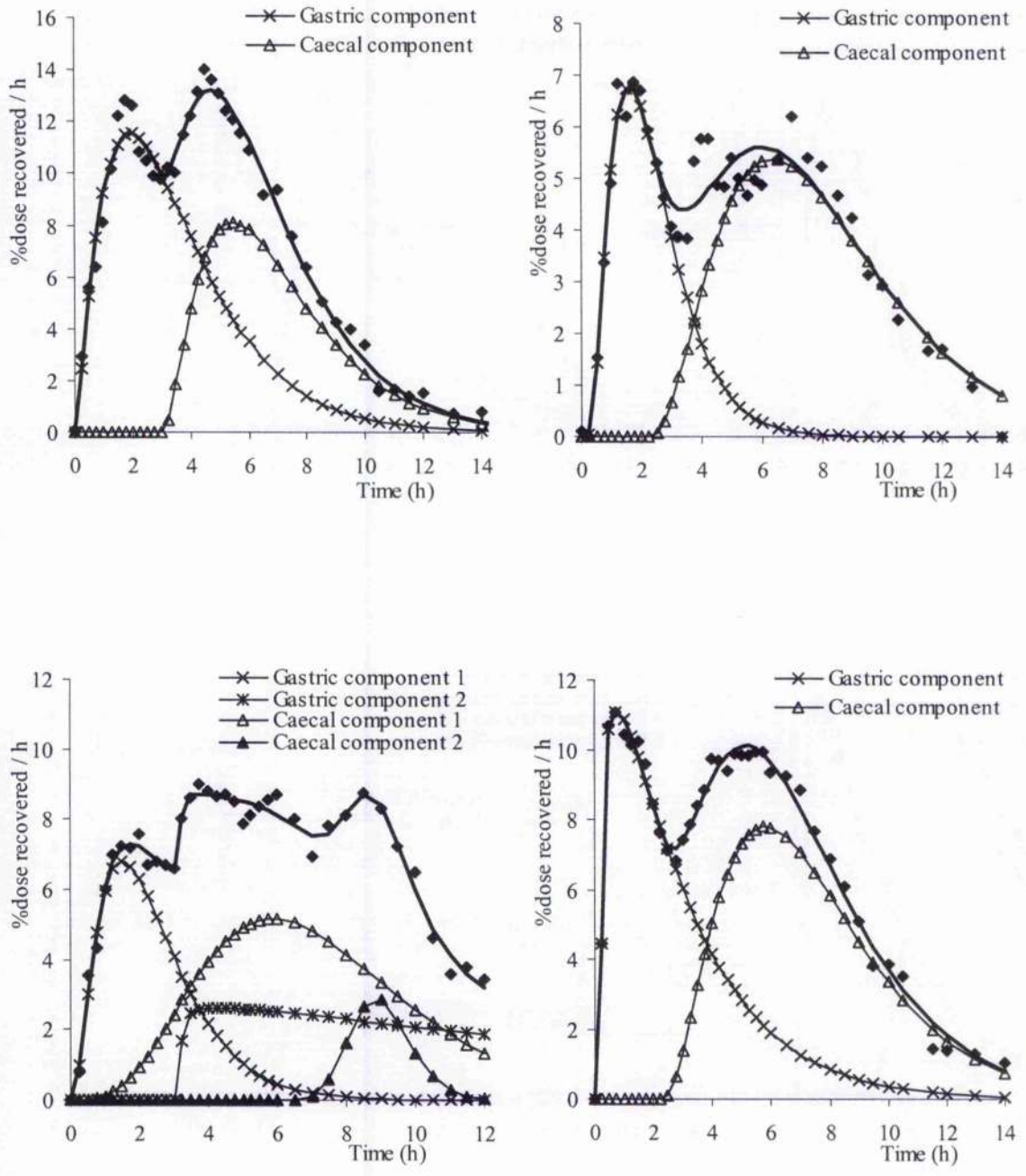


Figure 8.10 ^{13}C-OABT/^{13}C-LUBT results in subject 105, showing test results from weeks 1 to 4 clockwise from top left. Isotopic dose recovery in week 4 required a 4-peak model, possibly due to discrete gastric emptying events. In the remaining weeks, a dual peak model gave best fit to the data. The weekly intestinal transit parameters are given in Table 8.3.

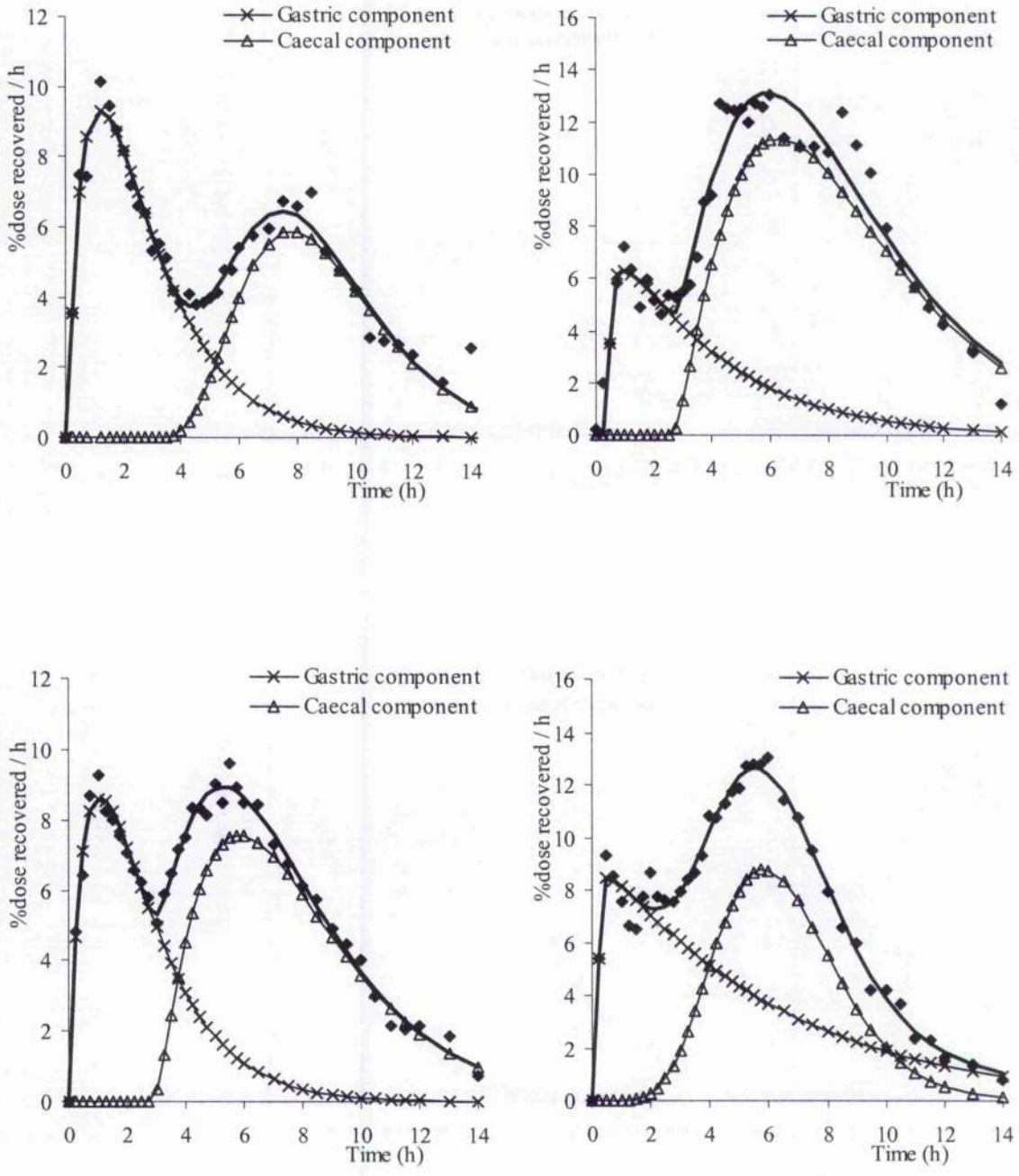


Figure 8.11 ^{13}C-OABT/^{13}C-LUBT results in subject 106, with weekly test results (weeks 1 to 4) given clockwise, starting from top left. The gastric and caecal isotope recovery components are modelled separately, and the combined modelled $^{13}CO_2$ recovery is shown (thick line). The diamonds represent the actual isotopic dose recovery data. The weekly intestinal transit parameters are given in Table 8.3.

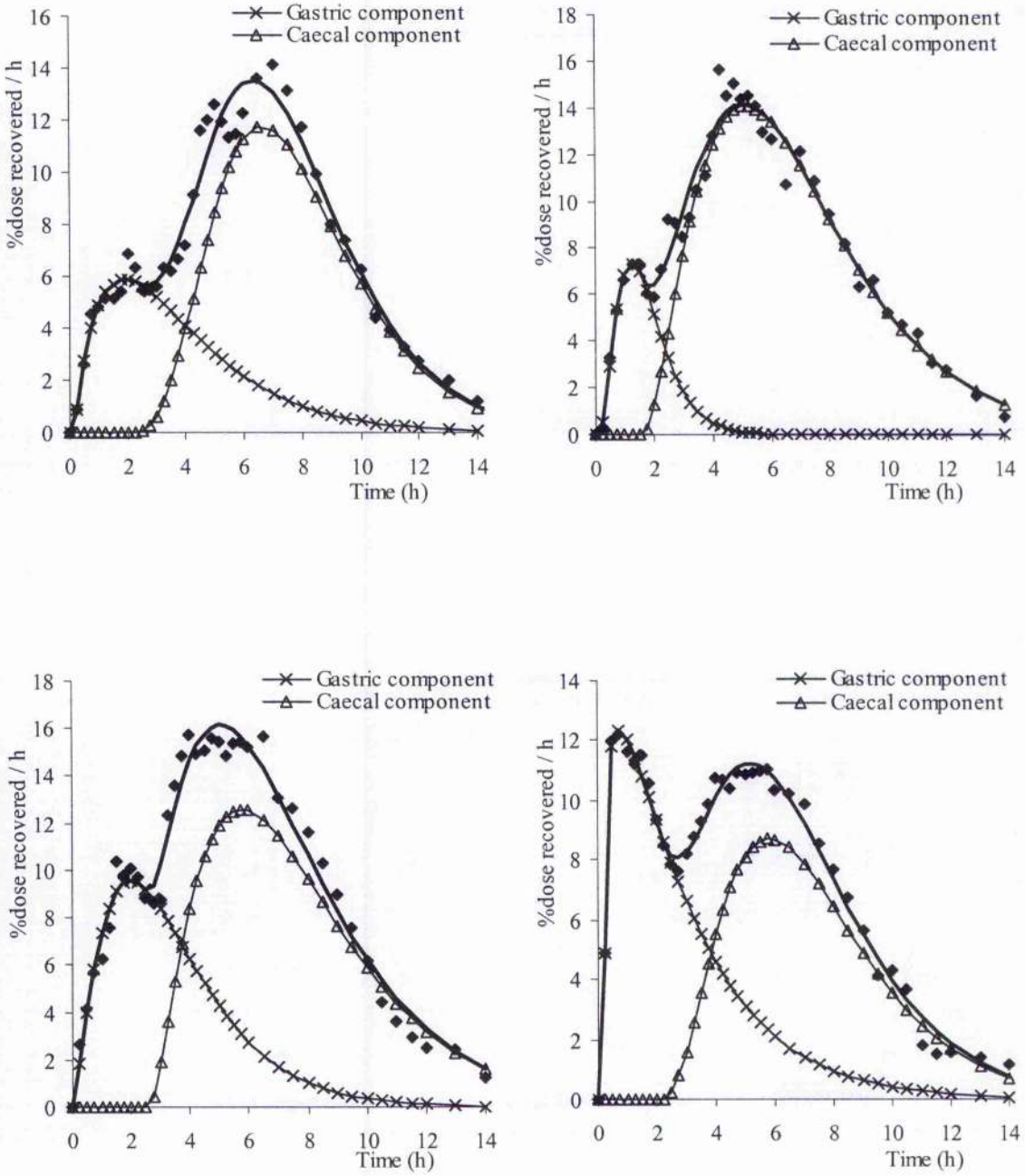


Figure 8.12 Combined ^{13}C-OABT/^{13}C-LUBT results in subject 108, with weekly test results (weeks 1 to 4) given clockwise, starting from top left. The gastric and caecal isotope recovery components are modelled separately, and the combined modelled $^{13}CO_2$ recovery is shown (thick line). The weekly intestinal transit parameters are given in Table 8.3.

In the remaining test (week 4 in subject 105), a 4-peak model was required to fit the ^{13}C recovery data. This was likely caused by two discrete gastric emptying events, and 2 subsequent waves of small intestinal transit, as the subject had paused for 15 min between eating the first and second halves of the test meal. The dual peak model was generally most accurate and simple to apply when gastric emptying was rapid and complete, due to wider separation of the two peaks in ^{13}C output. When gastric emptying was prolonged or had a short lag phase, there was less spatiation between ^{13}C peaks due to coincidence of small intestinal ^{13}C-octanoate absorption with arrival of the head of ^{13}C-LU-labelled chyme in the caecum. This made mathematical fitting of the individual peaks more difficult as a greater number of permutations were possible e.g. week 3 data in subject 102. However, manual assistance of the Solver function to find the minimum RMS values for the model produced in every case a successful solution to the ^{13}C recovery data.

A summary of the mean intestinal motility parameters generated by the dual isotope breath tests is presented in Table 8.3. Individual test results for each subject are given in Table 8.4. A number of interesting points are seen in these results. Gastric emptying rate was the most variable function, with wide inter- and intra-individual fluctuations in both t_{lag} and $t_{1/2}$. Gastric $t_{1/2}$ ranged from 1.46 to 4.73 h within the same individual, and t_{lag} ranged from just 0.46 h up to 2.13 h. Small bowel $t_{1/2}$ time was also variable, ranging from 2.04 to 6.32 h, and with a mean CV% of 24.67. By contrast, the caecal transit parameters were relatively well conserved within and between individuals. The mean CV%s for caecal $t_{1/2}$ and OCTT were 11.20 and 16.43% respectively.

Table 8.3 Mean intestinal transit parameters in 4 individuals using ^{13}C-OABT/^{13}C-LUBT

All tests	Gastric parameters (h)			Caecal Parameters (h)				SB Transit (h)	
(n = 16)	$t_{10\%}$	t_{lag}	$t_{1/2}$	OCTT	$t_{10\%}$	t_{lag}	$t_{1/2}$	$t_{10\%}$	$t_{1/2}$
Mean	0.87	1.26	2.87	3.66	4.51	6.28	7.32	3.65	4.45
s.d.	0.20	0.54	0.98	0.60	0.65	0.84	0.82	0.60	1.10
CV%	22.67	42.94	34.17	16.43	14.37	13.41	11.20	16.35	24.67

To investigate the relationship between gastric emptying and small intestinal transit rate, a series of linear regressions were performed, some of which are shown in Figures 8.13 – 8.15. As seen in Figure 8.13, there was no significant correlation between gastric $t_{10\%}$ and small bowel $t_{10\%}$, nor between gastric $t_{1/2}$ and OCTT, suggesting that the early part of small bowel transit was independent of solid phase gastric emptying rate.

Table 8.4 Summary of intestinal motility parameters as determined using the combined lactose ^{13}C-ureide/^{13}C-octanoic acid breath test in subjects 102, 105, 106 and 108. Weekly parameters are shown, together with the mean (n = 4) values for each individual.

Subject	Gastric parameters (h)			Caecal Parameters (h)				SB Transit (h)	
102	$t_{10\%}$	t_{lag}	$t_{1/2}$	OCTT	$t_{10\%}$	t_{lag}	$t_{1/2}$	$t_{10\%}$	$t_{1/2}$
week 1	0.55	0.92	1.46	2.50	3.39	5.34	6.02	2.84	4.56
week 2	1.17	2.13	4.28	4.25	5.33	7.68	8.24	4.16	3.96
week 3	0.99	0.66	4.73	4.29	5.39	7.79	8.36	4.40	3.63
week 4	0.70	0.85	3.58	3.51	4.44	6.49	7.00	3.74	3.42
Mean	0.85	1.14	3.51	3.64	4.64	6.83	7.41	3.79	3.89
s.d.	0.28	0.67	1.45	0.84	0.94	1.15	1.11	0.69	0.50
CV%	32.79	58.69	41.22	23.06	20.23	16.88	14.98	18.14	12.78

Subject	Gastric parameters (h)			Caecal Parameters (h)				SB Transit (h)	
105	$t_{10\%}$	t_{lag}	$t_{1/2}$	OCTT	$t_{10\%}$	t_{lag}	$t_{1/2}$	$t_{10\%}$	$t_{1/2}$
week 1	1.05	1.94	3.01	3.83	4.38	5.46	6.53	3.33	3.52
week 2	0.99	1.63	2.13	3.70	4.55	6.34	7.54	3.56	5.41
week 3	0.66	0.76	2.31	3.50	4.25	5.81	6.96	3.59	4.65
week 4	0.92	1.69	2.33	3.51	4.51	6.44	8.53	3.59	6.20
Mean	0.91	1.51	2.45	3.64	4.42	6.01	7.39	3.52	4.95
s.d.	0.17	0.51	0.39	0.16	0.14	0.46	0.87	0.13	1.14
CV%	18.98	34.19	15.84	4.38	3.07	7.66	11.71	3.58	23.08

Subject	Gastric parameters (h)			Caecal Parameters (h)				SB Transit (h)	
106	$t_{10\%}$	t_{lag}	$t_{1/2}$	OCTT	$t_{10\%}$	t_{lag}	$t_{1/2}$	$t_{10\%}$	$t_{1/2}$
week 1	0.73	1.20	2.27	5.10	5.95	7.79	8.59	5.22	6.32
week 2	0.97	0.95	3.10	3.74	4.60	6.30	7.93	3.63	4.83
week 3	0.92	0.47	4.45	3.16	4.01	5.84	6.30	3.09	1.85
week 4	0.63	1.08	2.11	3.76	4.47	5.88	7.24	3.84	5.13
Mean	0.81	0.93	2.98	3.94	4.76	6.45	7.52	3.95	4.53
s.d.	0.16	0.32	1.07	0.82	0.83	0.92	0.98	0.91	1.90
CV%	19.65	34.60	35.88	20.86	17.54	14.19	13.04	22.99	41.93

Subject	Gastric parameters (h)			Caecal Parameters (h)				SB Transit (h)	
108	$t_{10\%}$	t_{lag}	$t_{1/2}$	OCTT	$t_{10\%}$	t_{lag}	$t_{1/2}$	$t_{10\%}$	$t_{1/2}$
week 1	1.10	1.91	3.25	3.96	4.81	6.64	7.41	3.71	4.15
week 2	0.74	1.29	1.58	2.78	3.53	5.12	6.42	2.79	4.84
week 3	0.65	0.75	2.31	3.47	4.23	5.83	6.87	3.58	4.56
week 4	1.07	2.00	2.98	3.57	4.31	5.78	7.14	3.25	4.16
Mean	0.89	1.49	2.53	3.45	4.22	5.84	6.96	3.33	4.43
s.d.	0.23	0.58	0.75	0.49	0.53	0.62	0.42	0.41	0.33
CV%	25.67	39.28	29.51	14.26	12.48	10.65	6.07	12.31	7.56

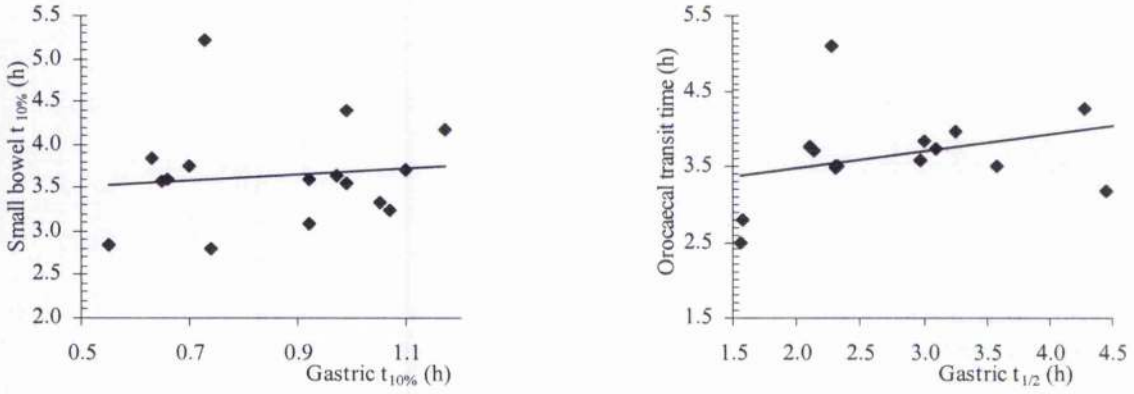


Figure 8.13 Linear relationships between gastric $t_{10\%}$ /small bowel $t_{10\%}$ (left) and between gastric $t_{1/2}$/OCTT (right) as measured by the ^{13}C-OABT/^{13}C-LUBT dual isotope breath test in 4 healthy adult individuals on 4 occasions. The line of 'best-fit' determined by linear regression is plotted. No significant correlation was present in either case.

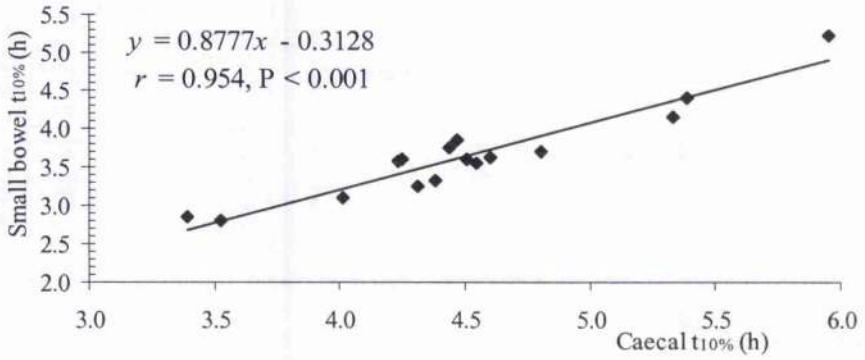


Figure 8.14 Linear relationship between caecal $t_{10\%}$ (h) and small bowel $t_{10\%}$ (h) as determined by dual isotope breath test in 4 healthy adult individuals. The combined test was performed in each subject on 4 occasions. The linear line of 'best fit' is shown, with a highly significant positive correlation (n = 16, r = 0.954, P < 0.001).

