ACE INHIBITORS - EVALUATION OF DISPOSITION CHARACTERISTICS AND CONCENTRATION EFFECT RELATIONSHIPS

by

Janet R. Harrigan

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DECLARATION

I declare that this thesis has been written by myself and is a record of work performed by myself. It has not been submitted previously for a higher degree.

This research was carried out in the department of Medicine and Therapeutics, University of Glasgow, under the supervision of Dr. P.A. Meredith and Professor J.L. Reid.

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LIST OF ABBREVIATIONS

A I	- Angiotensin I
A II	- Angiotensin II
ACE	- Angiotensin Converting Enzyme
ADH	- Antidiuretic Hormone
AES	- Automated External Standard
ANOVA	- Analysis Of Variance
AUC	- Area Under the plasma Concentration time curve
Bmax	- Maximum number of binding sites
C(50)	- Concentration of ACE inhibitor required to inhibit ACE by 50%
CHF	- Congestive Heart Failure
CI	- Confidence Interval
CL	- Clearance
Cmax	- Maximum Plasma Concentration
Cov	- Covariance
CPM	- Counts Per Minute
CV	- Coefficient of Variation
DPM	- Disintegrations Per Minute
F	- Bioavailability
GC-MS	- Gas Chromatography - Mass Spectrometry
GFR	- Glomerular Filtration Rate
HHL	- Hip-His-Leu
Ho	- Null Hypothesis
HPLC	- High Pressure Liquid Chromatography
IBA	- Inhibitor Binding Assay
i.p.	- Intraperitoneal (dose)
i.v.	- Intravenous (dose)
Ke	- Elimination rate constant
Ki	- Inhibitory rate constant
Km	- Michaelis Menton rate constant
LHRH	- Luteinising Hormone Releasing Hormone
Ln	- Natural Logarithm
MLQ	- Minimum Limit of Quantitation
NEM	- N-Ethylmaleimide
NSB	- Non Specific Binding
PMT	- Photomultiplier Tube
PRA	- Plasma Renin Activity
QC	- Quality Control
RAS	- Renin Angiotensin System
RBC	- Red Blood Cell
RIA	- Radioimmuno Assay
SD	- Standard Deviation
SHR	- Spontaneously Hypertensive Rats
Tabs	- Length of time over which absorption takes place
Tlag	- Lag time
TLRC	- Thin Layer Radio Chromatography
Tmax	- Time of maximum plasma concentration
UV	- Ultra Violet detection
Var	- Variance
Vc	- Volume of the central compartment
Vd	- Volume of distribution

SUMMARY

Extensive study of the active site of angiotensin converting enzyme (ACE) has led to the development of specific inhibitors which are now successfully used in the treatment of hypertension and cardiac failure. The work carried out within this thesis has been concerned with the evaluation of the disposition characteristics of the ACE inhibitors and their concentration effect relationships.

Characterisation of the pharmacokinetics of any compound is dependent upon the availability of accurate, precise, sensitive and specific assay methodology. Three groups of methods have been compared within this thesis, two different methods to measure plasma ACE activity, three methods to measure plasma enalaprilat levels and three methods to measure plasma benazeprilat levels. The methods to measure plasma ACE activity yielded results in good agreement. One of the three methods to measure plasma enalaprilat levels gave results that were significantly lower than the remaining two methods. No obvious explanation could be found for this. Of the three methods to measure benazeprilat, the specific method gave significantly lower results than the two non specific methods, the possible existence of unstable glucuronides contributing to the discrepancy between the methods. These results highlight the need to know the specificity of any assay technique being used.

The concentration effect relationship of two different series of ACE inhibitors was assessed in plasma from

individual rabbits and volunteers. Differences were found in both the rank order of potency of the compounds between rabbit and man, and also, there were significant differences in the potency of single compounds between rabbit and man. These differences may reflect variations in the tertiary structure of ACE between rabbit and man and indicate that different doses would be needed to obtain the same degree of ACE inhibition.

The in vivo/in vitro relationship for plasma ACE inhibition was assessed for perindoprilat and quinaprilat. For both compounds the in vivo values were significantly lower than those found in vitro, indicating greater inhibition of ACE in vivo. Any calculations based upon the in vitro values would overestimate the amount of drug needed to elicit the same response in vivo.

The third group of in vitro studies examined the effect of the presence of parent compound on the in vitro potency of the metabolite. In vitro dose response curves for five metabolites were characterised in the presence and absence of parent compound. The presence of parent compound brought about a significant decrease in potency for S-10211 (the active metabolite of S-9650) and quinaprilat in rabbit, and for enalaprilat and perindoprilat in man. The decrease in potency seen for some of the metabolites studied may be due to the parent compound binding to a second active site sequence on the ACE molecule where it causes steric hindrance but not ACE inhibition.

The rabbit was used as a model to characterise the effect of saturation of ACE binding sites on the pharmacokinetics of radiolabelled spiraprilat. Rabbits were pretreated with either placebo or unlabelled spiraprilat and the pharmacokinetics of a tracer radiolabelled intravenous dose of spiraprilat characterised. The pharmacokinetics of radiolabelled spiraprilat were best described by a three compartment model with a terminal elimination half life of the order of 2.5 hours. The effect of saturation of plasma and tissue ACE binding sites caused an increase in the rate of elimination during the second phase, the effect was small and did not contribute to a change in total clearance of the radiolabelled dose. Despite higher plasma ACE activity values in rabbit, the long terminal half life, a characteristic feature of ACE inhibitor drugs in man, was not observed in rabbit for radiolabelled spiraprilat. Thus, the rabbit would appear not to be a good model for the disposition of ACE inhibitors in man.
The pharmacokinetics of enalaprilat, formed after an oral dose of enalapril, were characterised in the presence and absence of saturated plasma and tissue ACE binding sites. Captopril was the agent used to saturate ACE binding sites. Eight volunteers received a single 10mg dose of enalapril. A washout period ensued then the volunteers were pretreated with captopril, 50mg twice daily, followed by a second 10mg dose of enalapril. Analysis of enalaprilat pharmacokinetics revealed no differences in the presence and absence of captopril. Analysis of the

plasma ACE activity data indicated that induction of plasma ACE had occurred during the pretreatment with captopril. Thus, the lack of any detectable change in enalaprilat pharmacokinetics could have been due to the induction of ACE by pretreatment with captopril. The resulting increase in the number of ACE binding sites may have negated the effect of captopril's occupancy of original ACE binding sites.

The pharmacokinetics of benazeprilat, formed after the acute and chronic administration of benazepril hydrochloride, were characterised in young and elderly volunteers. Nine young and nine elderly healthy volunteers were studied after the first and last doses of 10mg benazepril hydrochloride, once daily, for eight days. A series of models were fitted to the combined acute and chronic data, standard one and two compartmental models, and, models based upon a one compartment model which incorporated either binding to plasma ACE, binding to tissue ACE or binding to both plasma and tissue ACE. The vast majority of the subjects were best described by the one compartment model which incorporated tissue binding only. The value for the half life of elimination of free drug obtained from the model which best described the data for any one subject, was of the order of 2.5 hours. This is in keeping with the accumulation characteristics of benazeprilat.

CHAPTER 1

INTRODUCTION

1.1 INTRODUCTION

The use of Angiotensin Converting Enzyme (ACE) inhibitors in the treatment of hypertension and heart failure is increasing. Since the major mechanism of action of the ACE inhibitors is the competitive inhibition (Ondetti and Cushman, 1982) of the conversion of angiotensin I (AI) to angiotensin II (AII), it is appropriate to discuss the renin angiotensin system (RAS) in some detail.

1.2 Renin Angiotensin System

1.2.1 Renin Substrate

Renin substrate or angiotensinogen is an alpha$_2$ globulin synthesized in the liver. The liver would appear to have a dual role in controlling angiotensinogen levels in that it synthesises the substrate and regulates its consumption through its control of renin metabolism.

1.2.2 Renin

Renin, first discovered in 1896 by Tiegerstedt and Bergman who observed that a crude saline extract of kidney could elicit a pressor response, is found predominantly in the kidney although it has also been found within other body tissues such as vascular wall. Within the kidney renin is localised in the outer zone of the renal cortex and is

synthesised and stored in juxtaglomerular cells. Renin release by the kidney is influenced and controlled by a multiplicity of interrelated factors which work directly or indirectly on the kidney. Factors which increase renin secretion are those which decrease blood pressure or increase sympathetic output. These factors in turn depend on the status of Na+ reabsorption across the macula densa, AII, antidiuretic hormone (ADH), circulating catecholamines and an intrarenal baroreceptor mechanism which responds to the level of intraarteriolar pressure at the juxtaglomerular cells.

Renin, an acid protease acts on angiotensinogen, a polypeptide, splitting the bond between two leucine amino acids.

Renin has a molecular weight of about 40000 and a half life in the circulation of 15-30 minutes. It is metabolised mainly by the liver. Larger inactive forms of renin ("big renins") have been isolated from the kidney and found in plasma. The significance of these inactive forms of renin which can be activated by trypsin, acidification or storage of plasma in the cold is not yet known.

1.2.3 Angiotensin I

Angiotensin I, a decapeptide, is the product of the action of renin on angiotensinogen. It has little or no

intrinsic pharmacological activity.

1.2.4 ACE

Angiotensin converting enzyme was discovered by Skeggs and co-workers (Skeggs et al, 1956). It is a single chain glycoprotein and has a molecular weight of 140000-480000, depending on the source of the enzymes and the techniques used to solubilise them. ACE is a metallopeptidase with zinc in the active centre, hence its inhibition by metal binding agents (Erdös, 1975). Activation of enzyme activity by chloride and other monovalent anions is possibly due to the protection from methylation of a single lysine residue at or near the active site (Shapiro and Riordan, 1983). It is now recognised that ACE is very widely distributed in the body and in addition to cleaving AI by the removal of a dipeptide in vascular beds, it catalyses the hydrolysis of peptides in ways other than by the release of the C-terminal dipeptide.

1.2.4.1 Purification And Substrate Specificity

Angiotensin converting enzyme was first purified from animal tissues (Erdös, 1975) and then from human tissues (Erdös and Skidgel, 1985). The human enzyme appeared to be more hydrophobic than that extracted from animal lung or kidney. Its substrates include enkephalins (Palenker et al, 1984), the precursors of the enkephalins (Norman, 1985, Yang et al, 1981) and bradykinin (Erdös and

Yang, 1967), all of which have a free C-terminus. In addition to substrates with a free C-terminal amino acid, ACE also cleaves peptides with a protected C-terminus such as Substance P (Skidgel et al, 1984) and luteinising hormone releasing hormone (LHRH) (Skidgel and Erdös, 1985). In addition, LHRH has a blocked N-terminus which can also be cleaved by ACE.

1.2.4.2 Distribution

Immunohistochemical techniques have been used to demonstrate the cellular and subcellular localisation of the enzyme. In man the enzyme is detected in the vascular endothelium of most organs and in the epithelium of the renal proximal tubule and intestine (Takada et al, 1981). At the ultrastructural level the enzyme is located on the luminal surface of the vascular endothelial cells (Takada et al, 1982), thus ACE maintains direct contact with the blood in the pulmonary circulation, one of the major sites of conversion of AI to AII. Within the kidney the enzyme is localised mainly on the brush border membrane and to a lesser extent, on the intercellular and basal infolding membrane of proximal tubular cells (Takada et al, 1982). Kinins which enter the kidney from plasma are eliminated by ACE in the proximal tubules (Erdös, 1979). Human prostate contains a high amount of the enzyme (van Sande et al, 1985) as does the placental microvilli (Johnson et al, 1984). The function of ACE in these locations is as

yet unknown.

1.2.4.3 Regulation Of ACE Biosynthesis

The regulation of ACE is not well known but three stimuli to ACE biosynthesis have been documented, treatment with glucocorticoids, by macrophage activating factors and treatment with ACE inhibitors. Fyhrquist et al (1983a) treated Wistar-Kyoto rats for 14 days with either a) control b) captopril c)dexamethasone or d) captopril + dexamethasone. Both the dexamethasone and captopril increased the ACE content in purified lung plasma membrane but only captopril increased serum ACE. Macrophage activating stimuli such as E. Coli lipopolysaccharide B have been shown to potentiate the cellular increase of ACE in response to captopril in adherent macrophages prepared from peripheral defibrinated blood (Fyhrquist et al, 1983b). Both captopril and enalapril have been shown to induce biosynthesis in rats (Fyhrquist et al, 1980, and Forslund et al, 1982) and in man (Fyhrquist et al, 1983b). The precise mechanism of ACE induction and its possible biological relevance await further clarification.

1.2.5 Angiotensin II

Angiotensin II is the octapeptide released from AI after the removal of histidyl-leucine by ACE. AII is rapidly removed from the circulation by uptake into tissues and metabolism by the angiotensinases to form angiotensin III

and then to inactive peptide fragments.

AII is the most potent vasoconstrictor substance known. On a molar basis it has approximately forty times the potency of noradrenaline. The effect is exerted by a direct action on vascular smooth muscle, however, the action is not uniform for all blood vessels. The most potent vasoconstriction occurs in the vessels of the skin, kidneys and splanchic region. Less pronounced effects are seen in brain and skeletal muscle blood vessels. Arterioles exhibit a more pronounced vasoconstriction than that in veins (Kostis et al, 1987). In addition to its direct vasoconstrictor action, AII releases catecholamines from the adrenal medulla, acts on the cells of the zona glomerulosa in the adrenal cortex to release aldosterone and contracts smooth muscle in the gastrointestinal tract, the urogenital system and the bronchi. Within the central nervous system AII increases arterial pressure by increasing sympathetic nervous activity and can produce marked changes in hydration.

1.2.6 Tissue ACE Systems

An increasing number of studies suggest the existence of local angiotensin generating systems within individual tissues (Dzau, 1987 and Unger et al, 1988). The local tissue systems may be involved in blood pressure regulation, either independently of, or in conjunction

with the circulating RAS. The existence of discrepancies between levels of renin in the plasma and blood pressure levels during long term use of the ACE inhibitors (Waeber et al, 1980) indicates the involvement of the tissue systems during the response to chronic ACE inhibitor therapy (Dzau, 1989). It is clear that the physiological control of blood pressure, whether by a circulating endocrine system, by a series of local tissue systems or a synergistic action of the two types of system, remains to be established.

1.3 The Development Of Captopril And Enalapril

The action of most drugs is initiated by the interaction of the drug molecule to specific receptors, the chemical constitution of which is unknown. For ACE inhibitors the receptor is the active site of the enzyme and the development of these drugs was highly dependent on an understanding of the active site of ACE.

The isolation of specific inhibitors of ACE from the venom of Bothrops Jaracara (Ferreira, 1965) and the widespread study of the substrate specificity of ACE with simple peptide substrates enabled a picture of the active site of ACE to be built up (Ondetti et al, 1977). Early studies indicated that peptide substrates bound to the active site of ACE via their carboxyl-terminal tripeptide residues and this region of the active site was designated the 'obligatory binding site' (Figure 1.1). Three discrete

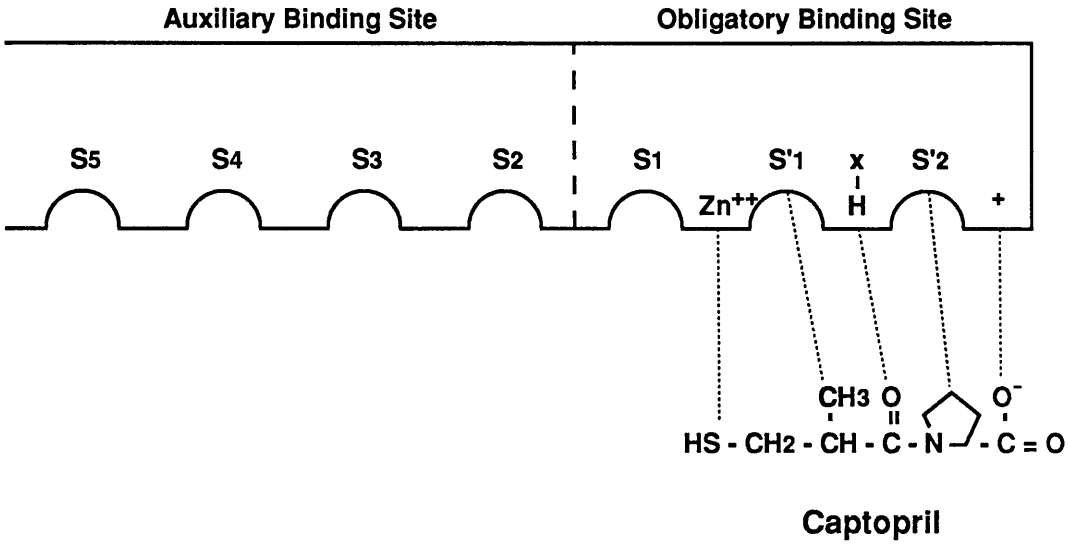


FIGURE 1.1

A Diagramatic Representation Of The Binding Site Of ACE

areas within the the obligatory binding site that specifically interact with the amino acid side chains of substrates were named subsites S_1, S_1' and S_2'. It was also clear from these early studies that a positively charged group on the active site of ACE formed an ionic bond with the negatively charged terminal group of the substrate and thus initiated the proper alignment of the terminal amino acid residues of the substrates with subsites of the active site. In addition to the subsites, it was seen that the zinc ion of ACE was located between two of the subsites and participated in the hydrolytic cleavage of the peptide bond between antipenultimate and penultimate amino acid residues of the substrate. A further interaction between ACE and various substrates led to the inclusion in the obligatory binding site model of a hydrogen bond donating group, also located between the subsites, this group could bind the terminal nonscissile bond of a peptide substrate (Cushman et al, 1981). An effective inhibitor of ACE must bind to these sites although binding to other sites on the enzyme is also possible (auxiliary binding).

Teprotide (SQ 20,881), was first isolated from the venom of Bothrops Jaracara, sequenced and then synthesised. Although it is an effective antihypertensive drug its lack of oral activity would severely restrict its use. Thus, in early 1974 the efficacy of ACE inhibitors as antihypertensive drugs had been demonstrated and awaited

the development of an orally active agent.

As the understanding of the nature of the active site grew, it led to the design and synthesis of hundreds of specific active site binding agents and eventually in 1980, to the introduction onto the market of captopril, the first orally effective ACE inhibitor available for medical use. Early studies with captopril were associated with a high incidence of side effects, some of which were potentially quite severe (Vlasses et al, 1982). Although these early studies were with very high doses of the drug it was postulated that since captopril contains a sulfhydryl moiety in its chemical structure, this was thought to be at least partly responsible for certain of these effects. The search for compounds that would inhibit ACE but were devoid of the sulfhydryl group led to the synthesis and evaluation of enalapril (MK421), itself a weak inhibitor of ACE but rapidly converted by ester hydrolysis to enalaprilat (MK422), a potent ACE inhibitor (Patchett et al, 1980). Enalapril was introduced onto the market in 1986.

With the increasing use of both captopril and enalapril there is now considerable interest in the pharmaceutical industry to provide additional compounds to compete in this lucrative market. Lisinopril was the third ACE inhibitor to become clinically available and was introduced onto the market in 1988. Many more potential ACE inhibitors are currently being developed.

1.4 The Pharmacokinetics Of Captopril, Enalapril And Lisinopril

The elucidation of the pharmacokinetics of these compounds depends entirely on the existence of analytical techniques to measure their concentration in a variety of biological fluids. Whilst this has not been a major problem for enalapril or lisinopril, the instability of captopril in biological media due to rapid autoxidation to form the disulphide dimer (Kripalani et al, 1980), created difficulties in describing the pharmacokinetic profile of captopril as comprehensively as that for enalapril, during development and early after release on to the market.

1.4.1 Captopril

The instability of captopril in biological fluids has made quantitation difficult and several approaches have been utilised to overcome this problem. Initial method development involved the use of antioxidants, chelating agents and derivatising agents. The addition of N-ethylmaleimide (NEM) to collection tubes and subsequent derivatisation of captopril will stabilise captopril in blood and urine samples (Duchin et al, 1982b).

Early pharmacokinetic studies utilised thin layer radiochromatography (TLRC) to quantify captopril but were limited in that only radiolabelled drug could be

administered. The development of gas chromatographic techniques with selective ion monitoring mass spectrometric detection (GC/MS) enabled quantitation of cold captopril doses. High performance liquid chromatography (HPLC) and RIA techniques have also been developed.

Methods utilised in pharmacokinetic studies usually measure free and total captopril. Total captopril comprises unchanged captopril, captopril disulphide dimers, disulphide conjugates of captopril with low molecular weight thiol-containing compounds (glutathione, cysteine) and captopril bound to protein (via covalent disulphide bonds).

1.4.1.1 Absorption

Captopril is rapidly absorbed from the gastrointestinal tract, peak captopril levels following a radiolabelled 10mg dose were reached at 0.7 hours (Duchin et al, 1982b) and then declined rapidly. Absolute oral bioavailability of captopril, calculated with reference to a 10mg intravenous dose, was 62% based on urinary excretion values and 65% based on plasma captopril AUC values. There is a linear relationship between oral dose (range 10-150mg) and the Cmax and AUC for unchanged captopril in plasma (Duchin et al, 1988).

The consumption of food prior to a single radiolabelled dose of captopril in healthy subjects has been reported to cause a 35 to 40% reduction in the bioavailability of

captopril (Singhvi et al, 1982b). However, in a long term study in patients with essential hypertension, whilst the Cmax and AUC values for total and free captopril were slightly reduced by food consumption, the blood pressure lowering effect was unchanged (Ohman et al, 1985).

1.4.1.2 Distribution

Captopril is not extensively bound to plasma proteins, only 23.0 and 30.6% captopril equivalents of a radiolabelled 5mg dose given orally were bound at 0.5 and 2 hours after dosing respectively (McKinstry et al, 1978). The drug is primarily bound to albumin via covalent disulphide linkages (Migdalof et al, 1984).

It is not known if captopril crosses the placenta in humans and only 1% of the peak blood concentration was detectable in human breast milk (Devlin and Fleiss, 1981). The volumes of distribution of unchanged captopril at steady state following intravenous administration of captopril, 2.5, 5 and 10mg, were 0.84, 0.80 and 0.80l/kg respectively (Duchin et al, 1988), indicating extensive partitioning of captopril into the tissues.

1.4.1.3 Metabolism And Excretion

Once absorbed captopril is metabolised to several forms including a disulphide dimer, mixed disulphides of captopril and endogenous sulfhydryl compounds and some polar metabolites (Kripalani et al, 1980). At 1 hour

after a radiolabelled dose of captopril, 100mg, unchanged captopril accounted for about 52% of total radioactivity in the blood and the disuphide dimer for about 10%. Excretion of captopril is rapid and primarily by the kidneys, with minor elimination in the faeces. Of the radiolabelled dose 86% was recovered, 67% of which was in the urine and 18% in the faeces. Urinary excretion of radioactivity was essentially complete within 24 hours, 66% of the dose being recovered in that time and very little further urinary excretion on subsequent days. Unchanged captopril accounted for over half of the radioactivity excreted in the first 24 hours. The primary mechanism of renal elimination is tubular secretion and accounts for 78% of radiolabelled captopril when the drug is given alone (Duchin et al, 1982a). The co-administration of probenecid with captopril reduced renal clearance by 44% and total clearance by 19% (Singhvi et al, 1982a).

The elimination half life for unchanged captopril in healthy subjects was found to be 1.7 and 1.9 hours following oral and intravenous administration of 10mg captopril respectively (Duchin et al, 1982b). The pharmacokinetics of captopril, 100mg t.i.d. to healthy subjects showed no accumulation of unchanged captopril, but there was a significant increase in AUC for total drug (Kubo and Cody, 1985). A similar accumulation of total but not unchanged captopril was also seen in patients with chronic heart failure who had received 25mg t.i.d. (Cody

et al, 1982c).

1.4.1.4 Effect Of Age And Disease States

The effect of age on the pharmacokinetics of captopril has been studied in twelve healthy male volunteers aged 65-76 years (Creasey et al, 1986). The mean values of Cmax and Tmax were essentially the same as those reported in an earlier study in which identical study design and analytical methods had been used (Kripalani et al, 1980), indicating that age alone does not significantly affect the pharmacokinetics of captopril.

Acute administration of captopril, 100mg, to patients with hypertension (Jarrott et al, 1982) gave rise to pharmacokinetic parameters which were in close agreement to those observed in healthy volunteers (Kripalani et al, 1980). These normal pharmacokinetic findings were confirmed in a further study in which 10 patients with essential hypertension were given captopril, 1mg/kg orally, in the fasting state (Richer et al, 1984).

Single dose pharmacokinetics have been studied following a 25mg oral dose of captopril to 12 patients with CHF in the fasting state (Cody et al, 1982c). The parameters obtained, with the exception of a slightly delayed Tmax, were similar to those from normal and hypertensive subjects.

Administration of a radiolabelled 100mg dose of captopril to patients with varying degrees of renal dysfunction

indicated that a linear relationship exists between the overall elimination rate constant and endogenous creatinine clearance (over the range 0-56ml/min) (Rommel et al, 1980).

A further study in patients with normal renal function, moderate chronic renal failure and severe chronic renal failure showed that degree of renal impairment had no effect on Cmax, total clearance or relative bioavailability. However, the elimination rate constant decreased and the elimination half life increased with degree of renal impairment. The authors proposed a decrease in the dosage of captopril or an increase in the dosage interval in patients with moderate to severe renal dysfunction (Guidicelli et al, 1984).

1.4.2 Enalapril

The analytical methodology used to measure enalapril, radio-immunoassay (RIA) (Hichens et al 1981), is specific for enalaprilat (the diacid metabolite of enalapril) but total drug (enalapril plus enalaprilat) can be measured after hydrolysis of intact enalapril to enalaprilat, levels of enalapril may then be obtained by subtraction. Radiometric methods utilising radiolabelled drug and a more general method which relies on enalaprilat's ability to inhibit ACE are also commonly used to measure drug levels.

1.4.2.1 Absorption

In man enalapril is well absorbed, 60-70% (Ulm, 1983), and is rapidly and extensively hydrolysed to enalaprilat. Following oral administration of 10mg enalapril maleate, peak serum concentrations (Cmax) of enalapril are observed 0.5-1.5 hours post dose and disappear from the circulation after about 4 hours (Ulm et al 1982). In contrast, the Cmax for enalaprilat is delayed, reaching a maximum at 4 hours before declining (Ulm et al, 1982) and detectable levels are still found after 72 to 96 hours. In healthy subjects the extent of enalapril absorption and hydrolysis to enalaprilat appears similar over the dose range 2.5-40mg as the maximum serum concentration is linearly related to dose (Davies et al, 1984a). Till et al (1982) also showed that the AUC of intravenous enalaprilat, after factoring out the terminal phase which represented approximately 1mg of the dose, was linearly related to dose.

The presence of food did not appreciably alter the absorption of 40mg enalapril administered to healthy volunteers or the bioavailability of enalaprilat when compared to data obtained after administration of the drug in the fasting state (Swanson et al, 1984).

1.4.2.2 Distribution

Enalaprilat is less than 50% bound to plasma proteins. Two types of binding site have been identified, a high

affinity low capacity site and a low affinity high capacity site (Davies et al, 1984b).

The nature of the distribution of enalaprilat in human tissue is unknown as is whether parent drug or metabolite can enter breast milk or cross the placental barrier. The intravenous administration of 10mg enalaprilat to 9 young and 9 elderly subjects enabled the volume of distribution at steady state, adjusted for weight, to be calculated (Hockings et al, 1986). The value of 0.278l/kg for the elderly was significantly less than the value for the young volunteers of 0.375l/kg suggesting that enalaprilat distribution may be altered by ageing.

1.4.2.3 Metabolism And Excretion

In man, the rapid and extensive metabolism of enalapril to enalaprilat occurs mainly in the liver (Ulm et al, 1982). There is no detectable metabolism of enalapril beyond its hydrolysis to enalaprilat (Ulm, 1983). A radiolabelled study in which 10mg of ^{14}C-enalapril was given orally showed that more than 90% of the dose was recovered in the urine and faeces over 72 hours (Ulm, 1983). Most of the drug (61% of the dose) was in the urine, a substantial fraction of which (70%) was enalaprilat. The main route of elimination is via the kidneys and in healthy subjects the renal clearance of enalaprilat is 9.5 l/h (Ulm et al, 1982) which is of the same order as glomerular filtration. Serum profiles of enalaprilat exhibit a polyphasic decline with a prolonged terminal phase of the order of 35 hours,

following both oral administration of enalapril (Ulm et al, 1982) and intravenous administration of enalaprilat (Hockings et al, 1986). Prolonged binding of enalaprilat to ACE and its subsequent dissociation may be responsible for the long half life of elimination. Despite the prolonged terminal phase, little accumulation of enalaprilat was seen following multiple dosing (Kelly et al, 1986, and Till et al, 1984) in subjects with a normal renal function.

1.4.2.4 Effect Of Age And Disease States

The influence of age on the pharmacokinetics of enalaprilat has been studied following acute (Hockings et al, 1986) and chronic (Lees and Reid, 1987) administration. Acute administration of 10mg enalapril maleate resulted in a significant increase in the enalaprilat area under the curve (AUC) for the elderly when compared to the young volunteers (997 v 772 ng.h/ml). Total systemic clearance (Cl/F) was significantly reduced in the elderly. Renal function as measured by creatinine clearance was also significantly less in the elderly and was positively correlated with enalaprilat clearance. There was no difference in the response of young and elderly to repeated administration of the drug.

The influence of various degrees of renal impairment on the pharmacokinetics of enalaprilat has been studied (Kelly et al, 1986 and Lowenthal et al, 1985). Renal

impairment resulted in elevated serum concentrations, delayed time to peak concentration (Tmax), decreased excretion rates and reduced urinary elimination. A threshold level of renal impairment (GFR less than $30ml/min/1.73m^2$) exists below which smaller doses of enalapril are required when compared to patients with normal or less severly impaired renal function (Kelly et al, 1986). A similar threshold for creatinine clearance (less than 60ml/min) has been reported (Lowenthal et al, 1985) below which serum concentrations of enalaprilat are related to kidney function.

The pharmacokinetics of enalapril have been studied in patients with CHF (Dickstein et al, 1987, and Swartz et al, 1985). Following single dose administration of enalapril there was a slower clearance of enalapril and reduced elimination of enalaprilat in patients with CHF compared to those with hypertension, however, the differences may be associated with age as well as disease state. After twice daily administration of enalapril for two weeks, plasma enalaprilat levels were similar in patients with CHF and hypertension (Johnston et al, 1984).

1.4.3 Lisinopril

Lisinopril, like enalaprilat, can be measured by specific RIA (Hichens et al, 1981, Worland and Jarrott, 1986) or any of the more general methods relying on the drugs ability to inhibit ACE.

Lisinopril has only recently been introduced onto the market and so the number of published studies describing the pharmacokinetics of the compound are fewer than for enalapril.

1.4.3.1 Absorption

Lisinopril is absorbed intact following oral administration and is not metabolised further, so, for this drug, bioavailability equals absorption. The absorption of lisinopril after oral administration is slower than that for enalapril, peak serum concentrations were attained at about 6 hours after oral administration of a 10mg dose to healthy subjects (Beerman et al, 1985, Mojaverian et al, 1986). Absorption, based upon urinary recovery following oral administration of 10mg to normal volunteers was 29% (Ulm et al, 1982). Similarly, bioavailability was found to be 25% when compared to the same dose administered intravenously (Beerman et al, 1986). Administration of lisinopril with food did not appreciably alter its absorption (Mojaverian et al, 1986).

1.4.3.2 Distribution

Ultrafiltration studies have shown that lisinopril binds to human serum only to a very small extent, possibly only to ACE present in the serum with little or no binding to other serum components ("Zestril". ICI Product Monograph). The volume of distribution after oral administration

(Vd/F) of 10mg to 19 healthy subjects was found to be 124 litres (Ajayi et al, 1985). Given that the bioavailability of this dose has previously been found to be approximately 25%, the value of Vd/F indicates extensive partitioning of lisinopril into the tissues. No information is yet available as to whether the drug enters breast milk or is transferred across the placenta.

1.4.3.3 Metabolism and Excretion

Lisinopril is not metabolised in man and is excreted unchanged, primarily in the urine. Following administration of 10mg to twelve healthy subjects 29 and 69% of the dose were recovered in the urine and faeces respectively (Ulm et al, 1982). Urinary recovery of an intravenous dose is essentially complete (Beerman et al, 1986).

The elimination of lisinopril from plasma is polyphasic with a prolonged terminal phase which has a half life of about 30 hours (Ulm et al, 1982). As with enalaprilat, the long terminal phase has been said to represent saturable binding to ACE. An effective half life of 12.6 hours has been reported, steady state concentrations being reached within three days with once daily administration (Beerman et al, 1985, 1986).

1.4.3.4 Effect Of Age And Disease States

The effect of age on lisinopril pharmacokinetics has been studied by Cirillo et al (1986) and Gautam et al (1987).

Elevated serum concentrations were found in the elderly following oral administration of 20mg lisinopril to two groups of 8 young and old healthy subjects. However, following administration of 5mg/day for 7 days to 6 young and old healthy subjects lisinopril clearance was found to be significantly correlated with creatinine clearance and so the elevated serum levels of lisinopril in the elderly possibly reflect a decreased renal function associated with increasing age.

The influence of various degrees of renal impairment on the pharmacokinetics of lisinopril has been studied after acute and chronic oral administration of 5mg (Kelly et al, 1987, van Schaik et al, 1987 and Jackson et al, 1988a). The effect of haemodialysis has also been examined (Kelly et al, 1988). In patients with severe renal failure peak concentrations were higher, the decline in serum concentrations was slower and the time to peak serum concentration extended. The changes in the pharmacokinetics were correlated with creatinine clearance. Haemodialysis had a large effect on the plasma levels of lisinopril, haemodialysis plasma clearance was of the order of 40ml/min.