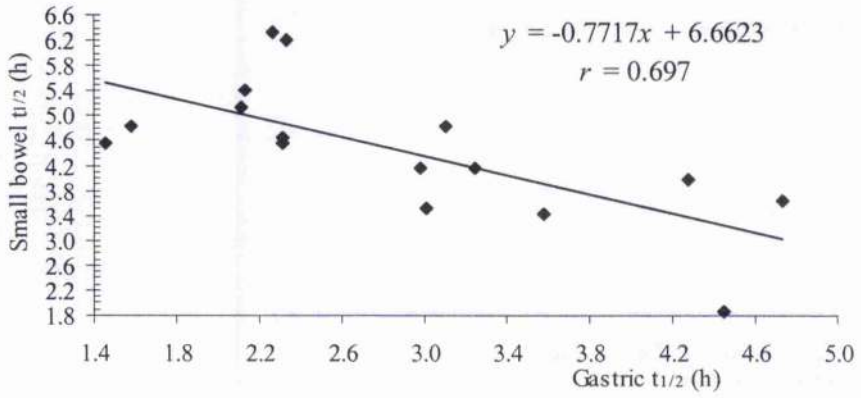


Figure 8.15 Linear correlation between gastric $t_{1/2}$ (h) and small bowel $t_{1/2}$ (h) as measured by ^{13}C-OABT/^{13}C-LUBT in 4 healthy individuals on 4 occasions. A significant negative correlation is present (n = 16, r = 0.697, P < 0.01).

Similarly, no significant correlation existed between gastric and caecal t_{lag} measurements, nor between gastric and caecal $t_{1/2}$. However, significant correlations were found between several indices of gastrointestinal motility, two of which are indicated in Figures 8.14 and 8.15. The correlation between caecal $t_{10\%}$ and small bowel $t_{10\%}$ was positive and highly significant ($n = 16$, $r = 0.954$, $P < 0.001$). Significant positive correlations were also present between $SBt_{10\%}T$ and OCTT ($n = 16$, $r = 0.937$, $P < 0.001$); $SBt_{10\%}T$ and caecal t_{lag} ($n = 16$, $r = 0.889$, $P < 0.001$); and between $SBt_{10\%}T$ and caecal $t_{1/2}$ ($n = 16$, $r = 0.802$, $P < 0.001$). A positive correlation was also seen between caecal $t_{1/2}$ and small bowel $t_{1/2}$ that reached significance ($n = 16$, $r = 0.497$, $P = 0.05$).

In addition to the positive correlations between small bowel motility and caecal transit parameters, there was also a significant negative linear correlation between small bowel $t_{1/2}$ and gastric $t_{1/2}$ ($n = 16$, $r = 0.697$, $P < 0.01$). Within each individual there was a tendency for small bowel $t_{1/2}$ to be relatively prolonged when gastric emptying was rapid, or shortened if gastric $t_{1/2}$ was extended. No significant correlations were detected between gastric and caecal transit parameters.

The mean (± s.d.) measurements for OCTT, caecal t_{lag} and caecal $t_{1/2}$ gained in this study (3.66 ± 0.60h, 6.28 ± 0.84 h, 7.32 ± 0.82 h) closely approximated those values gained with the induced LUBT in section 8.2 (3.24 ± 0.65 h, 5.62 ± 1.22 h, 6.31 ± 1.21 h respectively), further suggesting that the dual peak modelling technique used for the ^{13}C-OABT/^{13}C-LUBT data was accurate.

8.3.4 Discussion

In this study, the dual ^{13}C-OABT/^{13}C-LUBT was shown to be a useful modality for the evaluation of equine upper gastrointestinal tract motility. The developed test protocol appeared to be successful, allowing recovery of interpretable data in every test in each individual. Using the Maes' multi-peak power exponential model (Ghoos *et al.* 1993; Maes *et al.* 1994), it was possible to model the expiratory ^{13}C recovery data to gain information regarding not only concurrent gastric emptying rate and orocaecal transit time, but also small bowel transit rates. Consequently, using the dual isotope breath test, it was possible to study the regulatory interactions of the stomach and small intestine in controlling the delivery of ingesta to the caecum. This basic physiological information has previously been difficult to obtain in the horse, due to technical difficulties in assimilating the requisite data.

Using the dual isotope test in the 4 subjects in this study, it was demonstrated that small intestinal transit rate was highly variable between and within horses, as has been reported in man (Camilleri *et al.* 1991; Argenyi *et al.* 1995; Degen and Phillips 1996; Gryback *et al.* 2002). Intra-individual CV% for $SBt_{1/2}T$ ranged from 12.78 to 41.93% in this study, with a range in $SBt_{1/2}T$ from 1.85 to 6.32 h. Once again, equine gastric emptying rate was also confirmed to have high inter- and intra-subject variability with the mean $t_{1/2}$ CV% here of 34.17% similar to that reported for a different group of individuals in Chapter 3 (40.37%, see Table 3.1). Interestingly, initial small bowel transit rate was shown to be independent of gastric emptying rate, and in fact there was a significant negative correlation between $SBt_{1/2}T$ and gastric $t_{1/2}$. This suggests that, in the presence of nutrient-rich chyme, the equine proximal small intestine may exert negative feedback on gastric emptying rate, so as to slow subsequent emptying. Conversely, when calories are released only slowly to the small intestine, there is more rapid transit of these ingesta towards the caecum. This mechanism has not been proven to exist in the horse, but would be in agreement with the feedback theory of Hunt and Stubbs (1975) for maintenance of homeostatic nutrient delivery to the duodenum. Studies in man have also found small bowel transit to be independent of gastric emptying rate (Read *et al.* 1980, 1984a, 1986) but have not reported a specific negative correlation between these transit parameters.

The results of this study suggest that the equine small intestine has a major regulatory role in controlling the delivery of ingesta and nutrients to the caecum, which it does both by negative feedback on gastric emptying and variance of transit rate. The most significant positive correlations in this study were between $SBt_{10\%}T$ and the early caecal transit parameters (caecal $t_{10\%}$ (r = 0.954), caecal t_{lag} (r = 0.889) and caecal $t_{1/2}$ (r = 0.802)). Deriving small bowel transit time by subtracting gastric $t_{1/2}$ from colonic filling $t_{1/2}$, Read *et al.* (1984a) also found a significant correlation between this parameter and colonic filling $t_{1/2}$ in man (r = 0.87, $P < 0.001$). Controlled delivery of ingesta to the caecum is of particular importance in the horse, as excessive ileocaecal transfer of fermentable carbohydrate may result in decreased caecal pH, with consequent proliferation of lactic acid producing bacteria. Proliferation of organisms such as *Lactobacillus* spp. and *Clostridia* spp. leads to the death and lysis of Gram negative *Enterobacteriaceae* spp. with subsequent release and absorption of cell wall endotoxin. For this reason, one frequent sequel to carbohydrate overload in the horse is the development of laminitis (Garner *et al.* 1978). The close correlation observed here between small intestinal and caecal transit rates also further suggests the presence of an ileal brake mechanism in the horse, as reported in other species (Pappas *et al.* 1986; Brown *et al.* 1987; Spiller *et al.* 1988; Read and Houghton 1989;Treacy *et al.* 1990).

Molecular characterisation of equine carbohydrate digestion has shown that the disaccharidases sucrase, maltase and lactase are expressed in the small intestine, with maltase having the highest activity and equal expression throughout the duodenum, jejunum and ileum (Dyer *et al.* 2002). However, the sodium/glucose cotransporter isoform 1 (SGLT I), responsible for the uptake of glucose and galactose, is expressed with highest abundance in the duodenum, and at approximately half that abundance in the ileum (Dyer *et al.* 2002). As the SGLT 1 protein has a high affinity and low capacity for sugar substrates, this has physiological significance for the regulation of small intestinal motility. If the small intestine cannot compensate for high carbohydrate load by retardation of transit rate, then caecal lactic acidosis may follow due to over-saturation of the absorption transport molecules.

In previous equine studies, it was concluded that a 12 h period of fasting resulted in a dramatic reduction in orocaecal transit time, from the 3-4 h of normal feeding conditions to less than 1 h (Vigroux *et al.* 1975, Phaneuf and Ruckebusch 1983). Vigroux *et al.* (1975) considered that this phenomenon was attributable to 'hyperactivity' of the stomach and small intestine, induced by stimulation during prehension, mastication and insalivation of feed. The data collected here with the dual isotope breath test contradict the findings of Vigroux *et al.* (1975), which were based on interpretation of electromyographic data rather than transit of ingesta. Based on the evidence presented here and also in Chapter 3, gastric emptying rate is not accelerated in the horse after a period of 12 h food deprivation. Furthermore, if rapid emptying does occur, this is likely to be countered by a prolonged small bowel $t_{1/2}$, to minimise overall changes in orocaecal transit time. The data given here logically concur with the basic physiological requirement to minimise fluctuations in caecal pH (Garner *et al.* 1978). The conclusions made by Vigroux *et al.* (1975) also underscore the difficulties of relating electromyographic data to actual transit of ingesta.

Measurement of small bowel transit in the horse may be useful for the detection of both isolated small bowel dysfunctions/obstructions, and also for diffuse motor dysfunction (Parkman *et al.* 1995b). For example, transit of ingesta through the small intestine and colon is faster than normal in Irritable Bowel Syndrome (IBS) human patients with diarrhoea, but slower than normal in those with constipation (Read *et al.* 1986). Delayed small intestinal transit has also been demonstrated in idiopathic constipation (Stivland *et al.* 1991), whilst patients with carcinoid syndrome have increased transit rate (Von der Ohe *et al.* 1993). The possible involvement of disordered small intestinal transit in equine conditions such as chronic weight loss/malabsorption, recurrent impactions, ileal hypertrophy and unspecified causes of abdominal pain has yet to be determined. Such

information could yield useful clinical information regarding the diagnosis, management and prevention of a variety of conditions.

Specific factors that have been associated with an increased risk of colic include alterations in both diet and exercise (Cohen *et al.* 1996; Tinker *et al.* 1997), with changes in feeding practice being strongly associated (Hudson *et al.* 2001). To investigate this further, it would be particularly useful to monitor the spectrum of gastrointestinal transit parameters by dual isotope breath test during dietary manipulation. In preliminary work in this field, Nicholls and Freeman (2002) reported a significant initial increase in equine gastrointestinal motility in association with an abrupt change from pasture/forage management to stabling/concentrate feed. Small intestinal motility remained increased for 5 days after the management change, and was accompanied by a decrease in large intestinal motility (Nicholls and Freeman 2002). These findings should be interpreted with caution, as an unvalidated transcutaneous ultrasonographic technique (Freeman and England 2001) was used by this group to assess 'motility', rather than transit. However, the results indicate that further work should be performed in this important area. If specific dietary changes could be demonstrated by the dual isotope test to induce particular effects on intestinal motility, improved advice could be constructed to reduce dietary and managemental risk factors for colic.

The protocol developed and applied here for the combined ^{13}C-OABT/^{13}C-LUBT was successful and allowed valuable generation of hitherto unobtainable equine gastrointestinal transit data as reported in man (Van den Driessche 2001). In 15/16 tests, the acquired ^{13}C recovery data was readily modelled by a dual peak power exponential function, and a 4-peak model was required for the remainder. However, accuracy of curve fitting (and hence data generation) may have been improved by wider separation of the peaks in ^{13}C-recovery. This could have been achieved by combining ^{13}C-LU with a liquid phase marker for gastric emptying, such as sodium ^{13}C-acetate. Gastric liquid phase emptying data measured with this tracer have been reported in section 6.3.1 (see Table 6.2) and gastric $t_{1/2}$ was found to be shorter (1.72 ± 0.21 h) and less variable (intra-individual CV% of 8.83%) than solid phase $t_{1/2}$. The small intestinal transit of liquid has also been reported to be similar to that of solids (Malagelada *et al.* 1984; Bennink *et al.* 1999; Graff *et al.* 2001), such that incorporation of a liquid phase marker into the dual test may facilitate modelling and calculation of SBTT. However, such a test would potentially have the disadvantage of failing to detect abnormalities of gastric emptying (Thomforde *et al.* 1985; Chaudhuri and Fink 1991) and solid phase markers are generally recommended for the investigation of clinical disorders of gut motility (Read and Houghton 1989; Parkman *et al.* 1995).

In order to model expiratory ^{13}C recovery data accurately with the dual isotope test it was necessary to collect frequent breath samples, particularly in the first 6 h, which may limit clinical uptake of this test. As described in Chapter 9, a further aim of this research has been to produce minimised protocols for the ^{13}C-OABT and ^{13}C-LUBT so as to reduce both frequency and number of sample collection. This can be achieved for the individual tests as seen in the next Chapter, but is not likely to be possible for the dual isotope breath test without significant detriment to diagnostic accuracy. It is therefore envisaged that the ^{13}C-OABT/^{13}C-LUBT test be adopted principally as a useful, humane research tool by equine gastroenterologists for further elucidation of the pathophysiology of specific gastrointestinal transit disorders in this species.

8.4 Final discussion

In this Chapter it has been demonstrated that the induced ^{13}C-LUBT is a useful, non-invasive test for the measurement of equine OCTT and caecal transit of solid ingesta. Bacterial glycosylureide hydrolase activity appeared to be universally present in the equine caecum and, in contrast to the hydrogen breath test, arrival of ^{13}C-LU in the caecum resulted in reliable production of a tracer signal in all individuals. In all but 1 test, single peaks of $^{13}CO_2$ were produced following the ingestion of ^{13}C-LU, suggesting that fermentative activity of this compound was restricted to the large intestine as supported by the *in vitro* studies of ^{13}C-LU digestion. Should small intestinal cleavage of this compound occur, then it is likely to be characterised by an early transient peak in $^{13}CO_2$ recovery, prior to the main dose recovery.

In its own right, the induced ^{13}C-LUBT could form a useful diagnostic tool for both clinical and research applications in equine gastroenterology. Data collected in this study with the ^{13}C-LUBT suggested that equine caecal filling may be pulsatile in nature, and the possible presence of an ileal brake mechanism in this species merits further investigation. When paired with the ^{13}C-OABT in the dual Stable isotope breath test, further potential applications were revealed for the ^{13}C-LUBT. The dual isotope test was developed and applied on 4 occasions in 4 individuals, allowing successful concurrent generation of gastric, small intestinal and caecal solid phase transit data on each occasion.

Results gained from the dual isotope breath test suggested that equine small intestinal transit is independent of gastric emptying rate. Furthermore, a negative feedback mechanism may exist by which small intestinal nutrient receptors can retard gastric emptying, as a significant negative correlation was detected between $SBt_{1/2}$ T and gastric

$t_{1/2}$. Small bowel transit was also correlated significantly to early caecal transit of ingesta, suggesting that small intestinal motility plays a key role in regulating delivery of nutrients to this organ. As so much is yet to be discovered about the interactions in equine gastrointestinal motility and pathophysiology, there are widespread potential applications for the dual isotope test. With particular reference to investigating identified risk factors for the development of colic, further research into the effects of nutrient composition and exercise on intestinal motility are necessitated. The non-invasive, simple nature of these breath tests also makes them ideal for further studies on the effects of specific anaesthetic and prokinetic agents on equine gastrointestinal motility.

Chapter 9 CLINICAL APPLICATION OF THE STABLE ISOTOPE BREATH TESTS

9.1 Introduction

The breath and blood sampling protocols used for measurement of gastric emptying rate and orocaecal transit time in Chapters 3 – 5 and 8 of this thesis are extensive, and were designed to allow maximum expiratory recovery of the administered isotopes. This enabled the cumulative % dose recovery of the ^{13}C-octanoic acid and lactose ^{13}C-ureide tracers in the horse to be determined, and optimised the accuracy of the non-linear modelling functions used for calculation of the specific motility indices. However, the collection of 34 breath samples over 10 h (^{13}C-OABT) or 38 samples over a minimum 12 h (^{13}C-LUBT) is likely to be prohibitively expensive and labour-intensive for regular clinical use. Although decreasing in price, the costs of commercial ^{13}C:^{12}C analysis are variable, averaging £10 per sample in 2002. In order to maximise the utility of the newly validated tests, the effects of reducing both sampling duration and frequency are investigated in this Chapter with the aim of producing satisfactory protocols for clinical use.

In terms of minimising analytical costs, collection of the least number of samples for parameter calculation is desirable. The disadvantage of this approach alone is that inadequate collection or analysis of a single sample may then render the test non-viable, such that samples should be collected in duplicate. Reducing the duration of the sampling protocol may be more cost effective in terms of labour requirement. Both approaches have been evaluated in this Chapter. Minimised collection protocols for stable isotope tests of gastric emptying have recently been described in human medicine (Lee *et al.* 2000a, 2000b; Viramontes *et al.* 2001). Lee *et al.* (2000a) have recently reported a 'reduced model' [^{13}C] *Spirulina platensis* test, in which breath $^{13}CO_2$ at baseline, 90 and 180 min measured gastric emptying $t_{1/2}$ for solids with results that were comparable to those of scintigraphy.

9.2 Reduction of protocol sampling duration

9.2.1 Materials and Methods

The effect of reducing the duration of the sampling protocol was investigated using existing data sets. For the ^{13}C-OABT the 64 studies presented in Chapter 5 were used, thus including both normal and delayed gastric emptying data. As orocaecal transit time data has not yet been collected from horses with delayed small intestinal transit, a combination of 22 data sets from healthy horses performed using the final protocol for the induced ^{13}C-

LUBT was used. In each case, Maes' non-linear regression model (section 2.6.3.2) was used to compute gastrointestinal motility indices using the full protocol, and then repeated on sequentially reduced data samples. The effects of serial reductions in the protocols from 9 h to 2 h, and from 12 h to 5 h were examined for the ^{13}C-OABT and ^{13}C-LUBT data sets respectively.

9.2.2 Statistical analysis

The indices gained from the reduced sampling protocols were compared using a general linear model ANOVA. Pairwise comparisons were made by the Tukey method, using 95% confidence intervals (Minitab® 13, Minitab Inc., PA, USA). A probability of < 0.05 was taken to represent statistical significance. In some reduced data sets the curve-fitting formula was unable to estimate the motility indices due to the linear nature of the early pattern of tracer recovery. The proportion of data sets in which this occurred was recorded for each protocol.

9.2.3 Reduced protocol results

9.2.3.1 ^{13}C-OABT reduced protocol

The effect of reducing the duration of sample collection for measurement of both normal and delayed gastric emptying using the ^{13}C-OABT is presented in Figure 9.1. The boxplots represent the median value (horizontal bar), inter-quartile range (shaded box), and 10^{th}-90^{th} percentiles, with outlying data represented by an asterisk. The 2 h sampling protocol produced mean estimates of $t_{1/2}$ that were significantly different to the 4-9 h protocols ($P < 0.05$), and discrepancies in the 3 h protocol also verged on significance ($P = 0.06 - 0.08$). Mean $t_{1/2}$ estimated by the 4 h reduced protocol was not significantly different to the full protocol, but it can be seen in Table 9.1 that 6/64 (9.38%) data sets could not be modelled using this protocol. All of those data sets that could not be modelled using the 4 h protocol had a $t_{1/2}$ exceeding 5.35 h, approximating the 95th percentile of the entire sample ($n = 64$) and therefore classifiable as 'delayed' gastric emptying. Estimates of mean $t_{1/2}$ by the 8, 7, 6 and 5 h protocols were not significantly different from the full protocol, and allowed successful curve-fitting of 100, 100, 96.9 and 93.8% cases respectively (Table 9.1).

Estimation of mean t_{lag} and mean GEC using the reduced protocols yielded a similar pattern to that of $t_{1/2}$ and an identical curve-fitting outcome. Estimates produced from the 2 h protocol were significantly different in each case to those generated by the 4 – 9 h sampling duration. Once again, estimates of mean t_{lag} and GEC by the 8, 7, 6 and 5 h protocols were not significantly different to those of the full protocol.

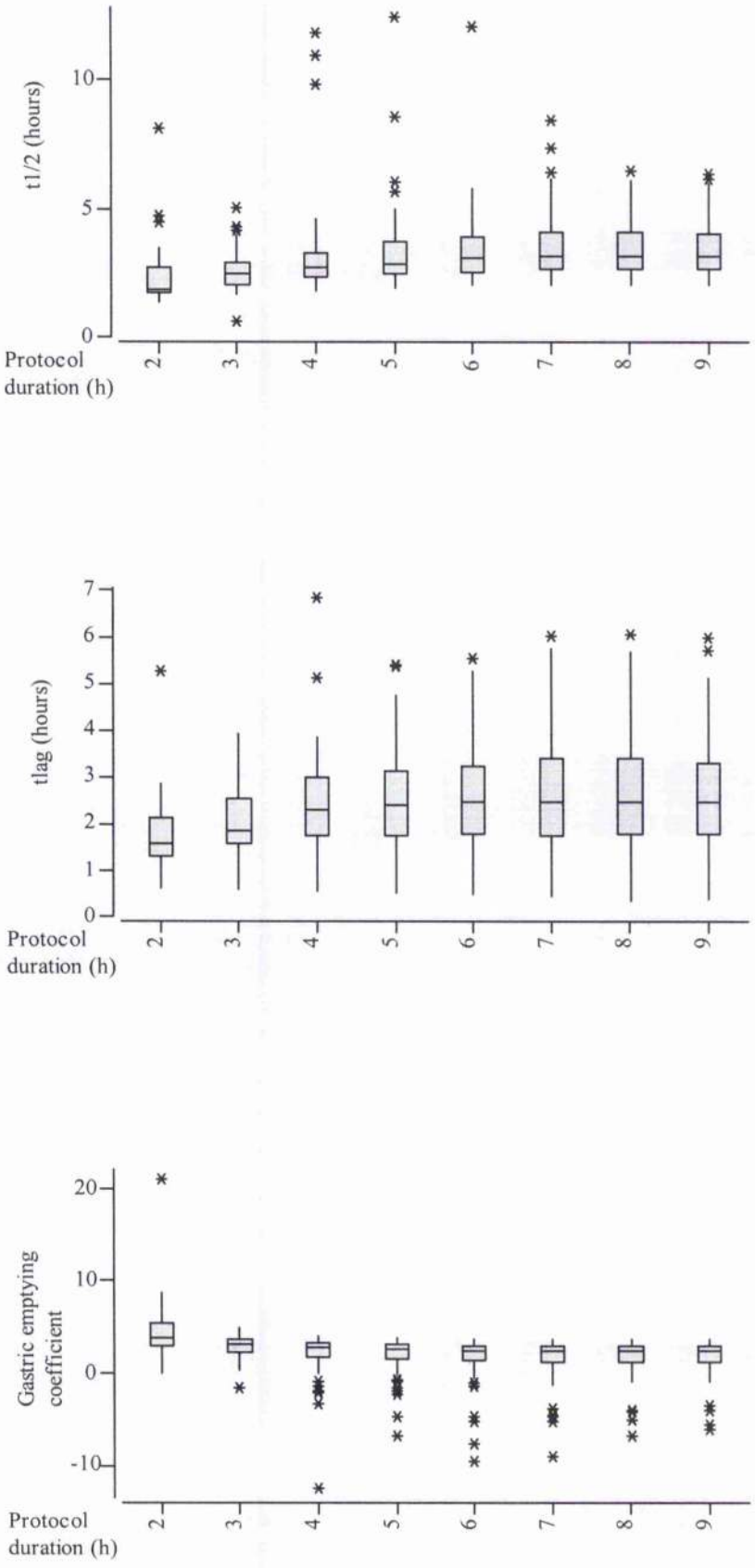


Figure 9.1 Boxplots demonstrating the effect of reduced protocol duration on estimation of gastric half-emptying time ($t_{1/2}$; top), gastric lag phase (t_{lag}; middle) and gastric emptying coefficient (bottom) using the Maes non-linear regression model. The median values, inter-quartile range (shaded area), 10^{th}-90^{th} percentiles and outlying values (*) are represented. Refer to Table 9.1 for sample size of each data set and mean numerical values.

Table 9.1 Effect of reducing duration of sample collection on estimation of indices of equine gastric emptying, using the ^{13}C-OABT and Maes' non-linear regression model (n = 64). Note that the model was often unable to calculate the required rate constants when the protocol was reduced to below 4 h.

Variable	Duration of Sampling (h)	Modelling Success	Mean (h)	s.d. (h)	Q1 (h)	Q3 (h)	Min. (h)	Max. (h)
$t_{1/2}$	9.00	64/64	3.47^{a}	1.05	2.70	4.08	2.08	6.41
	8.00	64/64	3.46^{b}	1.08	2.68	4.10	2.08	6.52
	7.00	64/64	$3.50^{c,g}$	1.27	2.67	4.13	2.07	8.42
	6.00	62/64	3.43^{d}	1.44	2.59	3.96	2.06	12.05
	5.00	60/64	3.37^{e}	1.64	2.52	3.77	1.95	12.47
	4.00	58/64	3.24^{f}	1.91	2.36	3.34	1.83	11.82
	3.00	50/64	2.58^{g}	0.77	2.08	2.92	0.63	5.09
	2.00	40/64	2.37^{a-f}	1.23	1.74	2.77	1.39	8.15
t_{lag}	9.00	64/64	2.63^{h}	1.22	1.79	3.29	0.37	5.99
	8.00	64/64	2.63^{i}	1.22	1.79	3.39	0.35	6.06
	7.00	64/64	2.63^{j}	1.25	1.77	3.41	0.44	6.02
	6.00	62/64	2.56^{k}	1.15	1.78	3.24	0.48	5.54
	5.00	60/64	2.46^{l}	1.05	1.76	3.14	0.52	5.41
	4.00	58/64	2.41^{m}	1.07	1.74	3.00	0.56	6.85
	3.00	50/64	2.03	0.71	1.60	2.53	0.58	3.91
	2.00	40/64	1.78^{h-m}	0.81	1.30	2.13	0.62	5.26
GEC	9.00	64/64	1.65^{n}	2.12	1.29	2.93	-6.07	3.68
	8.00	64/64	1.63^{o}	2.19	1.28	2.93	-6.78	3.68
	7.00	64/64	1.58^{p}	2.40	1.27	2.94	-9.04	3.70
	6.00	62/64	1.60^{q}	2.56	1.47	3.01	-9.46	3.72
	5.00	60/64	1.89^{r}	2.00	1.61	3.10	-6.68	3.83
	4.00	58/64	2.00^{s}	2.52	1.73	3.29	-12.51	4.03
	3.00	50/64	2.95^{t}	1.19	2.34	3.65	-1.61	4.90
	2.00	40/64	4.48^{n-t}	3.25	2.92	5.36	-0.05	21.03

Matching superscript letters denote significant differences between mean indices when calculated using the different sample collection protocols ($P < 0.05$). Note that the number of data sets for which gastric emptying indices could not be modelled increased with decreasing duration of sample collection.

When equine gastric emptying rate is not delayed, a 4 h sampling protocol should be sufficient for calculation of the specific gastric parameters using the ^{13}C-OABT. However, when emptying is delayed, or anticipated to be delayed, a longer sampling duration would be preferable. In this entire population, sample collection could have ceased at 7 h in even those cases with marked delays in emptying rate without loss of quantitative data from the modelling function.

9.2.3.2 *Induced ^{13}C-LUBT reduced protocol*

The effects of reducing the duration of sample collection for measurement of presumed normal equine small intestinal motility using the induced ^{13}C-LUBT protocol are demonstrated in Figure 9.2 (n = 22). It can be seen that for each of the mean parameters OCTT, orocaecal t_{lag} and caecal $t_{1/2}$ there is relatively little difference between the estimates produced by the reduced duration protocols. These differences, even between the 5 h and 12 h protocols, were not significant for any of the indices. However, as seen with the gastric emptying model, the ability of the Maes' model to calculate the required rate constants decreased with reducing duration of protocol (Table 9.2). For the 9, 8, 7, 6 and 5 h protocols, curve-fitting was successful in 95.7, 90.9, 86.4, 77.3 and 72.7% cases respectively. The success of curve-fitting depended not only on the speed of transit, but also on the pattern of isotope recovery. The mean (± s.d.) durations for OCTT and caecal $t_{1/2}$ in those data sets not compliant to protocol reduction were 4.19 (0.35) and 8.04 (0.37) h. As shown in Table 9.2, these values lay in the top quartile for each parameter.

The sampling duration for the induced ^{13}C-LUBT may be reduced to 9 h, with minimal loss of information even in abnormally slow small intestinal transit. However, further shortening of the protocol is likely to result in the loss of information in occasional individuals with delayed orocaecal transit of ingesta.

9.2.3.3 *Use of the Cusum approach for estimation of OCTT*

An alternative approach to the estimation of OCTT is the use of a cumulative sum (CUSUM) chart in which the cumulative sums of the deviations of each successive sample value from the target value may be used to determine when the first 'significant' change has occurred in the direction of the baseline $^{13}CO_2$ production. This method has been used to determine orocaecal transit time using both the ^{13}C-LUBT (Geypens *et al.* 1999; Van den Driessche 2001) and the hydrogen breath test (Brown *et al.* 1987; Muir *et al.* 1991), as the first significant change in $^{13}CO_2/H_2$ output has been considered to reflect the time of arrival of ingesta in the caecum.

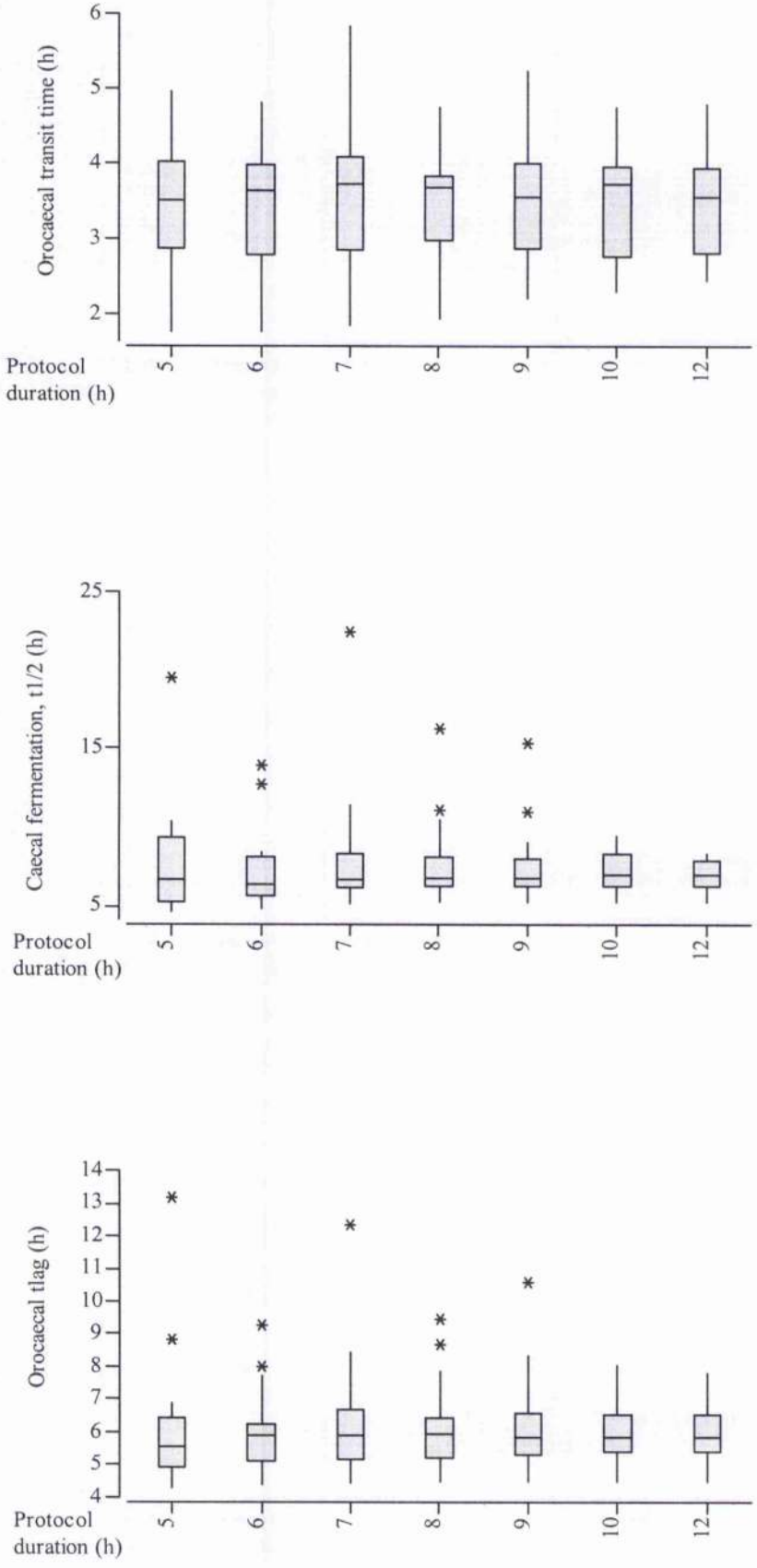


Figure 9.2 Boxplots demonstrating the effect of reduced sampling duration on estimation of orocaecal transit time (top), caecal half-fermentation time ($t_{1/2}$; middle) and orocaecal lag phase (t_{lag}; bottom) using the Maes non-linear regression model. Refer to Table 9.2 for sample size and mean numerical values.

Table 9.2 Effect of reducing duration of sample collection on estimation of indices of equine orocaecal transit time, using the induced ^{13}C-LUBT and Maes' non-linear regression model (n = 22). Note that the sample protocol may be successfully reduced in duration, but that calculation of the required rate constants ('Modelling Success') becomes increasingly difficult with reduced duration.

Variable	Duration of Sampling (h)	Modelling Success	Mean (h)	s.d. (h)	Q1 (h)	Q3 (h)	Min. (h)	Max. (h)
OCTT	12.00	22/22	3.50	0.64	2.81	3.95	2.30	4.79
	10.00	22/22	3.52	0.68	2.78	3.96	2.46	4.75
	9.00	21/22	3.54	0.77	2.88	4.00	2.21	5.25
	8.00	20/22	3.57	0.76	2.98	3.84	1.95	4.74
	7.00	19/22	3.66	0.92	2.85	4.09	1.86	5.84
	6.00	17/22	3.49	0.81	2.80	3.99	1.77	4.82
	5.00	16/22	3.46	0.83	2.88	4.03	1.77	4.96
Caecal t_{lag}	12.00	22/22	6.07	0.96	5.43	6.52	4.48	7.83
	10.00	22/22	6.11	1.03	5.40	6.52	4.47	8.07
	9.00	21/22	6.18	1.38	5.31	6.57	4.47	10.60
	8.00	20/22	6.17	1.30	5.21	6.45	4.47	9.44
	7.00	19/22	6.29	1.77	5.18	6.71	4.45	12.33
	6.00	17/22	6.01	1.26	5.13	6.25	4.37	9.26
	5.00	16/22	6.19	2.14	4.91	6.45	4.32	13.17
Caecal $t_{1/2}$	12.00	22/22	6.98	0.96	6.30	7.89	5.24	8.36
	10.00	22/22	7.19	1.21	6.33	8.28	5.24	9.47
	9.00	21/22	7.48	2.24	6.31	8.04	5.26	15.30
	8.00	20/22	7.69	2.56	6.31	8.10	5.24	16.26
	7.00	19/22	7.90	3.84	6.22	8.30	5.13	22.50
	6.00	17/22	7.38	2.50	5.72	8.16	4.90	13.90
	5.00	16/22	7.67	3.63	5.25	9.30	4.69	19.50

The potential advantage of the CUSUM approach is that sampling duration may be further reduced as there is no requirement to model the data beyond the first significant change in baseline, although this would require immediate sample analysis to be possible. Figures 9.3 and 9.4 demonstrate one-sided CUSUM determination of OCTT in 2 individuals (Minitab® release 13, Minitab Inc., PA, USA). In these examples, OCTT has been calculated as that point at which the pooled s.d. of successive PDR/h values first exceeds the basal centre line by 2.5 s.d. (Geypens *et al.* 1999). The values generated by the Maes' model (3% cumulative dose recovery) for OCTT are given for comparison.

The main disadvantage of this technique is that fluctuations in the baseline production of $^{13}CO_2/H_2$ have a relatively large influence on the calculation of OCTT. Furthermore, the remaining indices – orocaecal t_{lag} and caecal $t_{1/2}$ cannot be generated by this method. The Maes' model calculation of OCTT was therefore used in preference in this thesis.

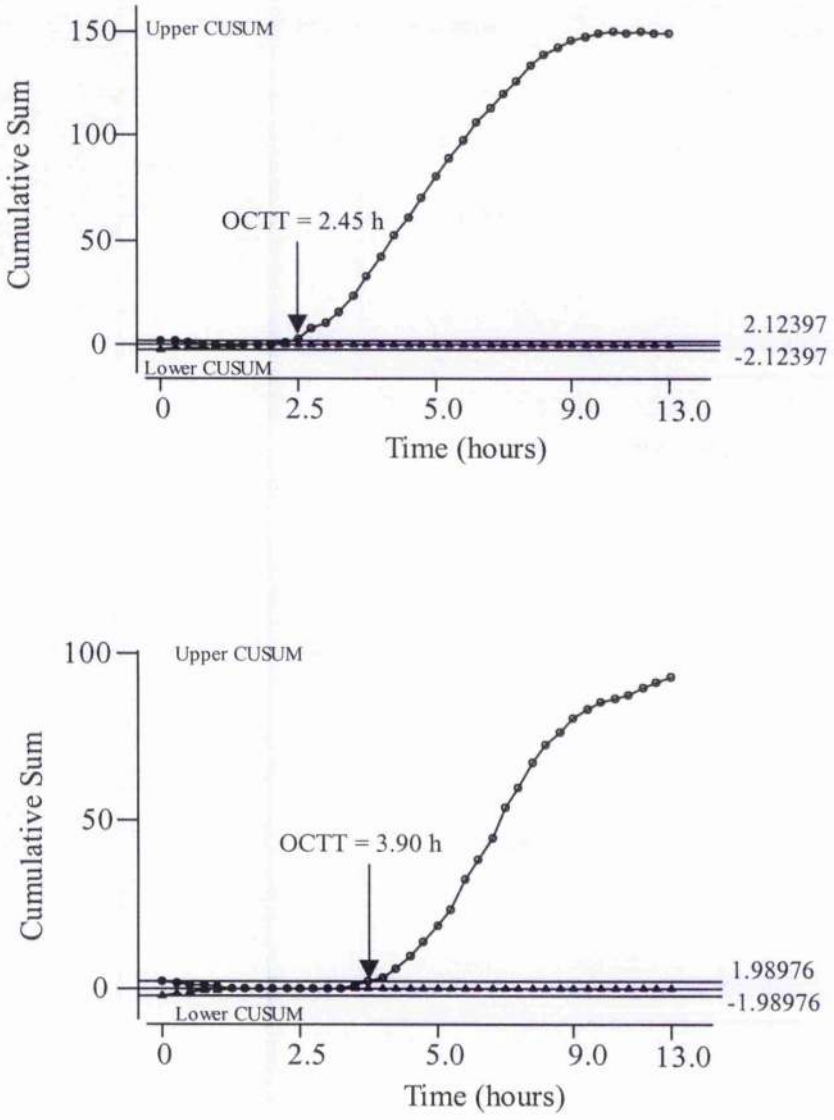


Figure 9.3 (Top) Cusum plot showing calculation of OCTT, based on time at which cumulative standard deviation of successive points (%dose recovered/h) first exceeds the central line by more than 2.5 s.d. In this example, OCTT = 2.45 h. When calculated as time to cumulative 3% dose recovery, OCTT = 2.48 h.

Figure 9.4 (Bottom) Example from individual with slower orocaecal transit of labelled ingesta. By the Cusum method, OCTT = 3.90 h. Using the Maes' model (cumulative 3% dose recovery), OCTT = 3.95 h.

9.3 Linear regression model reduced sampling protocol

9.3.1 Introduction

The objective of this present study was to develop a new mathematical model for equine breath data analysis that involved a shorter protocol and minimised sampling requirements, with the ultimate aim of producing a commercial test kit. Reduced linear regression models have been produced to directly estimate scintigraphic $t_{1/2}$ from a reduced number of breath samples in man (Choi *et al.* 1998; Lee *et al.* 2000a, 2000b). Collection of just 5 breath samples after ingestion of a ^{13}C-*Spirulina platensis*-labelled egg meal has recently been described to allow detection of delayed $t_{1/2}$ with a sensitivity of 86% and specificity of 80% in relation to scintigraphy (Viramontes *et al.* 2001). As sufficient numbers of simultaneous equine breath and scintigraphic studies were not available, the accuracy of reduced breath test regression models was investigated by direct comparison of the parameters gained with those of the full breath sampling protocol. The production of reduced Stable isotope breath test protocols would be of great value for routine measurement of equine gastrointestinal transit.

9.3.2 Materials and methods

Stepwise linear regression (Minitab® release 13) was used to develop models for predicting the gastric emptying and orocaecal transit parameters from the relevant breath test using the minimum number of expiratory $^{13}CO_2$ samples. Individual time points were entered into the model if their *F*-value exceeded 2.0. In each case the best model was selected from the alternatives generated. Model production was limited to the collection of a maximum of 5 samples, plus a baseline breath sample. The reduced ^{13}C-OABT models were developed using the 64 data sets previously reported in Chapter 5, and included several showing delayed gastric emptying. The final 3-, 4-, and 5-sample reduced models were compared to the full protocol results by both linear correlation and Bland Altman plots. Since evaluation of models may give an overly optimistic assessment when based on the same data (Viramontes *et al.* 2001), the final reduced ^{13}C-OABT models were also tested on a separate group of 20 ^{13}C-OABTs performed under the same conditions in 15 healthy adult horses.

The reduced linear regression models for estimation of OCTT, orocaecal t_{lag} and caecal $t_{1/2}$ were generated from 22 data sets in which the final induced protocol for the ^{13}C-LUBT had been applied, as described in Chapter 8. The final reduced models were re-applied to the

same data, and compared again by linear correlation and Bland Altman plots. Further application of these models to unrelated data sets has yet to be performed.

9.3.3 Results: Investigation of reduced linear regression protocols

9.3.3.1 ^{13}C-OABT reduced sampling protocol

Production of reduced model for ^{13}C-OABT

The best linear regression reduced models for the estimation of gastric $t_{1/2}$, t_{lag} and GEC based on a single baseline breath sample and 3, 4 or 5 subsequent breath analyses after ingestion of the labelled test meal in 64 horses are shown in Table 9.3. The equations are given in the form shown in equation 9.1:

$$Y = \alpha + \sum \beta i Xi \qquad \textbf{Equation 9.1}$$

where Y is the parameter of interest; α is the intercept value in the multiple regression analysis; βi are the weights (coefficients), and Xi are the values of the breath test samples (PDR/h) at time ti. The final 5-sample reduced model for calculation of gastric $t_{1/2}$ is given in equation 9.2.

$$t_{1/2}\,(h) = 3.122 - (0.044 * t_{1.00h}) - (0.045 * t_{1.50h}) - (0.059 * t_{2.75h})$$
$$+ (0.189 * t_{5.75h}) + (0.177 * t_{6.50h}) \qquad \textbf{Equation 9.2}$$

This model gave close approximation to the original values for $t_{1/2}$ with an R^2 (coefficient of determination) of 94.07. Application of the 5-sample reduced model to the original 64 data sets for calculation of $t_{1/2}$ resulted in an excellent correlation ($r = 0.968$, $P < 0.001$). An excellent correlation was also seen between the 5-sample model and full protocol for calculation of t_{lag} ($r = 0.977$, $P < 0.001$). Estimates of $t_{1/2}$, t_{lag} and GEC gained by the 3-, 4- and 5-sample reduced protocols were compared with those calculated from the full sampling protocol, by linear correlation and Bland Altman plots. These comparisons are shown in Figures 9.4 to 9.12. Reduced models were also produced using the Siegel estimates of the gastric emptying parameters (section 2.6.3.1). As anticipated, these were similar to those produced from Maes' formula, and are shown for completion in Appendix IV.

Table 9.3 Summary of the 3-, 4- and 5- sample reduced linear regression models produced for the estimation of gastric $t_{1/2}$, t_{lag} and GEC using the ^{13}C-OABT (n = 64; shown on left).

Table 9.4 Summary of the 3-, 4- and 5- sample reduced linear regression models produced for the estimation of orocaecal transit time (OCTT), caecal $t_{1/2}$ and orocaecal t_{lag} measurement using the induced ^{13}C-LUBT (n = 22; shown on right).