Till et al (1989) and Gautam et al (1987) have studied the pharmacokinetics of lisinopril in patients with heart failure. Absorption of an oral dose of lisinopril is reduced in patients with congestive heart failure when compared to normal subjects (16% vs 29%). Clearance of

intravenous lisinopril is reduced in congestive heart failure patients although this could be a function of age associated with a reduced renal clearance. It has been speculated that the reduced clearance is associated with a decrease in the apparent volume of distribution. Thus, in CHF patients given oral lisinopril, the plasma concentrations are a result of reduced absorption and clearance and could be reduced when compared to normal subjects. However, after intravenous administration elevated plasma levels may be seen.

1.4.4 Other ACE Inhibitors Under Development

Many other ACE inhibitors are currently under development, two that have been introduced onto the market are, quinapril and perindopril, both are non sulfhydryl containing molecules.

A new chemical class of ACE inhibitors has recently been developed (Petrillo et al, 1987), the phosphorus containing compounds such as fosinopril. This class of compounds have a somewhat different structure activity relationship when compared to the sulfhydryl containing compounds or the carboxyalkyldipeptides (Ondetti, 1988).

1.5 The Effect Of ACE Inhibition On The Renin Angiotensin System

The changes seen in the plasma RAS brought about by ACE

inhibitors depend on the state of activation of the system and the pathophysiological state of the patient prior to administration of the drug. Different results may therefore be obtained depending on the patient population, study design and analytical techniques employed.

Oral administration of captopril or enalapril causes a marked reduction in plasma AII and an increase in AI and plasma renin activity (PRA). After inhibition of ACE an increase in renin release rather than accumulation of AI (Nussberger et al, 1987) causes the rise in AI which is then degraded quickly to inactive compounds. The increase in PRA is probably due to the loss of feedback inhibition by AII and decreased renal perfusion pressure (Johnston et al, 1984, and de Leeuw et al, 1983). The fall in AII brings about a gradual reduction in plasma aldosterone concentrations although levels may slowly return to pretreatment values during chronic therapy (Johnston et al, 1984).

1.6 Haemodynamic Effects Of ACE Inhibitors In Hypertensive Patients

Acute interference with the RAS using ACE inhibitors has been shown to produce a substantial blood pressure lowering effect, not only in patients with high and normal renin levels but also in those exhibiting a suppressed renin secretion (Gavras et al, 1981). The reduction in

blood pressure is due to lowering of systemic vascular resistance secondary to a reduction in AII mediated vasoconstriction. The scale of the initial reduction in blood pressure has been found to be directly related to the pretreatment plasma renin and AII levels (Atkinson and Robertson, 1979). In some investigations carried out in small numbers of patients however, the magnitude of blood pressure reduction did not correlate with baseline plasma renin activity levels (Gavras et al, 1981). The relationship between pretreatment plasma renin activity and the resulting fall in blood pressure due to ACE inhibition tends to be weaker when the long term rather than the initial blood pressure response is considered (Case et al, 1980).

The time courses of the acute blood pressure lowering effects of captopril and enalapril are not the same. The first dose of captopril induces a rapid decrease that is evident within 15 minutes and reaches its peak by 0.5-1.0h post dose (Brunner et al, 1979). In comparison, the blood pressure reduction induced by enalapril is delayed, peak effect is approximately 4h post dose and persists for several hours (Gavras et al, 1981). Blood pressure can be kept at a low level throughout the day during chronic therapy with an ACE inhibitor even when 24h blockade of the plasma RAS is not achieved (Waeber et al, 1980).

Characteristically, heart rate does not accelerate when blood pressure is reduced with an ACE inhibitor (Campbell et al, 1982, and Millar et al, 1982). The lack of change

in heart rate may be due to the simultaneous fall in left ventricular filling pressure and/or an interaction with the autonomic nervous system. No change in noradrenaline levels or significant effects of drug treatment on a variety of tests of autonomic function were seen in normal subjects (Millar et al, 1982). However, enalapril has been shown to induce a resetting of the baroreflex in normotensive subjects (Guidicelli et al, 1985) and this could probably account for the lack of reflex tachycardia. Cardiac output is unchanged or slightly increased due to a rise in stroke volume.

Acute ACE inhibition with captopril consistently increased renal plasma flow with no parallel change in the GFR in patients on unrestricted sodium intake (Mimram et al, 1979). In one patient on a low sodium intake however, GFR was significantly increased. Cerebral blood flow measurements in 20 patients after 8 weeks of treatment demonstrated that good blood pressure control was achieved without reducing cerebral blood flow (Frei and Müller-Brand, 1986).

1.7 Haemodynamic Effects Of ACE Inhibitors In Patients With Cardiac Failure

Cardiac failure is manifested by reduced left ventricular systolic function and reduced cardiac output and stroke volume. As end diastolic volume increases to compensate

for decreased output, left ventricular wall stress and pulmonary capillary wedge pressure increase. Compensatory mechanisms that are activated to maintain perfusion pressure to vital organs include vasoconstriction and sodium retention by the kidney to increase total blood volume. The vasoconstriction although initially a beneficial compensatory response may contribute to the progression of heart failure. Circulatory levels of noradrenaline are also elevated and the heightened sympathetic tone contributes to the elevation of systemic vascular resistance and arterial vasoconstriction (Cody et al, 1982a). The decreased renal perfusion pressure, reduction in distal tubular sodium as sensed by the macula densa and enhanced sympathetic nervous system activity will all contribute to the stimulation of the RAS in chronic heart failure.

The magnitude of the haemodynamic response following the first dose of captopril (Cody and Laragh, 1982b, and Levine et al, 1980) and enalapril (Cody et al, 1983) appears to be closely related to the baseline level of RAS activity as estimated by plasma renin activity. In general the acute haemodynamic effects of enalapril and captopril are qualitatively similar with important differences in the time course of the changes. Oral administration of both drugs leads to a reduction in systemic vascular resistance due to a reduction in AII mediated vasoconstriction. The reduction in systematic resistance is usually accompanied by a reduction in mean

arterial pressure. The reduced afterload results in improvements in cardiac output and stroke volume and a reduction in pulmonary capillary wedge pressure. Heart rate is generally unchanged (Cody and Laragh, 1982b, and Cody et al, 1983) although profound bradycardia associated with hypotension has been seen during the initiation of captopril (Ader et al, 1980) and enalapril (Dicarlo et al, 1983). The first dose hypotension can usually be minimised by starting with low doses and titrating upwards.

1.8 Mechanism Of Action Of ACE Inhibitors

Although ACE inhibitors seem to exert their primary effect via inhibition of ACE there has been considerable indirect evidence that this may not be their only mode of action. In part this stems from the fact that ACE degrades the potent vasodilator bradykinin, the level of which has been reported to rise after captopril (Williams and Hollenberg, 1977). Other studies have failed to demonstrate a significant increase (Shoback et al, 1983). The conflicting results are difficult to assess because of the lack of accurate and reproducible bioassays for kinins. Also, as the principle site of action for the kinins is within the tissue it is not unreasonable that measurements of total kinin concentrations in circulating plasma necessarily reflect the true role of the kinin system in

blood pressure regulation.

Direct or indirect effects between the vasodilatory prostaglandins, especially PGE_2 and PGI_2 and the RAS have been proposed (Wennmalm, 1979). Although an association has been indicated (Moore et al, 1981) no consistent relationship between blood pressure effects of ACE inhibitors and changes in plasma or urinary metabolites of these prostaglandins has been observed (Hornych et al, 1982, and Freeman et al, 1984). Shoback et al (1983) and Swartz (1987) compared the effects of captopril and enalapril on PGE_2 metabolite production. In both studies increases in PGE_2 metabolite were demonstrated only with captopril and not with enalapril. Vlasses et al (1984), by demonstrating that captopril and enalapril reduce blood pressure to a similar extent and that adding the two ACE inhibitors together did not result in a further blood pressure reduction, suggested that the two drugs shared a common antihypertensive action. This further questions the relevance of the changes in prostaglandins reported for captopril.

The complex clinical effects of ACE inhibition appear to be a result of inhibition of both the kinin and renin angiotensin systems, both in plasma and in tissue. In most instances, the inhibition of the production of AII is the predominant mechanism whereby these drugs mediate their effects (Edwards and Padfield, 1985).

1.9 Scope Of Thesis

Despite the fact that the use of ACE inhibitors has become an established treatment for hypertension and heart failure, the exact nature of the pharmacokinetics and pharmacodynamics and the interaction of the two has not been adequately described. The thesis presented herein describes some studies designed to further evaluate the disposition characteristics and concentration effect relationships of the ACE inhibitors.

The background information and an introduction to the thesis are presented in chapter 1, materials and methods are described in chapter 2.

For any drug, the understanding and description of its pharmacokinetic properties is entirely dependent on the availability of analytical methodology to measure drug levels in a variety of biological fluids. Chapter 3 presents a comparison of several different techniques to analyse plasma samples for both ACE activity and active ACE inhibitor drug levels. The methods used are compared and contrasted by different statistical techniques.

For both captopril and enalapril the initial dose range was reduced after introduction onto the market, probably due to the initial doses being at the top of the dose response relationship. Much of the early work to elucidate the structure activity relationships and hence the dose response relationship was done in animal species.

For this reason, in chapter 4, comparisons of the in vitro potencies of the active species of several ACE inhibitors within individual rabbits and men have been made. The effect of parent compound on the in vitro potency of the metabolite has also been studied. In addition the in vivo/in vitro relationship for inhibition of plasma ACE has been examined with reference to perindoprilat and quinaprilat, the active metabolites of perindopril and quinapril respectively.

With the exception of captopril, for all ACE inhibitors currently in use, long terminal half lives have been reported which are of doubtful clinical significance. It has been proposed that drug persistence during this long terminal phase represents binding to ACE, a saturable phenomenon, and consequently makes no contribution to the accumulation of these drugs with chronic administration. The contribution of binding by spiraprilat to plasma and ACE tissue sites to the pharmacokinetics of the compound, in rabbit, was studied in chapter 5.

In chapter 6 a study was carried out with enalapril and its active metabolite enalaprilat. The study was designed to elucidate the contribution of the long terminal phase of elimination to pharmacokinetics of enalaprilat, in man. Administration of any drug to different subgroups of the population, for example the elderly or patients with renal disease, may give rise to a different pharmacokinetic profile. In chapter 7 the effect of age on the pharmacokinetics of benazepril, an ACE inhibitor currently

under development, was examined. The clinical relevance of induction of ACE following chronic administration of ACE inhibitors was also considered, again with reference to benazepril. In addition to standard compartmental models, pharmacokinetic models that take into account the binding of drug to ACE in plasma and/or tissue were used to describe the benazeprilat data.

Chapter 8 contains a discussion of the pharmacokinetics of the ACE inhibitors with reference to the work presented in this thesis.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

Di-potassium hydrogen orthophosphate tri-hydrate, sodium di-hydrogen orthophosphate, di-sodium hydrogen orthophosphate, sodium metabisulfite, hydrochloric acid (sp. gr. 1.18), glacial acetic acid and orthophosphoric acid (sp. gr. 1.75) were obtained from BDH Chemicals Ltd., Poole, Dorset. Sodium chloride was obtained from Formachem, Strathaven. Ethyl acetate, methanol and ammonium acetate were obtained from May & Baker Ltd., Dagenham. Potassium di-hydrogen orthophosphate (HPLC grade) was obtained from FSA Laboratory Supplies, Loughborough. Charcoal (Norit A) was obtained from Serva, Heidelberg. Phthalic acid, hippuric acid, bovine serum albumin, dextran (Mol. Wt. 9400), chloramine T, sodium iodide, rabbit gamma globulin, EDTA (di-sodium salt) and hippuryl-his-leu (Hip-His-Leu) (free base) were obtained from Sigma, Poole, Dorset. Donkey anti-rabbit globulin was obtained from Immuno Diagnostic Systems Ltd., Tyne & Wear. Ecoscint was obtained from National Diagnostics, New Jersey. Rabbit serum, used as a source of ACE activity, was supplied by the Scottish Antibody Production Unit (Law Hospital, Carluke). Hionic Fluor and Soluene were obtained from Packard, Pangbourne, Berks.

2.1.2 Radiochemicals

(Glycine-1-^{14}C)Hippuryl-His-Leu, ^{125}I-Sodium iodide and ^{3}H-n-hexadecane were obtained from Amersham International plc, Amersham.

2.1.3 Consumables

SP-Sephadex C25 was obtained from Pharmacia Fine Chemicals, Uppsala. Dialysis membranes (Visking Tubing, size 9-36/32") were obtained from Medicell International Ltd., London. Polypropylene tubes were obtained from Sarstedt, Leicester. HPLC vials and caps were obtained from Chromacol Ltd., London. Polythene tubing (ID 1.50mm, OD 2.70mm) was obtained from Portex, Hythe, Kent. Aqueous filters, 0.8 micron, were obtained from Millipore, Molsheim, France.

2.1.4 Drugs

Enalapril, enalaprilat, MK-351A and MK-422 antibody were gifts from Merck Sharp & Dohme Research Laboratories, Pennsylvania, USA. Benazepril (CGS 14824A) and benazeprilat (CGS 14831) were gifts from Ciba-Geigy Ltd., Basle, Switzerland. Captopril was a gift from E.R. Squibb and Sons Ltd., Moreton, England. Quinapril and quinaprilat were gifts from Warner-Lambert, Michigan, USA. Perindopril, perindoprilat, S-9650 and S-10211 were gifts

Perindopril, perindoprilat, S-9650 and S-10211 were gifts from Servier Institute of International Research, Neuilly sue Seine Cedex, France. Trandolaprilat (RU 44403) was a gift from Roussel UCLAF, Romainville, France. Spiraprilat was a gift from Sandoz, Basle, Switzerland. 3H-Spiraprilat was gift from Sandoz, Hanover, New Jersey, USA.

2.1.5 Equipment

2.1.5.1 HPLC Apparatus

Two HPLC systems were used. The first system consisted of a Hewlett Packard 1084B Liquid Chromatograph connected to a Pye Unicam variable wavelength UV detector set at 228nm and a Hewlett Packard 79850B integrator. The second system consisted of a Waters 510 pump, Waters WISP 710B autosampler, Waters 481 Spectrophotometer set at 228 nm and a Waters 740 integrator. The column used in both systems measured 10cm by 4mm (i.d.) and was packed with Spherisorb 5 micron ODS-1.

2.1.5.2 Protein Binding Apparatus

The binding of drugs under study was determined using the Dianorm Equilibrium Dialyser (MSE) equipped with Macro D1000 Teflon cells (volume 1.0ml per half cell). The advantages of this system were that standardised equilibrium dialysing experiments could be performed in up to 20 cells simultaneously, short dialysis times could be

achieved due to thorough mixing brought about by the slow rotation of the cells and that the teflon cells minimised adsorption to the cell wall. Dialysis was carried out in a temperature controlled water bath at 37 ^{o}C with a constant rotational speed of 8rpm.

2.1.5.3 Gamma Counter

The gamma counter used was a Packard Cobra, model 5005, an automated counter system with five detectors for the quantitative measurement of gamma radiation. The system was controlled by an IBM compatible computer. The detectors consist of 1.5 inch thallium-activated, sodium iodide crystals of the through-hole design. Crystal design is important in assuring that when gamma rays fall on the crystal, most of the energy is absorbed by the crystal and is not scattered and lost. Gamma rays which traverse the sodium iodide crystal interact with electrons in the crystal to produce scintillations of light which are detected at the end of the photomultiplier tube (PMT). Output at the end of the PMT is proportional to the gamma ray energy absorbed by the detector crystal.

2.1.5.4 Hamilton Microlab M

The Microlab M is a programmable microprocessor controlled diluter/dispenser for fast accurate diluting and dispensing. Its ability to be used as a multi reagent diluter lends it to be used in radioimmunoassays (RIA).

2.1.5.5 Scintillation Counter

The scintillation counter used was a Packard Model 3255 Tri-Carb liquid scintillation spectrometer system. The detection system consists of two matched bialkali PMT's. Background noise from the detector was reduced by the presence of a high speed coincidence circuit. Beta energy released from samples excites the scintillation fluid causing it to emit photons of light which interact with the bialkali cathode and eject photoelectrons. These are then multiplied by a series of diodes and detected as an electrical pulse at the end of the PMT. The height of the pulse is proportional to the energy of the beta particles in the sample.

2.2 METHODS

The quality control data and details of accuracy and precision of the methods described will be presented, compared and discussed in Chapter 3.

2.2.1 Iodination Of MK-351A

2.2.1.1 Solutions

2.2.1.1.1 MK-351A

MK-351A was supplied in 0.98mg quantities. The compound was taken up in 1ml distilled water to give a solution of 0.98mg/ml which was then diluted 1:19.6 to give a working

solution of 0.05mg/ml, 5 μl of which was taken for iodination.

2.2.1.1.2 0.5M Phosphate Buffer pH 7.5

Solutions of NaH_2PO_4 and Na_2HPO_4 were prepared by dissolving 7.8g and 7.1g respectively in 100ml distilled water. The NaH_2PO_4 was added to the Na_2HPO_4 using a pasteur pipette until the pH reached 7.5.

2.2.1.1.3 0.1M Phosphate Buffer pH 7.5

The 0.5M Phosphate buffer was diluted 1:5 to give 0.1M buffer and the pH checked to ensure it was still 7.5.

2.2.1.1.4 Chloramine T

This reagent was freshly prepared prior to use. 10mg of chloramine T was weighed into a glass vial and 10mls of 0.1M phosphate buffer pH 7.5 added.

2.2.1.1.5 Sodium Metabisulfite

This reagent was freshly prepared prior to use. 100mg sodium metabisulfite was weighed into a glass vial and 20ml distilled water added.

2.2.1.1.6 Sodium Iodide/ Bovine Serum Albumin

This reagent was freshly prepared prior to use. 100mg of sodium iodide was weighed into a glass vial and 10ml distilled water added. 100mg of bovine serum albumin was

weighed into a further glass vial and the sodium iodide added by pasteur pipette.

2.2.1.1.7 0.1M Ammonium Acetate Buffer pH 3.5

3.854g of ammonium acetate was dissolved in approximately 150-200mls distilled water. The pH was adjusted to 3.5 using glacial acetic acid and the volume made up to 500mls. The pH was then rechecked.

2.2.1.1.8 0.1M Ammonium Acetate Buffer pH 3.0

The buffer was made as above except that the pH was adjusted to 3.0.

2.2.1.1.9 0.01M Ammonium Acetate Buffer pH 3.0

The 0.1M ammonium acetate buffer pH 3.0 was diluted 1:10 with distilled water and the pH rechecked.

2.2.1.2 Setting Up Of Separation Column

Approximately 5-8g of SP-sephadex C25 was mixed with 0.01M ammonium acetate buffer pH 3.0. A 5ml disposable plastic syringe was clamped by a retort stand behind a lead shield. A glass wool plug was inserted inside the bottom of the syringe and a thin plastic tube with a gate clamp attached to the syringe nozzle. The syringe was filled with the sephadex C25 up to the 5ml mark and washed with 0.01M ammonium acetate buffer pH 3.0.

2.2.1.3 Reaction Step For Iodination

The 10 μl 1mCi Na Iodide was opened according to the Amersham instruction sheet, within a fume hood, behind a lead shield. Added to the radioactive compound in succession were 5μl (250ng) MK-351A, 25μl 0.5M phosphate buffer pH 7.5, 10μl chloramine T, the mixture vortexed and the stop clock started. The reaction was terminated after 45 seconds by the addition of 10μl sodium metabisulfite and 50μl sodium iodide/ bovine serum albumin.

2.2.1.4 Separation Of Radiolabelled MK-351A

The reaction mixture was transferred to the 5ml syringe and washed with 20mls 0.01M ammonium acetate buffer pH 3.0. The wash buffer was collected in a beaker for safe disposal. The radiolabelled compound was then eluted with 0.1M ammonium acetate buffer pH 3.5 and collected as 1ml fractions. The fractions were counted in a gamma counter and the fractions with the highest counts pooled and stored (approximately fractions 12-25) in a universal container at room temperature. The elution of radioactive MK-351A is shown in Figure 2.1.

2.2.2 Determination Of ACE Activity By The Measurement Of The Formation Of Hippuric Acid From Hip-His-Leu

Hip-His-Leu is a synthetic substrate for ACE. By quantifying the amount of hippuric acid formed by the action of ACE on Hip-His-Leu an indirect index of ACE

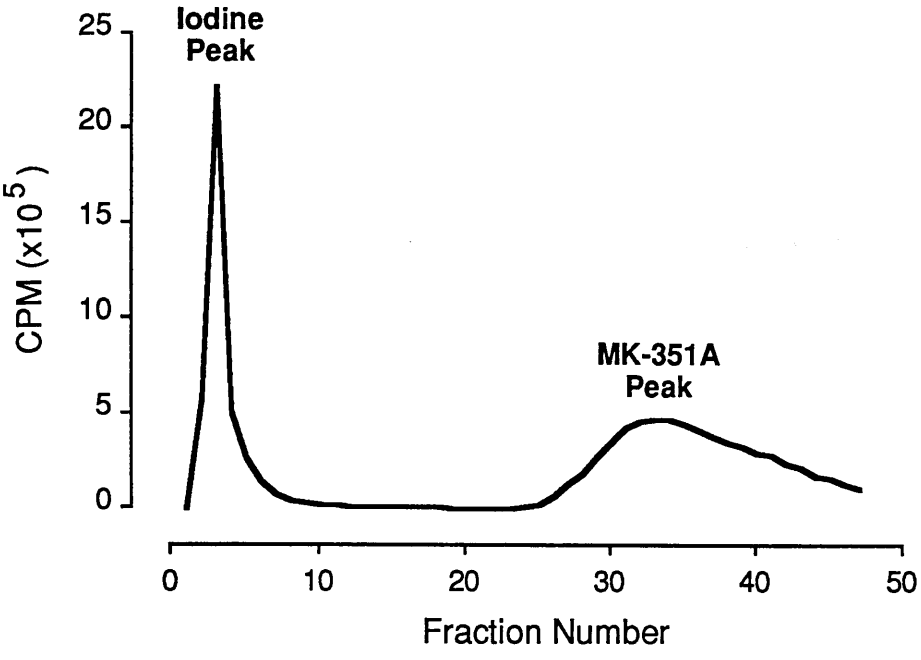


FIGURE 2.1

The Elution Of Radiolabelled MK-351A

activity can be calculated. One unit of ACE activity (1EU/l) is that which produces 1 Mole hippuric acid per minute at 37 ^{o}C.

2.2.2.1 Solutions

2.2.2.1.1 100mM Potassium Phosphate Buffer pH 8.3 (Substrate Buffer)

5.705g of K_2HPO_4 and 4.375g NaCL were dissolved in 250mls distilled water. 1.361g of KH_2PO_4 was dissolved in 100mls distilled water. The monopotassium salt was then added to the dipotassium salt until the pH reached 8.3.

2.2.2.1.2 5mM Hip-His-Leu (Substrate)

21.48mg Hip-His-Leu was dissolved in 10mls assay buffer. This was freshly made for each assay since there is a gradual degradation of the substrate to hippuric acid, the reaction product.

2.2.2.1.3 Internal Standard

20.4mg phthalic acid was dissolved in 20mls methanol and made up to 100mls with distilled water. The solution was then diluted 1:2.5 with distilled water to give the working solution (0.40mMol/l)

2.2.2.1.4 Hippuric Acid Standards

179.2mg of hippuric acid was added to 100mls of distilled

water, 2mls of which was taken and made up to 20mls with drug free plasma (5mMol/l). A standard calibration line of hippuric acid over the range 0.05 to 1.0mmol/l was prepared.

2.2.2.1.5 Mobile Phase

5.44g KH_2PO_4 (HPLC grade) was dissolved in 1.5L distilled water and the pH adjusted to 4.0 with orthophosphoric acid. The volume was made up to 2.0L with distilled water, 140mls discarded and replaced with 140mls methanol. The mobile phase was filtered through a 0.8 micron aqueous filter and finally degassed by bubbling helium through for a minimum of 10 minutes.

2.2.2.2 Analytical Procedure

2.2.2.2.1 Procedure For Standards

Into 4ml polypropylene tubes in duplicate was placed;

a) 200μl substrate buffer.

b) 50μl 50%HCL (v/v). Vortex briefly.

c) 20μl standard plasma.

d) 50μl internal standard solution. Vortex briefly.

e) 50mg (approximately) NaCl. Vortex briefly.

f) 1ml ethyl acetate. Vortex for 15 seconds.

g) Centrifuge at 2000rpm for 5 minutes.

h) Remove 500 μl of the organic layer to a clean 4ml polypropylene tube and concentrate under air/N_2 at 37 ^{o}C.

i) When dry add 100μl of mobile phase and vortex briefly.

j) Inject 20μl onto HPLC system.

2.2.2.2.2 Procedure for Unknown Samples

Into 4ml polypropylene tubes in duplicate were placed;

a) 200μl 5mMol Hip-His-Leu in substrate buffer.

b) 20μl unknown plasma sample. The reaction was started by vortexing briefly.

c) Samples were incubated in a water bath at 37 ^{o}C for 30 minutes.

d) Reaction stopped by the addition of 50μl 50% HCl and vortexing.

e) Procedure as from step d) in section 2.2.2.2.1.

2.2.2.3 Results

Typical chromatograms for a blank plasma extract and a sample containing both hippuric acid and phthalic acid are shown in Figure 2.2. A typical calibration line is shown in Figure 2.3.

2.2.3 Determination Of ACE Activity By Inhibitor Binding Assay (IBA).

IBA is a specific assay for the measurement of ACE activity and is based on the specific and avid binding of ^{125}I-MK-351A to the active site of the enzyme. The ACE activity value is quantified using standards assayed previously by the technique described in section 2.2.2.

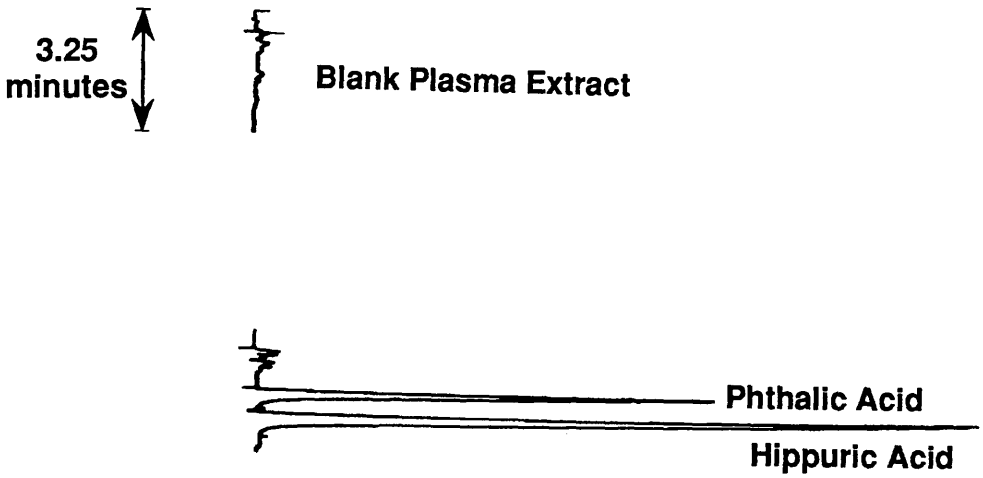


FIGURE 2.2

Typical Chromatograms For A Blank Plasma Extract
And A Sample Containing Hippuric Acid And Phthalic Acid

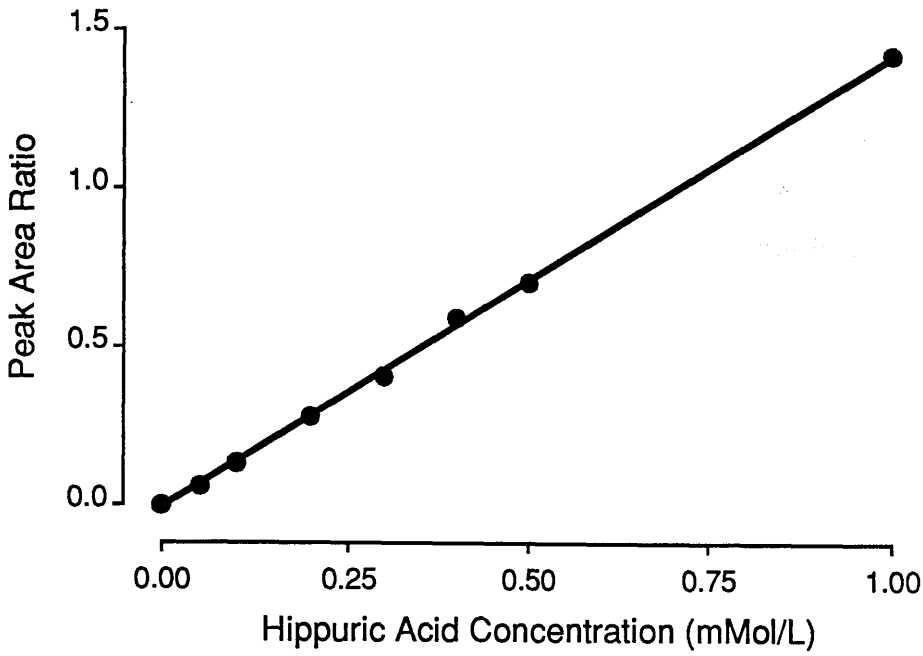


FIGURE 2.3

A Typical Calibration Line For The Measurement Of
ACE Activity By The Formation Of Hippuric Acid From Hip-His-Leu

2.2.3.1 Solutions

2.2.3.1.1 0.15M Phosphate Buffer pH 7.4 (Assay Buffer)

34.2g K_2HPO_4 and 26.3g NaCl were dissolved in approximately 700mls distilled water and the pH adjusted to 7.4 using concentrated hydrochloric acid. The buffer was then made up to 1 litre and 1g bovine serum albumin allowed to slowly dissolve. The pH was rechecked.

2.2.3.1.2 Coated Charcoal

0.4g dextran (Mol. Wt. Cutoff 9400) was allowed to dissolve in 100mls assay buffer overnight prior to the addition of 1g charcoal. The mixture was thoroughly mixed by means of a magnetic stirrer in order to keep the charcoal in suspension.

2.2.3.1.3 ^{125}I-MK-351A

The radiolabelled MK-351A was prepared as described in section 2.2.1. A working solution was prepared prior to use in each experiment by diluting the label approximately 1:300-700 with assay buffer such that 300μl of the diluted label contained 20000cpm. The dilution was calculated after each synthesis of ^{125}I-MK-351A and the same dilution used for all experiments with a particular batch of label. Each batch had a shelf life of 6 weeks, the half life of ^{125}I.

2.2.3.1.4 ACE Activity Standards

Human plasma previously assayed for ACE activity by the method described in section 2.2.2 was used to prepare the calibration standards for the IBA assay. Typically, the plasma would have an ACE activity of 20EU/l. Blank plasma with no ACE activity was prepared by heating control plasma to 60 ^{o}C for 1 hour to inactivate any endogenous ACE activity. The progressive inactivation of ACE with time at 60 ^{o}C is shown in Figure 2.4. The standards prepared in section 2.2.2 utilised plasma previously assayed to have a value of 20.3 EU/l. Three quality control samples were prepared in an identical fashion from a second batch of plasma such that their ACE activity values were at the bottom, middle and top of the standard calibration line.

2.2.3.2 Non Specific Binding Estimation

The degree of non specific binding (NSB) of the ^{125}I-MK-351A was estimated in the presence of a high concentration of MK-422 (1mg/ml) and individual results corrected.

2.2.3.3 Assay Procedure

Standards and quality control samples (QC's) were prepared as described previously and diluted 1:100 with assay buffer prior to use. Unknown samples were also diluted by a minimum of 1:5 prior to assay. The dilution factor was calculated such that the unknowns could be read from the linear portion of the standard calibration line. Duplicate tubes were set up containing 100 μl diluted

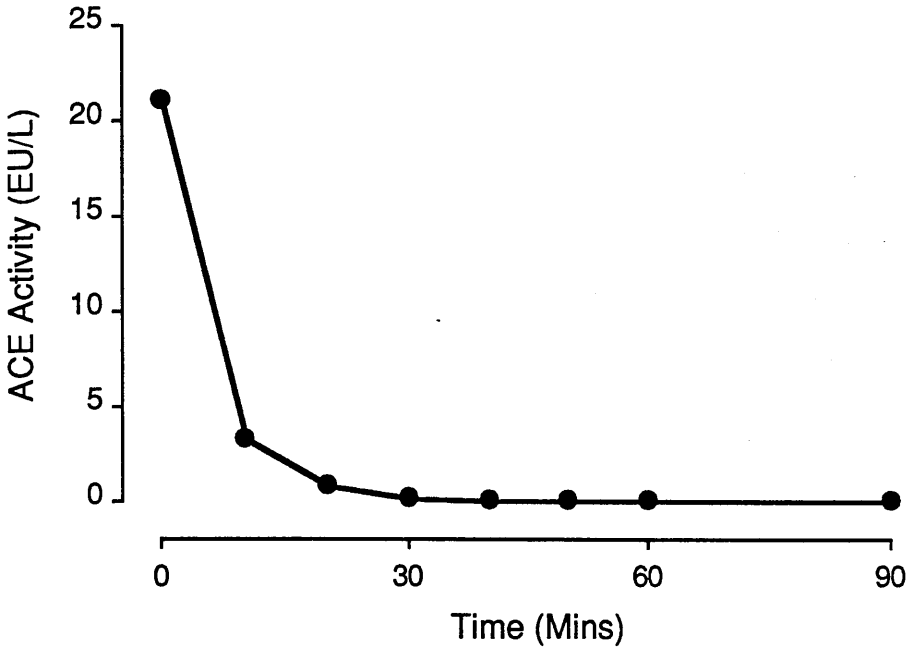


FIGURE 2.4

The Progressive Inactivity Of ACE By Heating

standard, QC or unknown and 300μl of diluted ^{125}I-MK-351A. Two tubes containing 100μl of MK-422 (1mg/ml) and 300 μl label were used to estimate NSB and two tubes containing only 300μl label were prepared to estimate total counts. All tubes were capped, mixed, and incubated in a water bath at 37 ^{o}C overnight (16-18 hours). Tubes were then centrifuged briefly, uncapped, 400μl of coated charcoal added and vortexed for 15 seconds. Following further centrifugation (20 minutes, 3000rpm, 4 ^{o}C) the supernatant was removed to waste and the charcoal pellets counted in a gamma counter for 1 minute.