^{13}C-Octanoic acid breath test				
Parameter	Samples	3	4	5
$t_{1/2}$ (h)	Constant	2.274	2.787	3.122
	$t_{5.75\ h}$	0.245	0.233	0.189
	$t_{1.50\ h}$	-0.070	-0.075	-0.045
	$t_{6.50\ h}$	0.194	0.159	0.177
	$t_{2.75\ h}$		-0.037	-0.059
	$t_{1.00\ h}$			-0.044
	R^2 value	92.200	93.020	94.070
tlag (h)	Constant	2.176	2.263	2.594
	$t_{5.75\ h}$	0.363	0.253	0.278
	$t_{0.75\ h}$	-0.118	-0.125	-0.130
	$t_{2.00\ h}$	-0.070	-0.072	-0.077
	$t_{7.50\ h}$		0.183	0.146
	$t_{4.00\ h}$			-0.043
	R^2 value	94.20	95.08	95.38
GEC	Constant	1.153	-0.096	0.282
	$t_{5.75\ h}$	-0.611	-0.572	-0.155
	$t_{2.50\ h}$	0.281	0.225	0.218
	$t_{2.75\ h}$	0.113	0.151	0.144
	$t_{1.00\ h}$		0.173	0.197
	$t_{5.75\ h}$			-0.420
	R^2 value	89.28	91.24	93.74

R^2 = coefficient of determination (Regression sum of squares / total sum of squares)
GEC = Gastric Emptying Coefficient
OCTT = orocaecal transit time

Induced lactose ^{13}C-ureide breath test				
Parameter	Samples	3	4	5
OCTT (h)	Constant	3.949	4.022	4.051
	$t_{10.50\ h}$	0.132	0.111	0.126
	$t_{3.00\ h}$	-0.162	-0.144	-0.165
	$t_{5.50\ h}$	-0.057	-0.059	-0.087
	$t_{1.00\ h}$		-0.810	-0.870
	$t4_{.00\ h}$			0.036
	R^2 value	83.550	84.770	85.310
Caecal	Constant	7.360	7.573	7.708
$t_{1/2}$ (h)	$t_{4.00\ h}$	-0.111	-0.058	
	$t_{11.00\ h}$	0.466	0.457	0.509
	$t_{6.00\ h}$	-0.108	-0.156	-0.223
	$t_{1.00\ h}$		-2.270	-2.260
	$t_{1.75\ h}$			0.006
	$t_{2.5\ h}$			-0.097
	R^2 value	92.34	96.45	98.12
Orocaecal	Constant	7.343	7.242	7.070
t_{lag} (h)	$t_{6.00\ h}$	-0.266	-0.279	
	$t_{0.50\ h}$	-3.230	-3.380	-4.750
	$t_{9.50\ h}$	0.265	0.780	10.710
	$t_{10.00\ h}$		-0.550	-9.000
	$t_{8.00\ h}$			-2.250
	$t_{12.00\ h}$			0.340
	R^2 value	87.84	88.50	91.88

Testing of reduced model for ^{13}C-OABT

In order to further investigate the validity of the reduced models for clinical use, they were then applied to 20 unrelated gastric emptying data sets collected from healthy adult horses. The comparisons of the estimates gained for $t_{1/2}$, t_{lag} and GEC using these new data are shown in Figures 9.13 to 9.21.

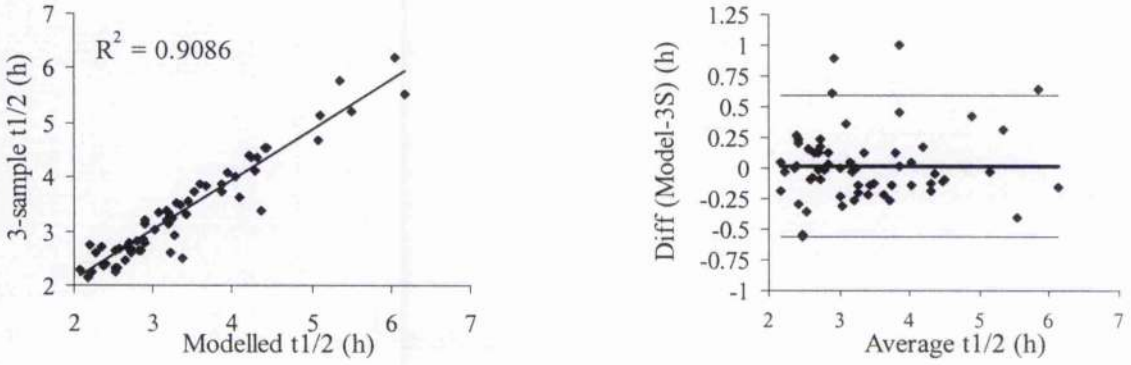


Figure 9.4 Comparison of gastric $t_{1/2}$ measurements derived from the 3-sample regression model with those of the full sampling protocol (n = 64). The best linear regression line and Pearson correlation coefficient are shown on the left, with the corresponding Bland Altman plot (difference versus average) on the right. The mean difference and 95% limits of agreement between the full and reduced models are plotted.

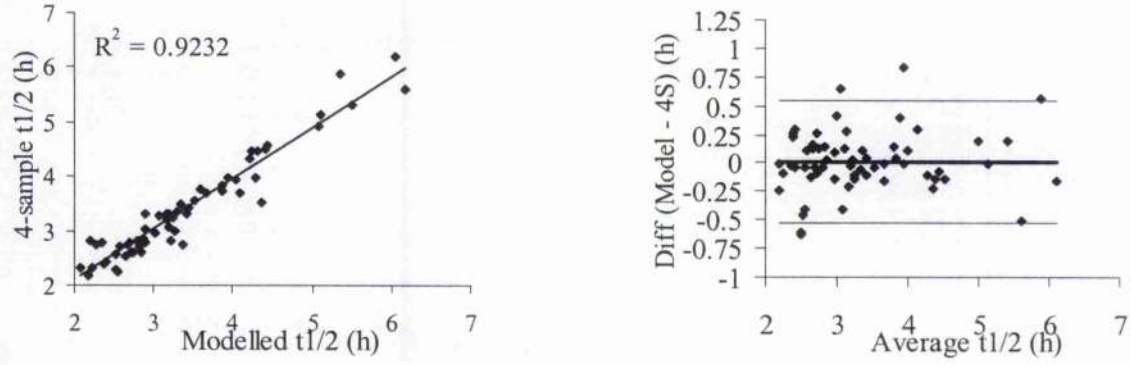


Figure 9.5 Comparison of 4-sample reduced regression model and full 32-sample model for measurement of gastric $t_{1/2}$ using the ^{13}C-OABT (n = 64). The linear correlation is demonstrated on the left and the Bland Altman (difference vs average value) on the right.

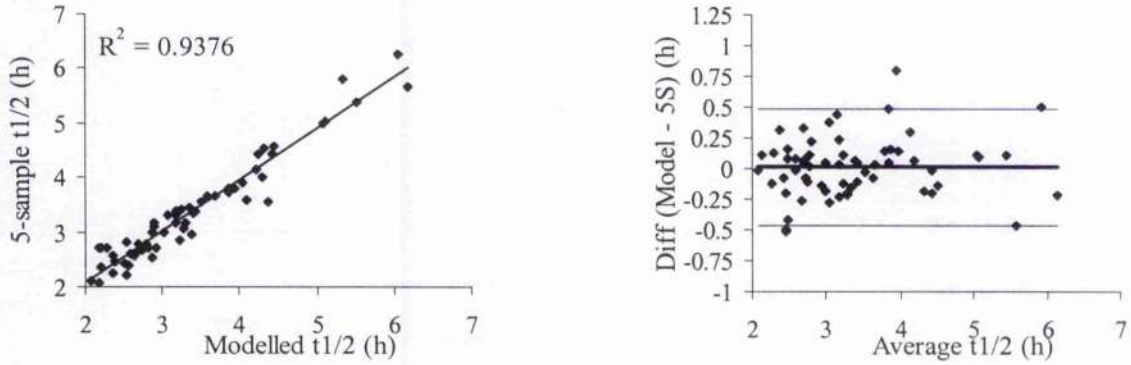


Figure 9.6 Final 5-sample regression model for estimation of gastric $t_{1/2}$ using the ^{13}C-OABT. The model is presented in Table 9.3. An excellent correlation exists between the full model and the 'reduced' 5-sample version, which is uniform across an extended range of values. The linear correlation line approximates parity.

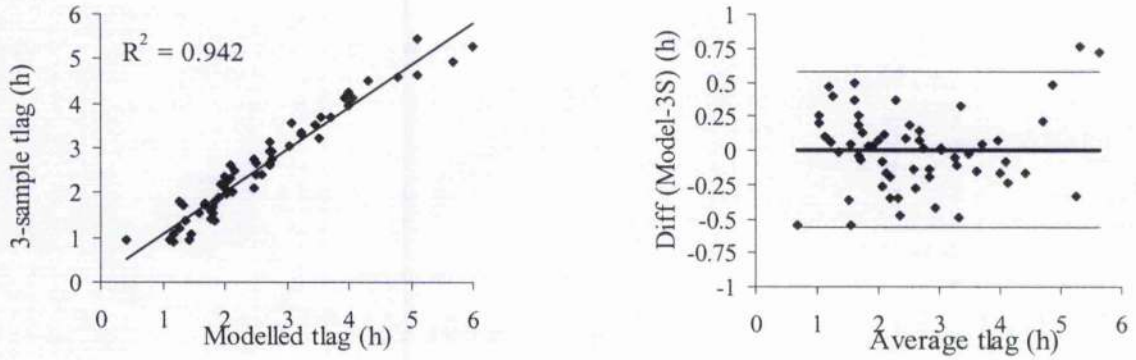


Figure 9.7 Comparison of gastric t_{lag} measurements derived from the 3-sample regression model with those of the full sampling protocol (n = 64). The best linear regression line and Pearson correlation coefficient are shown on the left, with the corresponding Bland Altman plot (difference versus average) on the right. The mean difference and 95% limits of agreement between the full and reduced models are plotted.

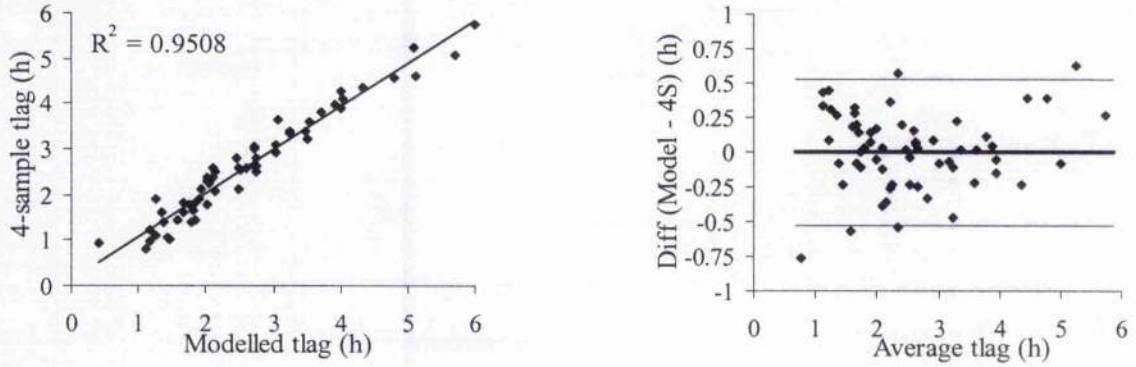


Figure 9.8 Comparison of 4-sample reduced regression model and full 32-sample model for measurement of gastric t_{lag} using the ^{13}C-OABT (n = 64). The linear correlation is demonstrated on the left and the Bland Altman (difference vs average value) on the right.

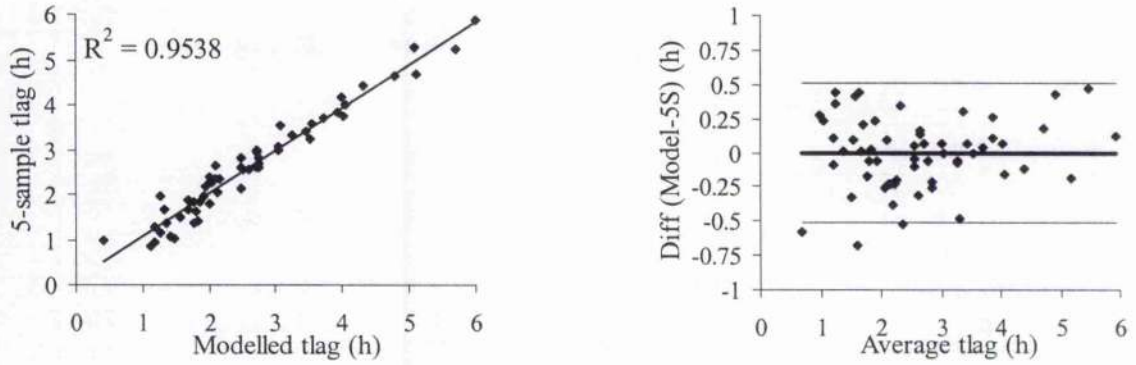


Figure 9.9 Final 5-sample regression model for estimation of gastric $t_{1/2}$ using the ^{13}C-OABT. The model is presented in Table 9.3. Again, an excellent correlation is demonstrated between the full model and the 'reduced' 5-sample version, which is uniform across an extended range of values.

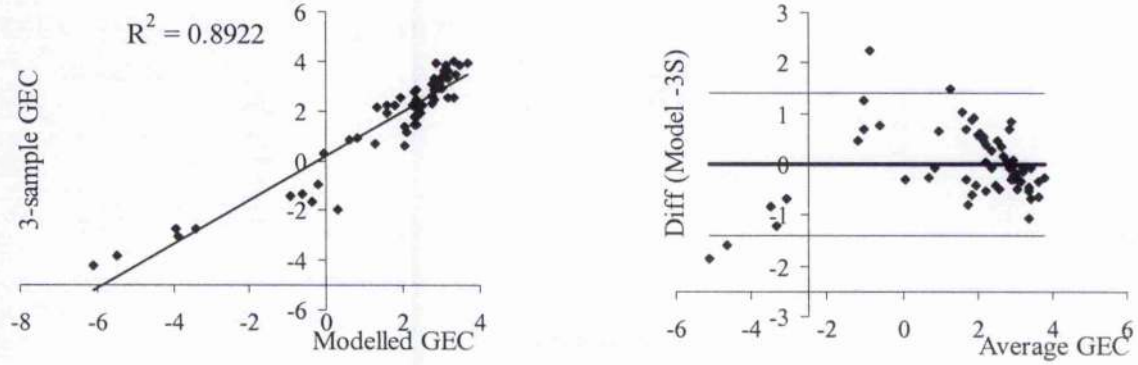


Figure 9.10 Comparison of GEC estimates derived from the 3-sample regression model with those of the full sampling protocol (n = 64). The best linear regression line and Pearson correlation coefficient are shown on the left, with the corresponding Bland Altman plot (difference versus average) on the right. The mean difference and 95% limits of agreement between the full and reduced models are plotted.

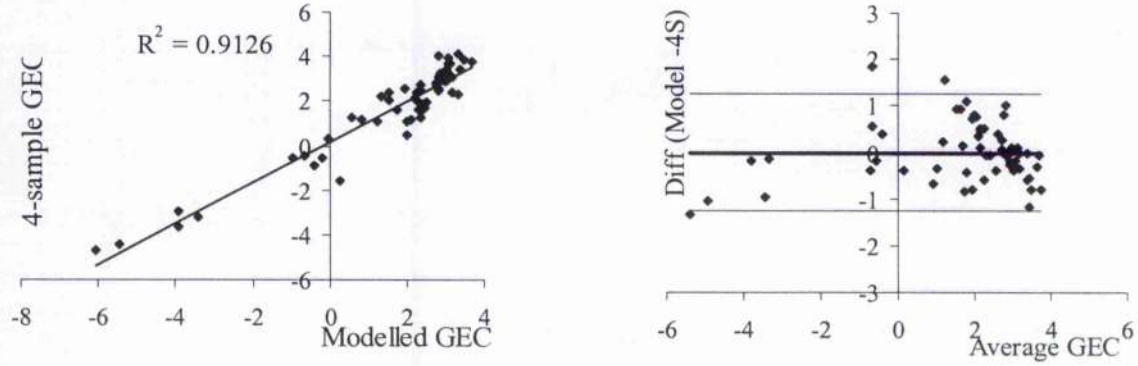


Figure 9.11 Comparison of 4-sample reduced regression model and full 32-sample model for measurement of GEC using the ^{13}C-OABT (n = 64). The linear correlation is demonstrated on the left and the Bland Altman plot (difference vs average value) on the right.

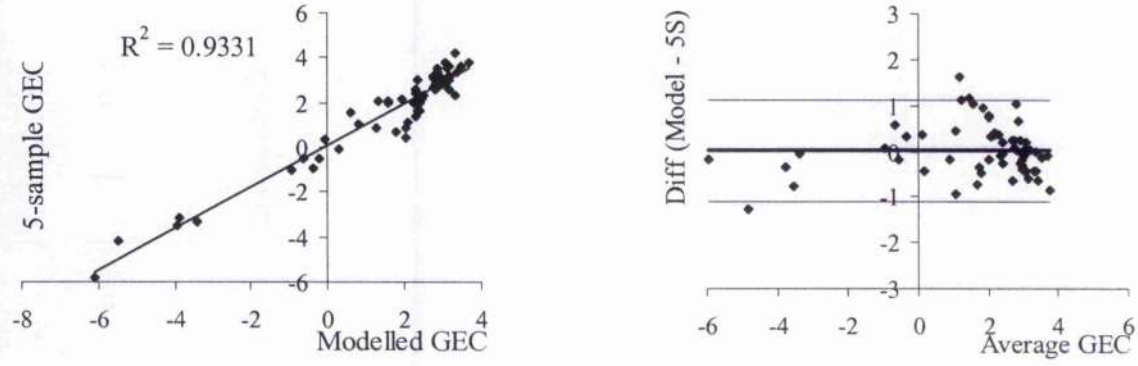


Figure 9.12 Comparison of final 5-sample reduced model with full 32-sample model for calculation of GEC using the ^{13}C-OABT (n = 64). The reduced model is presented in Table 9.3.

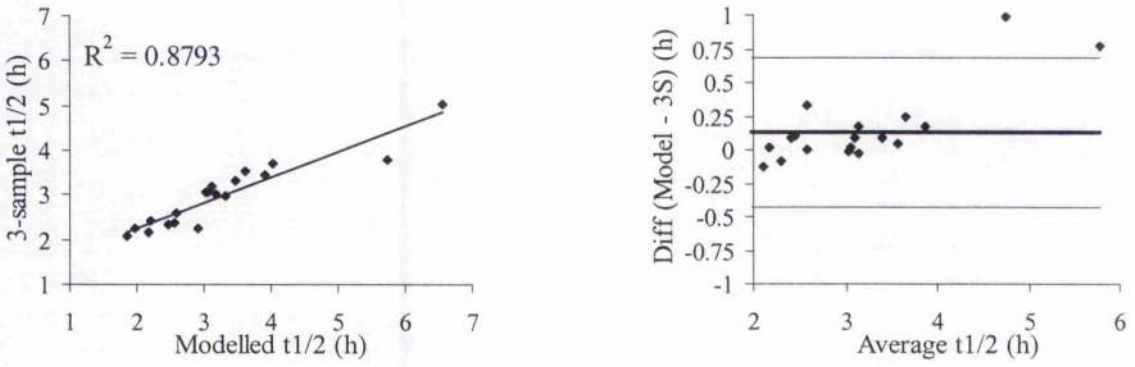


Figure 9.13 Test of developed linear regression model: 3-sample reduced model applied to 20 separate gastric emptying data sets from healthy adult horses. There is good linear correlation but the Bland Altman plot (right) shows that there is progressive underestimation of $t_{1/2}$ by the reduced model as $t_{1/2}$ increases in duration.

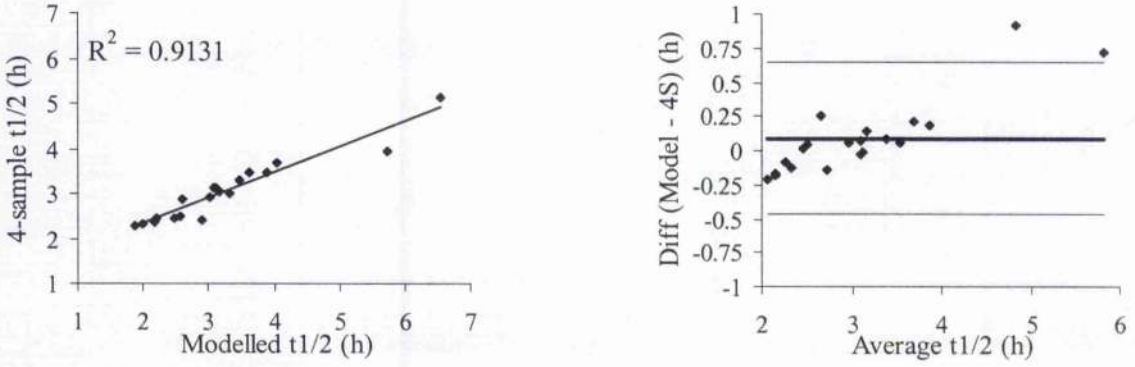


Figure 9.14 Application of the reduced 4-sample linear regression model to estimation of $t_{1/2}$ in 20 separate ^{13}C-OABT data sets. Again, the reduced model leads to under-estimation of $t_{1/2}$ in 2 individuals with prolonged gastric emptying.

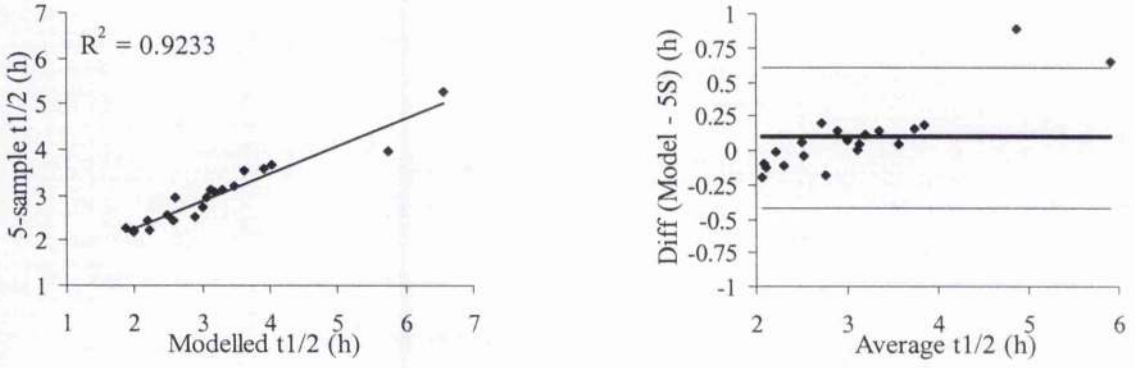


Figure 9.15 Testing of the final developed 5-sample reduced protocol for the estimation of gastric emptying using the ^{13}C-OABT (n = 20). The Bland Altman plot demonstrates minimal scattering of the inter-model differences about the mean value. However, the accuracy of the developed model is reduced when gastric emptying is very rapid or more prolonged.

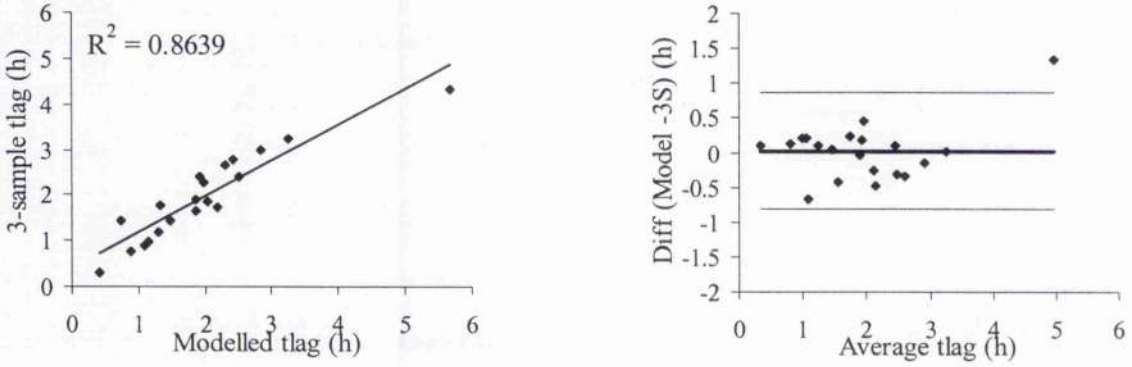


Figure 9.16 Test of developed linear regression model: 3-sample reduced model applied to 20 separate gastric emptying data sets from healthy adult horses. Linear correlation demonstrated on the left and Bland Altman plot on right. The reduced model underestimates t_{lag} in one case of delayed gastric emptying.

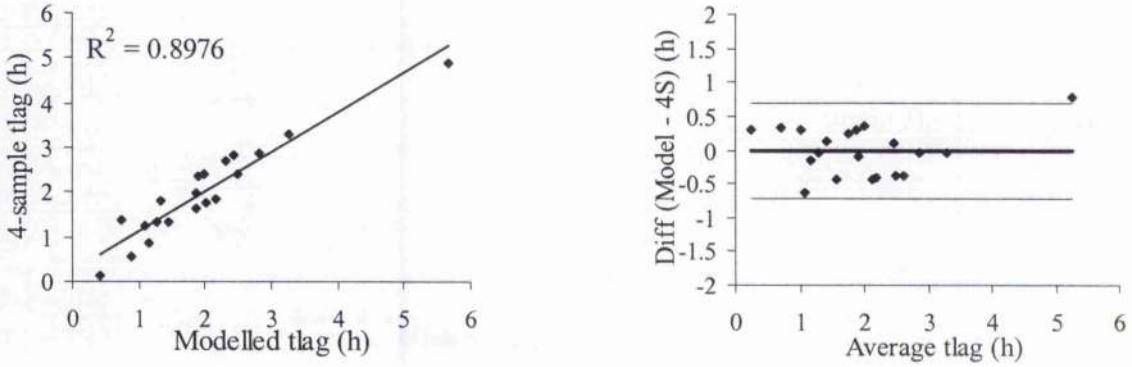


Figure 9.17 Application of the reduced 4-sample linear regression model to estimation of t_{lag} in 20 separate ^{13}C-OABT data sets. The 95% limits of agreement for the mean difference between the models are reduced compared with the 3-sample model, and the mean difference between the results approximates zero.

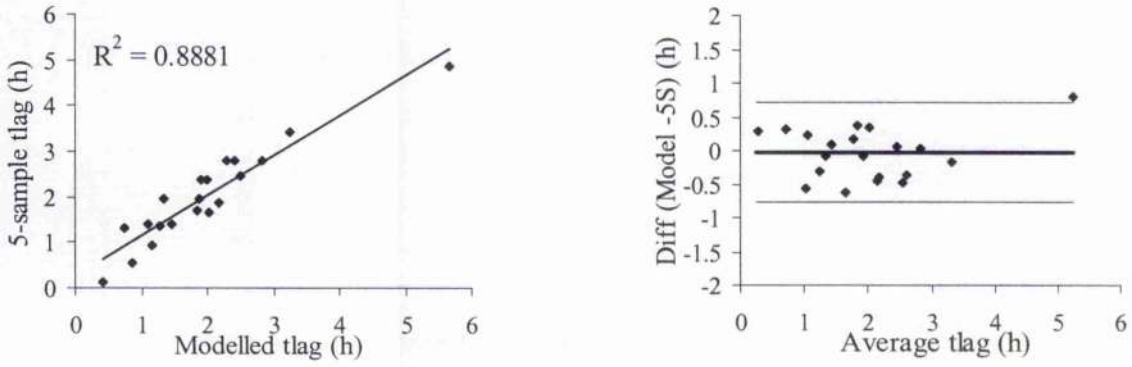


Figure 9.18 Comparison of the developed 5-sample model with the full model for calculation of t_{lag} using the ^{13}C-OABT. The best linear regression line approximates parity, and the mean difference in calculation of t_{lag} approximates zero. However, the reduced model underestimates duration of more prolonged t_{lag} in a single case.

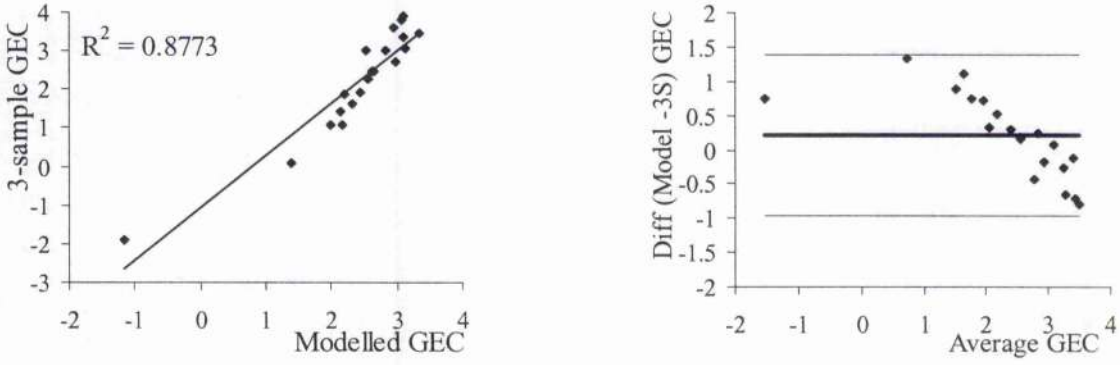


Figure 9.19 Test of developed linear regression model: 3-sample reduced model applied to 20 separate gastric emptying data sets from healthy adult horses. Linear correlation demonstrated on the left and Bland Altman plot on right. Although there is good linearity, the correlation line does not approximate parity, and the mean difference is variable.

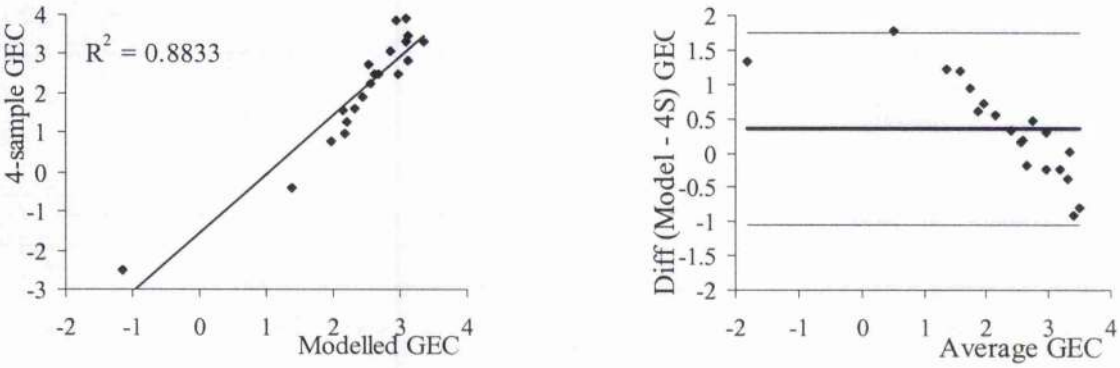


Figure 9.20 Comparison of the 4-sample reduced model with the full protocol model for the calculation of GEC using the ^{13}C-OABT (n = 20). The reduced model leads to overestimation of GEC when gastric emptying is rapid, and under-estimation when gastric emptying is prolonged.

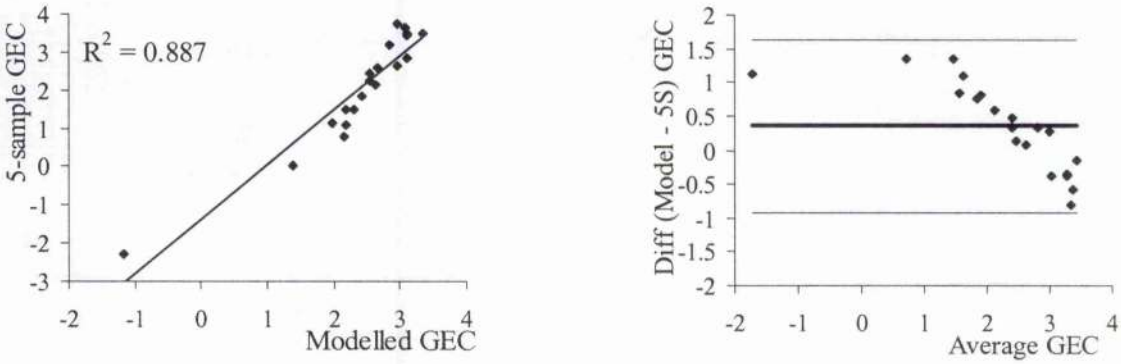


Figure 9.21 Comparison of final 5-sample model with full protocol for determination of GEC (n = 20) using the ^{13}C-OABT. Although there is a close linear relationship, the numerical estimation of GEC by the reduced model is seen on the Bland Altman plot to have poor similarity to the full model.

Excellent correlations were gained for both $t_{1/2}$ ($r = 0.961$, $P < 0.001$) and t_{lag} ($r = 0.939$, $P < 0.001$) using the 5-sample models on the unrelated data sets, with correlation lines that were close to parity. However, it can be seen in Figures 9.15 and 9.18 that the final reduced models underestimate $t_{1/2}$ and t_{lag} in 2 and 1 cases respectively where gastric emptying is delayed. In the selected population this would lead to mis-classification of 1 delayed $t_{1/2}$ value as normal by the reduced 5-sample model. Despite the significant linear correlation, the reduced model is less satisfactory for the estimation of GEC. Figure 9.21 shows a fluctuation about the mean difference between the models, with initial under-estimation by the reduced model, followed by over-estimation as GEC increases in magnitude.

9.3.3.2 ^{13}C-LUBT reduced sampling protocol

The reduced protocols for calculation of the parameters relating to small intestinal transit were produced using 22 induced ^{13}C-LUBT data sets from healthy adult horses. Again, model construction was restricted to the collection of a baseline breath sample before labelled meal ingestion, followed by a further 3, 4 or 5 breath samples. The best linear regression formulae for calculation of OCTT, caecal $t_{1/2}$ and orocaecal t_{lag} are shown in Table 9.4. The final 5-sample formula for OCTT estimation is given by equation 9.3:

$$\text{OCTT (h)} = 4.051 + (0.126 * t_{10.50\,h}) - (0.165 * t_{3.00\,h}) - (0.087 * t_{5.50\,h}) - (0.870 * t_{1.00\,h}) - (0.036 * t_{4.00\,h}) \qquad \textbf{Equation 9.3}$$

Due to the relative scarcity of equine induced ^{13}C-LUBT data, the developed models were reapplied to the same data set to determine the goodness of fit (n = 22). The best linear regression lines and Bland Altman plots for estimation of OCTT, caecal $t_{1/2}$ and orocaecal t_{lag} by the 3-, 4- and 5-samples models are illustrated in Figures 9.22 to 9.30. The final 5-sample models for both caecal $t_{1/2}$ ($r = 0.991$, $P < 0.001$) and orocaecal t_{lag} ($r = 0.958$, $P < 0.001$) showed excellent correlations to the full data sets and approximated parity. The final reduced model for OCTT measurement also demonstrated good linear correlation with the 34-sample model ($r = 0.924$, $P < 0.001$). However, this latter model underestimated atypical prolonged values for OCTT such that 1 delayed sample would have been mis-classified as normal in this data set.

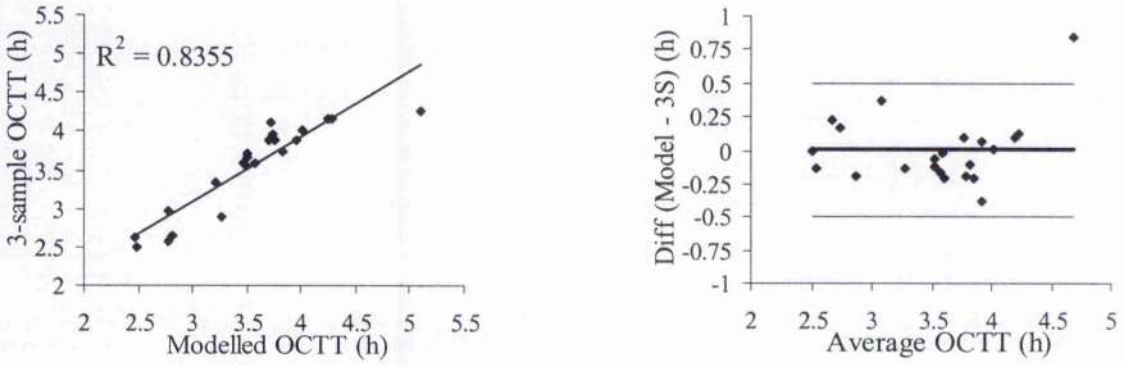


Figure 9.22 Comparison of the reduced 3-sample linear regression model with the full 34-sample protocol for the calculation of orocaecal transit time (OCTT) using the ^{13}C-LUBT (n = 22). The reduced protocol leads to underestimation of OCTT in a single individual with delayed small intestinal transit.

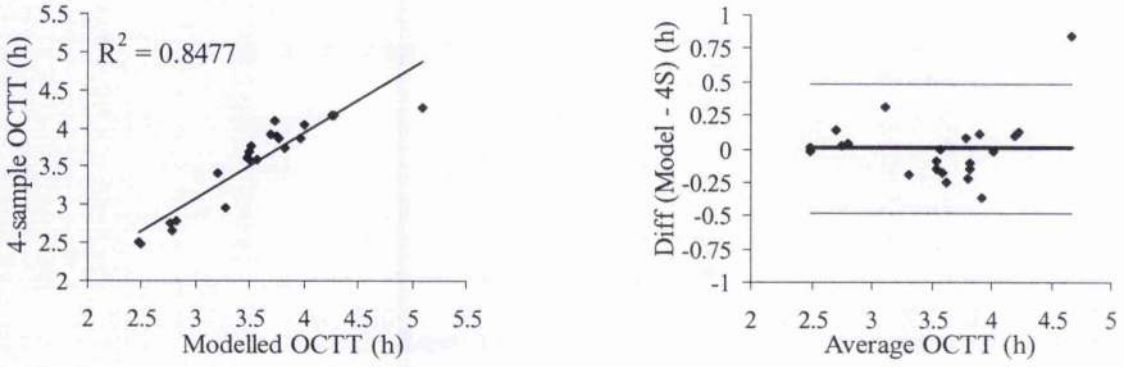


Figure 9.23 Comparison of the reduced 4-sample linear regression model with the full sampling protocol for the calculation of OCTT using the ^{13}C-LUBT (n = 22).

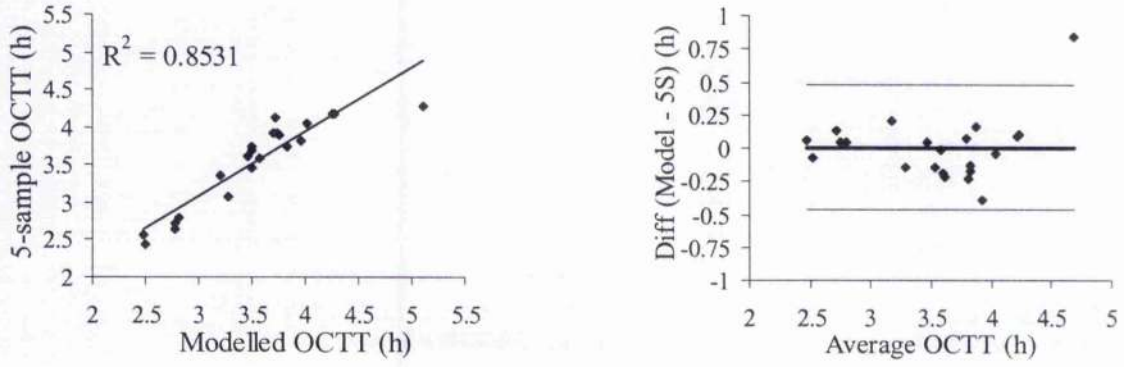


Figure 9.24 Final reduced 5-sample protocol for OCTT measurement: comparison with the full model. The reduced protocol values approximate those of the full model, with the correlation line falling close to equivalence. However, prolonged values for OCTT may be underestimated by the reduced model.

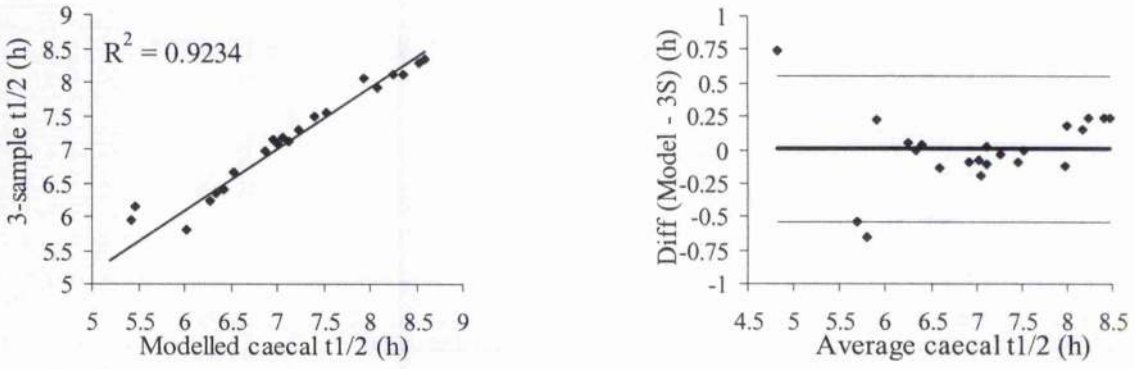


Figure 9.25 Comparison of 3-sample reduced protocol and full 34-sample protocol for estimation of caecal $t_{1/2}$ using the ^{13}C-LUBT (n = 22). The best linear regression line is shown on the left and the Bland Altman plot (difference vs average) on the right. The line of best fit is seen to approximate equivalence.

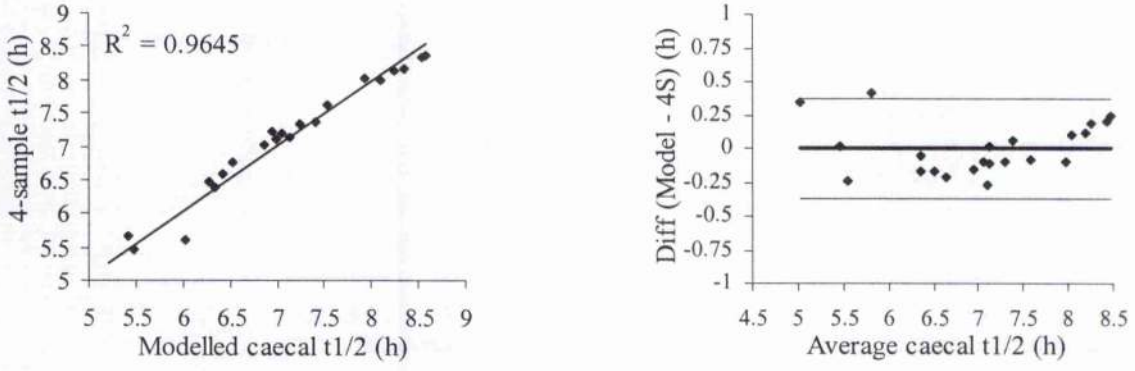


Figure 9.26 Comparison of the developed 4-sample protocol with the full 34-sample protocol for measurement of caecal $t_{1/2}$ using the ^{13}C-LUBT (n = 22).

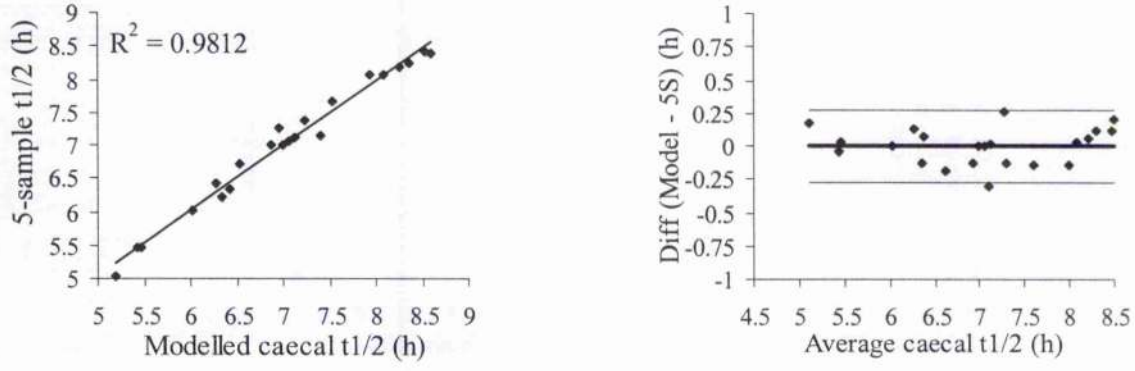


Figure 9.27 Final 5-sample model for measurement of caecal $t_{1/2}$ using the ^{13}C-LUBT: comparison with the full 34-sample model. An excellent correlation is demonstrated, with an even distribution of mean difference values across the range of values for caecal $t_{1/2}$.

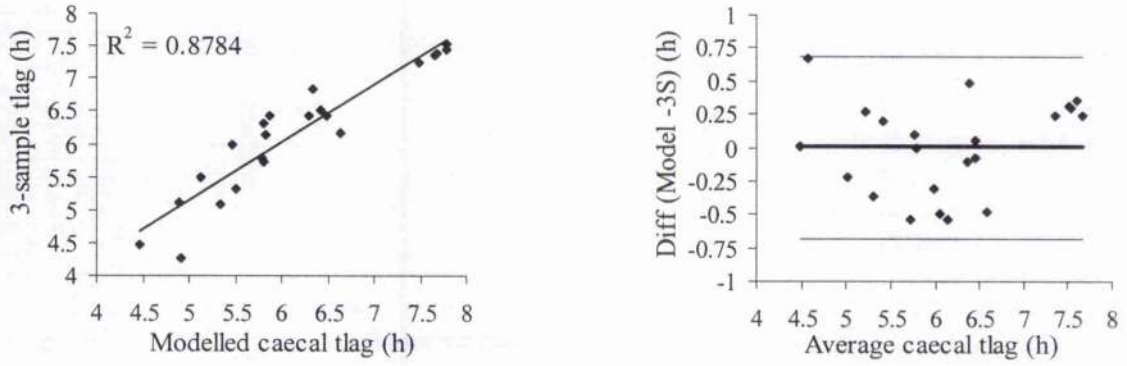


Figure 9.28 Comparison of reduced 3-sample protocol with full 34-sample protocol for estimation of orocaecal t_{lag} using the ^{13}C-LUBT (n = 22). The 95% limits of agreement are relatively wide on the Bland Altman plot (right).