2.2.3.4 Results

The results were calculated for each duplicate sample as shown;

$$\% \text{ NSB} = \frac{\text{Mean Counts in NSB samples} \cdot 100}{\text{Mean Total Counts}}$$

$$\% \text{ Bound} = \frac{(\text{Mean Counts per Sample} \cdot 100)}{\text{Mean Total Counts}} - \% \text{ NSB}$$

A typical calibration line for ACE activity versus % Bound is shown in Figure 2.5. The calculation of the % NSB and the % Bound is shown in Table 2.1.

2.2.4 Assay Of Enalaprilat By Radioimmunoassay (RIA)

The RIA method described is specific for enalaprilat and has been reported by Hichens et al (1981). RIA is a

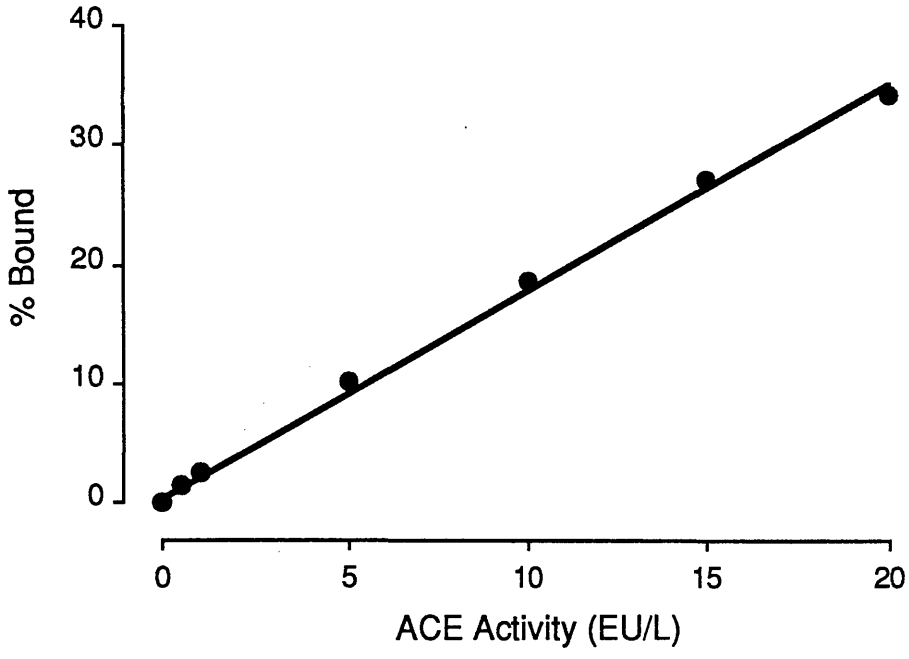


FIGURE 2.5

A Typical Calibration Line For ACE Activity Measured By IBA

TABLE 2.1

Calculation Of % Bound For ACE Activity Standards.

Sample	ACE Activity (EU/L)	Mean Counts	% Bound	% Bound-NSB
Total	-	20402	-	-
NSB	-	721	3.53	-
Std 1	0	590	-	0
Std 2	2.9	886	-	0.81
Std 3	6.8	1400	-	3.33
Std 4	9.6	1660	-	4.61
Std 5	12.5	1833	-	5.45
Std 6	16.4	2032	-	6.43
Std 7	19.3	2214	-	7.32

competitive reaction of antibody with labelled MK-351A and unlabelled enalaprilat. The binding that takes place results in both labelled and unlabelled drug being bound to the antibody in a competitive manner, the greater the amount of enalaprilat present the smaller the amount of MK-351A that is bound. The second antibody binds to the first antibody-drug complex causing it to precipitate out and enabling quantification.

2.2.4.1 Solutions

2.2.4.1.1 0.1M Phosphate Buffer pH 7.5 (Buffer (1))

11.41g K_2HPO_4 and 9.306g EDTA (Na_2 salt) were dissolved in 500mls of distilled water. After dissolving 0.5g of bovine serum albumin in the buffer the pH was checked and adjusted to 7.5.

2.2.4.1.2 Buffer Gamma Globulin

5mg of rabbit gamma globulin was dissolved in 100mls of buffer (1). The buffer was stored at 4 ^{o}C between use.

2.2.4.1.3 Label And Second Antibody

Donkey anti-rabbit globulin (supplied in solution) was diluted 1:10 with buffer (1). 10μl of the radiolabelled ^{125}I-MK-351A was counted in a gamma counter. The counts in one particular experiment were 578580 CPM. For the label 20000 CPM were required in 100μl of a 1:10 dilution

of the second antibody.

Therefore; $10\mu l$ = 578480 CPM

$100\mu l$ = 5784800 CPM

Approximately 20000 CPM are required in $100\mu l$

$$\frac{5784800}{20000} \text{ CPM} = 289.24 \text{ in } 100\mu l$$

Only $50\mu l$ are used so, $\frac{289.24}{2} = 144$

The label must be diluted 1:144 thus $100\mu l$ of label was made up to 14.4mls with buffer (1).
Equal volumes of the diluted (1:10) second antibody and diluted (1:144) label were then mixed (approximately 5mls of each) to give a final solution containing second antibody and 20000 CPM label in $100\mu l$.

2.2.4.1.4 First Antibody (Anti MK-521)
Vials were supplied containing $50\mu l$ anti MK521. The vial was reconstituted with 5mls of buffer (1) and stored in $100\mu l$ aliquots at -70^{o} C.

2.2.4.2 Preparation Of First Antibody Titre Curve
Dilutions of the first antibody were prepared over the range 1/500 to 1/100000. The range of dilutions of first antibody were incubated overnight at 4^{o} C with $100\mu l$ of the label/second antibody. The samples were then centrifuged at 3000rpm at 4^{o} C for 30 minutes. The supernatant was decanted off and the precipitate counted for 1 minute in a

gamma counter. The percent first antibody bound was calculated and plotted against the dilution. A first antibody titre curve is shown in Figure 2.6. The dilution of first antibody which gave closest to 50% binding and the dilution used in all subsequent assays was 1/16000.

2.2.4.3 Preparation Of Enalaprilat Standards And Quality Control Samples

Two enalaprilat solutions were prepared, the first solution was used to prepare the standards and the second solution the quality control samples. For both solutions approximately 20mg of enalaprilat was weighed out and dissolved in 20mls distilled water. The solutions were diluted 1:10 with distilled water prior to an appropriate dilution with buffer (1) to give a final concentration of 1μg/ml. The enalaprilat standards were prepared over the range from 0.4 to 200ng/ml.

The first of the three QC's was prepared by diluting the second solution by 1:10 with buffer (1) to give a concentration of 100ng/ml (QC No.1). The second QC was prepared by diluting QC No.1 by 1:10 with buffer (1) to give a concentration of 10ng/ml. The third QC was prepared by diluting QC No.2 by 1:5 with buffer (1) to give a concentration of 2ng/ml.

2.2.4.4 Assay Procedure

All samples were prepared in triplicate using a Hamilton

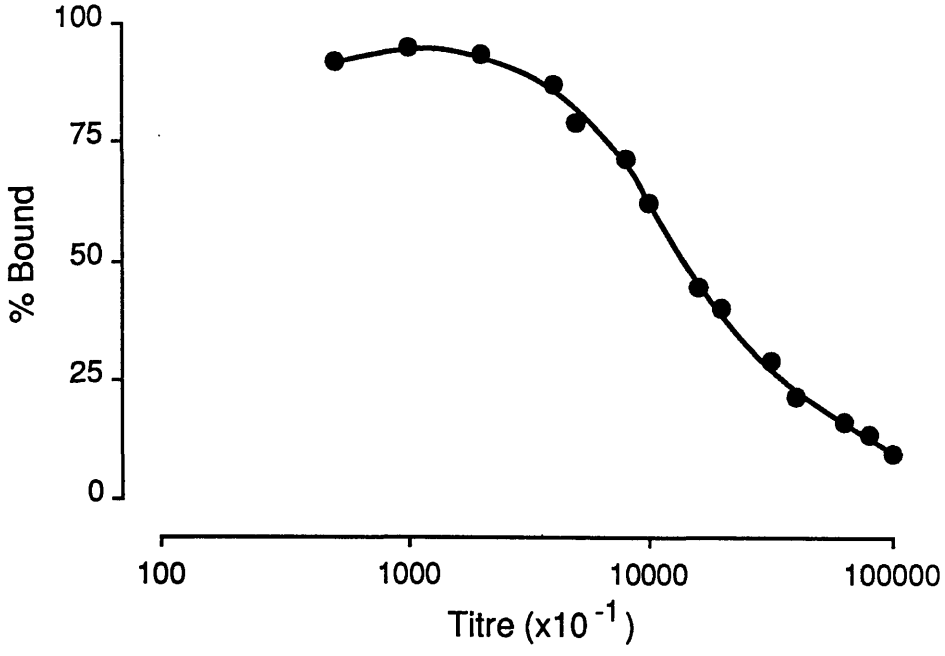


FIGURE 2.6

The First Antibody Titre Curve For Enalaprilat Measured By RIA

micro lab M for pipetting. Tubes 1-3 contained 100 μl label/second antibody to estimate total counts. Tubes 4-6 contained 100μl label/second antibody plus 700μl of buffer (1) to estimate nonspecific binding (NSB). The remaining tubes contained 100μl label/second antibody, 590μl buffer (1), 100 μl first antibody and 10μl of standard, QC or unknown. All tubes were placed in foam racks and kept at 4^{o}C overnight to equilibrate. Following centrifugation at 3000rpm, 4^{o}C for 30 minutes the supernatant was removed to waste and the pellet counted for 1 minute in a gamma counter.

2.2.4.5 Results

The % bound and % NSB were calculated as shown;

$$\%NSB = \frac{\text{Mean CPM (NSB Samples)}}{\text{Mean Total CPM}} .100$$

$$\% \text{ Bound} = \frac{(\quad \text{Mean CPM} \quad .100)}{\text{Mean Total CPM}} - \% \text{ NSB}$$

The % bound was then plotted against the concentration of enalaprilat and the QC values and unknowns read off the curve. A typical standard line is shown in Figure 2.7.

2.2.5 Determination Of Benazeprilat Levels By Gas Chromatography With Mass Spectrometry Detection

2.2.5.1 Assay Procedure

The assay of benazeprilat samples by gas chromatography

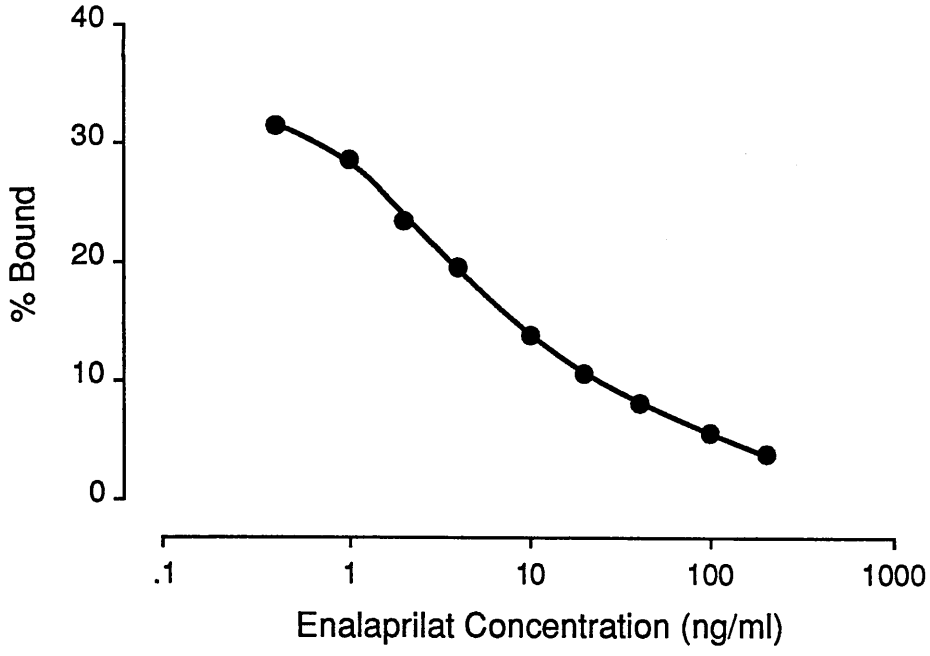


FIGURE 2.7

A Typical Calibration Line For Enalaprilat By RIA

with mass spectrometry detection was kindly carried out by Ciba-Geigy Limited (CRB, Rueil Malmaison, France). The method used was according to Kaiser et al, 1987. The method is specific for benazeprilat only.

2.2.6 Assay Of Enalaprilat And Benazeprilat By IBA

The method relies on the inhibition of the specific and avid binding of ^{125}I-MK351A to the active site of ACE by enalaprilat or benazeprilat.

2.2.6.1 Solutions

All solutions used were prepared as described in section 2.2.3.1.

2.2.6.2 Preparation Of Standards And Quality Control Samples

The methods used to prepare the standards and QC's were the same for both enalaprilat and benazeprilat. Two solutions were prepared for each drug, one to prepare the standards and one to prepare the QC's. In the case of benazeprilat the drug was dissolved in 0.5% ammonia, for enalaprilat, distilled water was used. All solutions were diluted with assay buffer and then spiked into plasma to give standard concentrations ranging from 0 to 100ng/ml. Three QC's were prepared for each compound, one each at the top, middle and bottom of the standard line.

2.2.6.3 Assay Procedure

All standards, QC's and unknowns were heated to 60°C for 1 hour prior to use to inactivate any endogenous ACE activity. All samples (plasma) were then diluted 1:100 with assay buffer and stored frozen between assays. Rabbit plasma, used as the source of ACE activity, was diluted 1:10 with assay buffer before use. ^{125}I-MK351A was prepared as described as in section 2.2.1 and an appropriate dilution made with assay buffer to give 20000 CPM in 200µl when the label was freshly prepared. All samples were prepared in duplicate. Tubes 1-2 contained only 200 µl diluted label to estimate total counts. To estimate non specific binding tubes 3-4 contained a high concentration of the drug under assay (typically 1mg/ml), 200µl label and 100µl diluted rabbit plasma. The remaining tubes contained 100 µl diluted sample, 100µl label and 100µl diluted rabbit plasma. All tubes were capped and allowed to equilibrate at 37°C overnight in a water bath. Following brief centrifugation (2 minutes, 500rpm) the caps were removed and 400µl coated charcoal added to separate the free from bound label. The tubes were recapped, centrifuged for 30 minutes at 4°C, 3000rpm, and the supernatant removed to waste. The charcoal pellet containing only free label was then counted for 1 minute in a gamma counter.

2.2.6.4 Results

The results were calculated as shown;

$$\% \text{ NSB} = \frac{\text{Mean Counts in Charcoal (C) (Samples 3\&4)}}{\text{Mean Total Counts (T)}} .100$$

$$\% \text{ Bound} = \left(\frac{(T-C).100}{(T)}\right) - \% \text{ NSB}$$

$$\% \text{ Maximum Bound} = \frac{\% \text{ Bound}}{\% \text{ Bound for Zero Standard}} .100$$

Typical standard curves of % Maximum Bound versus drug concentration are shown in Figures 2.8 and 2.9 for enalaprilat and benazeprilat respectively.

2.2.7 Assay For Enalaprilat And Benazeprilat By The Inhibition Of Formation Of Hippuric Acid From Hip-His-Leu

The principle of this method is the same as that described in section 2.2.2. The inhibition of formation of hippuric acid from a known amount of Hip-His-Leu by enalaprilat or benazeprilat enables the quantification of these drugs.

2.2.7.1 Solutions

All solutions used were prepared as described in section 2.2.3.1. The source of ACE activity used in the assay was rabbit plasma diluted 1:3 with 0.9% saline.

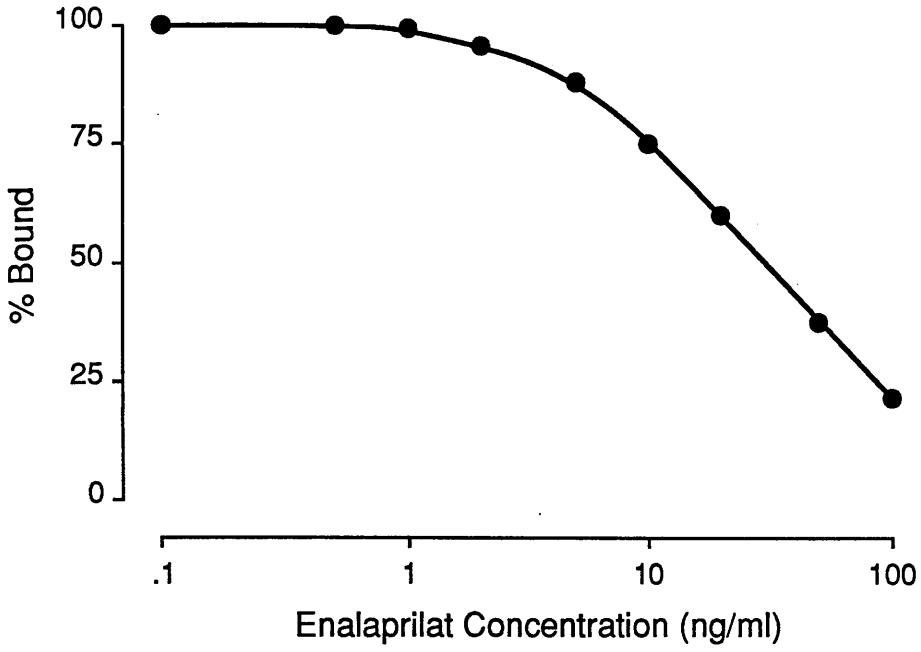


FIGURE 2.8

A Typical Calibration Line For Enalaprilat By IBA

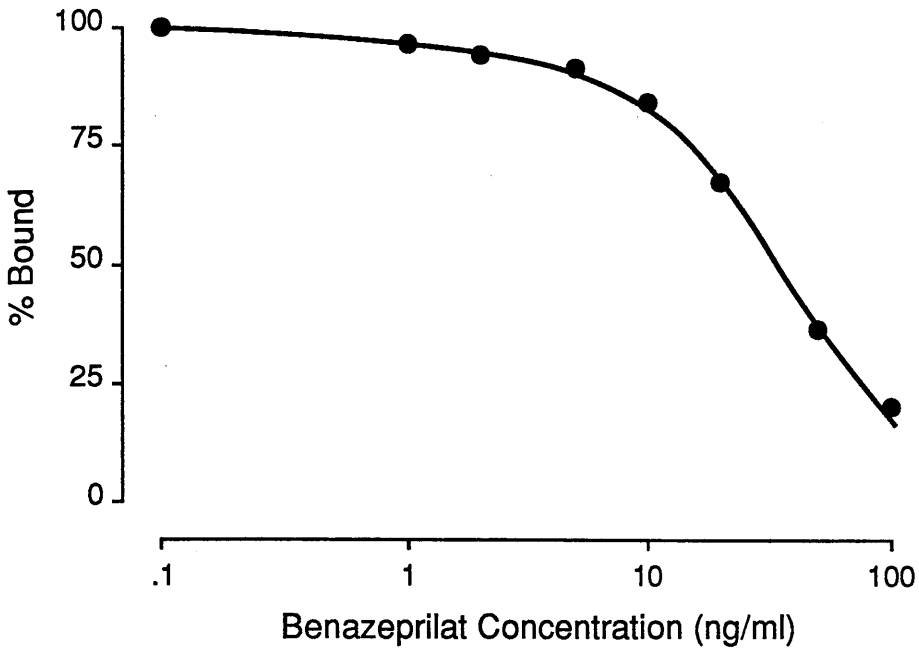


FIGURE 2.9

A Typical Calibration Line For Benazeprilat By IBA

2.2.7.2 Preparation Of Standards And Quality Control Samples

The methods used to prepare the standards and QC's were the same for both enalaprilat and benazeprilat. Two solutions were prepared for each drug, one to prepare the standards and one to prepare the QC's. In the case of benazeprilat the drug was dissolved in 0.5% ammonia, for enalaprilat, distilled water was used. To prepare the standards for both drugs a 500ng/ml solution was diluted 1:25 with blank plasma giving a concentration of 20ng/ml which was then further diluted with blank plasma to give standards ranging in concentration from 20 to 0.3ng/ml. Two QC's for each compound were prepared from solutions of 100 and 50ng/ml which were diluted 1:25 with blank plasma to give final concentrations of 2 and 4ng/ml.

2.2.7.3 Assay Procedure

The following method was used with all samples being assayed in duplicate;

a) 100μl sample plasma (standard, QC or unknown). This was heated for 1 hour at 60^{o}C to inactivate endogenous ACE activity.

b) Allow to cool for 10 minutes.

c) Add 400μl substrate in buffer.

d) Add 50μl diluted rabbit plasma. Mix.

e) Incubate for 1 hour at 37^{o}C in a water bath.

f) Add 100μl 50% (v/v) HCl. Mix.

g) Add 100μl internal standard (500 Mol/l phthalic acid).

Mix.

h) Add approximately 50mg NaCl. Mix.

i) Add 1ml ethyl acetate. Mix for 10 seconds.

j) Centrifuge for 5 minutes at 2000rpm, room temperature.

k) Remove 500 μl ethyl acetate to a clean tube and concentrate under air at 37 ^{o}C.

l) Redissolve in 200μl mobile phase and inject aliquot into HPLC system.

m) For all assays an aqueous blank (150μl 0.9% saline) and a plasma blank (100μl plasma used to prepare the standard curve) were also processed.

2.2.7.4 Results

Typical calibration lines for enalaprilat and benazeprilat are shown in Figures 2.10 and 2.11 respectively. All unknown samples were diluted by an appropriate amount such that they could be read from the linear section of the standard line.

2.2.8 Assay For Enalaprilat By The Inhibition Of Formation Of ^{14}C-Hippuric Acid From Radiolabelled Hip-His-Leu

2.2.8.1 Assay Procedure

All standards and QC's were prepared as in section 2.2.7.2. The method was the same as that described in section 2.2.7.3 with the following exceptions;

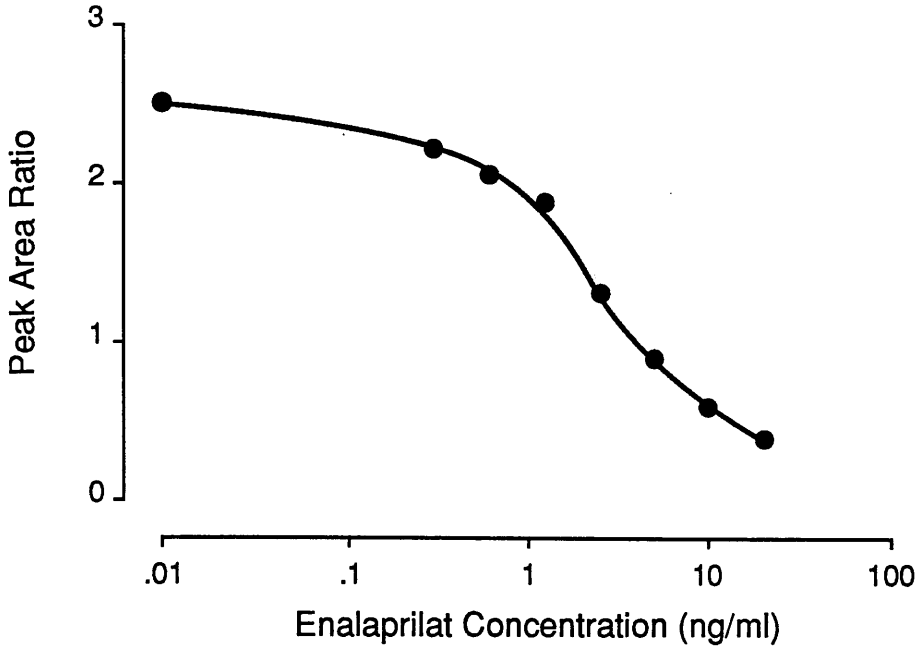


FIGURE 2.10

A Typical Calibration Line For Enalaprilat Measured By The Inhibition Of Formation Of Hippuric Acid From Hip-His-Leu

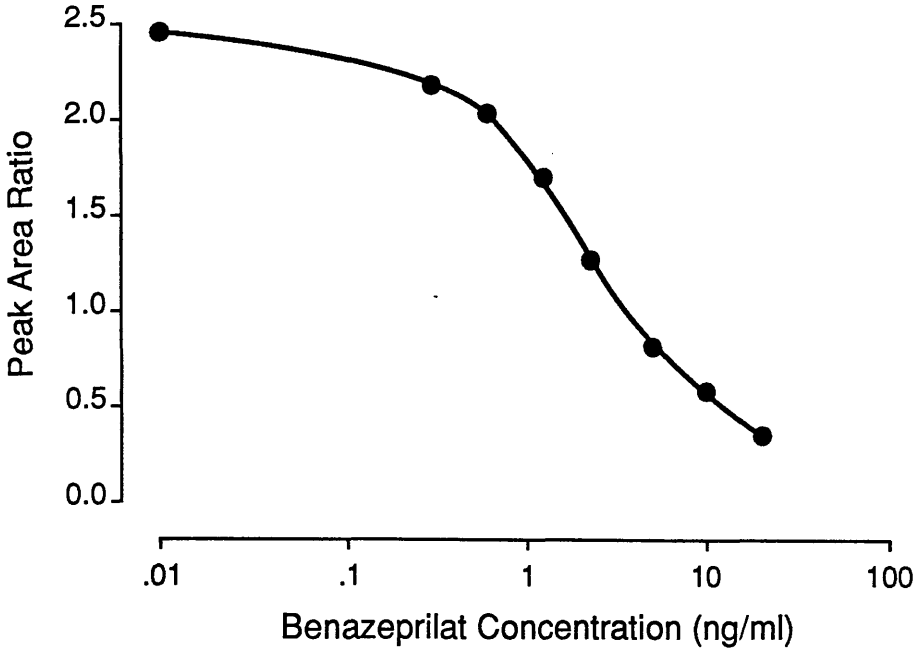


FIGURE 2.11

A Typical Calibration Line For Benazeprilat Measured By The Inhibition Of Formation Of Hippuric Acid From Hip-His-Leu

a) No internal standard was used.

b) The substrate in buffer also included a 1% solution of (glycine-1-^{14}C)Hip-His-Leu (approximately 10μCi/ml).

c) Instead of transferring 500μl ethyl acetate to a clean tube and subsequent steps, 800μl was taken and 400μl acid wash (4 volumes substrate buffer to 1 volume concentrated HCl) added to reduce background counts. The mixture was vortexed and then centrifuged for 2000rpm for 5 minutes. 500 μl ethyl acetate was taken into a scintillation vial and 10mls of Ecoscint added. The samples were then counted for 5 minutes.

2.2.9 Protein Binding

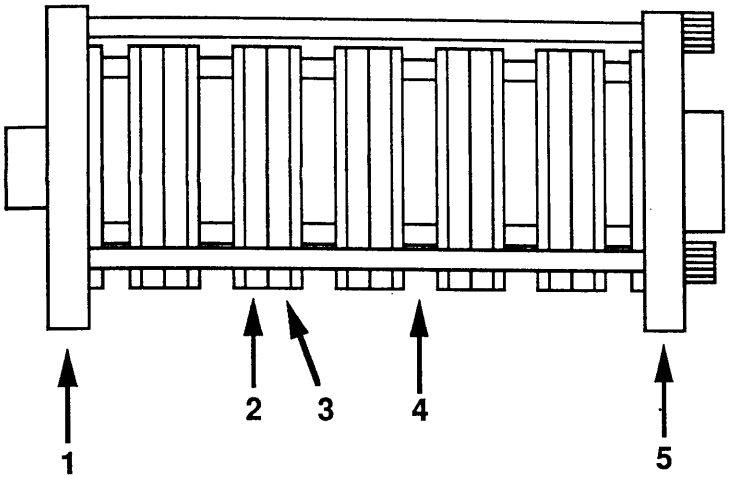
Protein binding studies were carried out for benazeprilat.

2.2.9.1 Preparation Of Membranes

Dialysis membranes were prepared by soaking in three changes of distilled water over a 24 hour period. The membranes were then transferred to phosphate buffer (0.15mol/l, pH 7.4, containing NaCl 0.15mol/l) and soaked in a further three changes of the buffer over 24 hours.

2.2.9.2 Dialysis Procedure

A diagram of the cell unit is shown in Figure 2.12. Each half cell had three holes. The two located close together were for filling, one accepted the pipette tip and the other acted as an air vent. The single hole was for



1 - Drlving flange with guide rods

2 - Teflon cell base
3 - Teflon cell lid
} 5 pairs in complete stack

4 - Spring loaded spacers, 6 in complete stack

5 - Bearing flange secured with 3 knurled nuts

FIGURE 2.12

A Diagram Of The Cell Unit Used For Protein Binding Determinations

emptying the cell. To set up the cell unit the two clean, dry halves of each cell (lid and base) were placed on a clean surface and the membrane gently stretched over the lid. The base was then lowered onto the lid making sure that the filling and emptying holes were aligned. The assembled cell was then picked up, inverted and placed on top of the spring loaded spacer within the driving flange. The previous steps were then repeated four more times until the driving flange was full then the bearing flange was secured in position with three knurled nuts and the unit mounted onto a clamp for filling. The emptying holes were stoppered and the half cells filled in quick succession. For all experiments plasma or buffer containing drug was placed on the left hand (lid) side of the cell and drug free buffer placed on the right hand (base) side of the cell. The filling holes were then quickly stoppered, the cell unit mounted in the drive unit and immersed in the water bath at 37 ^{o}C for the requisite length of time. To empty the cells the cell unit was placed back in the filling clamp, The emptying holes rotated to the top, stoppers removed and a piece of polythene tubing inserted. The cell unit was then rotated round so that the emptying hole was at the bottom and the cell emptied with the assistance of blowing air through the filling hole if necessary.

2.2.9.3 Determination Of Time To Equilibrium

Preliminary work had demonstrated that benazeprilat was very highly protein bound and so the determination of the time to equilibrium was carried out by dialysing benazeprilat in plasma (200ng/ml) against drug free plasma for times ranging between 30 minutes and 24 hours. A graph of the time to equilibrium for benazeprilat is shown in Figure 2.13. A dialysis time of 16 hours (usually overnight) was used in all subsequent experiments.

2.2.9.4 Analysis Of Drug Levels

The levels of benazeprilat were determined using the IBA technique described in section 2.2.6.

2.2.9.5 Recovery Of Drug From The Cells

The recovery of drug from each cell was measured during the determination of time to equilibrium experiments. The mean recovery of drug was 101% (SD=12.4%, n=12). Thus, there was no problem with the drug binding to either to the teflon cell or the membrane. In each experiment a control cell was included in which a known amount of drug in plasma was dialysed against drug free plasma enabling the recovery and whether equilibrium had been reached to be checked.

2.2.10 Preparation Of Tritium Quench Curve

The non quenching standard used was 3H-n-hexadecane, 5.04

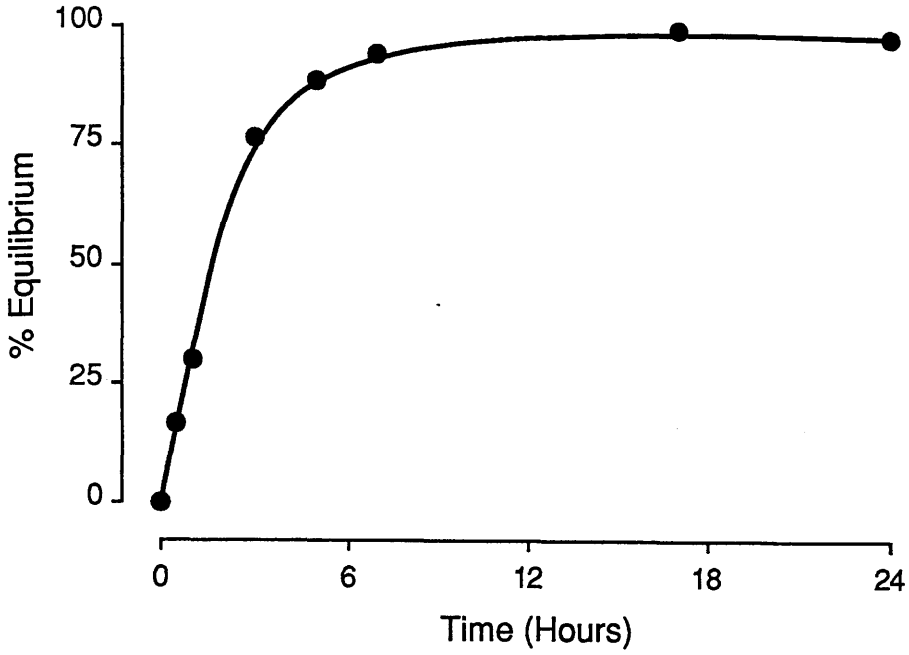


FIGURE 2.13

Determination Of The Time To Equilibrium
For Benazeprilat Protein Binding Analysis

* 10^6 dpm/g (+ or - 3%). The quenching agent used was $CHCl_3$. A series of vials were prepared, each containing 20 μl of 3H-n-hexadecane (10800 DPM) in 10mls of Hionic Fluor and an increasing volume of $CHCl_3$. The volumes of $CHCl_3$ used were 0, 5, 10, 20, 50, 75, 100, 150, 200, 300, 400 and 500μl. The vials were counted for five minutes using the external standard channels ratio method which produces an AES value. The counting efficiency was calculated as shown;

$$\text{Efficiency (\%)} = \frac{\text{Sample CPM - Background CPM}}{\text{Sample DPM (10800)}} * 100$$

A quench curve was drawn by plotting AES against efficiency and is shown in Figure 2.14. All samples CPM values were corrected to DPM using the AES value and the quench curve.