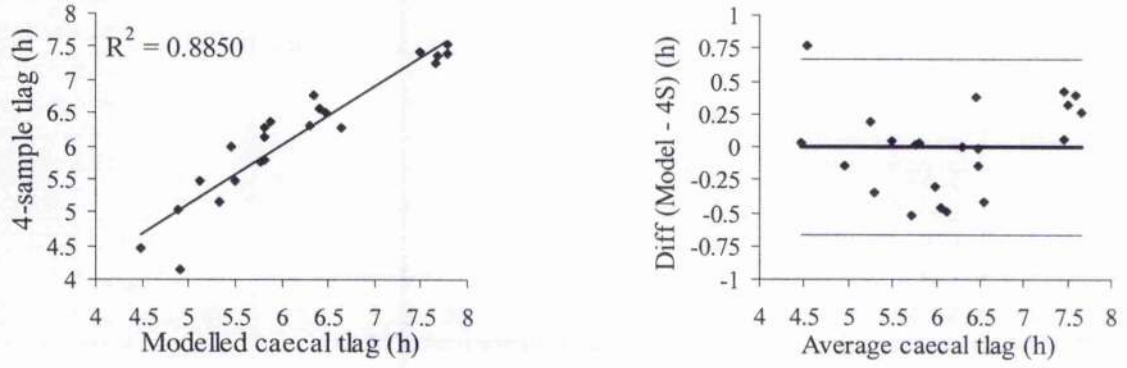


Figure 9.29 4-sample reduced model for estimation of orocaecal t_{lag}: comparison with the 34-sample protocol. Linearity and Pearson correlation coefficient are improved, compared with Figure 9.28.

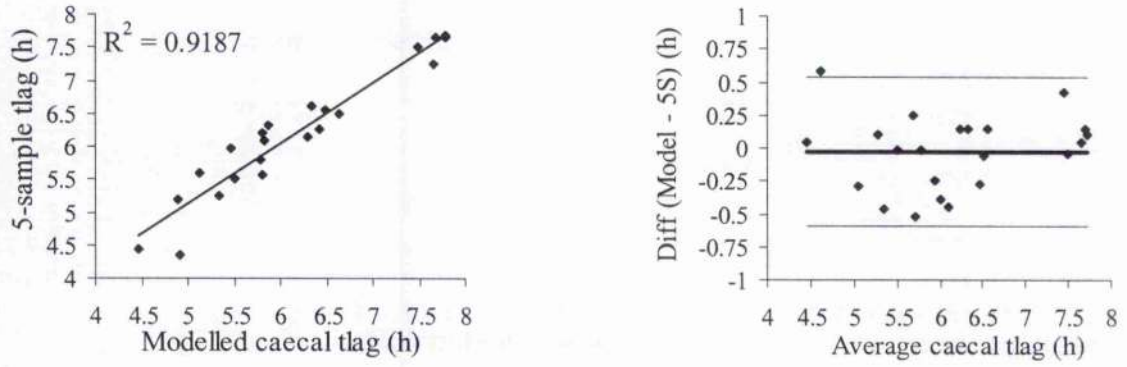


Figure 9.30 Final developed 5-sample model for estimation of orocaecal t_{lag} from the ^{13}C-LUBT. There is close agreement between the models, with the best linear regression line approximating equivalence.

9.4 Discussion

It has been shown in this Chapter that the protocols for the measurement of equine gastric and small intestinal function using the ^{13}C-OABT and induced ^{13}C-LUBT respectively may be reduced significantly with minimal loss of accuracy. Duration of sample collection may be safely reduced to 4-5 h for gastric emptying measurement using the ^{13}C-OABT or to 9 h for small intestinal transit measurements using the induced ^{13}C-LUBT. Alternatively, depending on preference, a minimal set of breath samples (baseline $^{13}CO_2$ and a further 5 samples) may be collected for each test, and the PDR/h term modelled according to the developed linear regression formulae. The reduced 5-sample formulae minimise labour and analytical costs, and may aid commercial development of the stable isotope breath tests for routine equine use.

Whilst the reduced test protocols have obvious potential benefits, they are not entirely without error. The reduced linear regression models are accurate for parameter estimation when these values lie in the inter-quartile range of the population, but become less accurate for quantitative measurement of delayed values, which could lead to the possible misclassification of some individuals with delayed motility. This problem could be minimised by the development of specific reduced regression models for gastroparesis or small intestinal ileus, as suggested in human medicine (Viramontes *et al.* 2001) but this approach would necessitate prior clinical knowledge of the condition. When information about OCTT alone is required in equine small intestinal ileus cases, the most appropriate reduced protocol for investigation may involve use of the Cusum approach for analysis (Muir *et al.* 1991; Geypens *et al.* 1999).

Utilising a reduced sampling protocol the Stable isotope breath tests should not prove prohibitively expensive to perform in equine practice. The cost of a ^{13}C-OABT gastric emptying study for example is far below that of a similar gastric scintigraphic study. Analytical costs of ^{13}C analysis are also likely to reduce due to the development and increased use of non-dispersive isotope-selective infrared spectroscopy (NDIRS, Braden *et al.* 1994, 1996). NDIRS appears to be a promising, easy to operate, and low cost potential alternative to conventional IRMS. This instrument provides on-line results and has been reported to have equal sensitivity and specificity to IRMS for the diagnosis of *H. pylori* infection in humans using the ^{13}C-urea breath test (Koletzko *et al.* 1995; Savarino *et al.* 1999). NDIRS units are also cheaper, lighter and smaller than IRMS units, with an enclosed reference gas. However, larger samples of breath (1200 ml) are required for each

analysis using NDIRS, and the process has yet to be automated, such that it is more time consuming than IRMS.

A further advance for the analysis of $^{13}CO_2$ breath samples is the development of automated continuous expiratory breath analysis by molecular correlation spectroscopy (BreathID®, Oridion BreathID Inc., Needham, MA, USA). In this process, expiratory $^{13}CO_2$ content is continuously monitored via a nasal prong before and after test meal ingestion, and the indices of gastric emptying displayed on a monitor. The test procedure for gastric emptying can be terminated as soon as the machine indicates that emptying is complete. The automated breath collection for this machine has yet to be modified for equine use.

Having previously validated the ^{13}C-OABT and the induced ^{13}C-LUBT for the measurement of equine gastric emptying and orocaecal transit times, it has been further shown in this Chapter that the tests are practical diagnostic modalities for clinical use. The collection protocols may be minimised in extent in order to maximise clinical utility. In addition, with the continual evolution of spectroscopic technology these stable isotope breath tests will become more cost-effective for equine diagnostic use.

Chapter 10 GENERAL DISCUSSION AND CONCLUSIONS

10.1 Stable isotope breath tests: equine validation studies

The accuracy of all ^{13}C-isotope breath tests is dependent on a constant basal production of $^{13}CO_2$ during the test period so that expiratory enrichment provides a true measure of the metabolism of the administered tracer. Preliminary studies confirmed that equine diet had a significant influence on basal $^{13}CO_2$ output, and showed that it was preferable to offer a test meal of equivalent ^{13}C:^{12}C ratio to the normal diet in order to avoid fluctuations in metabolic $^{13}CO_2$ production during the test period. In practical terms this was achieved throughout the study by avoiding diets that were particularly high in ^{13}C content. If this proved impractical in the clinical setting, then increased tracer could be added to the test meal to minimise inaccuracies resulting from fluctuations in resting $^{13}CO_2$ production.

The technique developed for collection of equine expiratory breath and measurement of its ^{13}C:^{12}C ratio was highly repeatable with almost perfect concordance between data sets. A method developed for measurement of peripheral blood ^{13}C content was also shown to generate values that were highly correlated to those of simultaneous breath samples. These results confirmed the robust nature of the method used for sample collection and analysis and suggested that breath could be substituted by blood for tracer analysis if required.

The ^{13}C-OABT was validated against the predicate method of scintigraphy for the measurement of $t_{1/2}$ and t_{lag} in 12 healthy horses and also in 8 individuals with atropine-induced gastroparesis. In both studies a comparable, constant real-time difference was present between the techniques for estimation of $t_{1/2}$. This was an initial cause of concern, but was shown to correspond to the $t_{1/2}$ for $^{13}CO_2$ recovery after direct duodenal instillation of the tracer. Hence it was concluded that the real-time delay in the ^{13}C-OABT GE results was caused by the time taken for the post-gastric handling of the isotope. This latter parameter was found to be constant in different individuals as noted in the first validation study in man (Ghoos *et al.* 1993), such that GE was the major rate-determining step in recovery of the isotope after test meal ingestion. Further proof that GE was the prime determinant of ^{13}C recovery was provided by deconvolution of the gastric emptying function in subjects with simultaneous ^{13}C-OABT and duodenal instillation and recovery data (Maes *et al.* 1998). The gastric function was found to show real-time homology to the simultaneous scintigraphic GE data. It was therefore concluded that the ^{13}C-OABT was an effective indirect test for the measurement of equine solid phase GE rate over a wide range of emptying times.

Validation of the ^{13}C-LUBT for the measurement of equine OCTT was more difficult to perform as there was no recognised standard technique reported for assessment of this parameter against which it could be compared. As the H_2BT had been described for estimation of OCTT in the horse (Bracher and Baker 1994; Murphy 1997; Murphy *et al.* 1998) an initial comparative study was performed between the H_2BT and the ^{13}C-LUBT. However, while discernible peaks in $^{13}CO_2$ output were observed in each individual after ingestion of the test meal, significant H_2 production proved to be unreliable. Furthermore, there was high intra-individual variability in the H_2BT and interaction with a number of dietary factors. It was concluded that the H_2BT was not suitable for clinical measurement of equine OCTT and an alternative *in vitro* technique was developed for validation of the ^{13}C-LUBT.

Using a series of anaerobic bacterial fermentation chambers containing aliquots of intestinal content collected from along the length of the small intestine and caecum, it was shown that the ability to ferment lactose ureide was principally restricted to equine caecal microbes. The rate of microbial lactose ^{13}C-ureide digestion was significantly increased by prior exposure to lactose ^{12}C-ureide, and it was hypothesised that this was caused by selective proliferation of the target organisms or increased production of an inducible glucoseureide hydrolase (Mohr *et al.* 1999). This induction process was characterised *in vitro* and a ratio of 2 to 5:1 for induction dose:^{13}C-tracer dose was found to be optimum. Duration of the induction period was also found to be critical and a minimum period of 14 h was required to maximise subsequent $^{13}CO_2$ production rate. The *in vitro* incubation results showed that occasional ileal microbial populations were able to digest lactose ^{13}C-ureide. However, this was not considered likely to compromise the accuracy of an *in vivo* test as the rate of ileal hydrolysis was significantly slower than that of caecal microbes, and ileal microbial volume is considerably smaller than that of the caecum.

Application of the induced ^{13}C-LUBT *in vivo* was successful, with production of a well-defined peak in $^{13}CO_2$. The prior feeding of lactose ^{12}C-ureide ('induction') was found to be an essential part of the test protocol, and a dose was administered the day prior to ingestion of the ^{13}C-LU test meal. A single test in one individual resulted in production of a small early peak in $^{13}CO_2$ output that was considered to correspond to small intestinal fermentation of the substrate. However, this was distinguishable from the mean peak in $^{13}CO_2$ production, and the induced ^{13}C-LUBT was considered overall to be an effective and reliable indirect means of assessing equine OCTT. Equine orocaecal transit of ingesta was revealed to be relatively rapid, with a mean (± s.d.) time of 3.24 (± 0.65) h.

Limitations of both validation studies included the relatively small size of the study populations, which prohibited the production of a standard reference range for intestinal transit parameters as measured by each test. However, for the ^{13}C-OABT a cut-off point was selected for detection of delayed gastric emptying as compared to the available scintigraphic data, using a ROC curve and TG-ROC plot. Based on the gastric scintigraphic data collected during the combined studies, a cut-off point for breath test $t_{1/2}$ of approximately 5.27 h was shown to have a sensitivity of 1.0 and specificity of 0.90 for the diagnosis of 'delayed gastric emptying rate' in this population.

The *in vitro* validation procedure for the ^{13}C-LUBT produced convincing significant differences between caecal and small intestinal fermentation rates for the ^{13}C-tracer. It should be remembered however that direct comparison between *in vitro* microbial systems and the *in vivo* scenario might not be valid for a variety of reasons. Metabolic products accumulate in anaerobic chambers as they are not absorbed, and may prove bacteriostatic to certain populations. Similarly, accumulations of excessive gas within the chambers may cause differential suppression of bacterial multiplication due to increased pressure. A superior method of validating the ^{13}C-LUBT may have been to use animals fitted with intestinal cannulae, but this method was not chosen for ethical reasons.

Before widespread application of the ^{13}C-OABT for equine gastric pharmacological studies, the effect of substrate competition on the rate of hepatic β-oxidation of medium chain fatty acids should be investigated. As suggested in section 5.4.5.3, certain compounds requiring hepatic β-oxidation have been shown to cause a significant reduction in octanoic acid metabolism *in vivo* (Kalbag and Palekar 1988; Fromenty *et al.* 1989) which would confound interpretation of test results. Similarly, the test has not been validated for the measurement of solid phase GE in the presence of significant small intestinal infiltrative disease, such that the coexistence of this condition should be considered, particularly when using the test as part of the diagnostic work up for horses presenting with weight loss. As previously discussed, relatively severe hepatic pathology has been demonstrated not to reduce hepatic oxidation of octanoic acid, as has reduction of hepatic blood flow (Van de Casteele 2002). Concurrent hepatic pathology is therefore unlikely to have a significant effect on interpretation of the breath test results in the horse.

Further validation procedures may also be required for the ^{13}C-LUBT prior to its clinical use, since it has not been demonstrated in any species whether bacterial fermentation of the tracer is compromised by concurrent antibiotic administration or recent changes in diet. Certain samples used for the *in vitro* fermentation studies were collected from individuals

that had received antibiotics prior to death, and no specific effect was noted on the rate of tracer digestion. However, a controlled study would be preferable to investigate the possible interactions of these compounds with microbial action. Application of the ^{13}C-LUBT in young foals may also be limited by the absence of the requisite microflora prior to weaning, as noted in human neonatal studies (Van den Driessche 2000).

The sodium ^{13}C-acetate breath test and the ^{13}C-bicarbonate breath test produced exponential patterns of $^{13}CO_2$ recovery after ingestion of a labelled test solution which were certainly consistent with liquid phase gastric emptying. However, neither of these tests has been validated against scintigraphy for the measurement of this parameter in the horse, and the ^{13}C-BBT has previously been shown not to be valid for this purpose in man (Bjorkman *et al.* 1991). Comparative validation studies are therefore required before further application of these tests for measurement of liquid phase emptying rate.

10.2 Effect of pharmacological agents on equine gastrointestinal tract

The ^{13}C-OABT proved to be a valuable diagnostic tool for investigating the effect of common sedative agents on equine GE. As expected from previous studies, detomidine was shown to delay solid phase GE rate, and a relatively small dose of butorphanol had a synergistic effect on its mechanism of action. However, a significant dose-related effect was also noted on the magnitude of induced gastroparesis, and detomidine was seen to have a significantly greater effect than an equipotent dose of xylazine. Acepromazine did not modulate gastric emptying rate, suggesting that preanaesthetic medication with this compound should not be a risk factor for the development of post-operative ileus.

Atropine administration had an interesting effect on the gastrointestinal tract, causing a marked delay in transit of ingesta through the terminal oesophageal smooth muscle, and altering the gastric contour by inhibition of resting tone. An average intravenous dose of 0.035 mg/kg atropine resulted in a significant delay in GE due to decreased antral peristalsis and loss of antropyloroduodenal coordination, but was transient in action and did not cause further gastrointestinal complications.

10.3 Physiological interpretations of study results

A number of interesting physiological features were noted during the course of these studies. Somewhat anecdotally, apprehension appeared to cause complete cessation of GE in one individual as determined by both scintigraphy and ^{13}C-OABT. GE resumed upon mental relaxation of the subject. Prolonged stressful situations may therefore predispose

subjects to development of gastroparesis or fluctuations of intragastric pH. Of basic physiological importance was the evidence gained using the combined ^{13}C-OABT/^{13}C-LUBT that equine small bowel transit times were independent of GE rate and highly variable between and within individuals. Furthermore a negative correlation was observed between small intestinal transit rate and GE rate, which suggested the presence of a small intestinal negative feedback mechanism on GE as has been noted in other species (Hunt and Stubbs 1975). Early equine small intestinal transit was also significantly correlated to the arrival of ingesta in the caecum, which was consistent with the theory that the equine small intestine has a major regulatory role in the delivery of ingesta and nutrients to the caecum. These findings may have been suspected, based on results in other species (Read *et al.* 1980, 1984a, 1986) but had not previously been proven for the horse and have important implications for future nutritional and prokinetic studies.

10.4 Clinical application of the ^{13}C-stable isotope breath tests

The results of this study showed that the ^{13}C-OABT and the induced ^{13}C-LUBT were both easy to perform, and produced quantitative data that were relatively simple to model for generation of intestinal transit parameters. As the tests require minimal equipment at the site of application it is hoped that there may be clinical uptake of these procedures for diagnostic use in addition to much needed research application. It was demonstrated in Chapter 9 that the sample collection protocol could be reduced significantly in duration for each test, without compromising test results in normal individuals. However, shortened sample duration may lead to underestimation of transit parameters in cases of gastroparesis or small intestinal ileus, and is therefore not recommended if the tests are being conducted for detection of these very same conditions. The linear regression models, based on the collection of a baseline sample and 5 subsequent expiratory breath samples gave an excellent correlation to the full sampling protocols for parameter estimation. However, the linear regression models were also less accurate for the estimation of parameters lying outwith the interquartile range.

The tests should not prove to be prohibitively expensive, particularly as cheaper methods of tracer analysis are constantly being sought and developed, and the ^{13}C-OABT is certainly cheaper to perform than scintigraphic assessment of gastric emptying. Samples may also be stored in Exetainers® for prolonged periods if necessary prior to analysis at the nearest stable isotope facility. As the uptake of stable isotope breath tests increases in veterinary medicine it is hoped that dedicated analysis and interpretation centres will be established for the handling of veterinary samples. Breath collection is simple to perform

in the horse. So long as a true expiratory breath sample of above 0.5% CO_2 is collected, absolute concentration of CO_2 in the sample is not critical as analytical technique depends on the relative measurement of the ^{13}C:^{12}C ratio. However, if breath collection cannot be performed, 1 ml blood samples stored in 10 ml Vacutainers® form an acceptable alternative for analysis.

10.5 Future applications of the ^{13}C-OABT and ^{13}C-LUBT

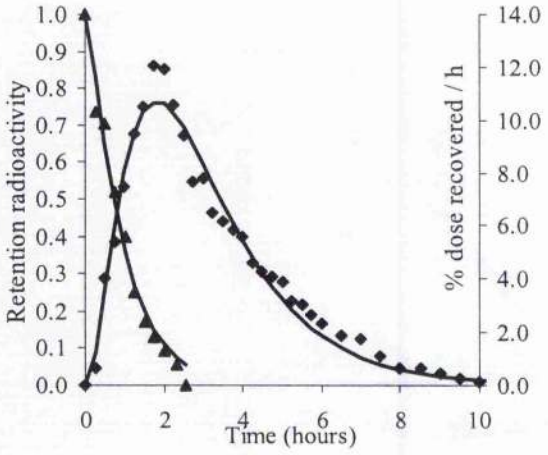
Having validated these stable isotope breath tests for the measurement of GE rate and orocaecal transit, the ^{13}C-OABT, ^{13}C-LUBT and dual isotope test would potentially by suitable for a number of applications. These would include diagnostic investigation of clinical cases presenting with abdominal pain or weight loss and the investigation of specific nutritional, managemental and pharmacological factors on equine gastrointestinal transit in controlled prospective trials. Application of the tests is also planned in future studies to determine the effect of prokinetic agents on parameters of intestinal transit in both healthy and diseased individuals. In conclusion, the stable isotope breath tests have been demonstrated to be valid modalities for the measurement of specific parameters of equine gastrointestinal transit of solids. As they are quantitative, simple to perform and non-invasive, they may provide a much needed technique for the implementation of an evidence-based medicine approach into the effect of multiple factors on intestinal motility that have been identified previously as risk factors for the development of equine colic.

APPENDIX I

Gastric emptying studies in 12 healthy adult horses, showing results of ^{13}C-OABT (% dose recovery/h) and simultaneous left lateral gastric scintigraphy (retention of radioactivity)

The full set of data discussed in Chapter 3, from the original validation of the ^{13}C-OABT against radioscintigraphy for the measurement of solid phase gastric emptying rate in healthy horses, is presented graphically in this appendix. Gastric emptying was measured simultaneously in each subject by scintigraphy and ^{13}C-OABT after the ingestion of a test meal labelled with ^{99m}technetium sulphur colloid and ^{13}C-octanoic acid. The results of each test are plotted on the same graph for each subject, against time. The retention of radioactivity in the stomach as measured by left lateral scintigraphy is shown on the left *y*-axis in each case, and the expiratory recovery of the ^{13}C isotope is presented as % dose recovered / h on the right *y*-axis. The correlations between the gastric emptying indices generated by these individual data sets have been presented previously in Chapter 3. It can be seen qualitatively in these graphs that rapid loss of radioactivity from the stomach is matched by rapid appearance of the ^{13}C-tracer in the breath (e.g. Figure AI.4) whilst prolonged retention of gastric radioactivity is matched by a lag phase in the expiratory appearance of the ^{13}C-isotope (Figure AI.12).

The scintigraphic data presented were modelled using Siegel's non-linear regression formula as given in section 2.6.3.1 (Equation 2.7) and the breath test data were modelled using the variant of Siegel's formula, based on the cumulative recovery of ^{13}C in the breath (Equation 2.8). This enabled direct comparison of the gastric emptying indices generated by each diagnostic imaging modality to be made.



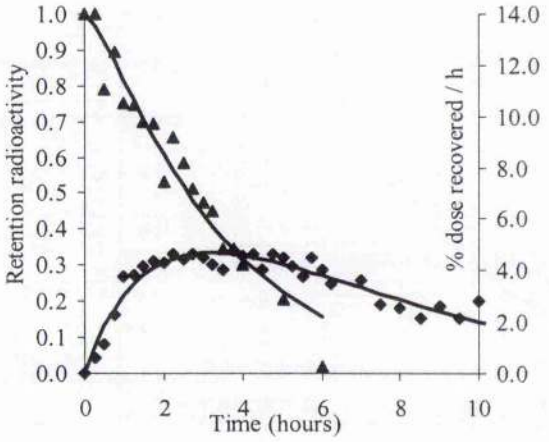
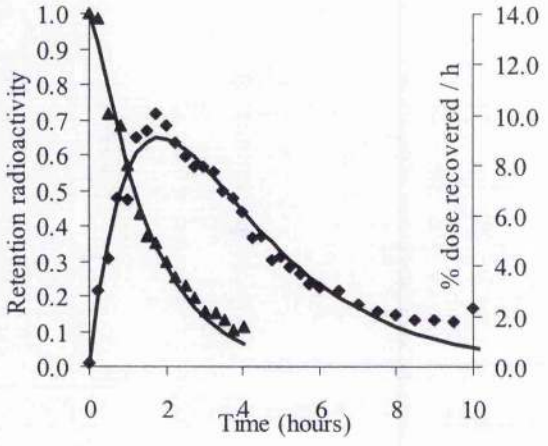


Figure AI.1 (left) Combined left lateral ^{99m}Tc-SC scintigraphic and ^{13}C-OABT gastric emptying study in horse 1891, a 3.5 y.o. Quarter Horse (QH) gelding (weight 461 kg). The retention of gastric radioactivity is shown on the left *y*-axis and the expiratory recovery of the ^{13}C isotope on the right *y*-axis.