2.2.11 Pharmacokinetic Analysis

Pharmacokinetics is the science of the relationships between the movement of a drug through the body and the processes affecting it. It is a discipline which describes the time-course of the movement of a drug into, around and out of the body. Mathematical models are used to describe and predict drug behaviour. Many different models have been used in this thesis and the methods used to fit the various models to the data will be described in

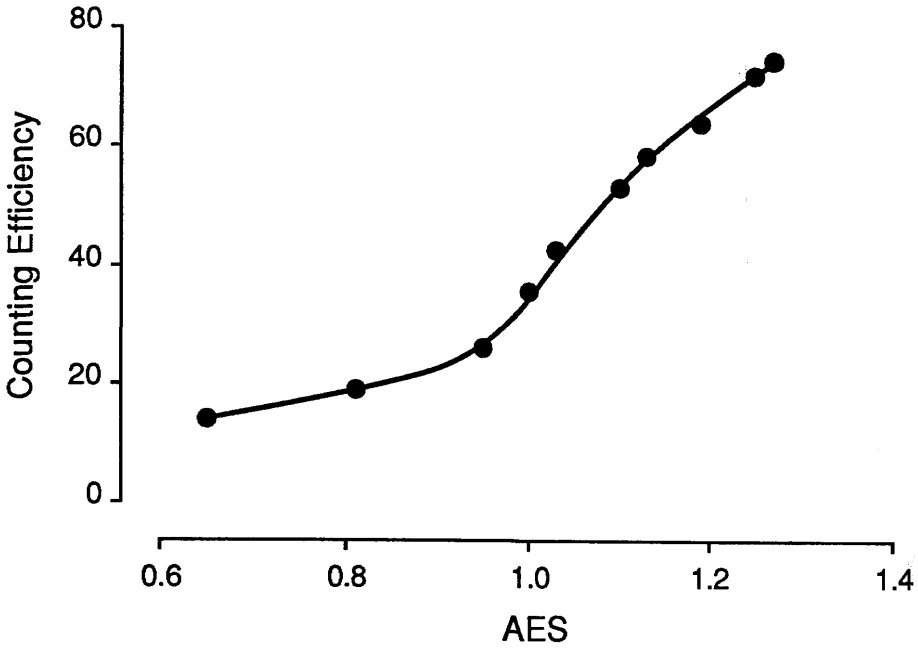


FIGURE 2.14

The Tritium Quench Curve

full in subsequent chapters.

2.2.12 Statistical Analysis

Statistics is the science of collecting, summarising, presenting and interpreting data, and of using them to test hypotheses. There is a great deal of intrinsic variation in most biological processes and it is the interpretation of data in the presence of such variability that the statistical techniques have been used for in this thesis. The methods used will be described in detail within the individual chapters.

Throughout this thesis the level at which statistical significance was achieved was $p<0.05$.

CHAPTER 3

COMPARISON OF DIFFERENT ANALYTICAL TECHNIQUES

3.1 INTRODUCTION

3.1.1 Scope Of Chapter

The work described in this chapter details four different analytical techniques and their application to the measurement of ACE activity and the ACE inhibitors enalaprilat (the active metabolite of enalapril) and benazeprilat (the active metabolite of benazepril). All but one of the techniques have been employed in later sections of this thesis. The four techniques were GC-MS (gas chromatography with mass spectrometry), RIA (radioimmunoassay), IBA (inhibitor binding assay) and HHL (the formation or inhibition of formation of hippuric acid from the synthetic substrate Hip-His-Leu). ACE activity was measured by HHL and IBA. Enalaprilat was measured by RIA, IBA and HHL. Benazeprilat was measured by GC-MS, IBA and HHL. All measurements were made in plasma. The analytical techniques have been described in detail in chapter 2.

3.1.2 Analytical Methodology To Measure ACE And Its Inhibitors

The introduction of ACE inhibitors onto the market has seen the development of many different analytical methods to measure both ACE itself and also ACE inhibitor drug levels.

The first methods reported to measure ACE activity used bioassay techniques. A bradykinin-potentiating factor present in the venom of Bothrops Jaracara was quantified by measuring the contractions induced in guinea pig ileum and rat uterus preparations (Ferreira, 1965). The converting enzyme activity in liver was also measured using the rat uterus, the contractions of the preparation were proportional to the amount of Angiotensin II (AII) present in the perfusate from the liver (Andersen, 1967). Rat colon has also been used to measure AII concentrations generated from the metabolism of Angiotensin I (AI) in isolated perfused tissues (Bakhle et al, 1969). Loyke (1970) estimated converting enzyme activity in rat serum by measuring the pressor response in the vagotomised, pentolinium blocked rat. The main disadvantages of all bioassay techniques are that they are time consuming to perform and lack sensitivity.

Bioassay techniques were replaced by direct measurement of an enzyme reaction product, either the natural product, AII, or hippuric acid, generated from the synthetic substrate Hippuryl-Histidyl-Leucine (Hip-His-Leu). A series of synthetic substrates has been developed, but Hip-His-Leu is one of the most commonly used. Hippuric acid generated during incubation of Hip-His-Leu with ACE can be extracted into ethyl acetate and quantified by spectrophotometry (Cushman and Cheung, 1971, Lieberman, 1975) or by High Pressure Liquid Chromatography (HPLC)

with Ultra Violet (UV) detection (Chiknas, 1979). Histidyl-Leucine, also a breakdown product of Hip-His-Leu, can be measured by fluorescence detection following derivatisation (Conroy and Lai, 1978, Cheung and Cushman, 1973) as can AII (Sakamoto et al, 1986).

A radiochemical assay for ACE measured the liberation of [Glycine-1-^{14}C] hippuric acid from radiolabelled Hip-His-Leu by liquid scintillation counting (Rohrbach, 1978). Advantages of the radiochemical assay were cited as increased sensitivity and lack of interference by nonionic detergents or lipids.

The introduction of radioimmunoassay (RIA) methods enabled the true ACE activity to be measured by using antibodies for AII (Hidaka et al, 1985) or ACE (Alhenc-Gelas et al, 1983). Both techniques are sufficiently sensitive to measure extremely small amounts of ACE activity accurately and may also be used during acute inhibitor therapy since synthetic inhibitors such as captopril or enalapril do not interfere with the RIA. An alternative to RIA utilises an enzyme in place of the radioisotope, thus simplifying the assay procedure whilst maintaining sensitivity and lack of interference from either serum components or pharmacological ACE inhibitors (Lanzillo and Fanbury, 1982).

The recent development of specific inhibitors of ACE as antihypertensive drugs has provided the stimulus for the development of a new assay principle, Inhibitor Binding Assay (IBA) (Tikkanen et al, 1984, Jackson et al, 1986).

Compound MK-351A, an analogue of enalapril (Patchett et al, 1980) retains the stability and potency of the parent substance after labelling with ^{125}I. The labelled analogue can then be used as a marker for ACE in biological fluids and tissues. The assay procedure is simple, specific for pharmacologically active drug, sensitive and is not subject to interference from other enzymes or immunologically similar substances. As one inhibitor molecule binds to one ACE molecule, the IBA could be modified to a stochiometric measurement of ACE. Thus, at equilibrium, the concentration of ACE could be calculated from the specific activity of ^{125}I-MK-351A, the degree of binding saturation and gamma counting efficiency. However, groups working with IBA use it mainly to measure ACE activity and not concentration. Changes in ACE "concentration" are usually ACE activity values measured after the removal of active ACE inhibitor which would mask any change in the amount of ACE present. Many of the methods used to measure ACE can, and have, been modified to measure specific drug levels by quantifying the degree of inhibition of ACE. In addition to these non specific methods, HPLC, RIA and GC-MS techniques have been developed to quantify drug levels with a much greater degree of specificity. The IBA method can be applied to the measurement of any pharmacologically active ACE inhibitor in blood (Fyhrquist et al, 1984), including captopril. Captopril is unstable in biological

fluids, but samples can be taken for assay by IBA and stored for 1-2 days at 4 °C with good stability (less than 5% degradation) (Gröhagen-Riska et al, 1987). Other compounds that have been reported to have been measured by IBA include lisinopril, perindoprilat (the active metabolite of perindopril), cilazaprilat (the active metabolite of cilazapril) (Jackson et al, 1987a) and enalaprilat (Jackson et al, 1987b).

3.1.3 Assay Validation

Characterisation of the pharmacokinetics of any compound depends entirely on the accurate measurement of the compound in body fluids or tissues. Experience has shown that all analytical results are subject to errors arising from a variety of causes. Many errors arising during analysis are due to chemical or instrumental causes although there are other, and often larger, errors. These may include human error and transcription or calculation errors which may be separated from other errors during statistical analysis.

The main emphasis of quality control is the monitoring of the performance characteristics of any given method. In the first instance this involves selection of a suitable method. Such selection involves assessing reliability by considering the accuracy, precision, specificity and sensitivity of the method. These parameters are defined as follows:

Accuracy. Agreement between the measured value of a known standard and its true value. This can be expressed as a percentage of the expected value.

Precision. Agreement between replicate measurements. This is often described by the percentage coefficient of variation (CV) of the results in a set of replicate measurements.

Specificity. The ability of an analytical method to determine solely the component(s) it purports to measure.

Sensitivity. The ability of an analytical method to detect small quantities of the measured component. There are two aspects to sensitivity. Firstly, the limit of detection, the smallest amount of analyte that can be measured above background. Secondly, the minimum limit of quantitation (MLQ), the lowest concentration of analyte measurable in the test matrix keeping within predefined limits of precision and accuracy.

Once methods have been examined and found to meet the desired specification, routine quality control (QC) samples may be included and analysed within batches of the unknown samples. Quality control samples are prepared separately from standard calibration samples and are analysed solely for quality control purposes, not for calibration. The results for these samples are accepted as they arise and are then used to assess the accuracy and precision of the method on a particular day (intra assay variation). Comparison of the results for the QC's over

several days permits the assessment of day to day variation (inter assay variation).

3.1.4 Statistical Comparison Of Different Analytical Methods

During the development of a new drug or class of drugs, ever more sophisticated or faster analytical techniques are often defined. Where more than one analytical technique is being used for the same measurement, a comparison of methods is essential to ensure the compatibility of results. Comparisons are usually made, inappropriately, by the use of correlation coefficients. The correct statistical approach is not obvious. To compare the groups of methods assessed in this chapter a dual approach has been adopted.

Firstly the groups were analysed by the paired t-test or repeated measures analysis of variance (ANOVA) depending on whether two or three methods were under comparison. These techniques take no account of the relative precision or bias of the methods under comparison. The second technique, the jackknife (Salsburg, 1975), compares the variance of the two methods under comparison and gives a confidence interval on the difference in the squared coefficient of determination.

3.2 METHODS

3.2.1 Quality Control Samples

The preparation of the QC samples has been described in detail in chapter 2. Intra assay variation was determined by repeated measurements of the QC's within a single assay. The inter assay variation was determined from the QC results measured in at least four assays, conducted on different days. The accuracy for each QC was calculated from the mean of the QC results, expressed as a percentage of the expected value. Throughout this thesis assays were only accepted if the QC results were within 10% of the target value otherwise the assay was rejected and subsequently repeated.

The minimum limit of quantitation (MLQ) was the lowest concentration of analyte measurable within the limits of precision and accuracy.

3.2.2 Samples

3.2.2.1 ACE Activity Samples

Thirty plasma samples containing varying degrees of ACE activity were measured by the IBA method and also by the formation of hippuric acid from Hip-His-Leu (HHL method). The 30 samples constitute 2 concentration time profiles from the study described in detail in Chapter 7.

3.2.2.2 Enalaprilat Samples

Thirty six plasma samples were assayed for enalaprilat by three different techniques. Firstly an RIA specific for enalaprilat, then by IBA and finally by the inhibition of formation of hippuric acid from Hip-His-Leu (HHL method). The latter two methods measure any active ACE inhibiting component. However, since it has been reported that enalaprilat is not metabolised further (Ulm, 1983), both methods should also be specific for enalaprilat. The 36 samples analysed were the 2 and 6 hour samples taken during the study described in greater detail in chapter 7.

3.2.2.3 Benazeprilat Samples

Three methods were used to assay 40 plasma samples containing varying concentrations of benazeprilat. The benazeprilat samples were first analysed by a specific GC-MS technique (Kaiser et al, 1987) (samples analysed courtesy of Ciba-Geigy) then by IBA, and finally by inhibition of the formation of hippuric acid from Hip-His-Leu (HHL method). Benazepril is a new ACE inhibitor currently under development, as yet there is very little published data relating to its further metabolism in man. Hence it is not known whether the latter two methods are specific for its active metabolite, benazeprilat. The 40 samples analysed constitute 3 concentration time profiles from the study described in detail in Chapter 7.

3.2.3 Statistical Analysis

In general the data were analysed by two different statistical approaches.

1) The data were assessed as to whether they were normally distributed. Analysis of the enalaprilat and benazeprilat data, where three methods of measurement were being compared, was carried out using the relevant parametric or nonparametric analysis of variance (ANOVA). The purpose of analysis of variance is to determine whether the means of several populations are equal, by partitioning the variation in the data and ascribing the variation to treatment or subject effects. In order to do this several assumptions must be made:

a) That the samples are independent of each other and are normally distributed (parametric analysis only).

b) The responses are generated by a linear model, thus the observed response is assumed to consist of an overall mean plus deviations due to the experiment (plus their interactions) plus a random error.

c) The random errors are independent and follow a normal distribution.

Analysis of the ACE activity data (measured by two different analytical techniques) was done using a paired t-test. A paired t-test examines the differences between two methods and tests whether the mean difference is significantly different from zero. Again an assumption is made that the differences between the data pairs come from

a normal distribution.

2) The data were also analysed using the jackknife technique (Salsburg, 1975). The jackknife is the name of the method for estimating the variance of a statistic by sample re-use. The method can be applied to parameter estimation and determination of the error of the estimated parameter. The jackknife has been applied in this thesis to estimating the difference in variance when comparing two methods of assay.

In general the method involves using all the data points to estimate a parameter, $\hat{\theta}$. The first data point is then deleted and the parameter reestimated, $\hat{\theta}^1$. A pseudo-value can then be calculated according to

$$\hat{\theta}_i = n\,\hat{\theta} - (n-1)\,\hat{\theta}^i$$

The pseudo-value can be thought of as the contribution of the data point to the overall variance. The first data point is then restored, the second data point deleted and the second pseudo-value calculated. The process is repeated sequentially until pseudo-values for all the data points have been calculated. The pseudo-values, which are assumed to follow a Students t-distribution, are then used to calculate the confidence limits of the parameter under estimation according to

$$\tilde{\theta} \pm tn,5\% * \frac{Si}{\sqrt{n}}$$

where $\tilde{\theta}$ and Si^2 are the mean and variance of the pseudo-values respectively.

The parameter being estimated in this case is the covariance between Log(method X * method Y) and Log(method X/method Y). Thus there are two methods, X and Y.

The null hypothesis (Ho) is that the variance (Var) of method X is equal to variance of method Y.

$$\text{Thus,} \quad \text{Ho;} \quad \text{Var}(X) = \text{Var}(Y)$$

$$\text{ie} \quad \text{Var}(X) - \text{Var}(Y) = 0$$

$$\text{But,} \quad \text{Var}(X) - \text{Var}(Y) = \text{Cov}(X + Y, X - Y)$$

where Cov is the covariance.

Thus, Ho becomes $\text{Cov}(X + Y, X - Y) = 0$

$$\text{If} \quad W = \text{Ln}(X)$$

$$\text{and} \quad V = \text{Ln}(Y)$$

$$\text{then} \quad W + V = \text{Ln}(XY)$$

$$\text{and} \quad W - V = \text{Ln}(X/Y)$$

and Ho becomes $\text{Cov}(\text{Ln}(XY), \text{Ln}(X/Y)) = 0$.

This makes use of the first order approximation that

$$\text{Var}(\text{Ln } X) \text{ is equivalent to } \frac{\text{Var}(X)}{X^2}$$

$$\text{Since} \quad \text{Var}(W) = \text{Var}(V)$$

$$\text{then,} \quad \text{Var}(\text{Ln } X) = \text{Var}(\text{Ln } Y)$$

$$\text{therefore} \quad \frac{\text{Var}(X)}{X^2} = \frac{\text{Var}(Y)}{Y^2}$$

$$\text{which is} \quad CV^2(X) = CV^2(Y)$$

where CV^2 is the squared coefficient of variation.

The technique does not involve a knowledge of the between measure correlation and can be applied in spite of

suspected non normality in the data.

The jackknife does not allow three methods to be compared at one time. In the case of enalaprilat and benazeprilat three separate comparisons were made, ie for each pair of methods within the group.

3.3 RESULTS

3.3.1 Quality Control Data

The results for the accuracy and MLQ of each method are shown in Table 3.1. The details of inter assay variation and intra assay variation (when measured) are shown in Table 3.2.

There were no obvious differences for MLQ within any of the sets of methods being assessed. The exception to this was the GC-MS technique used to measure benazeprilat levels which was shown to have much greater sensitivity, 2.4pg/ml compared to 0.1 and 0.5ng/ml for the HHL and IBA methods respectively.

In all cases the accuracy of the QC samples was within 10% of the target value, an acceptable variation. Again, there were no marked differences within the sets of methods.

The inter and intra assay variation figures showed slightly more variation within the sets of methods. In most cases there was a slight increase in both types of

TABLE 3.1

The Accuracy And Limit Of Detection For Each Method

Measurement	Method	QC	Accuracy	MLQ
ACE Activity	HHL	18EU/L[a]	104%	0.2EU/L
	IBA	2EU/L	109%	0.5EU/L
		5EU/L	102%	
		8EU/L	104%	
Enalaprilat	RIA	2ng/ml	100%	0.4ng/ml
		10ng/ml	103%	
		100ng/ml	101%	
	HHL	4ng/ml	96.8%	0.2ng/ml
		5ng/ml	97.2%	
		10ng/ml	97.8%	
		15ng/ml	94.5%	
	IBA	7ng/ml	95.1%	0.5ng/ml
		30ng/ml	96.7%	
		60ng/ml	98.0%	
Benazeprilat	GC-MS	15pg/ml	90.9%	2.4pg/ml
		77pg/ml	102.4%	
		155pg/ml	100.8%	
		309pg/ml	101.3%	
		513pg/ml	100.2%	
	HHL	2ng/ml	102%	0.1ng/ml
		4ng/ml	97.8%	
	IBA	10ng/ml	107%	0.5ng/ml
		30ng/ml	107%	
		60ng/ml	106%	

a - 1 EU/L is that which produces 1nmol hippuric acid per ml per minute at 37 ^{o}C.

TABLE 3.2

Intra and Inter Assay Variation For Each Method

Measurement	Method	QC	n	Variation	
				Intra	Inter
ACE Activity	HHL	18EU/L	10	2.0%	3.3%
	IBA	2EU/L	10	8.5%	14.2%
		5EU/L	10	5.4%	8.8%
		8EU/L	10	5.6%	7.7%
Enalaprilat	RIA	2ng/ml	5	4.8%	5.1%
		10ng/ml	5	5.4%	5.8%
		100ng/ml	5	1.7%	4.4%
	HHL	4ng/ml	18	-	6.7%
		5ng/ml	6	5.4%	-
		10ng/ml	7	3.9%	-
		15ng/ml	18	-	8.9%
	IBA	7ng/ml	10	5.1%	6.9%
		30ng/ml	10	7.6%	8.1%
		60ng/ml	10	4.4%	6.3%
Benazeprilat	GC-MS	15pg/ml	8	-	12.9%
		77pg/ml	8	-	2.8%
		155pg/ml	8	-	3.7%
		309pg/ml	8	-	2.1%
		513pg/ml	8	-	2.8%
	HHL	2ng/ml	6	4.0%	6.4%
		4ng/ml	6	3.8%	6.9%
	IBA	10ng/ml	10	5.2%	9.2%
		30ng/ml	10	4.8%	7.3%
		60ng/ml	10	5.0%	6.6%

n - number of samples used to determine intra assay variation. To determine inter assay variation n was repeated for at least four assays.

assay variation as the amount of analyte decreased. Whilst no intra assay variation data were reported for the benazeprilat GC-MS method, the inter assay variation (with the exception of the lowest QC) was two to three times lower than that for the IBA method, even though the QC's being measured were much lower in concentration than those assessed for the other two methods.

The inter and intra assay variation observed when measuring ACE activity by HHL was again two to three times lower than the values for the IBA method. However, a greater disparity in the actual values of the QC's may have contributed to this difference.

There were no marked differences in the values reported to measure variation in the three methods used to measure enalaprilat.

3.3.2 Measured Data

The means of the samples measured for ACE activity by HHL and IBA were both 8.0 EU/L. The means of the enalaprilat samples were 22.8, 20.7 and 16.2 ng/ml, measured by RIA, HHL and IBA respectively. The results of the means for the benazeprilat samples were 179, 302 and 324 mMol/L measured by GC-MS, HHL and IBA respectively. The means, SD's and ranges for all the samples analysed by the different methods are shown in Table 3.3.

There was no difference between the means of the samples assayed for ACE activity by HHL and IBA. There were 9.2,

TABLE 3.3

Results For The Mean, Standard Deviation And Range Of The Samples Measured By The Different Analytical Techniques

Measurement	Method	n	Mean	SD	Range Low	Range High
ACE Activity[a]	HHL	30	8.0	5.8	1.2	20.5
	IBA	30	8.0	5.9	0.8	22.1
Enalaprilat[b]	RIA	36	22.8	13.2	5.2	61.0
	HHL	36	20.7	11.9	3.7	55.0
	IBA	36	16.2	12.0	2.6	59.5
Benazeprilat[c]	GC-MS	40	179	182	0.0	644
	HHL	40	302	309	0.0	1033
	IBA	40	324	361	0.0	1303

a - All data have units of EU/L

b - All data have units of ng/ml

c - All data have units of mMol/L

28.9 and 21.7% differences between the means of the methods used to measure enalaprilat when comparing RIA - HHL, RIA - IBA and HHL - IBA respectively. For benazeprilat there were 68.7, 81.0 and 7.3% differences between the means of the methods when comparing GC-MS - HHL, GC-MS - IBA and HHL - IBA respectively.
The ACE activity data, enalaprilat data and the benazeprilat data are illustrated in the form of Box and Whisker plots in Figures 3.1, 3.2 and 3.3 respectively. The benazeprilat data (representing three concentration time profiles) have been averaged with respect to time for each method and are illustrated graphically in Figure 3.4.

3.3.3 Statistical Analysis

3.3.3.1 ACE Activity Data

The data were not normally distributed. Neither were the differences between the data. It was not possible to logarithmically transform the data since there were some zero differences. Therefore, the data were analysed by the Wilcoxon Matched Pairs Test which is the nonparametric equivalent of the paired t-test. There was no significant difference between the methods (P=0.767). There was also no significant difference in the squared coefficients of determination of the methods as measured by the jackknife technique. The confidence intervals for the difference in

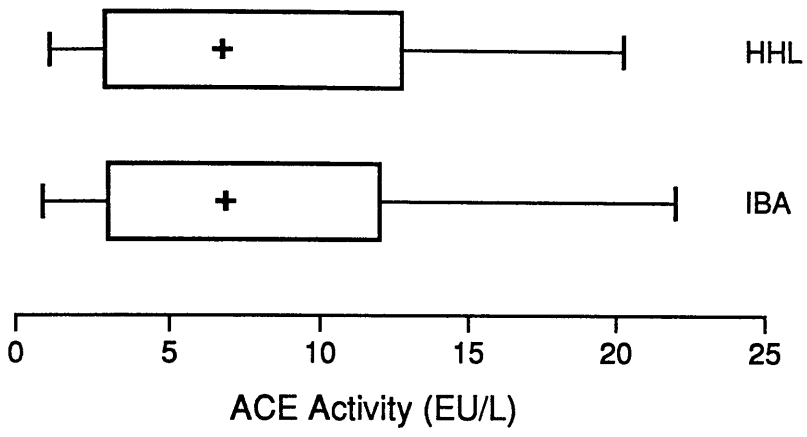


FIGURE 3.1

Box And Whiskers Plot Of ACE Activity Data
Measured By Two Different Analytical Techniques

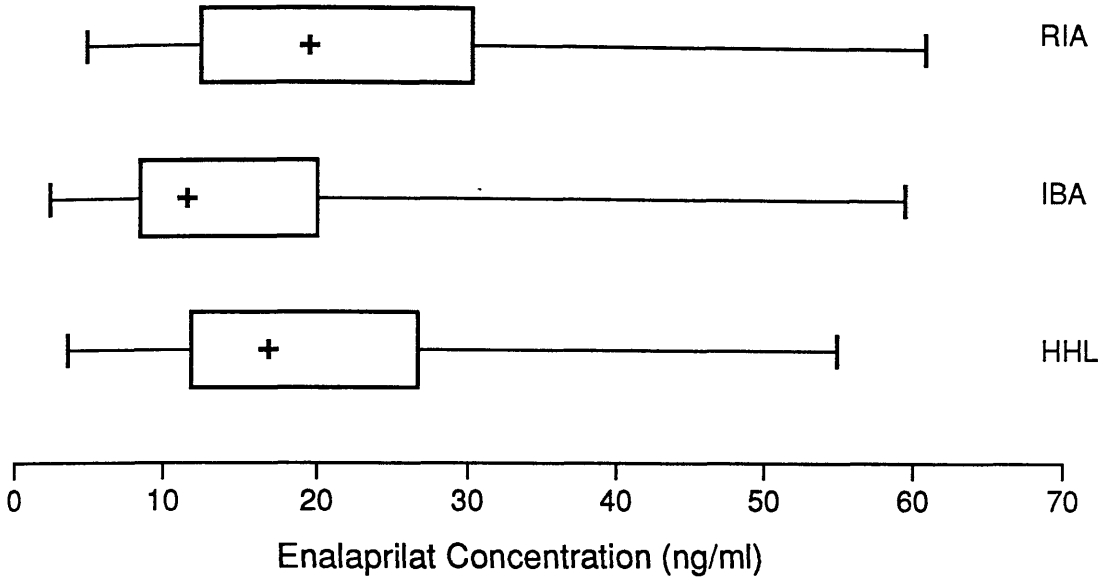


FIGURE 3.2

Box And Whiskers Plot Of Enalaprilat Data
Measured By Three Different Analytical Techniques

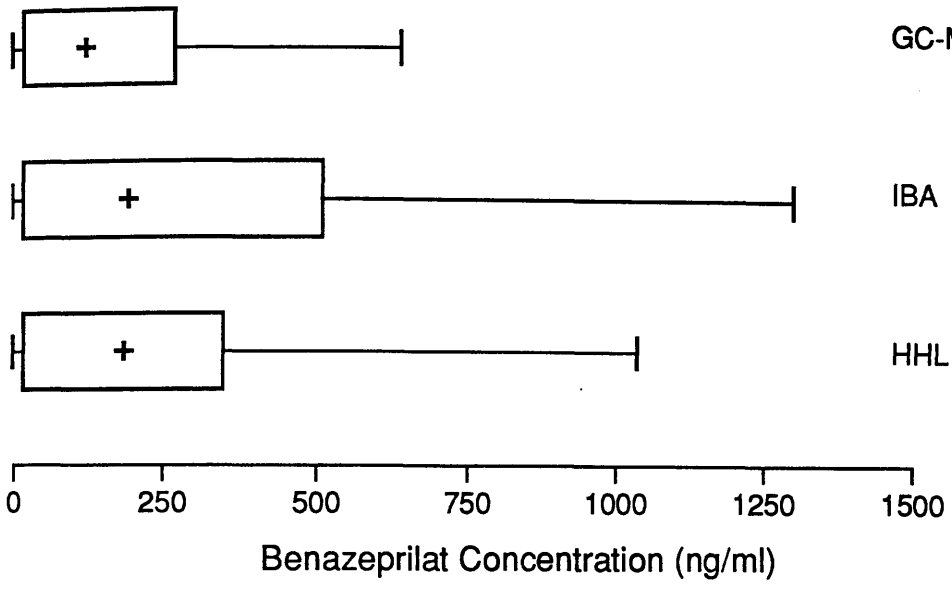


FIGURE 3.3

Box And Whiskers Plot Of Benazeprilat Data
Measured By Three Different Analytical Techniques

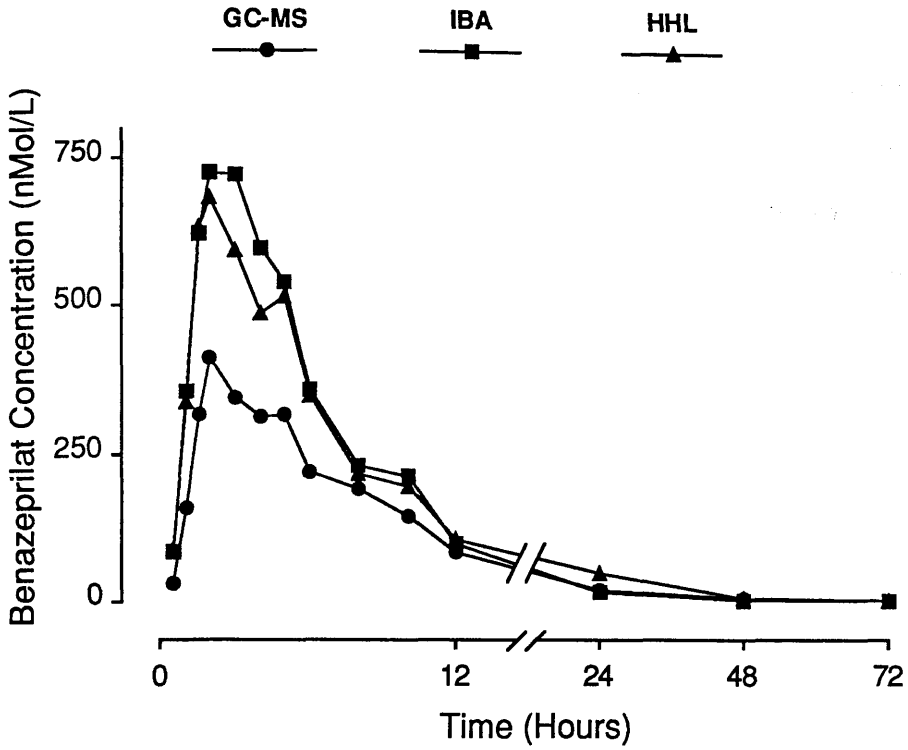


FIGURE 3.4

Analysis Of The Benazeprilat Data By Three Different Analytical Techniques With Respect To Time

mean and squared coefficient of determination between the methods are shown in Table 3.4.

3.3.3.2 Enalaprilat Data

The data were not normally distributed. However the natural logarithmically transformed data were normally distributed and so the transformed data were analysed by repeated measures ANOVA. This technique analyses the differences between the methods and assumes that the residuals are normally distributed. This was seen to be the case by the plot of the standardised residuals v normal scores of the data. The normal scores of the data are the expected values if the data come from a normal distribution (μ=0, σ =1). When the sampled scores normal data are plotted against the standardised residuals, a straight line is evidence that the original data were normally distributed.

Using repeated measures ANOVA, the RIA method and HHL methods were not significantly different, but the IBA method was significantly different from both the RIA and HHL methods. Bonferroni confidence intervals[a], which take account of the number of comparisons being made, are shown in Table 3.4. Analysis of the transformed data by the jackknife technique also revealed significant differences in the squared coefficients of determination of the IBA method when compared to the other two methods. No difference was found between the RIA and HHL methods. Confidence intervals for the difference in the squared

Miller, R.G. In 'Simultaneous Statisical Inference'. (1966). McGraw-Hill.

coefficients of determination for the methods are shown in Table 3.4.

3.3.3.3 Benazeprilat Data

The data were not normally distributed and again could not be logarithmically transformed due to the presence of zero values. Thus, the methods were analysed nonparametrically using Friedmans ANOVA. The test gave a significant result and so multiple comparisons were performed and confidence intervals for the ranks of the data calculated to discover where the differences lay. The IBA and HHL methods were not significantly different from each other, but both differed significantly from the GC-MS method. Jackknife analysis revealed a significant difference in the squared coefficient of determination between all three methods. The confidence intervals for the Friedmans ANOVA and the jackknife analysis of the untransformed data are shown in Table 3.4.

3.4 DISCUSSION

The correlation coefficient has often been used to assess agreement between two methods (Jackson et al, 1986, Grönhagen-Riska et al, 1986). However, a high correlation coefficient, r, does not necessarily mean that two methods agree, for a number of reasons (Bland and Altman, 1986). Firstly, r measures strength of a relation between two

TABLE 3.4

95% Confidence Intervals For The Differences Between The Means (Conventional Analysis) And The Squared Coefficients Of Determination (Jackknife Analysis) For The Analytical Methods

Method	Conventional Analysis[a]	Jackknife Analysis
ACE Activity		
HHL v IBA	-0.15 to 0.20	-0.22 to 0.05
Enalaprilat		
RIA v IBA	0.29 to 0.52*	-0.09 to -0.004*
IBA v HHL	-0.42 to -0.19*	0.01 to 0.06*
RIA v HHL	-0.01 to 0.22	-0.03 to 0.008
Benazeprilat		
GC-MS v IBA	-52.1 to -8.95*	-1.64 to -0.61*
IBA v HHL	-32.6 to 10.6	0.33 to 0.94*
GC-MS v HHL	-63.0 to -20.0*	-0.86 to -0.12*

* Denotes a significant difference

a - Parametric ANOVA or paired t-test for ACE Activity and enalaprilat methods. The benazeprilat methods were compared nonparametrically and so the confidence intervals are for the ranks of the data.

variables and not the agreement between them. Secondly, a significant correlation value shows that the two methods are related, not unexpected if they are both measuring the same sample! Thirdly, correlation depends on the range of values in the samples being compared, since methods are usually compared over the whole range of typically encountered values, high correlations are not unusual. Finally, the calculation of a correlation coefficient assumes no error in the X variable which is plainly not the case when two methods of analysis are being compared. The statistical approach employed to compare the methods under study within this thesis has sought to look at both the possible differences in the accuracy and the variability of the different methods. In all cases except one, the two statistical techniques were in agreement. The single instance where a discrepancy occurred was between the HHL and IBA methods to measure benazeprilat. The ANOVA found no difference but the jackknife showed a significant difference between the two methods. Whilst the ANOVA compares the means of the data (120 v 128ng/ml for HHL and IBA respectively) the jackknife looks at the variance of the two methods. The range of concentrations found by the IBA method was approximately 20% higher than that found by the HHL method. Since the samples at the top end of the range had been highly diluted prior to analysis, it is possible that variations in dilution could have contributed to the difference in the variance between the two methods.

Excellent agreement was found between the two methods used to measure ACE activity, either method could be used with equal confidence in the results. The same was not found for enalaprilat. Since enalaprilat has been reported as not being metabolised any further, all methods, specific (RIA) or specific for any pharmacologically active moiety (HHL and IBA), should give the same result. There was good agreement between the RIA and HHL methods. Comparison of the results from the IBA method gave consistently lower answers for the individual samples and this resulted in significant differences between this and the other methods. In the absence of any reported active metabolite of enalaprilat no obvious reason could be found for this phenomenon.

Agreement was found between the HHL and IBA methods to measure benazeprilat, but both were significantly different to the results for the GC-MS method. Since the GC-MS method measures specifically benazeprilat, and the HHL and IBA methods measure any pharmacologically active moiety, it could be inferred that there may be other active molecules present in the plasma. This supposition is further encouraged by examining Figure 3.4 where the three averaged concentration time profiles that formed the 40 samples are presented. The discrepancy occurs only between 0-8 hours, at the higher levels of benazeprilat. Later time points, where the levels are much lower, show very similar results regardless of the method of

measurement. These results imply that there are other pharmacologically active moieties present during 0 to 8 hours. There are very few published data relating to the possible further metabolism of benazeprilat in man, however, glucuronide conjugates have been identified in urine (Waldmeier et al, 1990). In baboon, unstable glucuronide conjugates have been identified in plasma (Waldmeier and Schmid, 1989). The observed differences for the methods used to measure benazeprilat highlight the need to know the specificity of any analytical method. Both the GC-MS and the IBA methods have been used during the work presented within this thesis, the implications of the particular method used will be discussed at the relevant time.

CHAPTER 4

IN VITRO STUDIES WITH THE ACE INHIBITORS

4.1 INTRODUCTION

4.1.1 Scope Of Chapter

In this chapter three groups of in vitro studies have been carried out. The first group examines two different series of ACE inhibitors and compares the rank order of potency of the drugs in rabbit and man. The second group characterises the in vivo/in vitro relationship of plasma ACE inhibition for perindoprilat and quinaprilat. The third group of studies examines the effect of the presence of parent compound on the in vitro potency of the active metabolite, in rabbit and man.

All studies described in this chapter are based on the evaluation of dose response curves in which known or analytically measured concentrations of an ACE inhibitor are plotted against the measured percent ACE inhibition. The resulting curves can then be described mathematically enabling comparisons of drugs or species to be made.

4.1.2 Rank Order Of Potency Of ACE Inhibitor Drugs

For any new drug entity much of the early work to elucidate structure activity relationships, and subsequently, to characterise dose response relationships, is undertaken in animal species. After captopril and enalapril were introduced onto the market the initial dose range for both drugs was reduced, probably due to the

initial doses being at the top end of the dose response relationship. Whilst comparisons of drug potency have been made between species (Ibarra-Rubio et al, 1989, and Chen et al, 1984) these experiments have generally used samples obtained from a group of subjects or animals and have not addressed possible inter individual variation between subjects or animals. Since inter individual variation may contribute to interspecies variation, the first group of studies described in this chapter compares, in vitro, two series of ACE inhibitors within individual rabbits and human volunteers. The potency of the drugs are compared between the two species and any possible differences in the rank order of potency of the two series of ACE inhibitors between the two species are characterised.

4.1.3 The In Vivo/In Vitro Relationship For Plasma ACE Inhibition

Many authors have cited the usefulness of in vitro tests to predict what will happen in vivo (Swanson et al, 1981, Ibarra-Rubio et al, 1989 and Unger et al, 1984). Swanson et al (1981) investigated the relationship between the in vivo and in vitro percent ACE inhibition response to enalaprilat. Whilst the results were similar, the data were obtained from different groups of subjects, the in vivo and in vitro responses had been characterised in mild

to moderate hypertensive patients and normal subjects respectively. A large amount of data has been published describing various in vivo and in vitro responses to ACE inhibitors but no data is available to describe the relationship between in vivo and in vitro values within individual subjects. The second group of studies described in this chapter examines the in vivo/in vitro relationship within individual subjects for two ACE inhibitors.

4.1.4 The Effect Of The Presence Of Parent Compound On The In Vitro Potency Of The Metabolite

Many of the new ACE inhibitors currently under development are prodrugs and require deesterification in the liver to form the active metabolite. Whilst the hypotensive action of the metabolite is a consequence of its inhibition of ACE, little work has been done to study the effect of parent compound on the efficacy of the metabolite. This type of interaction has been addressed in only a small number of studies. Work carried out by Funck-Brentano et al (1989) has shown a pharmacokinetic and pharmacodynamic interaction between procainamide and its metabolite N-acetyl procainamide. The presence of metabolite increased the procainamide elimination half life and potentiated procainamide induced QTc prolongation in the electrocardiogram. The study did not examine the effect of procainamide on the pharmacokinetics and

pharmacodynamics of the metabolite. Thomson et al (1987) examined the pharmacokinetics of lignocaine and its metabolite, MEGX, administered separately or together to eight healthy male volunteers. When lignocaine and MEGX were administered in combination, MEGX inhibited lignocaine clearance.

Previous work (Lees, 1986) has demonstrated the presence of hysteresis in the relationship between the plasma levels of the active metabolite perindoprilat and percent ACE inhibition following an oral dose of the parent compound, perindopril. This effect was not seen following an intravenous dose of perindoprilat. The presence of hysteresis after oral administration is unusual. Conventional thinking would predict the presence of hysteresis after an intravenous dose of a compound where high plasma drug levels are attained almost immediately but a lag time is observed before maximum effect can be measured. After oral administration a lag time would be predicted before the peak of both plasma drug levels and effect. The third group of studies described within this chapter examines the effect of parent compound on the in vitro potency of the metabolite for five ACE inhibitors, in rabbit and in man.

4.2 METHODS

4.2.1 Preparation And Reproducibility Of In Vitro Dose Response Curves.

In vitro dose response curves were prepared by obtaining plasma samples from each rabbit or volunteer, aliquoting it, the individual aliquots were then spiked with known amounts of drug such that the assayed ACE activity was inhibited from 0 to 100%. The drug concentrations used for the rabbit studies ranged from 0-1000 ng/ml for all compounds with the exception of captopril where the range studied was 0-20000 ng/ml. The range of drug concentrations studied in man again varied between 0-1000 ng/ml with the exception of captopril where a range of 0-2000 ng/ml was used. The resulting dose response curves were analysed as described in section 4.2.6.

Dose response curves were typically prepared by 'spiking' 10 μl of drug solution into 90μl plasma aliquots. To assess the variability in pipetting the 10μl spike, eight 10μl aliquots of water were weighed. In order to assess the combined variability in preparing the 10μl spike plus the 90 μl plasma aliquot, together with the assay variability, 10 μl water was spiked into eight 90 μl aliquots of plasma and the ACE activity assayed. In both experiments the mean, standard deviation (SD) and percent coefficient of variation (CV) were calculated.

4.2.2 Assessment Of The In Vitro Rank Order Of Potency Within Individual Rabbits And Human Volunteers.

Four series of studies were carried out, the first two series compared dose response curves for captopril, and the active metabolites enalaprilat, perindoprilat and trandolaprilat within each of eight individual volunteers and rabbits (study series 1 and 2 respectively). The third and fourth series of studies compared dose response curves for enalaprilat, perindoprilat, benazeprilat and S-10211 (the active metabolite of S-9650) within each of six individual volunteers and rabbits (study series 3 and 4 respectively).

4.2.3 The In Vivo/In Vitro Relationship For Perindoprilat And Quinaprilat.

The perindoprilat in vitro dose response curves were constructed using the pooled plasma for each volunteer from the placebo phase of an intravenous dose ranging study in which eight healthy volunteers received placebo, 1, 2 and 4mg of perindoprilat. The in vivo dose response curves for the individual subjects were prepared by plotting the percent ACE inhibition data against assayed perindoprilat plasma levels for the three active doses of the study.

Quinaprilat in vivo and in vitro dose response curves were prepared as described for perindoprilat, using plasma

samples generated from an oral dose ranging study in which ten healthy male volunteers received placebo, 0.5, 2.5, 5 and 20mg of quinapril.
All generated dose response curves were analysed as described in section 4.2.6.

4.2.4 The Effect Of Parent On The In Vitro Potency Of Metabolite In Rabbit And Man.

The effect of parent compound on the in vitro potency of the metabolite was studied for five different ACE inhibitors, enalapril, perindopril, benazepril, S-9650 and quinapril. Each study was carried out using plasma from rabbit and man. In addition, in man only, the effect of perindopril on the dose response curve for enalaprilat was studied. The studies were carried out using pooled plasma samples, plasma from at least ten individual rabbits or human volunteers contributing to the relevant pool.
Firstly, in vitro dose response curves for the individual metabolites were constructed as described in section 4.2.2. Secondly, in vitro dose response curves were constructed for the parent compounds, again using aliquots of plasma from either pool. The parent dose response curves were analysed as described in section 4.2.6 and the concentrations of parent compound that inhibited ACE by 5, 10 and 25% calculated. Finally, the dose response curves for the individual metabolites were repeated in the

presence of the three calculated concentrations of the respective parent compound. In addition, in man only, the enalaprilat dose response curve was repeated in the presence of the three concentrations of perindopril.

4.2.5 Analytical Methods.

In all the studies described in this chapter ACE activity was measured by the method detailed in chapter 2, section 2.2.2. The method involves the quantitation of hippuric acid by HPLC with UV detection after its liberation from the synthetic substrate Hip-His-Leu by the action of ACE. The in vitro studies described in sections 4.2.1, 4.2.2 and 4.2.3 involved the preparation of known concentrations of the various ACE inhibitors in plasma aliquots. This was done by dissolving a known amount of drug, and then by serial dilution, preparing a range of standards in distilled water, the concentration of which was ten times the desired concentration in the plasma aliquot. The individual drug standards were then diluted 1:10 with plasma to give the desired concentration.

Measurement of the perindoprilat and quinaprilat plasma levels, forming the in vivo dose response curves described in section 4.2.2, was a modification of the method detailed in section 2.2.7. Quality control data for the two compounds is shown in Table 4.1.

TABLE 4.1

Quality Control Data For Perindoprilat and Quinaprilat

Drug	QC (ng/ml)	Variation Intra (%)	Variation Inter (%)	Accuracy (%)	MLQ (ng/ml)
Perindoprilat	2	3.30 (n=7)	5.75 (n=30)	95.5	0.1
	4	2.52 (n=9)	5.09 (n=30)	98.2	
Quinaprilat	2	4.64 (n=8)	6.31 (n=12)	95.0	0.1
	4	3.68 (n=10)	5.33 (n=12)	98.5	

MLQ - Minimum limit of quantitation

4.2.6 Data Analysis.

All the dose response curves were of the form shown in Figure 4.1 which can be described by the equation;

$$E = \frac{C^{\gamma} * Emax}{C(50)^{\gamma} + C^{\gamma}}$$

where; E - percent ACE inhibition

Emax - maximum inhibition of ACE

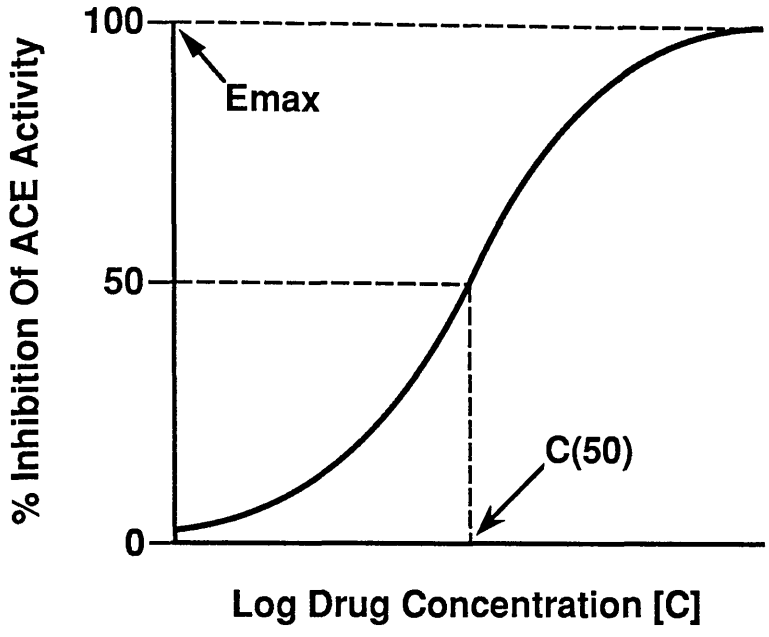
C - concentration of ACE inhibitor

C(50) - concentration of ACE inhibitor that produces 50% inhibition of ACE

γ - a parameter to describe the sigmoidicity of the curve

The equation is known as the Langmuir equation when γ is constrained to equal 1 and the Hill equation when γ is allowed to vary. Four models were fitted to each dose response curve, the first two were Langmuir models (γ constrained to equal 1), one allowing Emax to vary and one constraining Emax to equal 100%. In the third and fourth models the Hill equation was used (γ allowed to vary), again Emax was either allowed to vary or to be constrained to 100%.

The estimates of model parameters, Emax, γ and C(50) where appropriate, were obtained by non-linear least squares regression with unweighted data using the statistical package VASP on a Nodecrest V76 computer.



$$\% \text{ Inhibition} = \frac{[C]^{\gamma} \times \text{Emax}}{C(50)^{\gamma} + [C]^{\gamma}}$$

FIGURE 4.1

A Schematic Representation Of A Typical Dose Response Curve

All model comparison was done using the F-Ratio test. In the first instance the two Langmuir models and the two Hill models were compared to assess whether allowing Emax to vary significantly improved the fit of the model to the data. For the vast majority of the data this was not the case and so final model selection was made by comparing the Langmuir and Hill models where Emax was constrained to 100%.

All comparisons of potency, assessment of the in vivo/in vitro relationship or the examination of the effect of parent drug on the activity of the metabolite were made using the C(50) parameter, the concentration of drug which produced 50% inhibition of ACE.

4.2.7 Statistical Analysis.

Comparison of the rank order of potency of the two series of ACE inhibitors, each series characterised in rabbits and volunteers was carried out by repeated measures ANOVA. The two sets of in vivo/in vitro data were compared by paired t-test. Different numbers of data pairs were used to estimate the in vivo and in vitro model parameters (approximately 20 data pairs for the in vitro data and approximately 55 data pairs for the in vivo data). Since this may affect the degree of confidence with which the C(50) parameter was estimated, the in vivo/in vitro data were also compared using a weighted t-test in which the

inverse error in estimating C(50) was used as the weighting function.

Any possible effect of parent compound on the in vitro potency of the metabolite was assessed by firstly fitting the models to the individual dose response curves. The four data sets were then combined into one and the models refitted. The General Linear or F-Ratio test was used to test if there was any statistically significant justification for fitting with four individual C(50) parameters or whether one combined C(50) parameter was adequate.

4.3 RESULTS

4.3.1 Reproducibilty In The Preparation Of The Dose Response Curves.

The results for the mean, SD and CV of the eight weighed 10 μl aliquots of water were 9.82mg, 0.14mg and 1.41% respectively. The results for the mean, SD and CV of the eight assayed 10μl water plus 90μl plasma samples, in which ACE activity was determined, were 20.4EU/L, 0.316EU/L and 1.55% respectively. The value of 1.55% encompasses both variability in preparing the samples and the assay variability.

4.3.2 Assessment Of The Rank Order Of Potency Within Individual Rabbits And Volunteers.

In order to assess which model, Hill or Langmuir, best described the data the results of model choice from study series 1 and 3 and study series 2 and 4, in which volunteers and rabbits were studied respectively, were combined. For each species fifty six dose response curves had been prepared. For man, thirty two of the dose response curves were best described by a Hill model. In the case of rabbit all but four out of the fifty six dose response curves were best described by a Hill model. The individual C(50) values for each study are shown in Tables 4.2 to 4.5. Graphs of the four dose response curves within a single representative subject, one subject per study, are shown in Figures 4.2 to 4.5.

In the case of study series 1 where enalaprilat, perindoprilat, captopril and trandolaprilat were compared within eight individual subjects the rank order of potency for all eight subjects was trandolaprilat > perindoprilat > enalaprilat > captopril. The mean values for C(50) (SD) were 1.10(0.34), 2.59(1.48), 7.21(2.41) and 107(24.2) ng/ml respectively. In study series 2 where the same four compounds were compared in rabbit the mean rank order of potency was perindoprilat > enalaprilat > trandolaprilat > captopril, which is different to that seen in man. Six out of the eight rabbits exhibited the same rank order as that of the mean values, in one of the remaining rabbits,

TABLE 4.2

C(50) Values For Enalaprilat, Perindoprilat, Captopril And Trandolaprilat In Eight Human Volunteers.

Subject	C(50) Value (ng/ml)			
	Trandol.lat	Perind.lat	Enal.lat	Captopril
1	1.15	4.14	5.88	75.9
2	1.71	2.68	12.0	87.6
3	1.31	1.70	6.14	99.5
4	0.67	1.20	7.09	145
5	0.80	1.10	3.68	131
6	0.87	2.17	6.57	96.2
7	0.97	2.34	7.66	127
8	1.31	5.38	8.67	94.3
Mean	1.10	2.59	7.21	107
SD	0.34	1.48	2.14	24.2

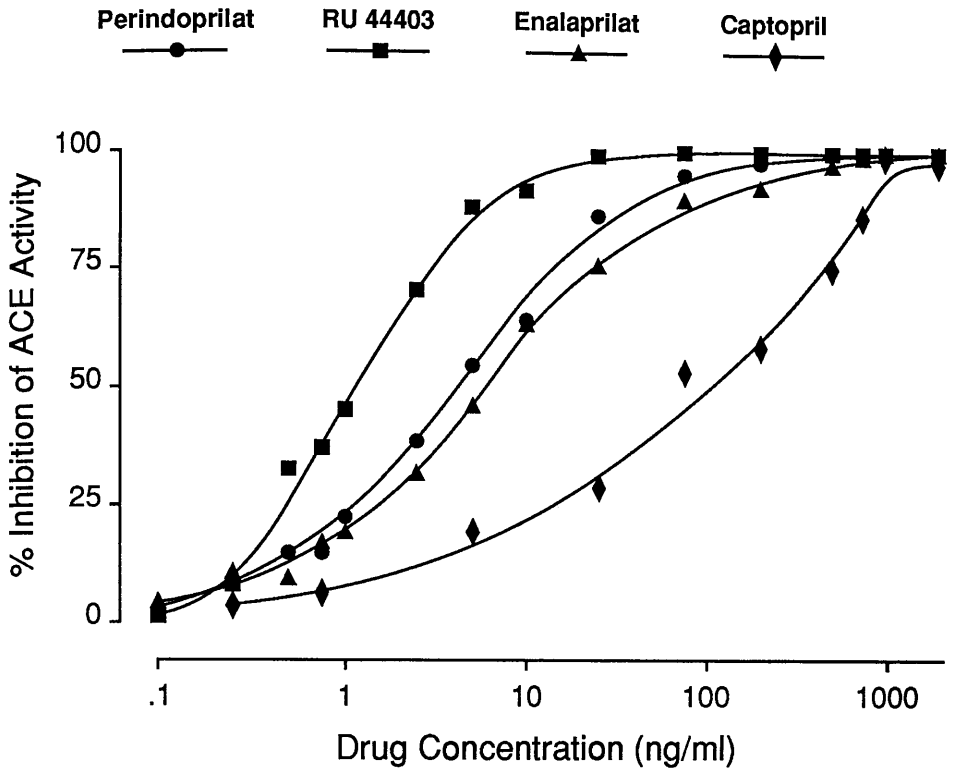


FIGURE 4.2

Dose Response Curves For Subject No. 1
From Study No. 1

TABLE 4.3

C(50) Values for Enalaprilat, Perindoprilat, Captopril And Trandolaprilat In Eight Rabbits.

Rabbit	C(50) Values (ng/ml)			
	Trandol.lat	Perind.lat	Enal.lat	Captopril
1	1.41	0.60	1.20	1333
2	1.72	0.76	2.35	1227
3	1.73	0.90	1.67	638
4	1.10	1.30	1.54	449
5	2.25	1.30	1.31	1382
6	2.85	1.35	2.10	1308
7	1.65	0.66	1.25	461
8	2.05	1.33	1.73	1436
Mean	1.84	1.02	1.64	1029
SD	0.54	0.33	0.41	433

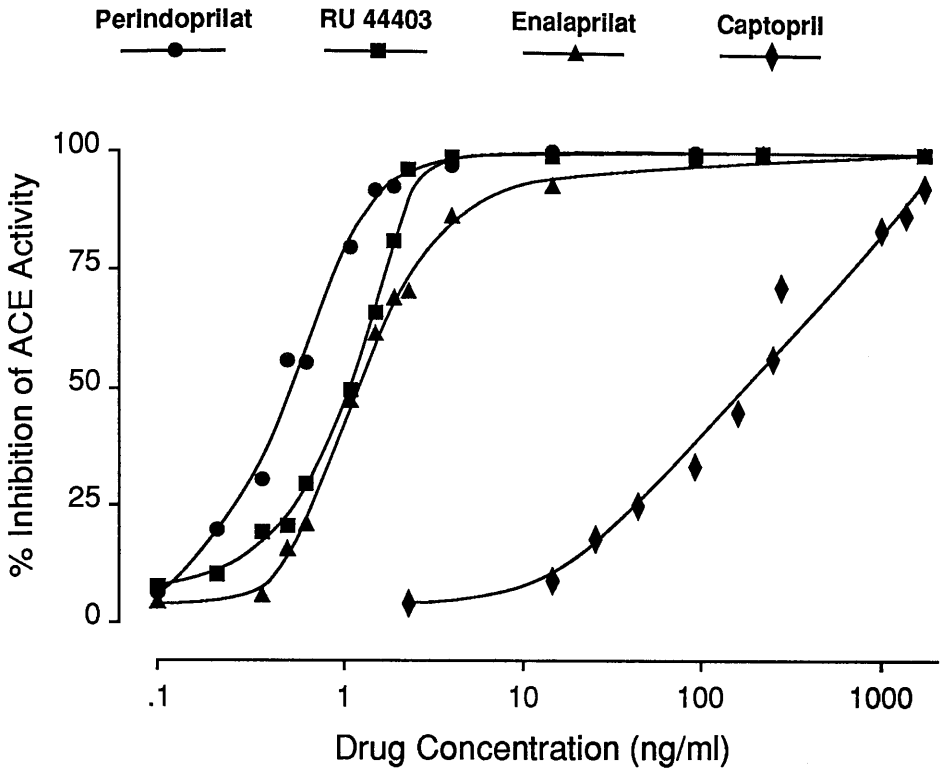


FIGURE 4.3

Dose Response Curves For Rabbit No. 3
From Study No. 2

TABLE 4.4

C(50) Values For Enalaprilat, Perindoprilat, S-10211 And Benazeprilat In Six Human Volunteers.

Subject	C(50) Values (ng/ml)			
	Enal.lat	Perind.lat	S-10211	Benaz.lat
1	2.43	1.09	0.81	5.81
2	7.62	1.23	2.73	6.91
3	7.91	1.68	4.81	5.26
4	6.34	1.36	2.31	4.24
5	6.13	1.25	1.78	4.55
6	7.31	1.56	2.12	4.09
Mean	6.29	1.36	2.43	5.14
SD	2.02	0.22	1.33	1.08

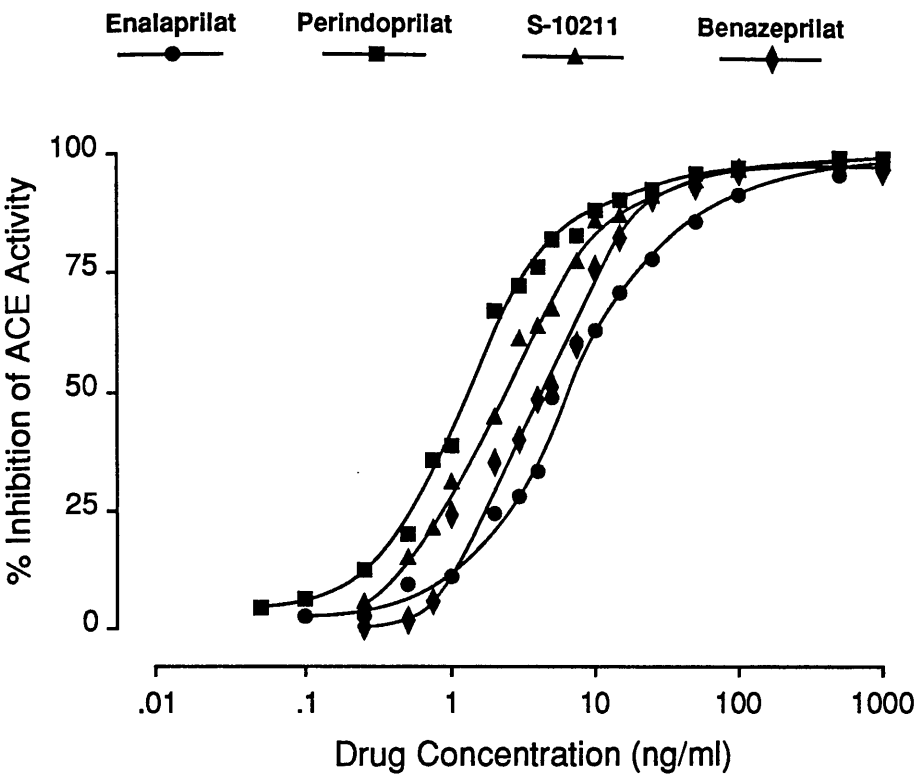


FIGURE 4.4

Dose Response Curves For Subject No. 4
From Study No. 3

TABLE 4.5

C(50) Values For Enalaprilat, Perindoprilat, S-10211 And Benazeprilat In Six Rabbits.

Rabbit	C(50) Values (ng/ml)			
	Enal.lat	Perind.lat	S-10211	Benaz.lat
1	1.47	0.69	0.78	0.99
2	1.58	0.78	0.71	1.13
3	2.33	0.83	1.12	1.22
4	2.11	1.04	0.99	1.27
5	1.92	0.90	1.06	1.00
6	1.74	0.98	1.02	1.21
Mean	1.86	0.87	0.95	1.14
SD	0.33	0.13	0.16	0.12

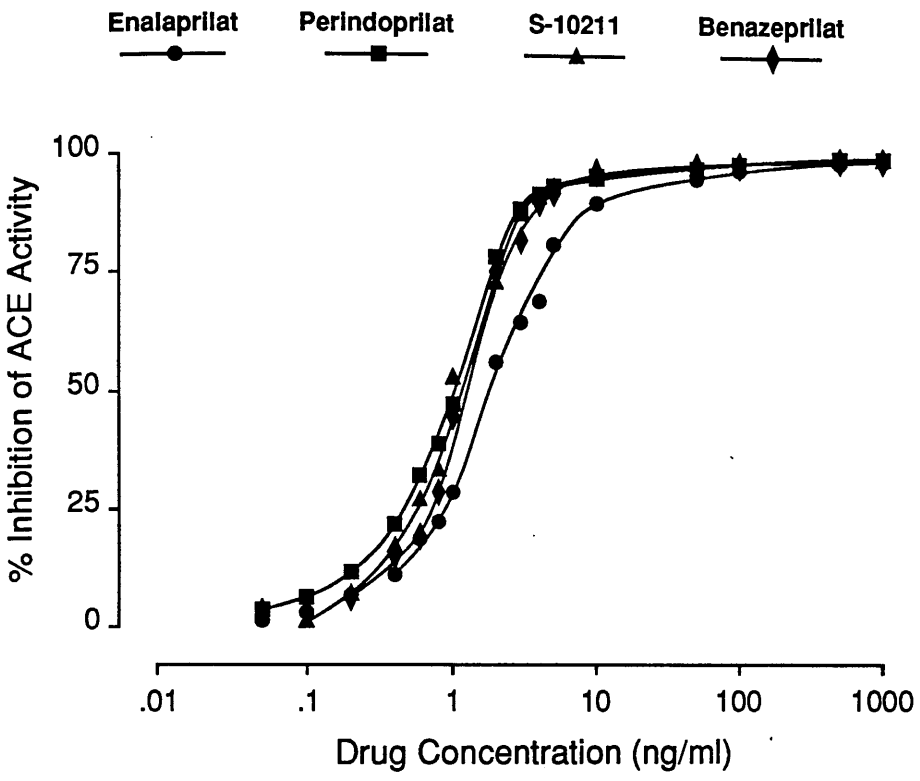


FIGURE 4.5

Dose Response Curves For Rabbit No. 1
From Study No. 4

enalaprilat and trandolaprilat were transposed and in the other rabbit the most potent compound was trandolaprilat. The mean values of C(50) (SD) in rabbit were 1.02(0.33), 1.64(0.41), 1.84(0.54) and 1029(433) ng/ml for perindoprilat, enalaprilat, trandolaprilat and captopril respectively.

The data from the four drugs studied in the eight individual rabbits and volunteers were assessed statistically by repeated measures analysis of variance. Analysis of the data revealed trends in the residual plots coupled with a non linear normal scores plot and so the analysis was repeated on the logarithmically transformed data. There were significant effects for both species and drug as well as an interaction between the two and so the results were considered with respect to the interaction by means of Bonferroni confidence intervals which make corrections for the number of comparisons made. There were significant differences between rabbit and man for all the drugs studied. There were also significant differences between all the drugs in man. In rabbit however, there were no significant differences between enalaprilat and trandolaprilat or enalaprilat and perindoprilat. There were significant differences between the remaining drugs. These statistical differences are in keeping with the rank order of potency results where, in man, where all the drugs had significantly different C(50) values, all eight volunteers exhibited the same rank order

of potency. In rabbit where the results for the drugs were not all statistically significantly different, six out of the eight rabbits showed the same rank order of potency, in the remaining two animals transpositions occured between enalaprilat, perindoprilat and trandolaprilat.

In study series 3 and 4 enalaprilat, perindoprilat, benazeprilat and S-10211 were compared within six individual volunteers and rabbits. In man the rank order of potency was perindoprilat > S-10211 > benazeprilat > enalaprilat where the mean values of C(50) (SD) were 1.36(0.22), 2.43(1.33), 5.14(1.08) and 6.29(2.02) ng/ml respectively. Five of the six subjects demonstrated the same rank order of potency as the mean values. In the remaining subject the rank order of potency was S-10211 > perindoprilat > enalaprilat > benazeprilat. In the six rabbits the mean rank order was the same as that seen in man, the mean values of C(50) (SD) were 0.87(0.13), 0.95(0.16), 1.14(0.12) and 1.86(0.33) for perindoprilat, S-10211, benazeprilat and enalaprilat respectively. Only half of the six rabbits exhibited the same rank order as that of the mean values, in two rabbits S-10211 and perindoprilat were transposed and in the remaining rabbit the rank order of potency was perindoprilat > benazeprilat > S-10211 > enalaprilat.

Again, statistical analysis of the original data for study series 3 and 4 revealed trends in the standard residual plots plus a non linear normal scores plot so the data

were logarithmically transformed. There was also one result which was far outside the range of the other results, the enalaprilat C(50) for subject number 1. Omission of this data point did not improve the standard residual plot and so the data point was restored and the data analysed as a complete set. Once again, due to an interaction between species and drug, the data were assessed by Bonferonni confidence intervals. There were statistically significant differences between rabbit and man in the C(50) values for all the drugs. In man there was no significant difference between enalaprilat and benazeprilat, the remaining drugs all showing significant differences. In rabbit, only enalaprilat was significantly different, the remaining three drugs showed no significant differences. These results are also in keeping with the rank order of potency results, one volunteer out of six had enalaprilat and benazeprilat reversed. In rabbit various orders of potency were seen but all rabbits had enalaprilat as the least potent drug, as measured by the C(50).

4.3.3 The In Vivo/In Vitro Relationship For Perindoprilat And Quinaprilat.

The placebo plasma used to prepare the in vitro perindoprilat dose response curves had been stored, frozen, for up to one year prior to pooling and subsequent

evaluation of the in vitro dose response curves. The original range of ACE activity values for the placebo plasma samples, measured up to one year prior to the pooling of the plasma samples, and the value for the pooled placebo plasma are shown in Table 4.6. For three of the volunteers the value for the pooled plasma was lower than the range originally encountered, and in one case, subject no. 4, had fallen so low such that the in vitro dose response curve could not be characterised. Of the seven in vitro dose response curves assessed three were significantly better described by the Hill model. All eight of the in vivo dose response curves were best described by the Hill model.

The quinaprilat in vitro dose response curves were prepared within days of the placebo phase of the study, allowing no time for any deterioration in the ACE activity in the placebo plasma. Of the ten volunteers, eight of the in vitro dose response curves and four of the in vivo dose response curves were best described by the Hill model.