Figure AI.2 (right) Simultaneous combined gastric emptying study in subject 1894, a 10 y.o. QHX gelding (weight 501 kg).



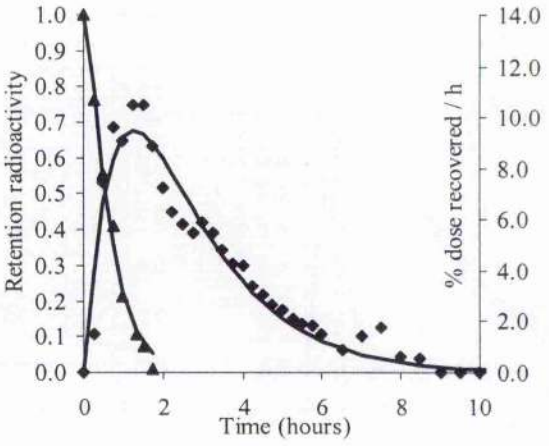
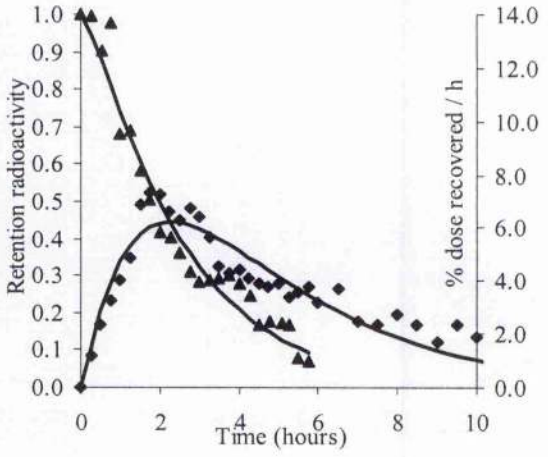


Figure AI.3 (left) Combined scintigraphic and ^{13}C-OABT gastric emptying study in horse 1884, a 9 y.o. QHX gelding (weight 513 kg).

Figure AI.4 (right) Simultaneous combined gastric emptying study in horse 1916, a 2 y.o. QH gelding (weight 347 kg).



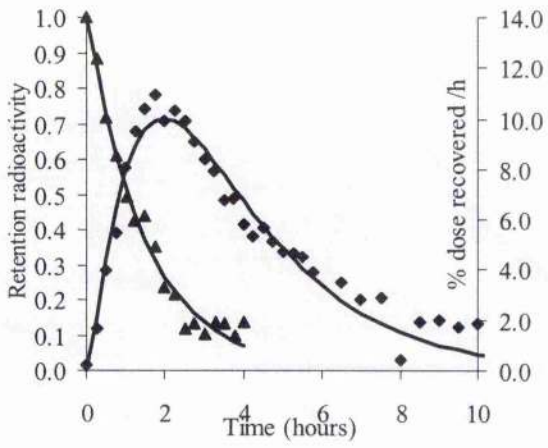
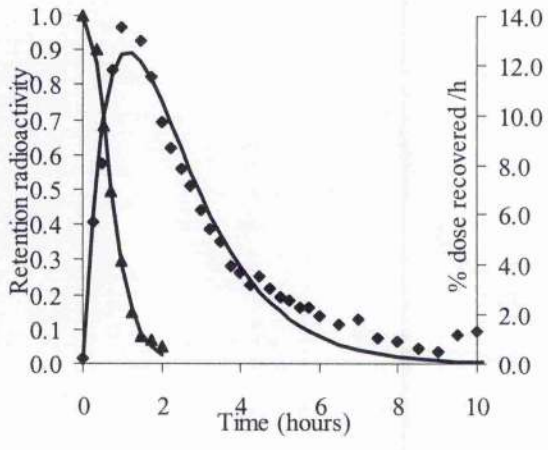


Figure AI.5 (left) Simultaneous scintigraphic and ^{13}C-OABT gastric emptying study in horse 1881, a 25 y.o. QH gelding (weight 472 kg).

Figure AI.6 (right) Combined gastric emptying study, showing gastric retention of radioactivity (left *y*-axis) and expiratory recovery of $^{13}CO_2$, after ^{13}C-OABT (right *y*-axis) in horse 1914, a 7 y.o. QH mare (weight 445 kg).



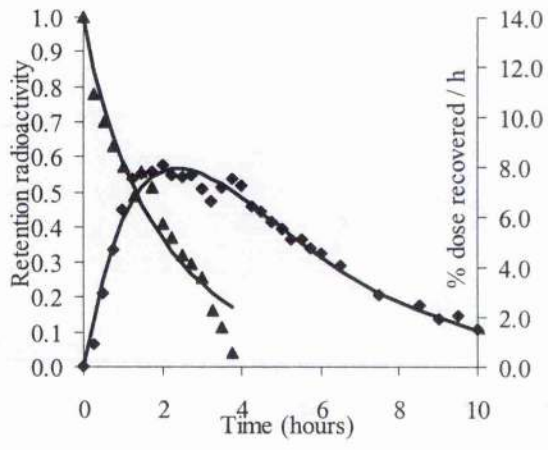
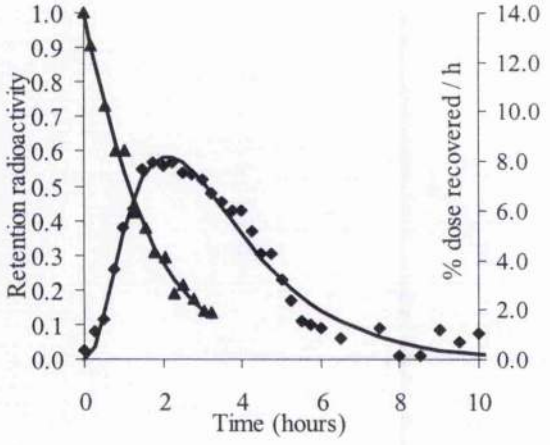


Figure AI.7 (left) Simultaneous scintigraphic and ^{13}C-OABT gastric emptying study in horse 1920, a 4 y.o. QH mare of body weight 463 kg.

Figure AI.8 (right) Combined gastric emptying study, showing scintigraphic detection of gastric radioactivity on left *y*-axis, and expiratory recovery of the administered ^{13}C isotope on the right *y*-axis. Study performed in horse 1928, a 25 y.o. QH gelding of body weight 454 kg.



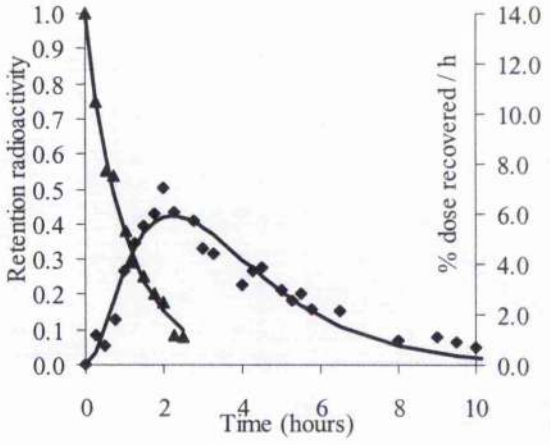
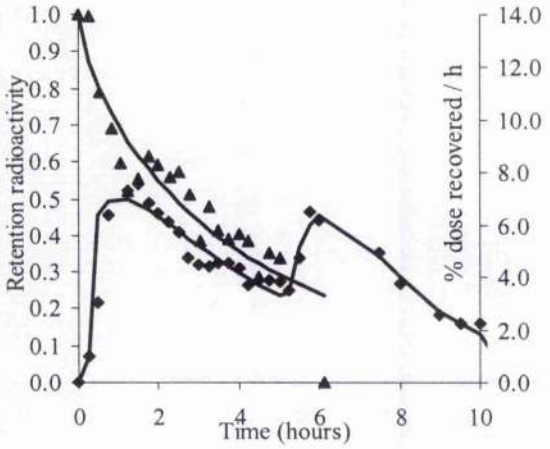


Figure AI.9 (left) Simultaneous scintigraphic and ^{13}C-OABT gastric emptying study performed in horse 1927, a 20 y.o. Thoroughbred mare, of body weight 528 kg. Gastric retention of radioactivity is plotted on the left *y*-axis and expiratory recovery of the ^{13}C-isotope on the right *y*-axis.

Figure AI.10 (right) Combined scintigraphic and ^{13}C-OABT gastric emptying study in horse 1929, a 7 y.o. QH gelding of body weight 564 kg.



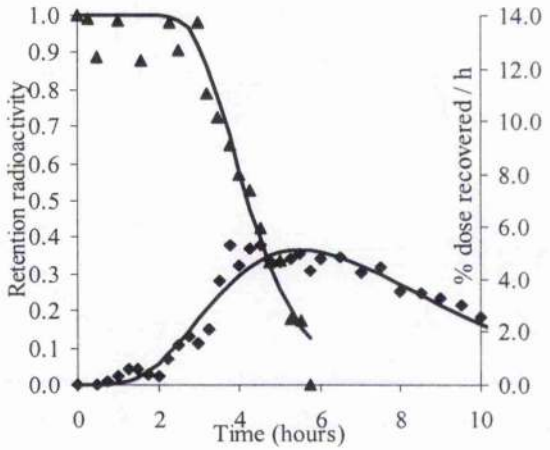


Figure AI.11 (left) Combined scintigraphic and ^{13}C-OABT gastric emptying study in horse 1925, a 3.5 y.o. Arab female, showing biphasic expiratory recovery of the exhaled ^{13}C isotope and a plateau in the loss of radioactivity from the left gastric region.

Figure AI.12 (right) Simultaneous gastric emptying study using radioscintigraphy and ^{13}C-OABT in horse 1889, a 17 y.o. QHX gelding of body weight 533 kg. There is a pronounced lag phase in both the loss of radioactivity from the left gastric region and the recovery of $^{13}CO_2$ in the breath after ingestion of the labelled test meal. A repeat of the gastric emptying study was performed in this individual with similar results.

APPENDIX II

Delayed gastric emptying in a horse presenting with a gastric impaction as demonstrated by the ^{13}C-OABT

AII.1 Case History

An 8 y.o. Thoroughbred gelding (Case 138250) was referred to the Glasgow University Veterinary School Equine Hospital for the further investigation and treatment of moderate abdominal pain of 48 hours' duration. The horse had been lethargic and lost condition over the previous month. In view of this, the owner had removed the horse from pasture and stabled it 2 weeks prior to presentation, with supplemental feeding of concentrates and *ad libitum* hay. Appetite had proved to be variable during this period. In addition to the apparent abdominal pain, faecal output had been decreased in the 48 hours before referral.

AII.2 Clinical Abnormalities

Although the horse displayed signs of moderate abdominal pain, pulse, respiratory rate and peripheral haematocrit were all within reference range on initial examination. Intestinal borborygmi were present in all 4 abdominal quadrants, and of normal frequency and intensity. However, a large doughy mass was palpable in the right dorsal abdomen on examination *per rectum*, and abdominal paracentesis yielded a serosanguineous sample of peritoneal fluid. A small amount of feed material was gained after gastric lavage with a nasogastric tube, but no excessive gastric fluid.

AII.3 Further investigation and treatment

In view of the chronicity of the symptoms, persistent pain and the abnormal peritoneal fluid, an exploratory laparotomy was performed. The stomach was found to be grossly enlarged and impacted with doughy material. The remainder of the small and large bowel appeared normal on inspection and palpation. In order to break up the impacted material, water was pumped into the stomach using a nasogastric tube and ciphoned out whilst its serosal surface was massaged directly. Only small quantities of food material could be refluxed via the stomach tube and therefore the softened mass was left to pass through the intestinal tract.

AII.4 Case Progression

The horse made a good recovery after surgery. Liquid paraffin was administered twice *per os* in the first 48 hours, but the horse was otherwise maintained on intravenous fluids and allowed nothing by mouth. Gastroscopic examination was performed after this interval and

showed that the stomach was empty, with no ulceration, nor obvious abnormality of the pyloric antrum or proximal duodenum. Small concentrate feeds were gradually reintroduced over the subsequent days and the horse remained free from signs of abdominal pain. In order to investigate the cause of the gastric impaction a ^{13}C-OABT was performed 9 days after surgery to measure gastric emptying rate. The test was performed according to the standard protocol outlined in section 2.4.4. The % dose recovery of the ^{13}C isotope in the breath is shown plotted against time in Figure AI.1. Gastric emptying rate was markedly delayed, with a prolonged triturative phase (t_{lag} = 6.30 h) and slow half-emptying time ($t_{1/2}$ = 7.03 h). These values lay beyond the previously determined cut-off points for 'delayed' emptying from data collected previously in healthy horses (see Chapter 4).

No further abnormalities were found in the horse's dentition or intestinal tract. The horse was discharged on a regimen of stall rest, small and frequent concentrate feeds, grass and grass hay via a fine hay net. The horse was re-examined after 6 weeks, and the owner reported that its appetite and faecal output had remained normal in the intervening period. The ^{13}C-OABT was repeated, again following the standard protocol. The results are presented in Figure AI.2. A biphasic recovery of isotope was noted, consistent with discrete gastric emptying events. However, gastric emptying rate was improved compared with 6 weeks previous (t_{lag} = 1.37 h, $t_{1/2}$ = 4.24 h) and using the criteria presented in Chapter 4, was classified as within normal reference range. No recurrence of symptoms has been noted in the two years since discharge.

AII.5 Discussion

Primary gastric impaction has been defined as an abnormal accumulation of dry, non-swelling, poorly fermentable ingesta held in the stomach (Barclay *et al.* 1982; Foerner *et al.* 1983; Owen *et al.* 1983). The condition has a different presentation and aetiology to gastric dilatation in which the stomach is acutely distended by intra-gastric fermentation or toxic insult (Schulman and Bolton 1998). Gastric impaction has been linked to extrinsic factors including ingestion of fibrous grasses or straw (Owen *et al.* 1987), persimmon seeds or mesquite beans (Honnas and Schumacher 1985; Kellam *et al.* 2000), and irregular water supply or poor dentition (Owen *et al.* 1987). It has been hypothesised that intrinsic factors such as defective gastric secretion or gastric atony may be implicated in the aetiology of primary impaction (Owen *et al.* 1987) although this has not been proven to the author's knowledge. Gastric impaction has also been recorded secondary to hepatic failure (McGorum *et al.* 1999) and specifically to ragwort toxicity (Milne *et al.* 1990).

None of the reported risk factors were found to be present in this case, apparently confirming a diagnosis of primary gastric impaction of unknown aetiology. Similar cases of unknown cause have been reported previously (Barclay *et al.* 1982). The presence of delayed gastric emptying in this horse as confirmed by the ^{13}C-OABT was therefore of considerable interest, and the possible causative aetiological factor. However, the possibility of secondary gastric dysmotility subsequent to an initial stretching injury cannot be discounted, particularly given the improved gastric emptying rate seen in this horse 6 weeks after recovery. It is concluded that reduced gastric motility may be an inciting cause in the development of equine primary gastric impaction. Further use of the ^{13}C-OABT in horses presenting with gastric impaction may help to elucidate the importance of delayed gastric emptying in the aetiology of this condition. Furthermore, the ^{13}C-OABT is a helpful diagnostic tool in such cases for assessment of prognosis and response to treatment.

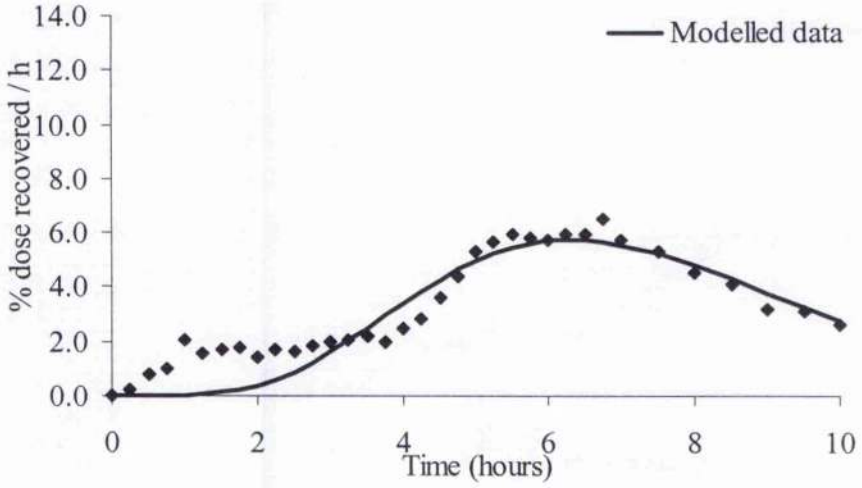


Figure AII.1 Gastric emptying profile generated by the ^{13}C-OABT in an 8 y.o. Thoroughbred gelding (case 138250), 9 days after presentation with, and treatment for, a gastric impaction. The % dose recovery of the administered ^{13}C isotope is plotted on the *y*-axis against time. Gastric emptying is delayed: half-emptying time ($t_{1/2}$) = 7.03 h; gastric lag phase (t_{lag}) = 6.30 h; Gastric Emptying Coefficient (GEC) = -3.16.

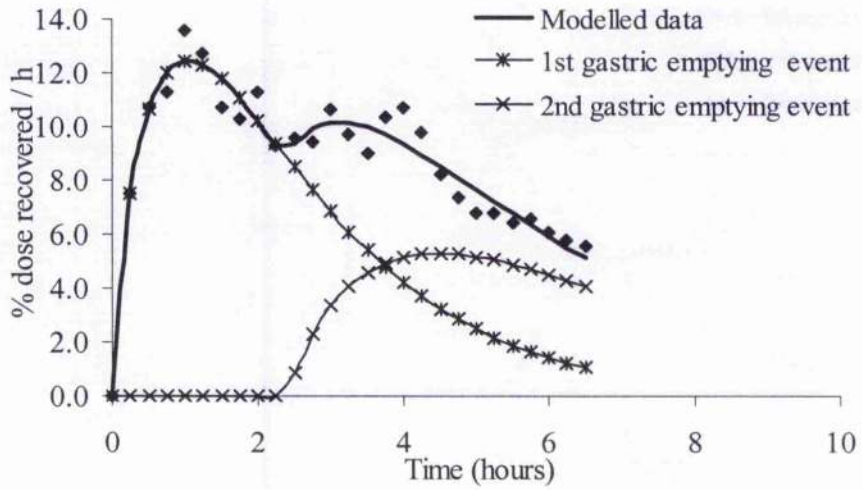


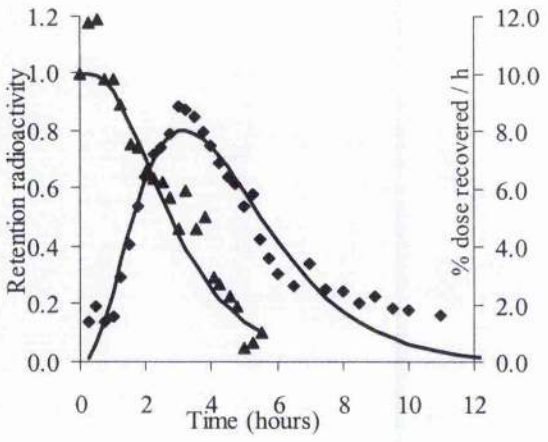
Figure AII.2 Results of a ^{13}C-OABT performed in the same horse 6 weeks after receiving treatment for a gastric impaction. The expiratory recovery of the stable isotope is best fitted by a dual peak model, possibly due to a second gastric emptying event. The overall rate of gastric emptying is improved compared with Figure AII.1: $t_{1/2}$ = 4.24 h; t_{lag} = 1.37 h; GEC = 2.67.

APPENDIX III

Gastric emptying studies in 8 adult horses with delayed gastric emptying induced by the administration of atropine. The results of ^{13}C-OABT and simultaneous left lateral gastric scintigraphy are shown.

The data discussed in Chapter 4, from the validation of the ^{13}C-OABT against radioscintigraphy for the measurement of atropine-induced delayed solid phase gastric emptying in healthy horses, are presented graphically in this appendix. Atropine was administered immediately after ingestion of the dual labelled test meal in 8 adult horses. A standard dose of 0.035 mg/kg bwt atropine was administered intravenously. Gastric retention of the ^{99m}Tc-SC labelled test meal was mapped scintigraphically and the rate of duodenal entry of the ^{13}C-octanoic acid was measured indirectly by determining the rate of increase in expiratory ^{13}C:^{12}C ratio. The results of each test are plotted on the same graph for each subject, against time. The retention of radioactivity in the stomach as measured by left lateral scintigraphy is shown on the left *y*-axis in each case, and the expiratory recovery of the ^{13}C isotope is presented as % dose recovered / h on the right *y*-axis. The correlations between the gastric emptying indices generated by these individual data sets have been presented previously in Chapter 4. The scintigraphic data presented were modelled using Siegel's non-linear regression formula as given in section 2.6.3.1 (Equation 2.7) and the breath test data were modelled using the variant of Siegel's formula, based on the cumulative recovery of ^{13}C in the breath (Equation 2.8) and also by Maes' formula (Equation 2.12). Where possible, the breath test data are shown modelled by a single peak Maes' model for ease of comparison. However, in one individual (Figure AIII.8) a double peak model was required.

In Figures AIII.1 - AIII.6, there is an apparent increase in the gastric radioactive counts per second after time 0 h as evidenced by the increase in gastric retention of radioactivity above 1.0. As discussed in more detail in Chapter 4, this may have been caused by a combination of factors related to the administration of atropine: prolonged oesophageal transit of the labelled ingesta; loss of proximal gastric tone, or a movement of ingesta from the distal to proximal stomach, due to a loss of coordinated antro-pyloric propulsion. In Figures AIII.2 – A.III.5 and AIII.7 – AIII.8, there is also biphasic recovery of the ^{13}C tracer in the breath. This may have been caused by delayed onset of action of the atropine or the possible presence of gastric ulceration in these individuals (see Chapter 4).