The values of C(50) for the in vivo and in vitro dose response curves are shown in Tables 4.7 and 4.8 for perindoprilat and quinaprilat respectively. The mean in vivo and in vitro values of C(50) (SD) for perindoprilat were 1.8(0.9) and 5.8(2.1) ng/ml respectively. The mean values of C(50) (SD) for quinaprilat were 1.5(0.8) and 3.9(1.3) ng/ml for the in vivo and in vitro dose response curves respectively. Graphs of the in vivo and in vitro

TABLE 4.6

ACE Activity Values Of Plasma Used To Prepare The Perindoprilat In Vivo And In Vitro Dose Response Curves

Subject	Original ACE Activity Range (In Vivo) (EU/L)	Pooled ACE Activity Value (In Vitro) (EU/L)
1	17.2 - 22.6	22.9
2	24.9 - 32.2	24.0
3	17.7 - 23.5	18.5
4	24.2 - 32.0	10.3
5	29.5 - 35.4	32.8
6	29.9 - 40.3	20.4
7	17.5 - 25.0	27.8
8	30.0 - 36.7	22.0

The original ACE activity was measured up to one year prior to the pooling of the placebo plasma

TABLE 4.7

Perindoprilat C(50) In Vivo/In Vitro Relationship

Subject	C(50) Values (ng/ml) In Vivo	In Vitro
1	1.2	5.0
2	0.7	6.4
3	1.1	9.6
4	1.4	nr
5	3.1	6.3
6	2.9	2.9
7	1.9	4.3
8	2.2	5.8
Mean	1.8	5.8
SD	0.9	2.1

nr denotes no result

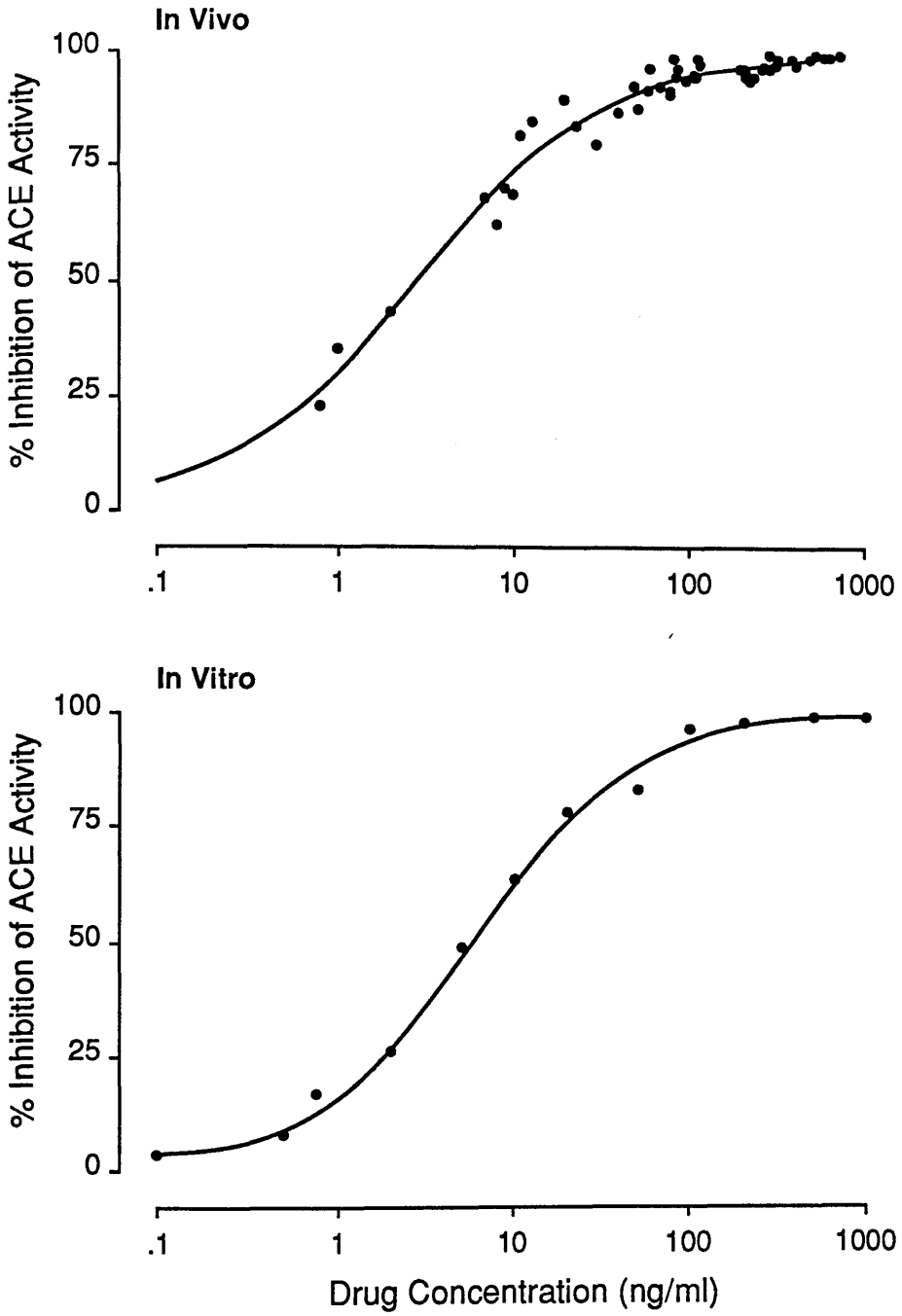


FIGURE 4.6

Perindoprilat In Vivo And In Vitro Dose Response Curves For Subject No. 8

TABLE 4.8

Quinaprilat C(50) In Vivo/In Vitro Relationship

Subject	C(50) Values (ng/ml) In Vivo	In Vitro
1	2.3	2.2
2	0.6	6.6
3	2.1	2.6
4	1.6	3.2
5	1.5	4.9
6	1.1	3.5
7	3.2	5.1
8	0.8	3.2
9	1.1	4.2
10	0.8	3.5
Mean	1.5	3.9
SD	0.8	1.3

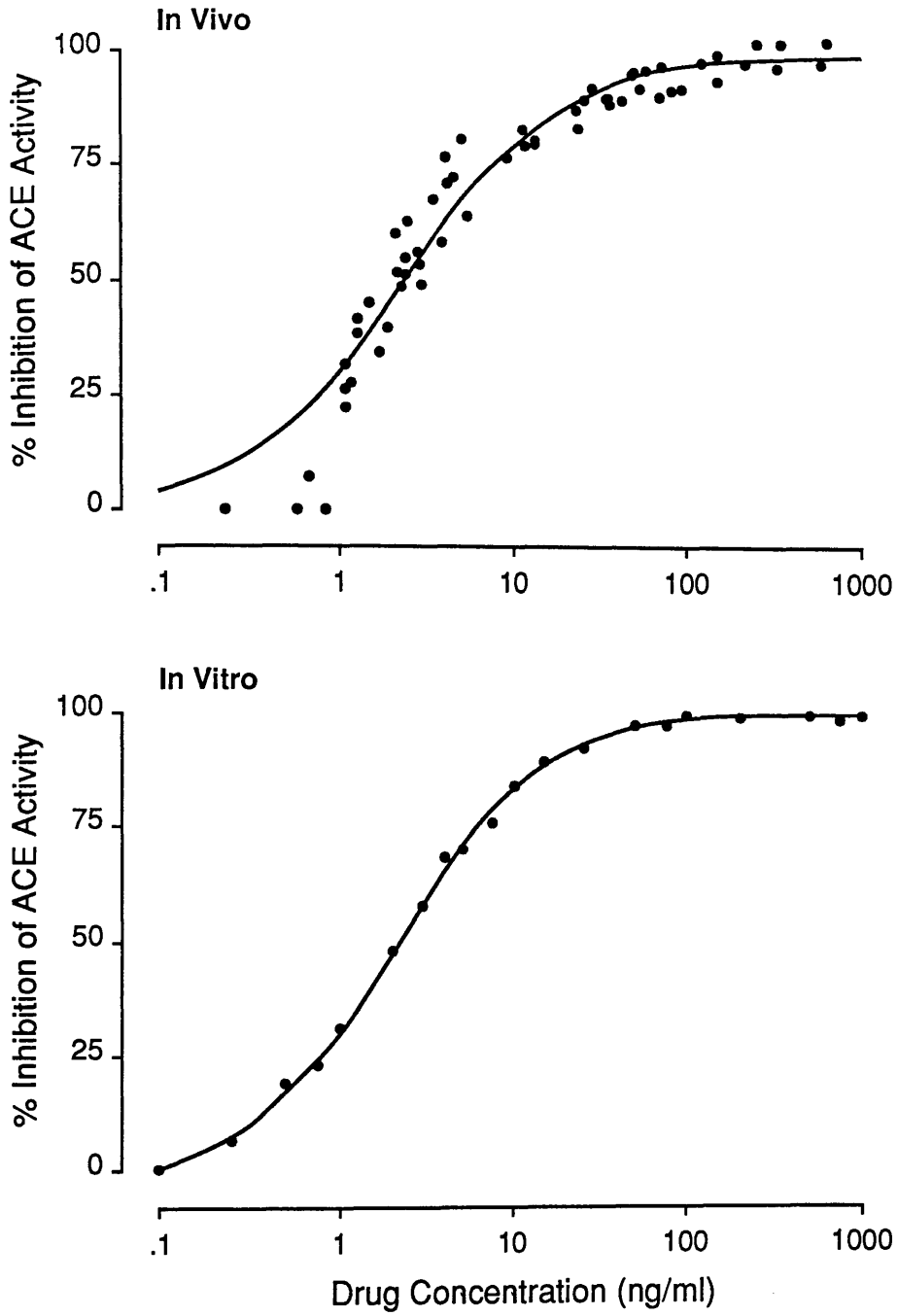


FIGURE 4.7

Quinaprilat In Vivo And In Vitro Dose Response Curves For Subject No. 1

dose response curves for a single representative subject for each compound are shown in Figures 4.6 and 4.7.
For both compounds, as assessed by the paired t-test, the in vivo values were significantly lower than the in vitro values ($p<0.01$ and $p<0.001$ for perindoprilat and quinaprilat respectively). When the inverse error in estimating the C(50) parameter was used as a weighting function in the t-test (see section 4.2.7), the in vivo values were still significantly lower than the in vitro values ($p<0.05$).

4.3.4 Effect Of Parent On The In Vitro Potency Of The Metabolite In Rabbit And Man.

The results presented in section 4.3.2 demonstrated a remarkable consistency in the rank order of potency for different ACE inhibitors within individual rabbits or volunteers. For this reason the evaluation of the effect of parent on the in vitro potency of the metabolite was undertaken in pooled plasma, samples from at least ten individual rabbits or volunteers contributing to each pool.
The effect of parent was assessed for five different compounds. For each compound a dose response curve for parent and four dose response curves for the metabolite were prepared, one for the metabolite alone and three with metabolite and parent compound. In rabbit, eighteen of

the twenty dose response curves were best described by a Hill model whereas, in man, this was the case in only ten of the dose response curves.

The dose response curves for the parent compounds are shown in Figures 4.8 and 4.9 for man and rabbit respectively. After characterising the parent compound dose response curves the concentrations of parent that inhibit ACE by 5, 10 and 25% were calculated. These values (for both rabbit and man) are shown in Table 4.9.

The results for the C(50) values of the in vitro dose response curves for the five metabolites, characterised in the presence and absence of parent compound, are shown in Table 4.10. The effects of enalapril and quinapril on enalaprilat and quinaprilat in rabbit plasma are shown in Figures 4.10 and 4.11 respectively. The effects of enalapril and perindopril on enalaprilat and perindoprilat respectively, in human plasma, are shown in Figures 4.12 and 4.13. The effect of perindopril on enalaprilat, again in human plasma, is shown in Figure 4.14.

The results of the F-Ratio test showed that each of the four curves for S-10211 and quinaprilat in rabbit plasma were significantly better described by individual C(50) values and not a single combined C(50) value. For both S-10211 and quinaprilat, the presence of parent compound caused a significant decrease in the potency of the metabolite, reflected by increased C(50) values. The value of C(50) increased from 0.82 to 1.27ng/ml (a 54.9% change) and from 1.03 to 2.10ng/ml (a 104% change) for S-

TABLE 4.9

The Concentrations Of Parent Compound Calculated To Inhibit ACE Activity By 5, 10 And 25%.

Drug	Species	Concentration (ng/ml) 5%	10%	25%
Enalapril	Man	200	500	2000
Perindopril	Man	5	10	50
Benazepril	Man	750	1500	5000
S-9650	Man	20	40	120
Quinapril	Man	1800	3900	1.16 *
Enalapril	Rabbit	50	100	200
Perindopril	Rabbit	15	50	100
Benazepril	Rabbit	15	25	50
S-9650	Rabbit	2	4	8
Quinapril	Rabbit	150	300	900

* Value in mg/ml

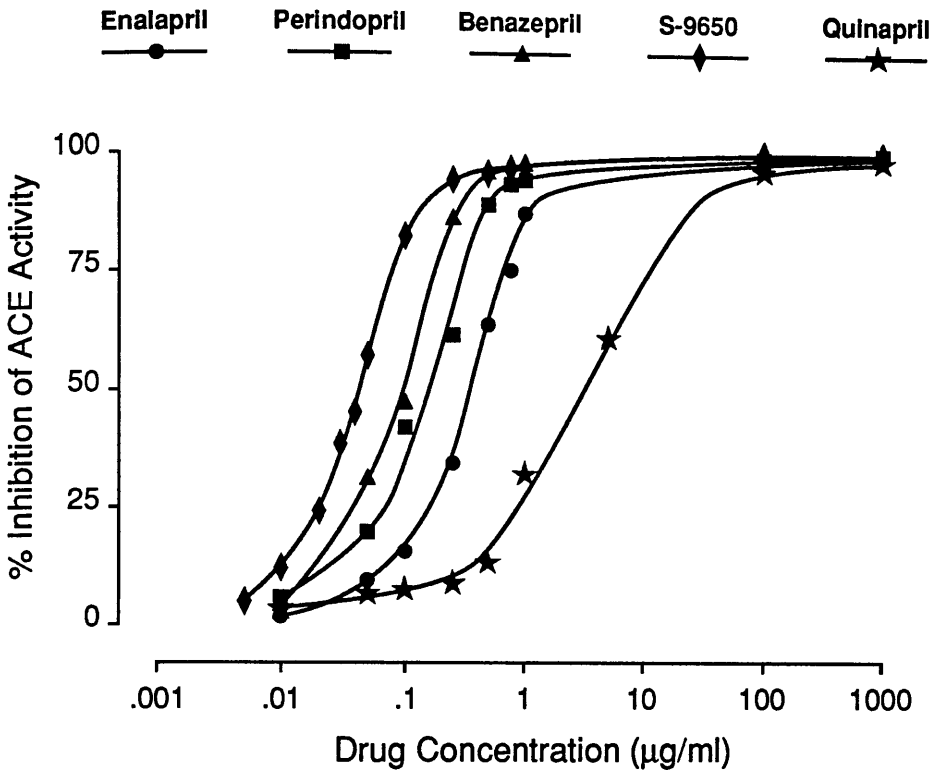


FIGURE 4.9

Dose Response Curves For Parent Compound In Rabbit

TABLE 4.10

The C(50) Values For The Metabolite In The Presence And Absence Of Parent Compound In Rabbit And Man.

Compounds		Species	C(50) Values (ng/ml)			
Parent	Metabolite		Alone	5%	10%	25%
Enalapril	Enalaprilat	Rabbit	2.17	2.04	1.97	2.38
Perindopril	Perindoprilat	Rabbit	0.99	1.14	0.86	0.98
Benazepril	Benazeprilat	Rabbit	1.28	1.20	1.18	1.02
S-9650	S-10211	Rabbit	0.82	1.18	1.01	1.27*
Quinapril	Quinaprilat	Rabbit	1.03	1.56	1.48	2.10*
Enalapril	Enalaprilat	Man	6.78	8.01	7.84	8.21*
Perindopril	Perindoprilat	Man	1.64	1.80	1.99	3.43*
Benazepril	Benazeprilat	Man	4.60	5.72	5.35	5.07
S-9650	S-10211	Man	2.25	2.48	2.47	2.35
Quinapril	Quinaprilat	Man	3.49	3.75	3.63	3.11
Perindopril	Enalaprilat	Man	7.33	6.21	6.52	9.23*

* - C(50) values statistically different ($p<0.05$) based upon the F-Ratio test

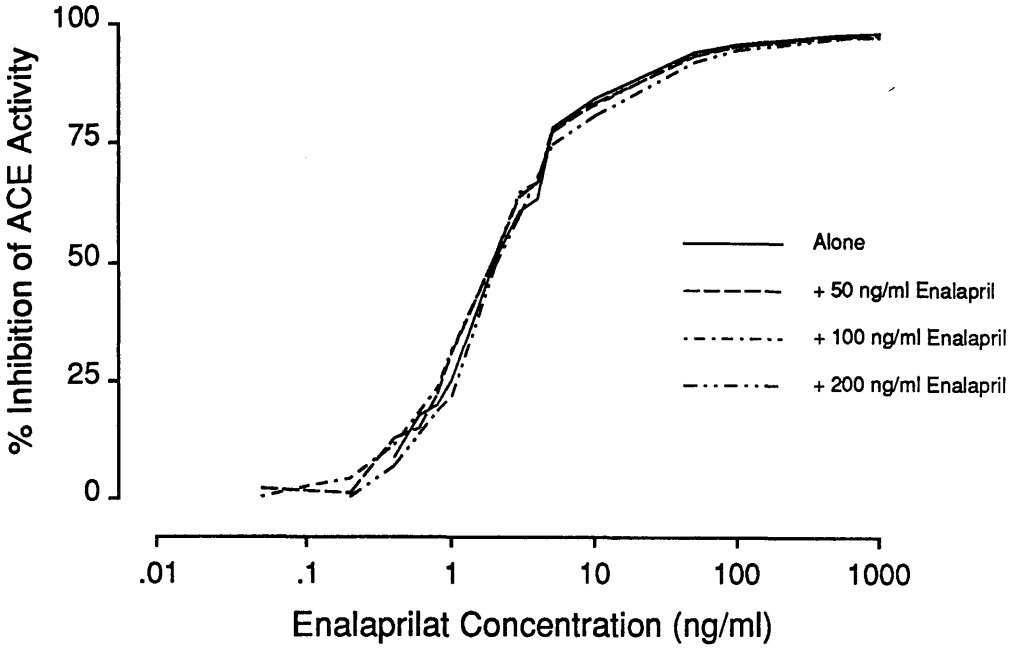


FIGURE 4.10

The Effect Of Enalapril On The Dose Response Curve For Enalaprilat In Rabbit

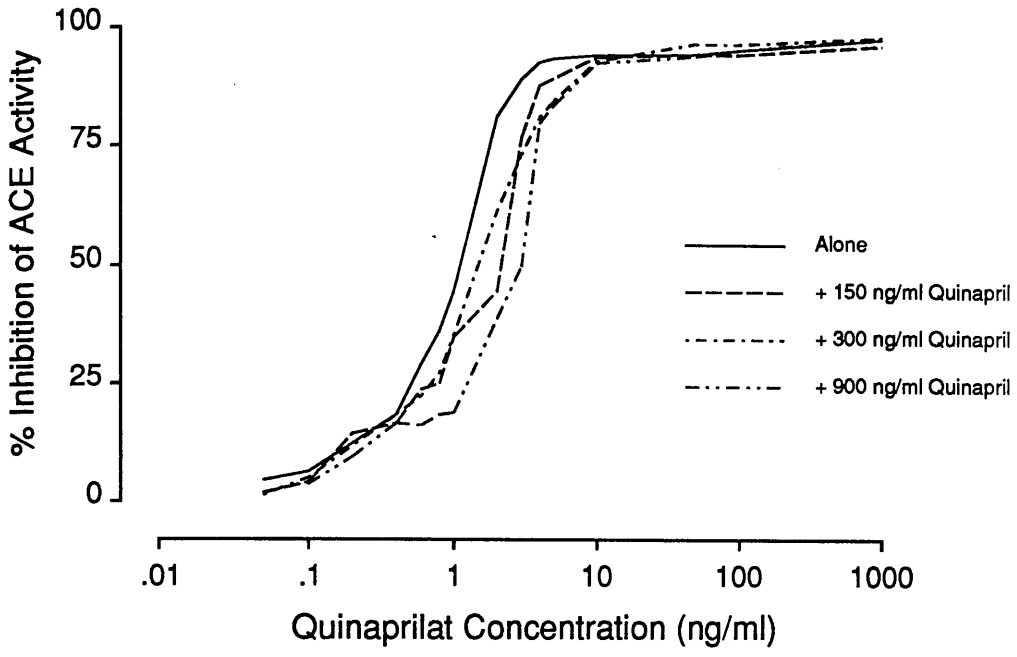


FIGURE 4.11

The Effect Of Quinapril On The Dose Response Curve For Quinaprilat In Rabbit

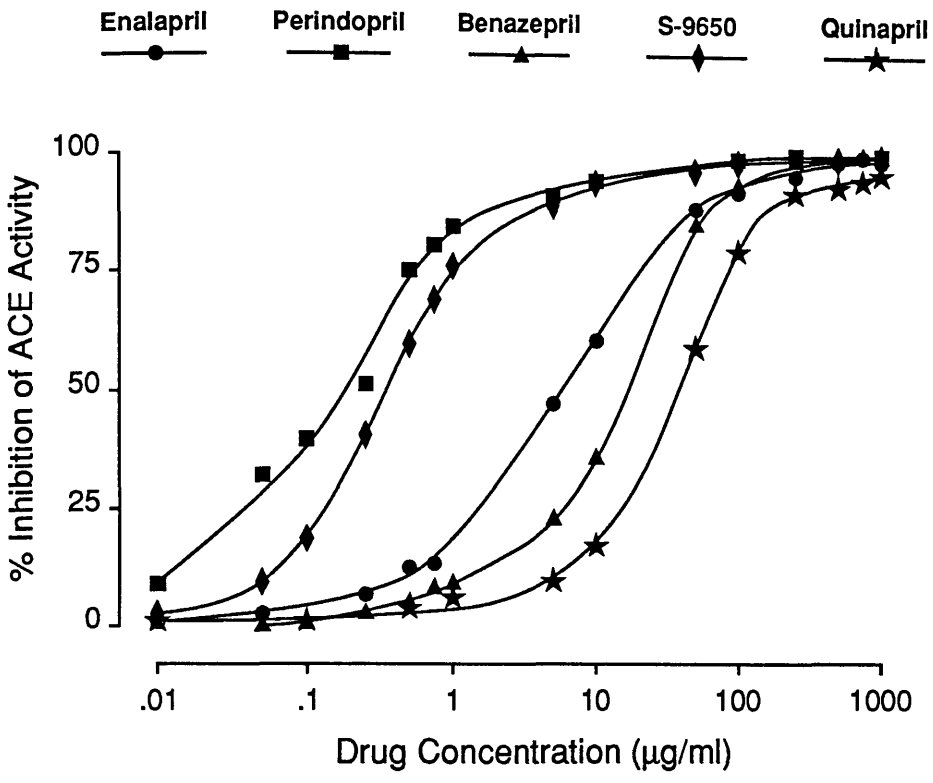


FIGURE 4.8

Dose Response Curves For Parent Compound In Man

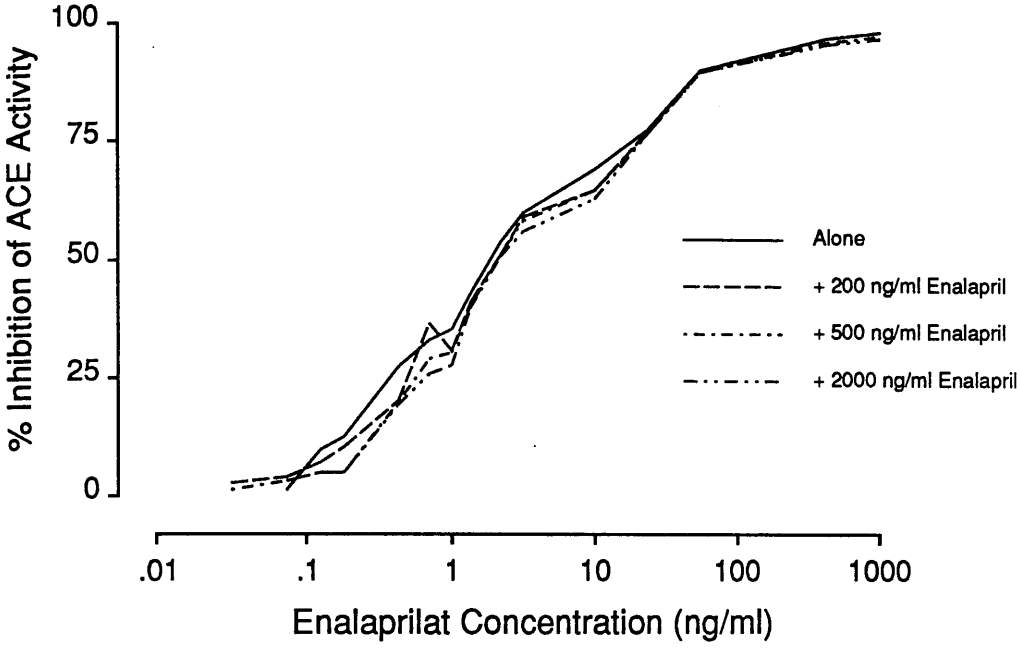


FIGURE 4.12

The Effect Of Enalapril On The Dose Response Curve For Enalaprilat In Man

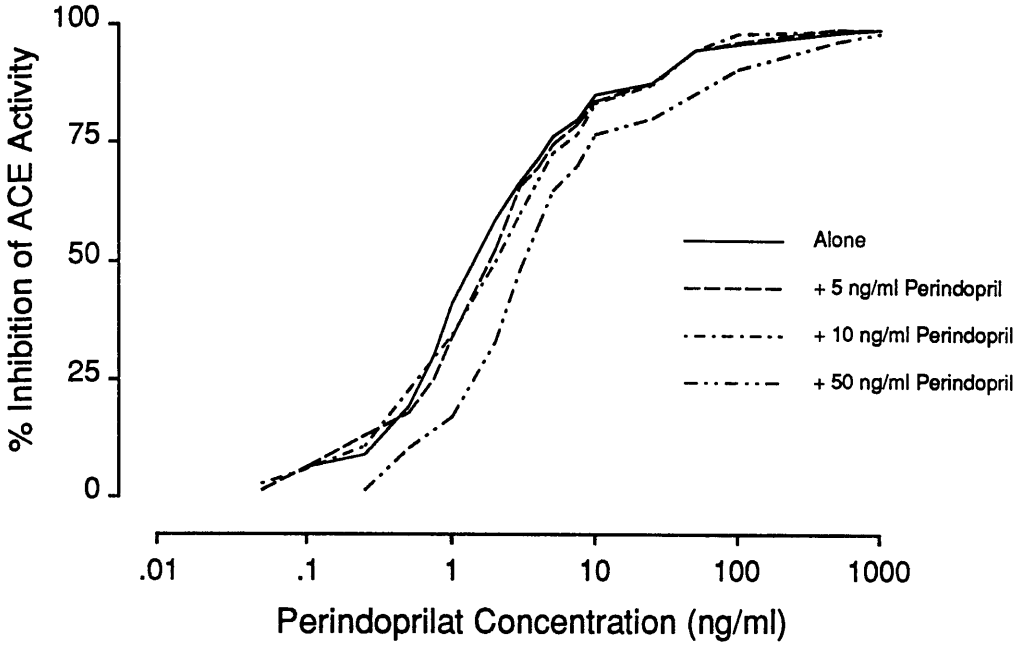


FIGURE 4.13

The Effect Of Perindopril On The Dose Response Curve For Perindoprilat In Man

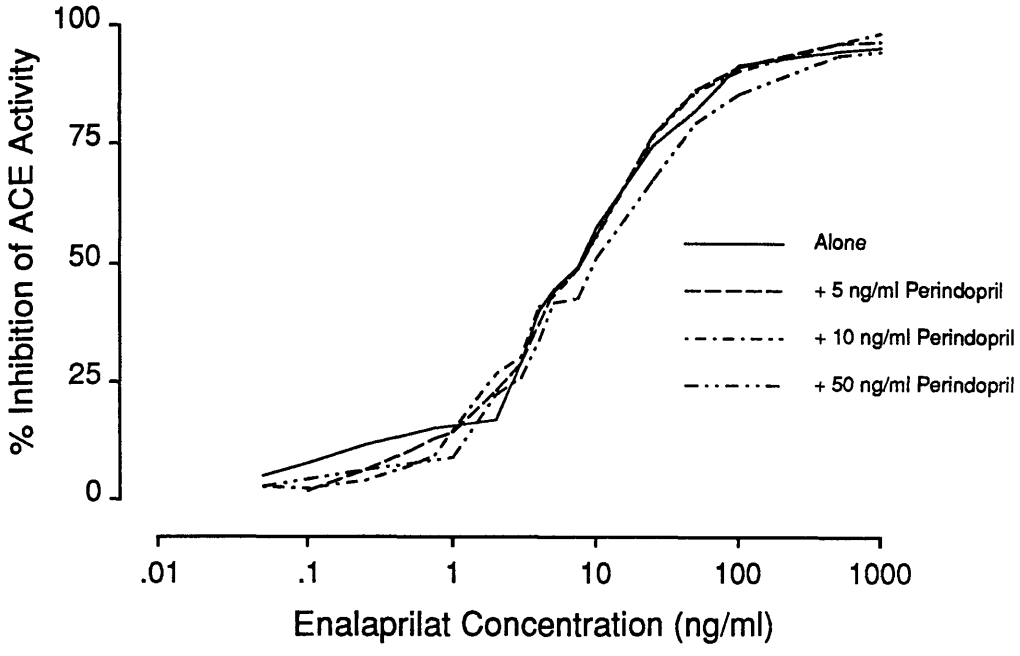


FIGURE 4.14

The Effect Of Perindopril On The Dose Response Curve For Enalaprilat In Man

10211 and quinaprilat respectively. No effect was observed for the presence of parent compound on the in vitro potency of enalaprilat, perindoprilat or benazeprilat in rabbit.

In man significant effects for enalapril and perindopril were observed on enalaprilat and perindoprilat. Enalapril caused a modest increase from 6.78 to 8.21ng/ml in C(50), (a 21.1% change), while perindopril caused a larger increase in C(50) from 1.64 to 3.43ng/ml, (a 109% change). Also in man, the presence of perindopril caused an increase in enalaprilat C(50) from 7.33 to 9.23ng/ml, a 25.9% change. Thus perindopril brought about a significant decrease in potency for both enalaprilat and perindoprilat, the decrease in potency for enalaprilat being of a similar order to that caused by enalapril. No effect of parent on the C(50) values for benazeprilat, S-10211 or quinaprilat was observed, in man.

4.4 DISCUSSION

Very low values for the coefficient of variation in the preparation and assay of the in vitro dose response curves were obtained. This indicates that any difference in the various C(50) values can be attributed to true variation and not to the relatively small variability within the experimental methods.

Two groups of compounds were compared within individual

rabbits and men. In the first series of drugs enalaprilat, perindoprilat, trandolaprilat and captopril were compared and in the second series enalaprilat, perindoprilat, S-10211 and benazeprilat were compared. All the drugs were significantly different across the species studied and exhibited a greater potency in rabbit than in man, except for captopril. The result for captopril is in agreement with previously published work where a similar, approximately ten fold greater potency was observed in man when compared to rabbit (Ibarra-Rubio et al, 1989).

There were variations in the rank order of potency for the two series of compounds in rabbit and man, especially in the first series of compounds. In the second series of compounds the mean rank order of potency was the same for the two species, however, in man a difference could only be detected between enalaprilat and benazeprilat whereas in rabbit no difference could be detected between any of perindoprilat, benazeprilat or S-10211. Thus, even though the mean rank order of the compounds was the same, there were differences in the relationships between the compounds for the two species. Ibarra-Rubio et al (1989) have found differences in the C(50), the kinetic constants Km and Vmax, and Ki, the inhibitory rate constant across ACE between many species including man and rabbit. They postulate that the differences in these constants could reflect variations in the tertiary structure of ACE. This may explain why the two series of compounds were handled

differently, with respect to rank order of potency, in the two species. Furthermore, they speculate that since ACE is present in many tissues serum ACE may represent ACE from more than one source. The differences in the kinetic parameters may then reflect different proportions of isoenzymes from the various tissues of the species studied.

The remarkable degree of consistency in the response of individuals within a species to the drugs indicates that interindividual variability is not high and so enables interspecies variability to be more easily detected. The recommended therapeutic dose range for both enalapril and captopril was reduced after the introduction of the drugs onto the market. The differences in C(50) found for the two drugs in rabbit and in man reflect the fact that different doses would be needed to obtain the same degree of ACE inhibition. Since the therapeutic range for any drug is based upon the dose response relationship and these are initially characterised in animals, then the differences in potency for enalapril and captopril between man and rabbit may have contributed to the post marketing dosage reduction.

The in vivo/in vitro relationship for plasma ACE inhibition was assessed for two drugs, perindoprilat and quinaprilat. For both compounds the in vivo values were significantly lower than those found in vitro, indicating greater inhibition of ACE in vivo. In the first instance,

the lower potency observed for the in vitro perindoprilat study was attributed to the fact that the plasma used to prepare the in vitro dose response curves had been stored frozen, for up to one year. Thus, the decrease in potency seen in vitro may reflect a deterioration in the plasma ACE activity. However, in the case of quinaprilat the dose response curves were prepared almost immediately after obtaining the plasma allowing no change in the ACE activity to occur and again, the in vitro response was less potent than that characterised in vivo. A possible reason for the difference between the in vivo and in vitro responses is that the in vitro dose response curves characterise a "snap shot" in a changing picture whereas the in vivo curves represent the whole dynamic situation. In vivo, a combination of different factors such as the presence of the ACE inhibitor within the system or the ramifications of alterations in blood pressure (therapeutic effect) may be altering the plasma ACE activity response, and so contributing towards the discrepancy between the in vivo and in vitro values.

The renin angiotensin system has been traditionally viewed as an endocrine system, however an increasing body of evidence suggests that the ACE found in tissues may be the primary site of action (Moursi et al, 1986, Sakaguchi et al, 1988 and Unger et al, 1985). In the light of the proposed tissue site(s) of action the relevance of the plasma ACE inhibition to the hypotensive action of the ACE inhibitors must be considered. Indeed, it has been shown

that the antihypertensive action of the ACE inhibitors does not always directly relate to the inhibition of ACE in the plasma (Richer et al, 1984).

The discrepancy between the in vivo and in vitro values may further help to explain why the therapeutic dose range for both enalapril and captopril was reduced after marketing. The in vitro results have been shown to be less potent than those obtained in vivo. Any calculations based upon the in vitro values would have overestimated the amount of drug needed to elicit the same response in vivo.