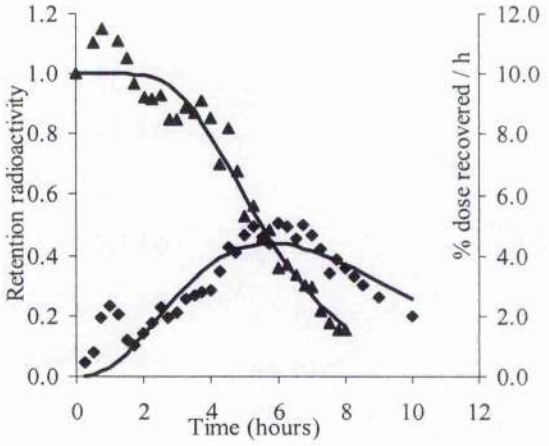
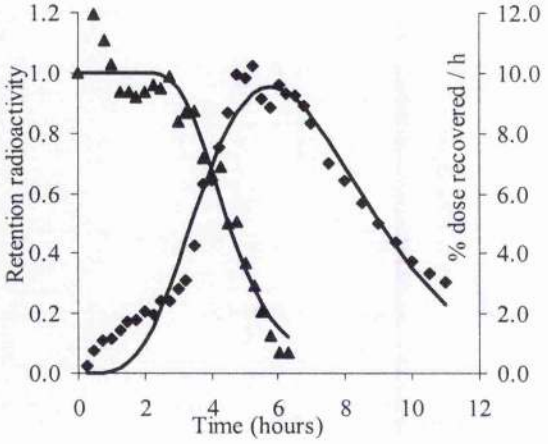


Figure AIII.1 (left) Simultaneous scintigraphic and ^{13}C-OABT gastric emptying study in a horse with delayed gastric motility, induced by the intravenous administration of atropine 0.035 mg/kg bwt immediately after test meal ingestion. The gastric retention of ^{99m}Tc-SC is shown on the left *y*-axis and the expiratory recovery of $^{13}CO_2$ on the right *y*-axis. Subject 1953, a 13 y.o. QH gelding of body weight 502 kg, is shown.

Figure AIII.2 (right) Combined scintigraphic and ^{13}C-OABT gastric emptying study in subject 1937, a 10 y.o. QH gelding (425 kg bwt), after administration of atropine 0.035 mg/kg bwt at time 0 h.



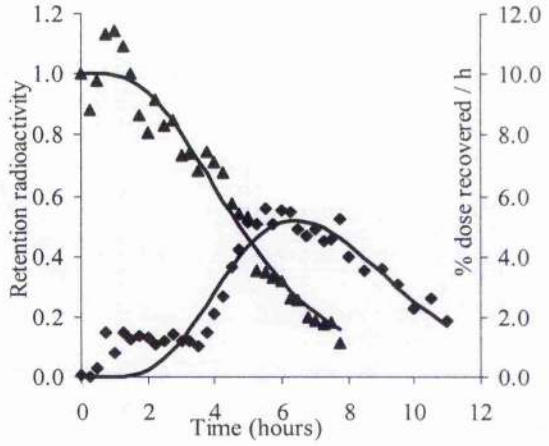
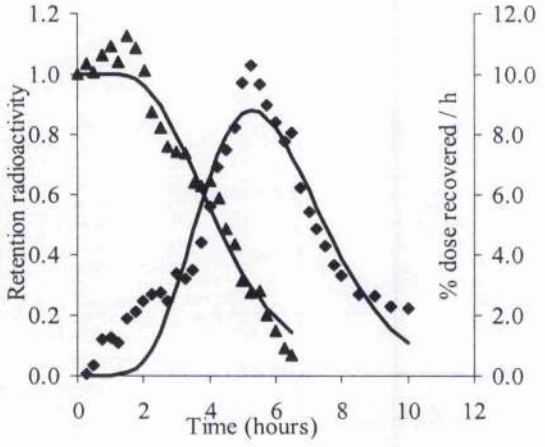


Figure AIII.3 (left) Simultaneous scintigraphic and ^{13}C-OABT gastric emptying study in horse 1944, a 12 y.o. QH gelding of body weight 519 kg, after administration of atropine 0.035 mg/kg bwt. The individual gastric emptying parameters are detailed in Chapter 4.

Figure AIII.4 (right) Combined gastric emptying study in a healthy subject with delayed gastric emptying rate induced by the intravenous administration of atropine 0.035 mg/kg. Subject shown is 1965, a 25 y.o. Thoroughbred mare of body weight 526 kg.



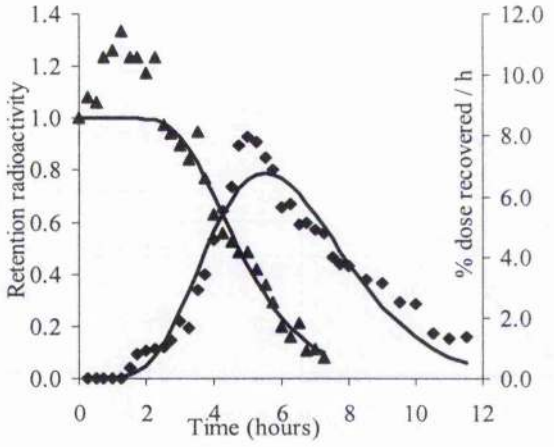
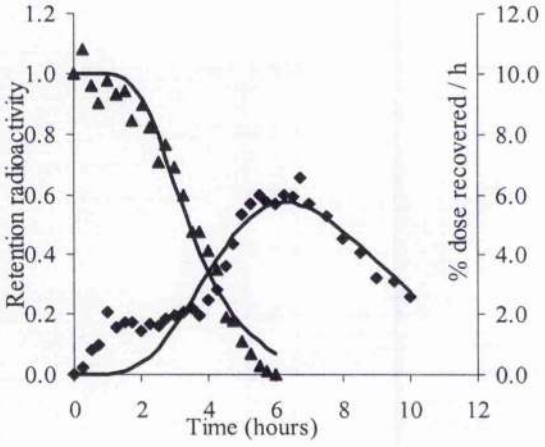


Figure AIII.5 (left) Combined scintigraphic and ^{13}C-OABT gastric emptying study in a 17 y.o. Thoroughbred gelding (subject 1947) with delayed gastric emptying induced by the administration of atropine 0.035 mg/kg IV. The gastric retention of radioactivity as determined by left lateral scintigraphy is shown on the left *y*-axis and the expiratory recovery of the ^{13}C-tracer on the right *y*-axis.

Figure AIII.6 (right) Combined gastric emptying study in subject 1936, an 11 y.o. QH gelding of body weight 510 kg, after intravenous administration of atropine at 0.035 mg/kg bwt.



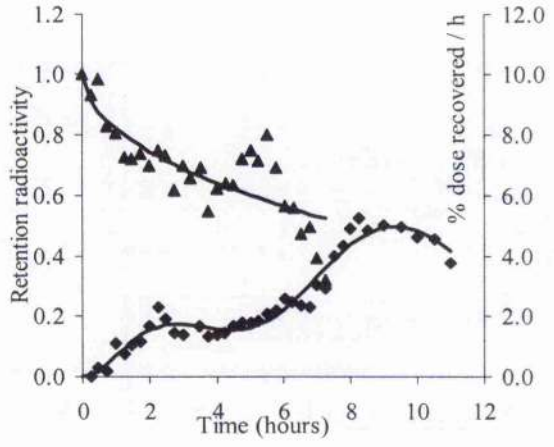


Figure AIII.7 (left) Demonstration of delayed gastric emptying by combined scintigraphic and ^{13}C-OABT modalities in subject 1949, a 14 y.o. QH gelding of body weight 522 kg, after intravenous treatment with atropine at 0.035 mg/kg bwt at time 0 h.

Figure AIII.8 (right) Markedly delayed gastric emptying in subject 1970, a 7 y.o. Thoroughbred gelding (body weight 530 kg) as demonstrated both by gastric scintigraphy (left *y*-axis) and ^{13}C-OABT (right *y*-axis). A biphasic expiratory recovery of the ^{13}C-tracer is seen, together with a biphasic loss of gastric radioactivity. Gastric emptying was delayed in this subject by administration of 2 doses of atropine 0.035 mg/kg at times 0 h and 1.75 h after ingestion of the labelled test meal.

APPENDIX IV

Reduced sampling protocols: development of linear regression models for calculation of equine ^{13}C-OABT gastric indices, based on Siegel's non-linear regression estimates

As described in section 9.3.2, stepwise linear regression (Minitab® release 13) was used to develop mathematical models for the prediction of equine gastric emptying parameters based on the ^{13}C:^{12}C ratio in a minimum number of expiratory breath samples after ingestion of ^{13}C-octanoic acid. Regression models designed to approximate equine $t_{1/2}$ and t_{lag} as generated by Maes' formula from the full complement of 32 breath samples were described in Chapter 9. For completion, the 3-, 4- and 5-sample regression models developed for estimation of $t_{1/2}$ and t_{lag} as generated by Siegel's formula are shown in this Appendix. As stated previously, modelling of equine gastric emptying data with these two different non-linear regression formulae produces similar results. The linear regression formulae described here for the Siegel's model reduced protocols are also similar to those described in Chapter 9 for the Maes' model reduced protocols. The best models for Siegel's $t_{1/2}$ ($St_{1/2}$) and t_{lag} (St_{lag}) estimation, based on the initial $^{13}CO_2$ output and the ^{13}C:^{12}C ratio of a further 3, 4 or 5 breath samples, are given in Table AIV.1.

Table AIV.1. Linear regression formulae for estimation of equine $t_{1/2}$ and t_{lag}, derived from ^{13}C-OABTs performed in 64 horses, with original expiratory isotopic recovery modelled by Siegel's formula (Equation 2.7).

^{13}C-Octanoic acid breath test - Siegel's formula									
	Samples	3	4	5		Samples	3	4	5
$St_{1/2}$ (h)	Constant	2.430	2.540	3.278	St_{lag} (h)	Constant	2.129	2.119	2.448
	$t_{5.75\ h}$	0.228	0.180	0.147		$t_{5.75\ h}$	0.351	0.202	0.192
	$t_{1.75\ h}$	-0.084	-0.064	-0.051		$t_{0.75\ h}$	-0.107	-0.112	-0.123
	$t_{6.50\ h}$	0.226	0.267	0.237		$t_{2.00\ h}$	-0.069	-0.066	-0.057
	$t_{1.00\ h}$		-0.035	-0.057		$t_{6.50\ h}$		0.194	0.176
	$t_{2.75\ h}$			-0.057		$t_{2.75\ h}$			-0.029
	R^2 value	91.92	93.06	94.38		R^2 value	93.39	94.41	94.68

The final 5-sample model for the best estimation of gastric t_{lag} when compared to the Siegel's formula estimate in 64 horses is given by Equation AIV.1:

$$St_{lag} = 2.448 + (t_{5.75\,h}*0.192) - (t_{0.75\,h}*0.123) - (t_{2.00\,h}*0.057) + (t_{6.50\,h}*0.176) - (t_{2.75\,h}*0.029) \quad \textbf{Equation AIV.1}$$

The final 5-sample model for the best estimation of gastric $t_{1/2}$, compared to the Siegel's formula estimates from 64 horses is given in Equation AIV.2:

$$St_{1/2} = 3.278 + (t_{5.75\,h}*0.147) - (t_{1.75\,h}*0.051) + (t_{6.50\,h}*0.237) - (t_{1.00\,h}*0.057) - (t_{2.75\,h}*0.057) \quad \textbf{Equation AIV.2}$$

The reduced model estimates for calculation of both St_{lag} and $St_{1/2}$ were compared to the results of the original 32-sample protocol ^{13}C-OABT in 64 healthy adult horses by both linear regression and Bland Altman plots. The results of the 3-, 4- and 5-sample models for each parameter are shown in Figures AIV.1 to AIV.6. It can be seen that the final 5-sample linear regression models produced results that were significantly correlated to the data from the 32-sample protocol as modelled by Siegel's formula. For St_{lag}, $r = 0.972$, $P < 0.001$. There was also an approximately constant difference between the models across a wide range of values: mean (± s.d.) difference = 0.003 ± 0.267 h. For $St_{1/2}$, $r = 0.970$, $P < 0.001$. The correlation between the final 5-sample model and the original results was particularly close in the upper and lower quartiles. The mean (± s.d.) difference between the techniques for $St_{1/2}$ estimation was 0.013 ± 0.252 h. The 3- and 4-sample protocols for each parameter also produced results that were significantly correlated with the original data sets, but the reduced sampling protocols increased the 95% limits of agreement of the mean difference between the modelled parameters.

It is concluded that reduced linear regression models may be used to model the results of equine ^{13}C-OABT gastric emptying studies. A minimum of the original baseline sample ^{13}C:^{12}C ratio, and the % dose recovery/h of a further 5 expiratory samples allows good approximation of the gastric emptying indices as modelled by Siegel's formula from an original complement of 32 breath samples. As stated in Chapter 9, the possibility of a reduced sampling protocol should maximise the utility of the ^{13}C-OABT for the measurement of gastric emptying rate in equine practice.

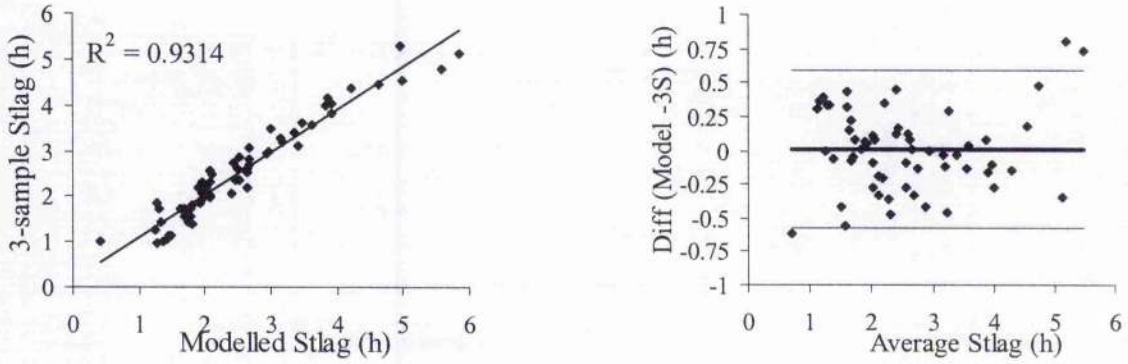


Figure AIV.1 Comparison of 3-sample reduced linear regression model with full 32-sample protocol for estimation of gastric t_{lag} using the ^{13}C-OABT and Siegel's non-linear regression model (n = 64). The Bland Altman plot shows the mean difference and its 95% limits of agreement (right).

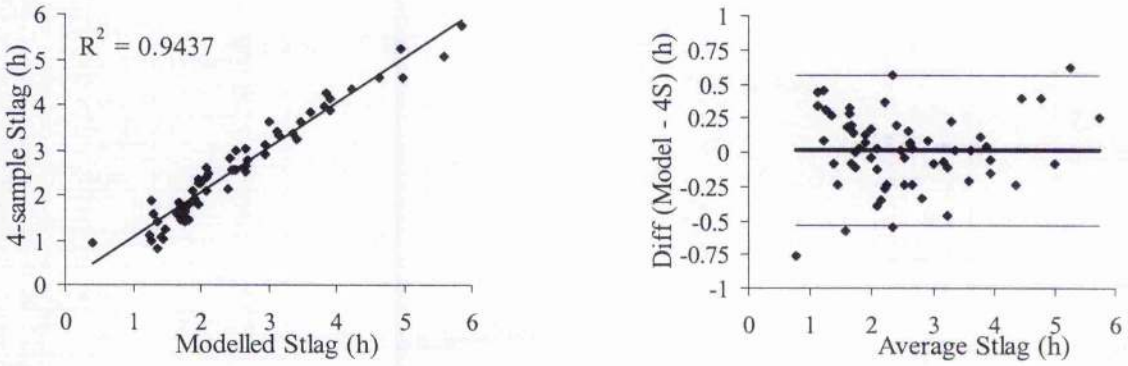


Figure AIV.2 Comparison of 4-sample reduced model results for gastric t_{lag} with those gained by the full 32-sample protocol and Siegel's non-linear regression formula. The specific linear regression formulae for the reduced protocol are given in Table AIV.1.

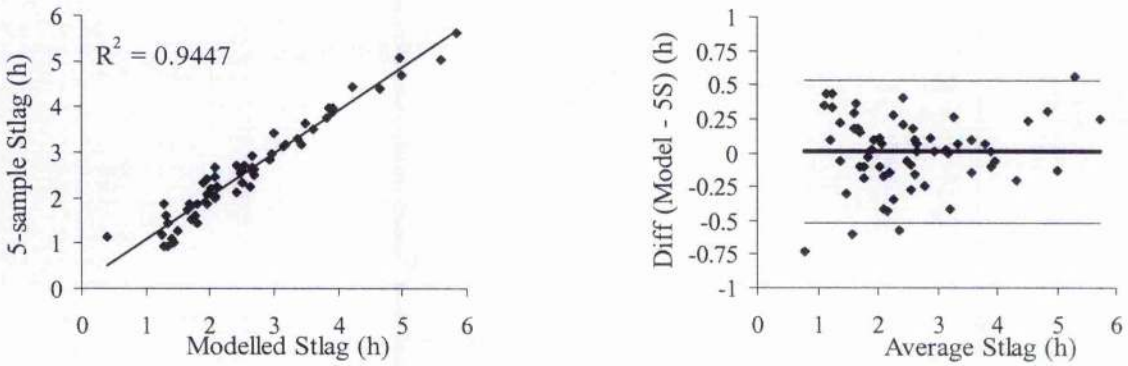


Figure AIV.3 Calculation of gastric t_{lag} using the ^{13}C-OABT: comparison of the results of the final developed 5-sample linear regression protocol with those of the full sampling protocol (n = 64). Siegel's formula was used to calculate t_{lag} (St_{lag}) from the original protocol. There is an excellent linear correlation between the calculation methods (r = 0.972, P < 0.001). The mean (± s.d.) difference = 0.003 ± 0.267 h.

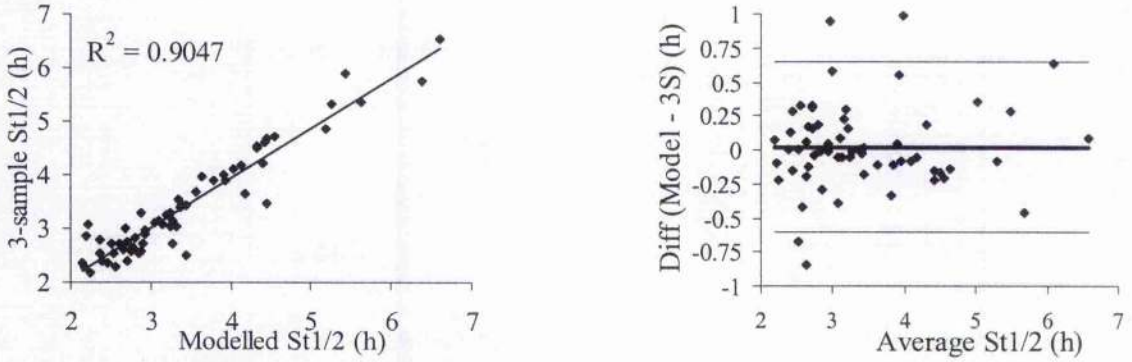


Figure AIV.4 Comparison of 3-sample reduced linear regression model with full 32-sample protocol for estimation of gastric $t_{1/2}$ using the ^{13}C-OABT and Siegel's non-linear regression model (n = 64). The Bland Altman plot shows the mean difference and its 95% limits of agreement for $St_{1/2}$ (right).

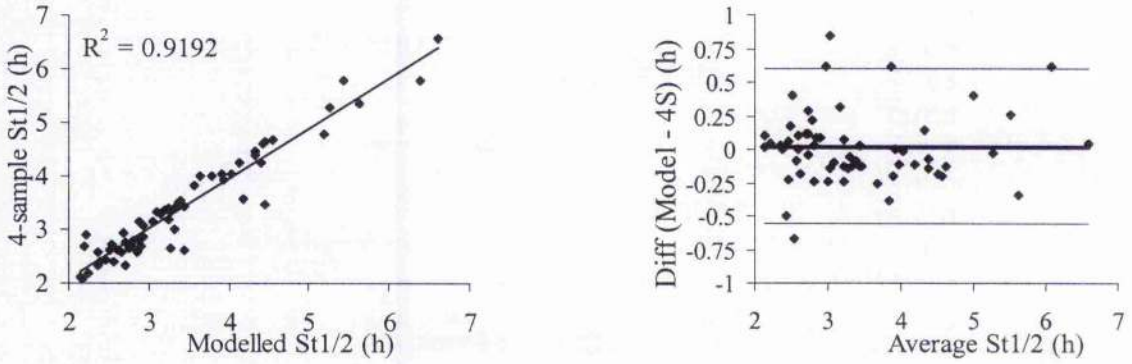


Figure AIV.5 Comparison of 4-sample reduced model results for gastric $t_{1/2}$ with those gained by the full 32-sample protocol and Siegel's non-linear regression formula. The specific linear regression formulae for the reduced protocol are given in Table AIV.1.

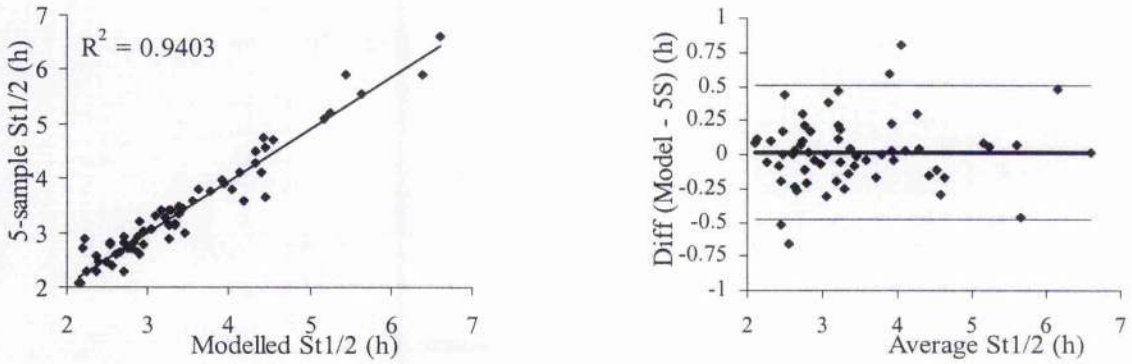


Figure AIV.6 Calculation of gastric $t_{1/2}$ using the ^{13}C-OABT: comparison of the results of the final developed 5-sample linear regression protocol with those of the full sampling protocol (n = 64). Siegel's formula was used to calculate $t_{1/2}$ ($St_{1/2}$) from the original protocol. There is an excellent linear correlation between the calculation methods (r = 0.970, P < 0.001). The mean (± s.d.) difference = 0.013 ± 0.252 h.

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