The third group of studies examined the effect of parent compound on the efficacy of the metabolite for enalapril, perindopril, benazepril, S-9650 and quinapril in human and rabbit plasma. The presence of parent compound brought about a significant decrease in potency for S-10211 and quinaprilat in rabbit and for enalaprilat and perindoprilat in man. In addition, in man, the presence of perindopril also decreased the potency of enalaprilat, by the same degree as the decrease caused by enalapril. Soubrier et al (1989) have carried out molecular cloning of the human ACE molecule and have demonstrated the presence of two regions with active site sequences similar to other metalloendopeptidases. It has been proposed that only one of these sites is capable of binding the ACE inhibitor molecule. The decrease in potency seen for some of the metabolites studied may be due to the parent

compound binding to the second active site sequence in the ACE molecule where it causes steric hindrance but not ACE inhibition.

The data can be extrapolated to the clinical situation and used to explain the reason for the apparently reduced incidence of first dose hypotension, observed after administration of perindopril when compared to that seen after enalapril. The decrease in potency seen for perindoprilat in the presence of perindopril could be the cause of the hysteresis seen after oral perindopril (Lees, 1986). The lag in appearance of effect behind the plasma drug levels may then explain the apparent reduced incidence of first dose hypotension. To further investigate this possibility the incidence of hysteresis after oral administration of enalapril was examined. The study and data are fully described in chapter 6 but, in summary, nine young volunteers received 10mg of enalapril orally, plasma enalaprilat levels and percent inhibition of plasma ACE were measured and then plotted against each other and the data points joined up in time sequence. The resulting graphs are shown in Figures 4.15-4.17. There is an obvious anticlockwise hysteresis loop for one volunteer and the possibility of hysteresis in a further volunteer. There was no evidence of hysteresis in the remaining seven volunteers. Since the effect did not appear to lag behind plasma drug levels in the majority of volunteers, these data further support the theory that the hysteresis seen after oral perindopril may be the reason for the reduced

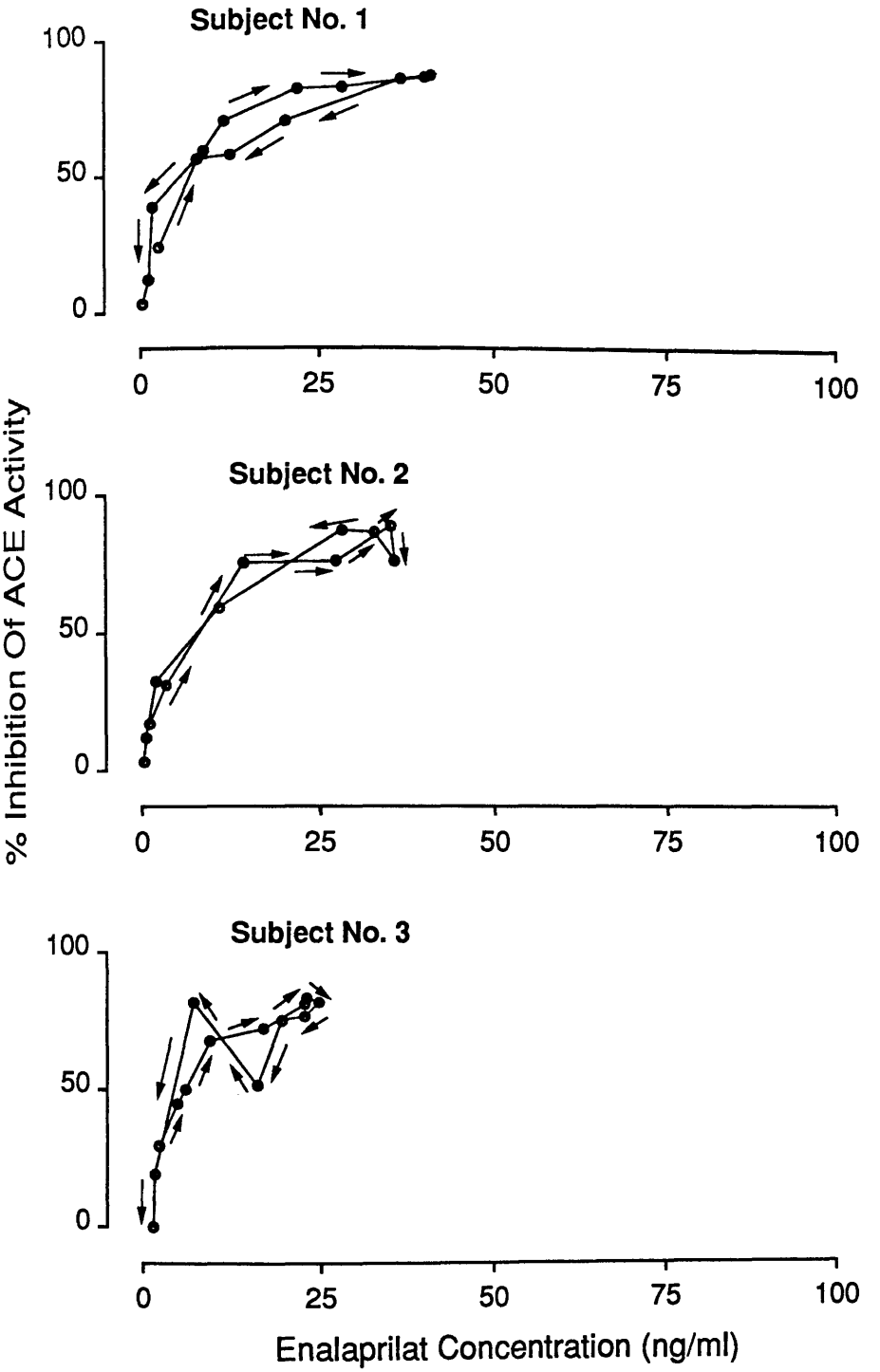


FIGURE 4.15

The Relationship Between Enalaprilat Plasma Levels And ACE Inhibition In Subjects 1, 2 And 3

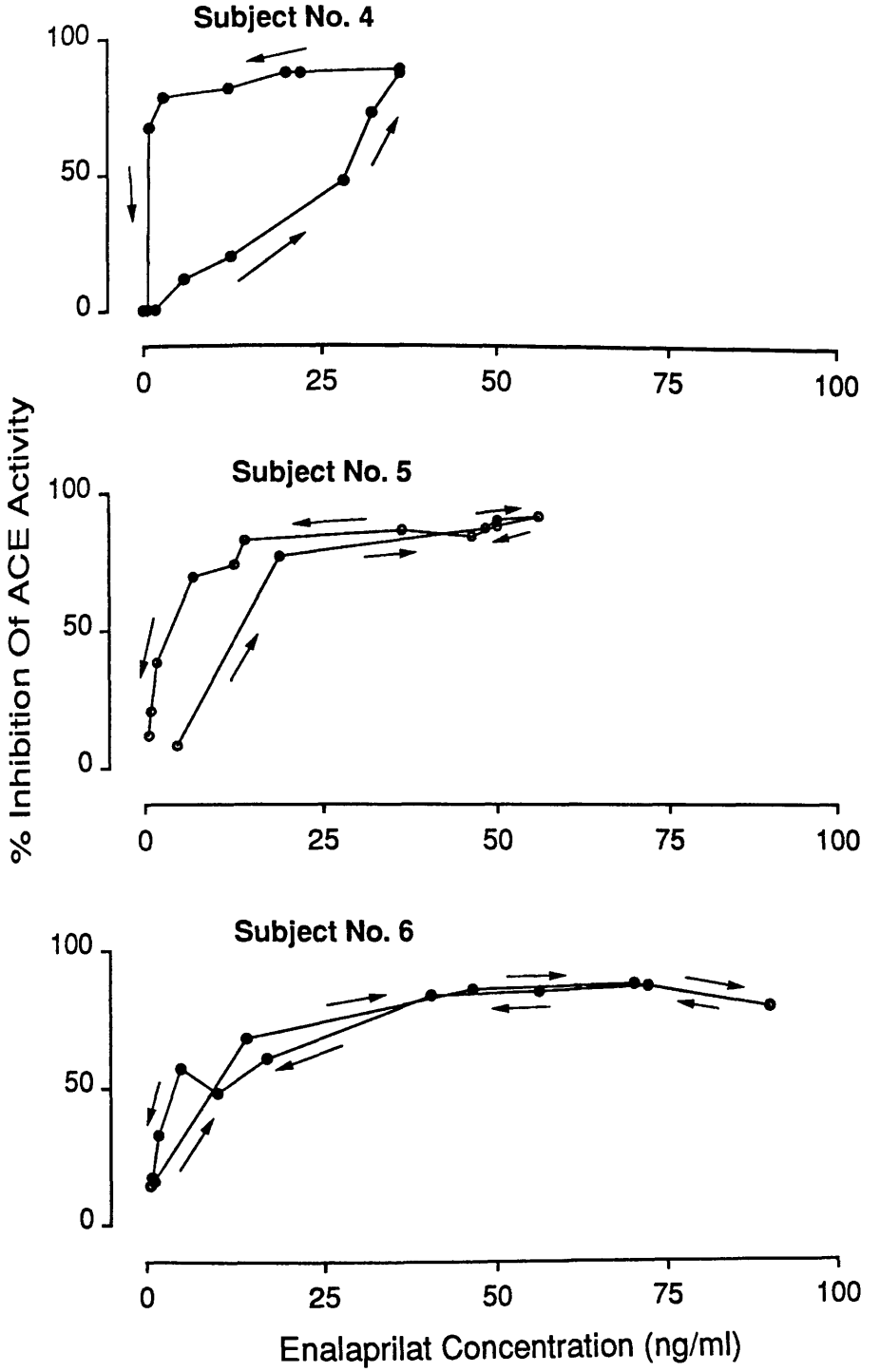


FIGURE 4.16

The Relationship Between Enalaprilat Plasma Levels And ACE Inhibition In Subjects 4, 5 And 6

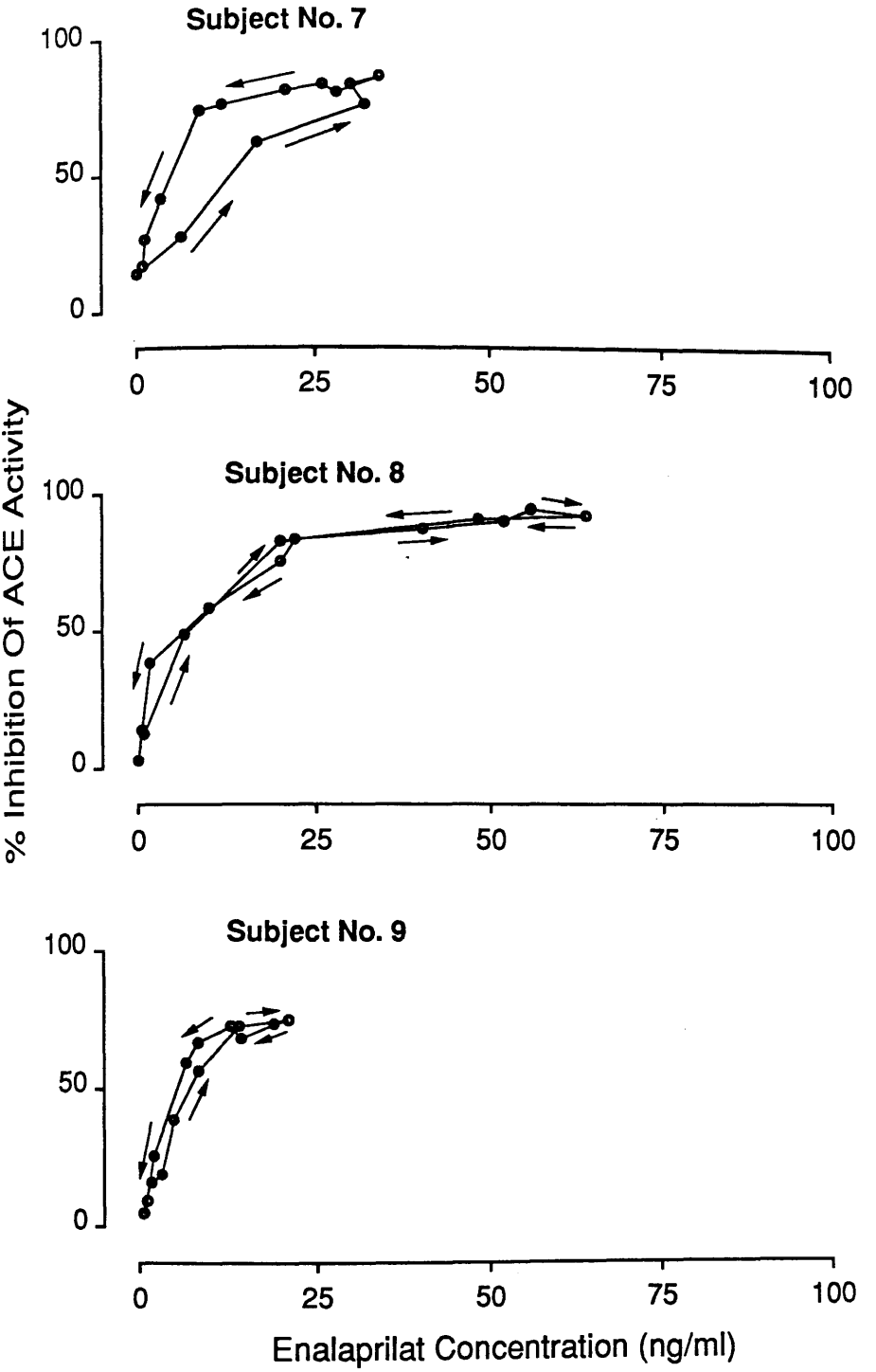


FIGURE 4.17

The Relationship Between Enalaprilat Plasma Levels And ACE Inhibition In Subjects 7, 8 And 9

incidence of first dose hypotension observed with this compound.

CHAPTER 5

THE EFFECT OF SATURATION OF PLASMA AND TISSUE ACE BINDING SITES ON THE PHARMACOKINETICS OF 3H-SPIRAPRILAT IN RABBIT

5.1 INTRODUCTION

5.1.1 Scope Of Chapter

In this chapter the pharmacokinetics of radiolabelled spiraprilat in rabbit are characterised, in the presence and absence of saturated plasma and tissue binding sites. In addition, at the end of the study, the rabbits were sacrificed and the levels of radiolabelled spiraprilat in various tissues quantified.

5.1.2 The Contribution Of Tissue ACE To The Pharmacodynamic and Pharmacokinetic Response To ACE Inhibitors.

The possibility that tissue sites and not plasma may be the major site of action for the ACE inhibitors was first proposed in the early 1980's. Work carried out in animals demonstrated that inhibition of ACE activity in certain tissues better correlated with the antihypertensive activity of ACE inhibitors than did the inhibition of plasma ACE activity. Cohen and Kurz (1982) studied the effect of administering captopril or enalapril to spontaneously hypertensive rats (SHR). Both drugs produced a prolonged inhibition of ACE in lung, aorta and kidney when compared to serum. Similar findings were reported by Unger et al (1985) after administration of enalapril and Hoe498 to SHR. Two weeks of treatment with

either drug produced markedly different responses in ACE inhibition within different tissues. After withdrawal of the drug blood pressure remained decreased for a further two weeks whereas plasma ACE activity returned to normal or elevated levels. Tissue ACE activity, measured one week after withdrawal, was still inhibited in aortic wall and kidney.

In man, a characteristic feature of the ACE inhibitors is a long terminal half life of elimination. The explanation for this phenomenon is thought to be due to binding to, and subsequent dissociation from, ACE in plasma and/or tissue. Pharmacokinetic information about the ACE inhibitors in animals is scant, although a slow terminal phase of elimination has been reported for benazeprilat after intravenous administration to rat, dog and baboon (Waldmeier and Schmid, 1989).

The study reported in this chapter sought to investigate the pharmacokinetics of spiraprilat, the active metabolite of spirapril, in rabbit. In a crossover design the animals were predosed with either unlabelled spiraprilat or dose vehicle to saturate plasma and tissue binding sites. The pharmacokinetics of intravenous radiolabelled spiraprilat were then characterised in the presence and absence of saturated ACE binding sites. After the second phase of the study the animals were sacrificed and the effect of the predosing regimen on tissue levels of spiraprilat assessed.

5.2 METHODS

5.2.1 Study Design

The study was an open, balanced, crossover design in which six New Zealand White male rabbits (Cheshire Rabbit Farm, Tarpoley, EW6 0ED) were pretreated with placebo or 3mg of unlabelled spiraprilat. Pretreatment doses were administered intraperitoneally (i.p.), twice daily, for three days. On the third day the rabbit received their morning i.p. dose and then the marginal ear vein and central ear artery were cannulated. One hour after the morning i.p. dose the rabbits received an intravenous (i.v.) dose of 3.1mg spiraprilat which contained a tracer dose of 0.1mg 3H-spiraprilat. Blood samples (1ml) were taken from the cannulated artery at 0, 2, 5, 10, and 30 minutes, 1, 2, 4, 6, 8, 10 and 24 hours post i.v. dose. Blood samples were separated by centrifugation (15 mins, 3000rpm, 4 ^{o}C), the resulting plasma and red blood cells (RBC) were stored frozen (-20 ^{o}C) until analysis.

In the second phase of the study the rabbits received the alternate pretreatment followed by a second i.v. dose containing 3H-spiraprilat.

Preliminary analysis of plasma levels of 3H-spiraprilat obtained during the first phase of the study indicated that the levels of radioactive counts had fallen to background by 8 hours post i.v. dose, and so, the 10 and 24 hour samples were not taken in the second phase of the

study.

At 8 hours post i.v. dose in the second phase of the study the animals were sacrificed by an i.v. overdose of barbiturate. The animals were quickly dissected and tissue samples taken and immersed in ice cold saline. The tissues removed were aorta, atria, lung, liver and kidney.

5.5.2 Preparation Of Spiraprilat Dose Solutions

Unlabelled spiraprilat was dissolved in 0.1M Phosphate Buffer, pH7.4, to give a final concentration of 6mg/ml.

The 3H-spiraprilat was supplied in ethanol. The ethanol was evaporated under air at 40 ^{o}C and the drug taken up in 7mls of the unlabelled spiraprilat solution. The final concentration was 6.2mg/ml, containing a tracer amount of 3H-spiraprilat, 0.2mg/ml.

The rabbits were predosed i.p. with 0.5ml unlabelled spiraprilat or 0.5ml 0.1M Phosphate Buffer. The i.v. dose, 3.1mg in 0.5ml, contained 0.1mg of 3H-spiraprilat.

5.2.3 Sample Analysis

5.2.3.1 Plasma And RBC 3H-Spiraprilat

Plasma aliquots, 0.4ml, were counted for 5 minutes in 10mls of Hionic Fluor. Samples were corrected for counting efficiency using the automated external standard channels ratio method and a quench curve (chapter 2,

section 2.2.10). Results were expressed in ng/ml of 3H-spiraprilat. The limit of detection for 3H-spiraprilat was 0.1ng/ml, as defined by counts due to 3H-spiraprilat being twice the counts due to background.

The RBC, 0.1g, were decolourised with 1.0mls hydrogen peroxide and counted for 5 minutes in 10mls Hionic Fluor. Samples were corrected for counting efficiency as described previously.

5.2.3.2 Tissue 3H-Spiraprilat

Tissues had been removed from the animals and stored in ice cold saline. The tissues were thoroughly rinsed to remove any residual blood, dried and finely chopped. Approximately 100-150mg of chopped tissue was weighed into a scintillation vial and 1ml of soluene added. Vials were placed in a shaking water bath at 37 ^{o}C and left overnight to allow the tissues to solubilise. The dissolved tissues were decolourised by the addition of 200μl of hydrogen peroxide and counted for 5 minutes in 10mls of Hionic Fluor. Results were corrected for counting efficiency and sample weight, and were expressed as ng 3H-spiraprilat/g tissue. The limit of detection for 3H-spiraprilat in tissue was 0.1ng/g.

5.2.4 Pharmacokinetic Analysis

Two and three compartment intravenous bolus dose models were fitted to the 3H-spiraprilat plasma data. The

estimates of the model parameters were obtained by non-linear least squares regression on a Nodecrest V76 computer using the statistical package VASP. Model selection was based on the Akaike criterion[a] and the F-Ratio test.

The AUC (0-8h) was calculated by the trapezoidal rule. The area from 8h to infinity was calculated by dividing the concentration at 8h by the terminal elimination rate constant. Clearance of 3H-spiraprilat was calculated by dividing the dose by the AUC (0 to infinity).

5.2.5 Statistical Analysis

The parameters of the pharmacokinetic model which best described the data were compared by paired t-test. The paired t-test was also used to compare the calculated clearance values.

5.3 RESULTS

5.3.1 Rabbits

During phase 1 (pretreated with placebo), at 8.5 hours post i.v. dose rabbit no. 2 died. This was not thought to be a drug related effect. Since results had only been collected for one phase of the study for this animal, it was excluded from the final analysis.

[a] Yamaoka, K. et al. (1978). J. Pharmacokinetics and Biopharmaceutics. 6(2), 165 - 175.

No other incidents occurred during either phase of the study.

5.3.2 Plasma 3H-Spiraprilat Analysis

After i.v. administration of 3H-spiraprilat, plasma levels declined rapidly in all animals and were no longer detectable by 10 hours post i.v. dose. Mean plasma levels after placebo and active pretreatment are shown in Table 5.1 and are illustrated in Figure 5.1.

Plasma levels declined in a multiphasic fashion. Two and three compartment intravenous bolus dose models were fitted to the data. On the basis of the Akaike criterion and the F-Ratio test the data for all animals, in both phases of the study, were best described by a three compartment model. The mean (SD) half lives for the three phases of decline were 1.8(1.0), 9.5(3.9) and 197(124) minutes following placebo pretreatment and 1.4(0.6), 7.5(1.9) and 147(10.6) minutes following unlabelled spiraprilat pretreatment. The individual parameter values of the model are shown in Table 5.2. The parameters of the model derived following placebo or active pretreatment were compared statistically. No significant difference was found between any of the parameters with the exception of beta, the rate constant associated with the second phase ($p=0.03$). The mean (SD) values of beta were $4.83(1.34)$ and $5.78(1.26) h^{-1}$ following placebo and active pretreatment respectively.

TABLE 5.1

Mean Plasma Levels Of Intravenous 3H-Spiraprilat Following Pretreatment With Placebo Or Unlabelled Spiraprilat

Time (h:min)	Plasma 3H-Spiraprilat (ng/ml) Placebo Pretreatment	Active Pretreatment
0:02	87.9 (24.0)	82.1 (22.0)
0:05	47.3 (14.3)	36.2 (9.80)
0:10	24.2 (12.2)	18.6 (5.76)
0:30	5.37 (2.50)	4.39 (2.79)
1:00	1.65 (0.44)	1.92 (1.02)
2:00	0.90 (0.21)	0.96 (0.29)
4:00	0.47 (0.20)	0.58 (0.22)
6:00	0.34 (0.18)	0.43 (0.15)
8:00	0.26 (0.13)	0.36 (0.18)

Data shown are mean (SD) values.

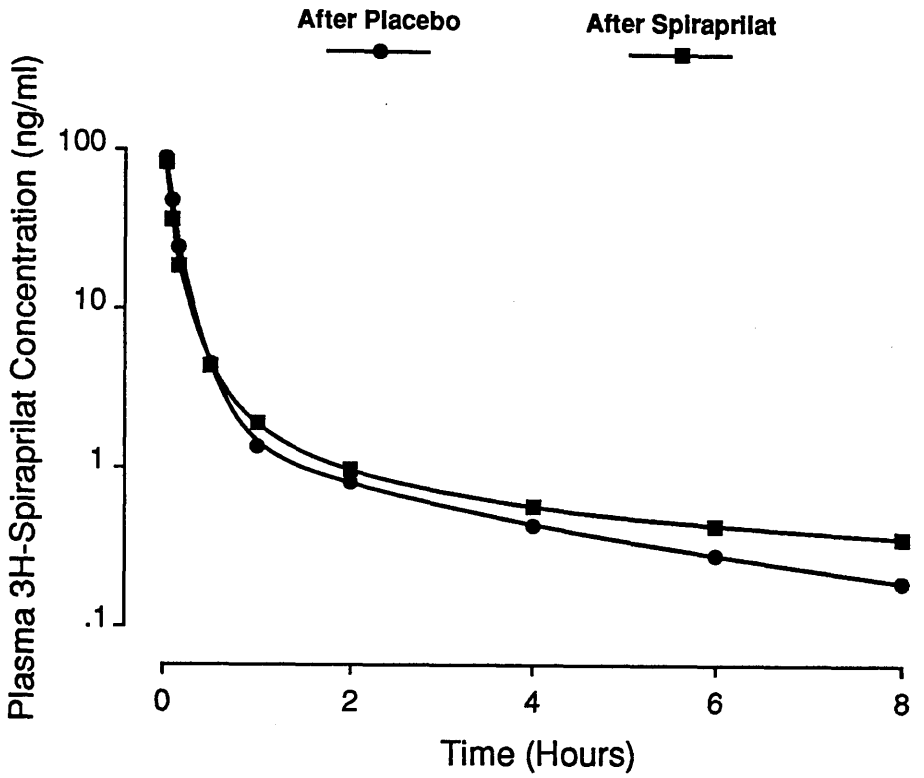


FIGURE 5.1

Mean Plasma 3H-Spiraprilat Levels In Rabbit

TABLE 5.2

Parameter Values For The Three Compartment IV Bolus Dose Model Used To Describe Plasma 3H-Spiraprilat Data In Rabbit

Rabbit	A (ng/ml)	Alpha (/h)	B (ng/ml)	Beta (/h)	C (ng/ml)	Gamma (/h)
Placebo Pretreatment						
1	126	23.2	43.5	5.44	1.79	0.40
3	269	61.9	90.8	5.96	2.21	0.25
4	135	27.0	27.5	5.35	1.64	0.28
5	102	12.2	22.3	2.54	0.46	0.10
6	106	23.6	20.7	4.88	1.23	0.29
Mean	148	29.6	41.0	4.83	1.47	0.26
SD	69.3	18.9	29.3	1.34	0.66	0.11
Active Pretreatment						
1	127	43.2	40.6	7.17	1.52	0.28
3	283	53.1	62.1	6.28	1.97	0.26
4	112	27.3	41.0	6.38	1.99	0.27
5	161	28.2	42.7	3.93	3.44	0.30
6	128	18.4	23.5	5.12	1.67	0.31
Mean	162	34.0	42.0	5.78	2.12	0.28
SD	70.0	13.9	13.7	1.26	0.77	0.02

The clearance of 3H-spiraprilat was calculated for both phases of the study. Individual data are shown in Table 5.3. The mean (SD) clearance values were 104(36.3) and 108(32.2)ml/min following pretreatment with placebo and unlabelled spiraprilat respectively. No significant difference was found between the clearance values.

5.3.3 RBC 3H-Spiraprilat Analysis

The methodology available to measure the concentration of 3H-spiraprilat in RBC involved solubilisation followed by decolourisation with hydrogen peroxide and finally, counting in 10mls of Hionic Fluor. The volume of hydrogen peroxide used, 1ml, was the maximum amount that the 10mls of Hionic Fluor could be added to and still form a stable emulsion. However, for the vast majority of the samples the solutions were still so coloured that the AES values lay outwith the range of the quench curve. For the few samples that were able to be quench corrected, a qualitative assessment revealed that the levels of 3H-spiraprilat were low, approximately 5ng/g, and appeared to remain fairly constant over the 8 hour sampling period.

5.3.4 Tissue 3H-Spiraprilat Analysis

Five tissues were sampled, aorta, liver, lung, atria and kidney. In the case of the aorta the weight of tissue

TABLE 5.3

Individual Clearance Data For 3H-Spiraprilat

Rabbit	3H-Spiraprilat Clearance (ml/min) Placebo Pretreatment	Active Pretreatment
1	110	139
3	65.0	86.0
4	107	118
5	80.2	64.2
6	160	134
Mean	104	108
SD	36.3	32.2

sampled was insufficient to enable any 3H-spiraprilat to be detected. The levels of 3H-spiraprilat measured in the remaining tissues were well above the limit of detection. Only five of the original six rabbits reached the end of the second phase of the study. Of these five, two had been pretreated with unlabelled spiraprilat and three had received placebo.

The mean levels of 3H-spiraprilat following unlabelled spiraprilat pretreatment were 1.5, 2.7, 0.62 and 23.7 ng/g in liver, lung, atria and kidney respectively. The mean levels of 3H-spiraprilat following placebo pretreatment were 1.0, 8.9, 0.60 and 8.7ng/g in liver, lung, atria and kidney respectively. The individual data are illustrated in Figure 5.2.

5.4 DISCUSSION

The aim of the study described within this chapter was to characterise the pharmacokinetics of an intravenous tracer dose of 3H-spiraprilat in the presence and absence of saturated plasma and tissue ACE binding sites. Only a limited amount of 3H-spiraprilat was available for use. To ensure a sufficient presence of spiraprilat within the plasma such that the 3H-spiraprilat could be detected, the 3H-spiraprilat was administered as a tracer dose within a thirty fold larger unlabelled spiraprilat dose.

The plasma pharmacokinetics of 3H-spiraprilat were best described by a three compartment model. No difference was

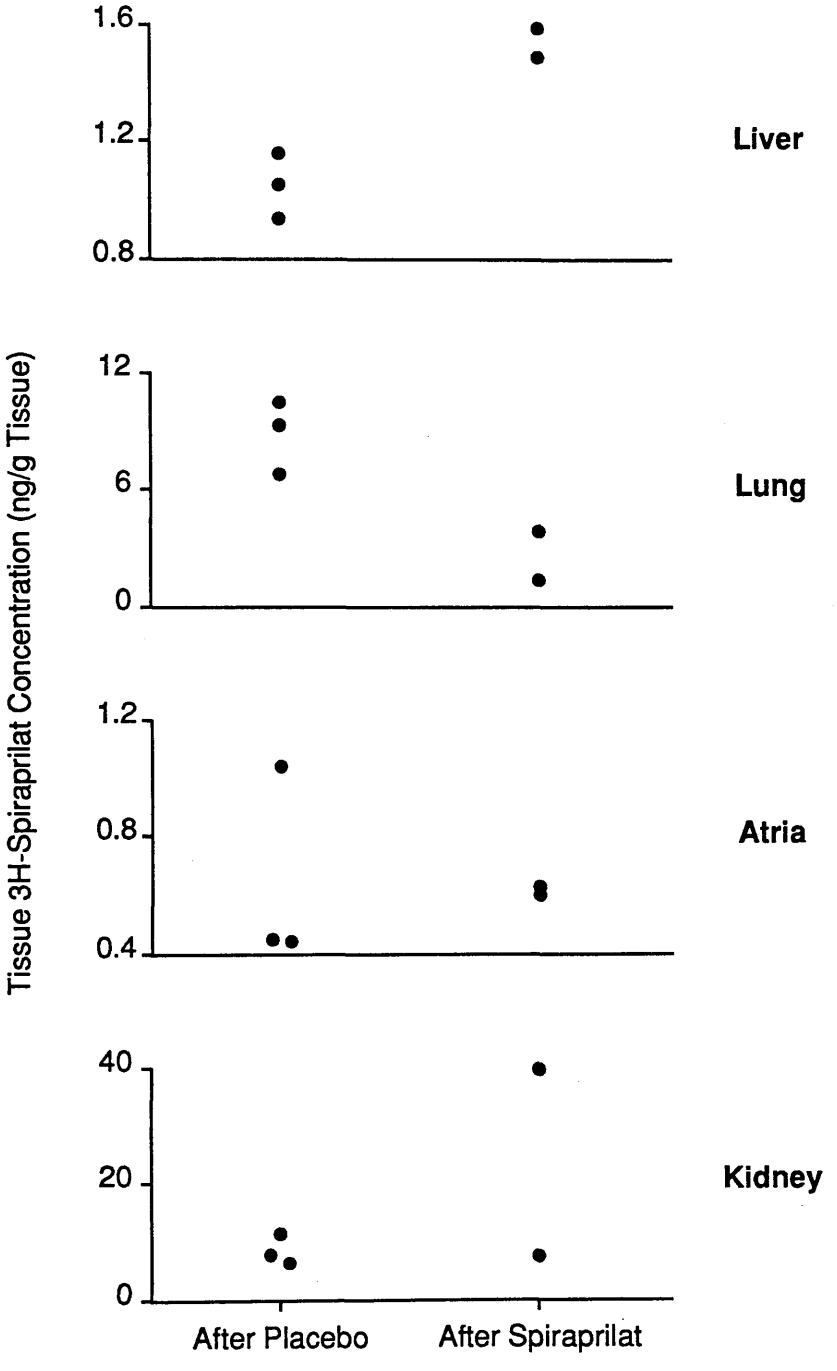


FIGURE 5.2

Individual Tissue Plasma 3H-Spiraprilat Levels In Rabbit

found between any of the parameters of the model, irrespective of pretreatment, with the exception of beta, the rate constant associated with the second phase of decline. The effect of saturation of plasma and tissue ACE binding sites caused an increase in the value of beta from 4.83 to 5.78h^{-1}. This was manifested as a decrease in the half life of elimination for that phase from 9.5 to 7.5 minutes. No difference was found in the calculated clearance values. Thus, in the presence of saturated ACE binding sites, while the rate of elimination was increased in the second phase, the effect was small and did not contribute to a change in total clearance.

The plasma ACE activity of rabbits is typically 40-60EU/L which is higher than the range normally found in man, 15-30EU/L. Despite the greater amount of ACE activity present in rabbit plasma, the terminal half life of elimination of 3H-spiraprilat was short, between 2 and 3 hours, irrespective of the pretreatment administered.

Due to the low number of animals studied with respect to characterisation of tissue levels of 3H-spiraprilat, the results can only be interpreted qualitatively. Despite this, some interesting trends were shown for some of the tissues studied. The highest levels of 3H-spiraprilat were found in the lung and the kidney. Both these organs are associated with high endogenous amounts of ACE. The presence and absence of saturated binding sites appeared to have no effect on the 3H-spiraprilat levels in kidney

or atria. However, in the lung, lower levels of 3H-spiraprilat were found when binding sites had been saturated. The opposite was found in the liver, the pretreatment to saturate binding sites led to an increase in the amount of 3H-spiraprilat present.

In conclusion, the rabbit would appear not to be a good model for plasma ACE inhibitor pharmacokinetics in man. Despite higher plasma ACE activity values, the long terminal half life, a characteristic feature of ACE inhibition in man, was not observed for 3H-spiraprilat in rabbit. It is possible that the long terminal half life may have been present at levels below the limit of detection for 3H-spiraprilat in rabbit plasma. The presence of saturated plasma and tissue ACE binding sites did not cause any significant alteration in the clearance of 3H-spiraprilat from rabbit.

CHAPTER 6

THE EFFECT OF SATURATION OF PLASMA AND TISSUE ACE BINDING SITES ON THE PHARMACOKINETICS OF ENALAPRILAT IN MAN

6.1 INTRODUCTION

6.1.1 Scope Of Chapter

In this chapter the pharmacokinetics of enalaprilat in man are characterised, in the presence and absence of saturated plasma and tissue ACE binding sites. The pharmacokinetics of enalaprilat were studied after two separate single oral doses of 10mg enalapril to eight healthy male volunteers. The ACE binding sites were saturated by predosing the volunteers with captopril prior to the second dose of enalapril.

6.1.2 The Contribution Of Tissue ACE To ACE Inhibitor Pharmacokinetics

It is only within recent years that the traditional view of the renin angiotensin system (RAS) as a circulating endocrine system has been expanded. It is now widely recognised that renin and angiotensinogen genes, and their products, are expressed at many local tissue sites.
An increasing body of evidence supports the theory that these local tissue sites play an important role in blood pressure regulation. That this is the case is supported by the relative independence of the ACE inhibitors from their plasma pharmacokinetics (Schoenberger and Wilson, 1986, de Cesaris et al, 1987 and Garanin, 1986).
Work has been carried out in animal species to investigate

the degree of inhibition of ACE within the tissues with respect to time. These data have shown that the ACE inhibition profile seen in plasma does not parallel that seen in tissue (Jackson et al, 1988b and Johnson et al, 1988).

The contribution of tissue sites of action to the plasma pharmacokinetics of the ACE inhibitors has not been investigated in man. It is a characteristic feature of the ACE inhibitors that they have a long terminal half life. Binding to ACE, in the plasma and/or in the tissues may be responsible for the long terminal phase (Till et al, 1982). The extended half life for these drugs appears to have no clinical significance since there is little accumulation after chronic dosing (Nussberger et al, 1987).

The study described in this chapter seeks to examine the pharmacokinetics of enalaprilat in the presence and absence of saturated plasma and tissue binding sites in man. Captopril was selected as the ACE inhibitor to saturate the binding sites because it is known to be unstable in plasma, undergoing rapid autoxidation to form disulphide dimers (Kripalani et al, 1980). As such, storage of the plasma samples containing both captopril and enalaprilat would eventually enable a 'clean' quantification of the enalaprilat plasma concentrations.

6.2 METHODS

6.2.1 Study Design

Eight young healthy male volunteers were recruited to the study. Their mean ages and weights (SD) were 27 (8) years and 74 (12) kg respectively. Full demographic data are shown in Table 6.1. These volunteers formed part of a study designed to investigate the effects of age on the pharmacokinetics of benazepril and its active metabolite, benazeprilat, and to compare the results with enalapril. The benazepril study will be described in full detail in chapter 7. Thus, for the first part of the present study, the eight volunteers (Subjects 2-9) had already received a single 10mg dose of enalapril and placebo as part of a double blind, randomised, crossover trial. The second part of the present study was an open design in which the eight volunteers received captopril, 50mg, twice daily, for five days. Two hours after the morning dose of captopril on the fifth day a second oral dose of enalapril, 10mg, was administered. Blood samples were taken predose and following both enalapril doses for a 72 hour period.

6.2.2 Sample Analysis

6.2.2.1 Captopril Analysis

Samples taken at 15 minutes before and at the time of the

TABLE 6.1

Volunteer Demographic Data For Enalapril

Pharmacokinetic Study In Man

Subject	Age (yrs)	Weight (kg)	Height (cm)	Creatinine Clearance (ml/min)
2	22	70	170	162
3	27	102	185	160
4	21	62	170	97
5	39	73	172	87
6	39	71	175	112
7	24	73	175	100
8	21	69	180	90
9	21	76	179	nd
Mean	27	74	176	115
SD	8	12	5	32

nd - not determined.

second dose of enalapril were assayed for captopril levels using a combination of the methods reported by Jarrot (1980) and Hayashi (1985). Mixed disulphides of captopril were reduced to form captopril which was then derivatised and assayed by HPLC with fluorescence detection. No internal standard was used so the results for total captopril are semi qualitative.

Measurement of 'clean' enalaprilat levels after pretreatment with captopril depended on the instability of captopril in biological media. To check that captopril does dissociate from any ACE molecules it may be bound to (since the presence of captopril would interfere with subsequent assays for plasma enalaprilat), two predose blood samples containing captopril, withdrawn from two different subjects in the second phase of the study were used. One sample was kept on ice and the other at room temperature. The samples were assayed for ACE activity eight times during the eight hours after sampling.

6.2.2.2 ACE Activity Analysis

ACE Activity was measured by the method described in chapter 2, section 2.2.2. ACE activity was measured on four different occasions, once each after placebo and enalapril alone and twice after enalapril plus captopril. Following the second dose of enalapril (taken as well as captopril), samples were first assayed within one hour of the sample being withdrawn such that the combined effect

of captopril and enalaprilat on ACE activity could be measured. The second analysis was carried out at least one month later, when any changes in ACE activity could be attributed to enalaprilat only.

6.2.2.3 Enalaprilat Analysis

Following administration of enalapril, enalaprilat levels were measured by the technique described in chapter 2, section 2.2.8.

6.2.3 Pharmacokinetic Analysis

The ACE activity data were placebo corrected and the percent inhibition of ACE calculated. The AUC for the percent inhibition of ACE with respect to time was calculated from 0 to 72 hours by the trapezoidal rule.
The area under the enalaprilat plasma concentration v time curve (AUC) for 0 to ∞ was calculated by the trapezoidal rule. The area from 72 hours to ∞ was calculated by dividing the concentration at 72 hours by the terminal elimination rate constant.
The terminal elimination half lives ($t_{1/2}$) for enalaprilat were calculated according to

$$t1/2 = \frac{0.693}{\beta}$$

where β is the terminal elimination rate constant.

One and two compartment models with zero order input were fitted to the enalaprilat data. The estimates of the model parameters were obtained by non-linear least squares regression on a Nodecrest V76 computer. The data were weighted by the reciprocal of the concentration. Model selection was based on the Akaike criterion and the F-Ratio test.

6.2.4 Statistical Analysis

The three ACE activity AUC values were compared by repeated measures one way analysis of variance (ANOVA).
The enalaprilat AUC data, the parameters of the model and the terminal elimination half lives were compared by paired t-test.
The four ACE activity profiles (placebo, enalapril alone and enalapril plus captopril analysed both immediately and after one month) were analysed by repeated measures ANOVA. Where appropriate, Bonferroni multiple comparisons were calculated to identify significant differences both within and across the different ACE activity profiles.

6.3 RESULTS

6.3.1 Volunteer Compliance

Seven of the eight volunteers completed the study. The remaining volunteer (subject no. 8) did not return for the 48 and 72 hour samples following the second dose of enalapril. This prevented the pharmacokinetics of enalaprilat and ACE activity from being correctly characterised in the second phase of the study.

6.3.2 Captopril Analysis

The results for the two samples analysed for captopril for each volunteer, taken 15 minutes before and at the time of administration of the enalapril dose are shown in Table 6.2.

The results for the two samples containing captopril, one stored on ice and the other at room temperature, are shown in Table 6.3. The ACE activity values for both samples increased over the eight hour analysis period indicating a rapid dissociation of captopril from ACE.

6.3.3. ACE Activity Data

The mean ACE activity data for the seven subjects who completed the study are shown on Table 6.4. The mean

TABLE 6.2

Plasma Captopril Levels In Enalapril

Pharmacokinetic Study In Man

Subject	Plasma Captopril Concentration (μg/ml)	
	Time(min)	
	-15	0*
2	nd	nd
3	0.8	0.6
4	0.1	0.1
5	0.8	0.4
6	0.4	0.5
7	1.2	0.4
8	0.8	1.0
9	0.4	0.3
Mean	0.6	0.5
SD	0.4	0.3

nd - not determined.

Times relate to the second dose of enalapril.

TABLE 6.3

The Dissociation Of Captopril From ACE

With Respect To Time

Sample Kept At Room Temperature		Sample Kept On Ice	
Time (min)	ACE Activity (EU/L)	Time (min)	ACE Activity (EU/L)
52	2.2	32	5.3
111	8.3	72	6.0
171	12.3	133	7.4
230	13.6	173	8.1
290	12.2	225	9.8
350	13.0	291	11.8
410	13.4	345	15.0
470	12.4	460	18.3

TABLE 6.4

Mean ACE Activity Data Measured In Enalapril Pharmacokinetic Study In Man

Time (h)	Placebo	Enalapril Alone	Enalapril plus Captopril	
			Analysed Immediately	Analysed After 1 Month
0	29.1 (5.6)	27.5 (6.5)	3.1 (2.6)	29.0 (4.9)
0.5	26.3 (5.2)	22.6 (5.0)	2.3 (0.8)	24.5 (5.8)
1.0	27.0 (5.1)	16.8 (8.3)	3.8 (1.8)	14.3 (7.1)
1.5	26.8 (5.7)	11.3 (7.2)	4.3 (2.3)	9.7 (5.5)
2.0	25.6 (5.8)	8.2 (5.8)	4.3 (2.5)	7.9 (4.5)
3.0	26.3 (6.4)	6.2 (3.4)	4.0 (2.2)	6.8 (4.0)
4.0	26.9 (6.2)	5.1 (1.8)	3.7 (2.1)	7.1 (4.4)
5.0	26.8 (5.6)	5.1 (2.3)	4.5 (2.2)	6.0 (2.4)
6.0	26.9 (5.3)	4.9 (2.7)	4.7 (2.1)	6.5 (2.3)
8.0	25.1 (4.4)	6.3 (2.6)	5.9 (2.0)	7.9 (2.3)
10.0	25.6 (5.0)	8.0 (3.5)	8.7 (2.4)	10.0 (2.4)
12.0	25.5 (4.4)	9.6 (4.0)	9.9 (2.8)	11.9 (2.5)
24.0	27.6 (5.4)	14.0 (7.1)	17.4 (5.4)	21.9 (5.2)
48.0	26.9 (6.3)	20.5 (7.8)	24.1 (7.3)	30.5 (6.3)
72.0	28.8 (5.6)	25.3 (6.2)	27.3 (5.5)	35.3 (6.8)

Values shown are mean ACE activity (SD) levels and have units of EU/L.

percent inhibition of ACE data for the eight subjects are shown in Table 6.5 and represented graphically in Figure 6.1.

The AUC for the percent inhibition of ACE with respect to time was determined from 0-72 hours on three occasions per volunteer. First, when enalapril had been given alone, thus all inhibition of ACE could be attributed to enalaprilat (AUC_1). The second ACE inhibition AUC was determined from the samples analysed immediately after the second dose of enalapril when inhibition of ACE activity was due to the both enalaprilat and captopril (AUC_2). The third ACE AUC was determined one month after the second dose of enalapril and so represents the contribution of enalaprilat only to the inhibition of ACE activity (AUC_3). Subject no. 8 did not return for the 48 and 72 hour samples following the second dose of enalapril and so his ACE AUC data were calculated from 0-24 hours only. The individual ACE AUC data are shown in Table 6.6.

The three ACE AUC's were analysed statistically by repeated measures one way ANOVA. The analysis was carried out twice, once including and once excluding the 0-24 hour data for subject no. 8. For both analyses the test gave the same qualitative result and so only the data excluding subject no. 8 are presented. There was a significant difference between the three ACE AUC's ($p=0.001$). Bonferroni confidence intervals (95% CI) were calculated to show where the differences lay. No difference was found between AUC_1 and AUC_2 (95% CI -665 to 1136) but

TABLE 6.5

Mean Inhibition Of ACE Activity Calculated

In Enalapril Pharmacokinetic Study In Man

Time (h)	Enalapril Alone	Enalapril plus Captopril	
		Analysed Immediately	Analysed After 1 Month
0	0 (0.0)	88.1 (11.5)	0 (0.0)
0.5	7.1 (8.0)	82.4 (7.2)	6.3 (15.5)
1.0	32.6 (26.2)	79.8 (9.4)	45.1 (18.7)
1.5	50.6 (29.5)	76.9 (9.7)	59.5 (16.3)
2.0	57.0 (25.9)	73.1 (9.3)	61.0 (14.5)
3.0	69.0 (17.7)	78.5 (7.9)	66.8 (10.6)
4.0	73.0 (10.6)	79.2 (10.1)	68.2 (14.9)
5.0	74.0 (6.1)	76.3 (10.2)	71.6 (11.2)
6.0	74.2 (10.5)	74.9 (11.5)	69.4 (12.4)
8.0	63.4 (13.6)	65.5 (9.4)	59.1 (10.3)
10.0	58.4 (16.0)	57.8 (10.3)	54.4 (11.6)
12.0	53.2 (13.6)	52.8 (8.3)	46.8 (9.7)
24.0	44.2 (23.9)	34.0 (13.7)	20.4 (10.4)
48.0	18.9 (27.3)	7.3 (3.9)	-12.6 (9.8)
72.0	6.2 (14.7)	2.5 (11.7)	-22.4 (15.5)

Values shown are mean (SD) percent inhibition of ACE activity.

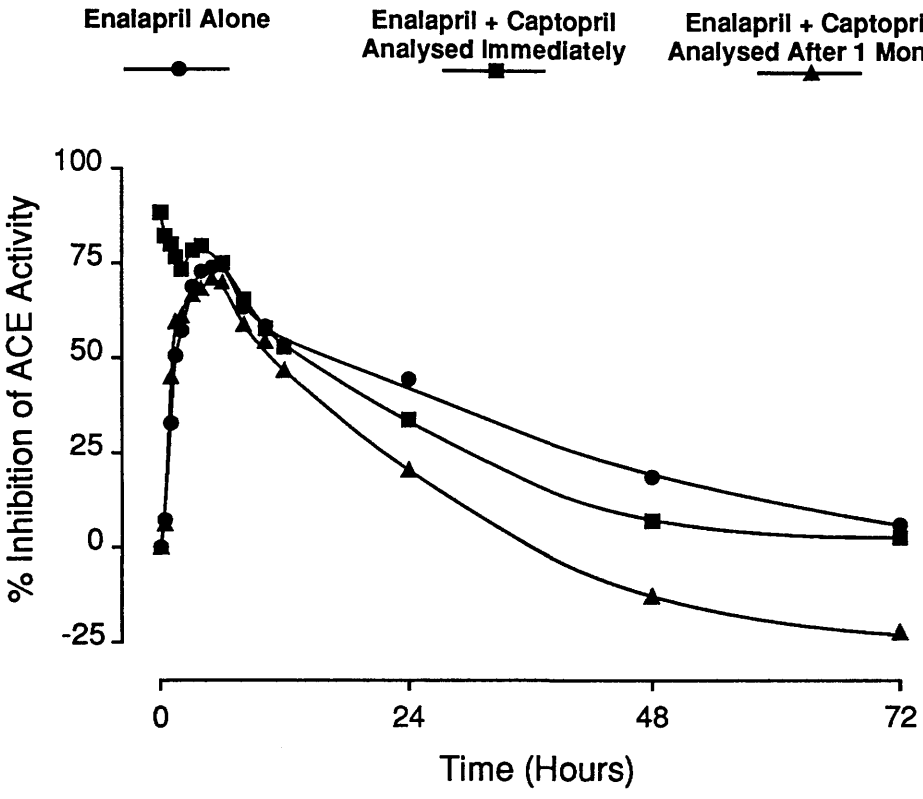


FIGURE 6.1

Mean % Inhibition Of ACE Activity

TABLE 6.6

Individual ACE Activity AUC Data

Subject	Enalapril Alone	Enalapril plus Captopril	
		Analysed Immediately	Analysed After 1 Month
	AUC 1	AUC 2	AUC 3
2	1308	2361	726
3	2376	1591	13.0
4	3916	2504	1756
5	3112	1987	787
6	1799	2920	452
7	2756	2166	730
8 *	1143	1397	999
9	1179	1270	867
Mean	2109	2114	808
SD	863	558	494
Mean **	2247	2024	781
SD **	831	575	527

Data have units of EU.h/ml and are calculated from 0-72 hours.

* - ACE Activity AUC calculated from 0-24 hours.

** - Values calculated excluding data for subject no. 8.

both were significantly greater than AUC_3 (95% CI's 687 to 2488 and 452 to 2254 respectively).

In order to try and account for the difference in AUC_3 when compared to AUC_1 and AUC_2, repeated measures ANOVA of the ACE activity data was carried out. Significant differences were found between the ACE activity profiles ($p<0.001$) and between individual time points ($p<0.001$). Bonferroni multiple comparisons were then calculated to show where the differences lay and the results are illustrated in Figure 6.2, a-d. The first graph, (a), in Figure 6.2 illustrates the significant differences between the ACE activity profile obtained after enalapril alone and the profile obtained after the second analysis of the enalapril plus captopril, when any change in ACE activity is attributable to enalaprilat alone. There were significant differences between the two profiles at 48 and 72 hours with ACE activity being higher when captopril and enalapril were co-administered. Figure 6.2 (b) illustrates the differences between the placebo and enalapril plus captopril (analysed after one month) profiles. There are obvious significant differences between the two profiles from 1 to 24 hours. The point of interest is at 72 hours where the ACE activity value is significantly higher for the enalapril plus captopril profile when compared to placebo. The elevated levels at 48 hours just failed to achieve statistical significance (95% CI -9.8 to 1.3). Figure 6.2 (b) also shows that for

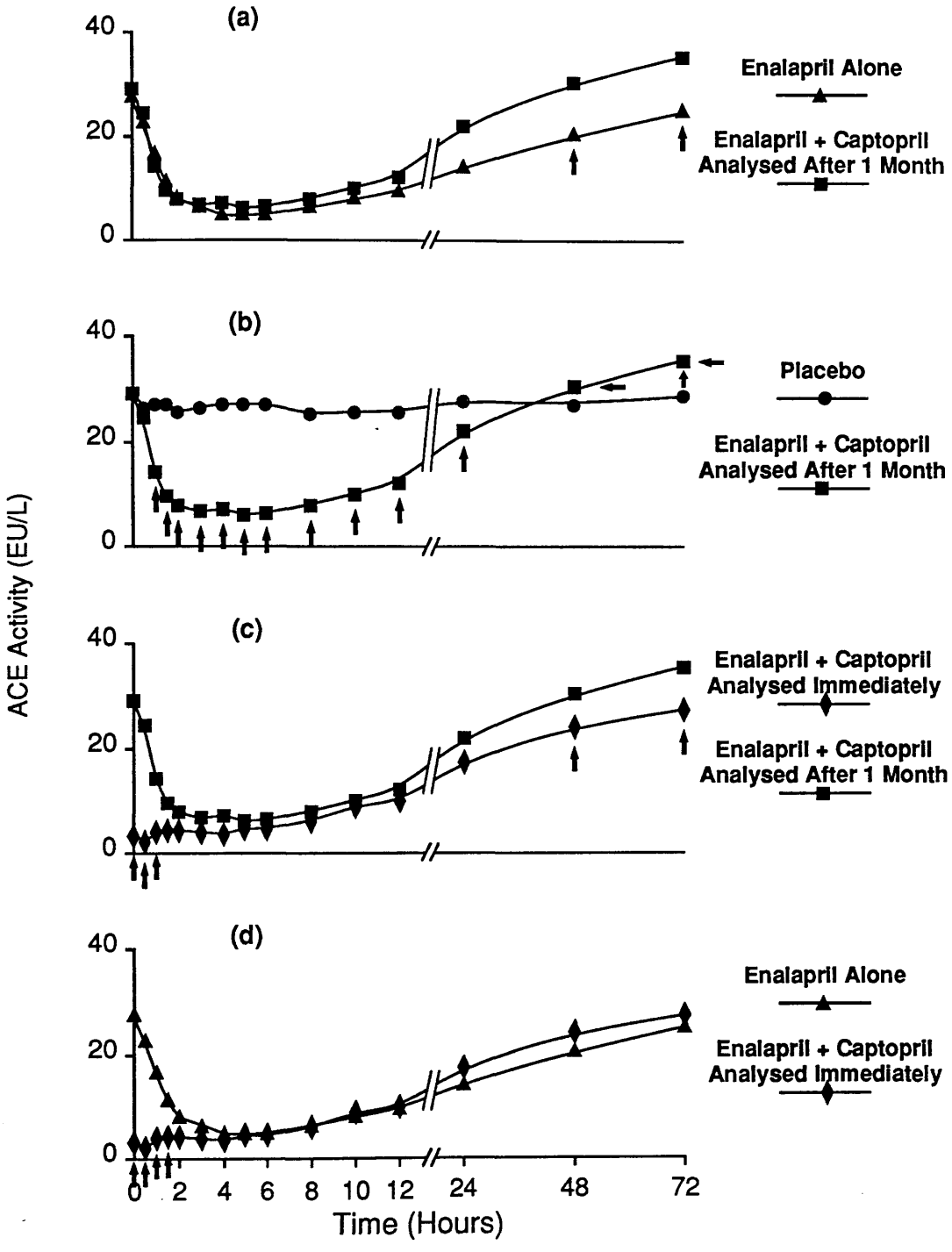


FIGURE 6.2

The Significant Changes In ACE Activity

the enalapril plus captopril analysed after one month profile, the 48 and 72 hour time points are significantly higher than their respective 0 hour time point. Figure 6.2 (c) highlights the difference between the two analyses of the enalapril plus captopril profile. Significant differences are indicated at the early time points when the ACE activity was already inhibited due to the prior dose of captopril. Significant differences were also found at 48 and 72 hours. Figure 6.2 (d) compares the profile due to enalapril alone and that due to enalapril plus captopril, analysed immediately. Significant differences were only found at the early time points, again due to the prior dose of captopril.

6.3.4 Enalaprilat Pharmacokinetic Analysis

The two enalaprilat profiles obtained for each subject are represented graphically in Figures 6.3-6.6.

The enalaprilat AUC data obtained in the presence and absence of captopril (0-∞) are shown in Table 6.7. The data for subject no. 8 are for 0-24 hours only. No difference was found between any of the pairs of enalaprilat AUC's.

One and two compartment models with zero order input were fitted to the enalaprilat data. The comparison of the models by Akaike criterion and F-Ratio test revealed that the majority of the data were best described by a two compartment model.

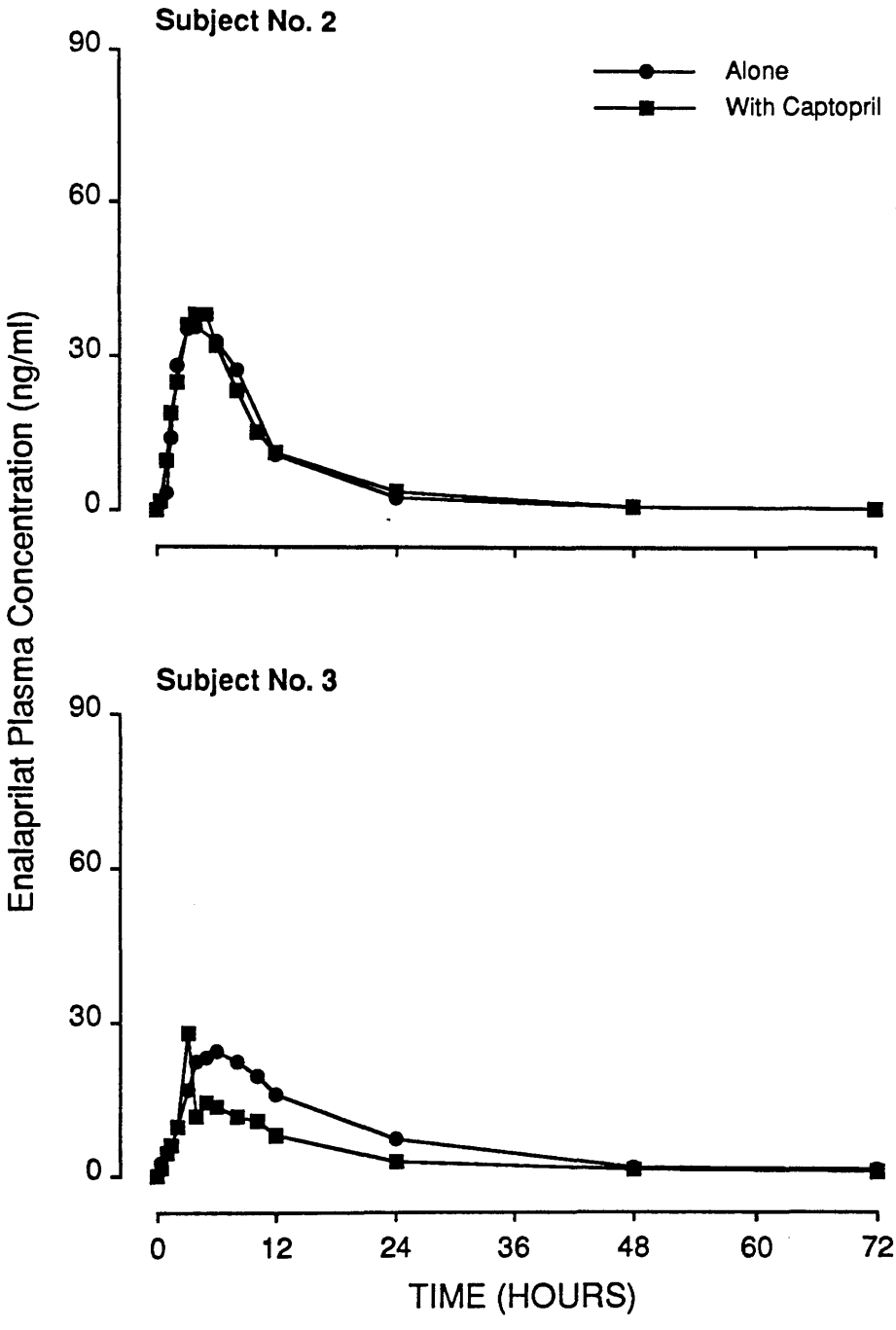


FIGURE 6.3

Enalaprilat Plasma Levels In Subjects 2 And 3

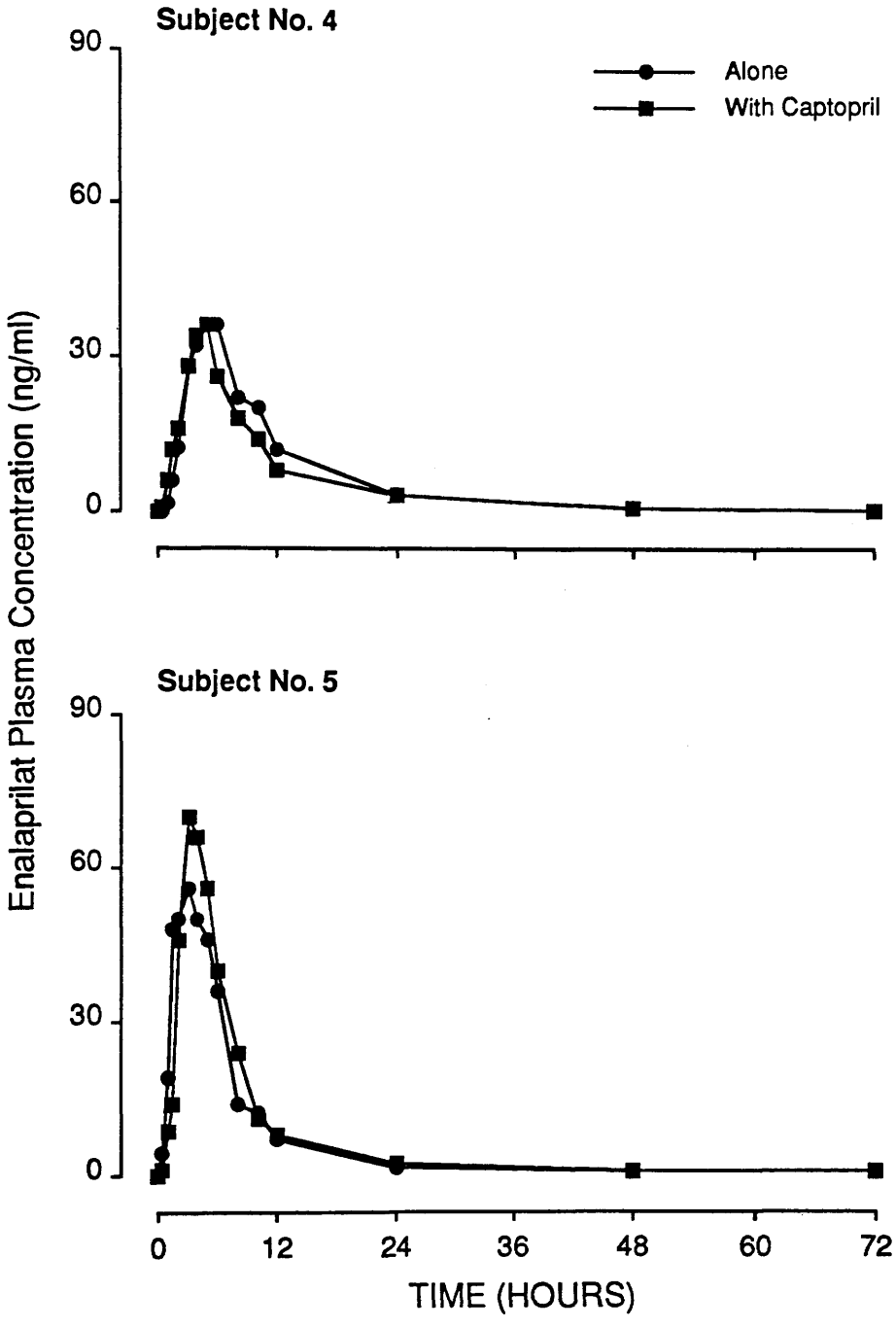


FIGURE 6.4

Enalaprilat Plasma Levels In Subjects 4 And 5

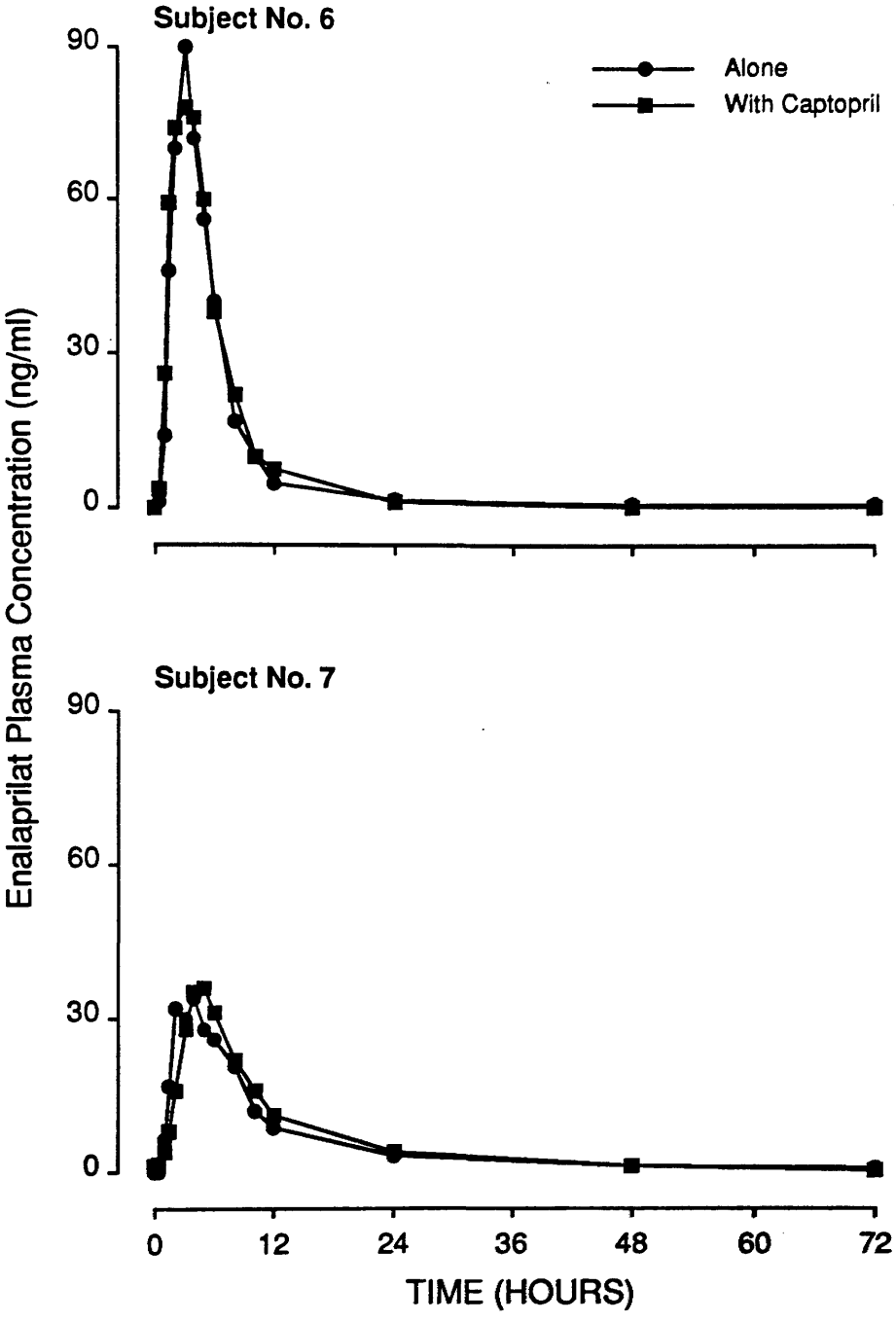


FIGURE 6.5

Enalaprilat Plasma Levels In Subjects 6 And 7

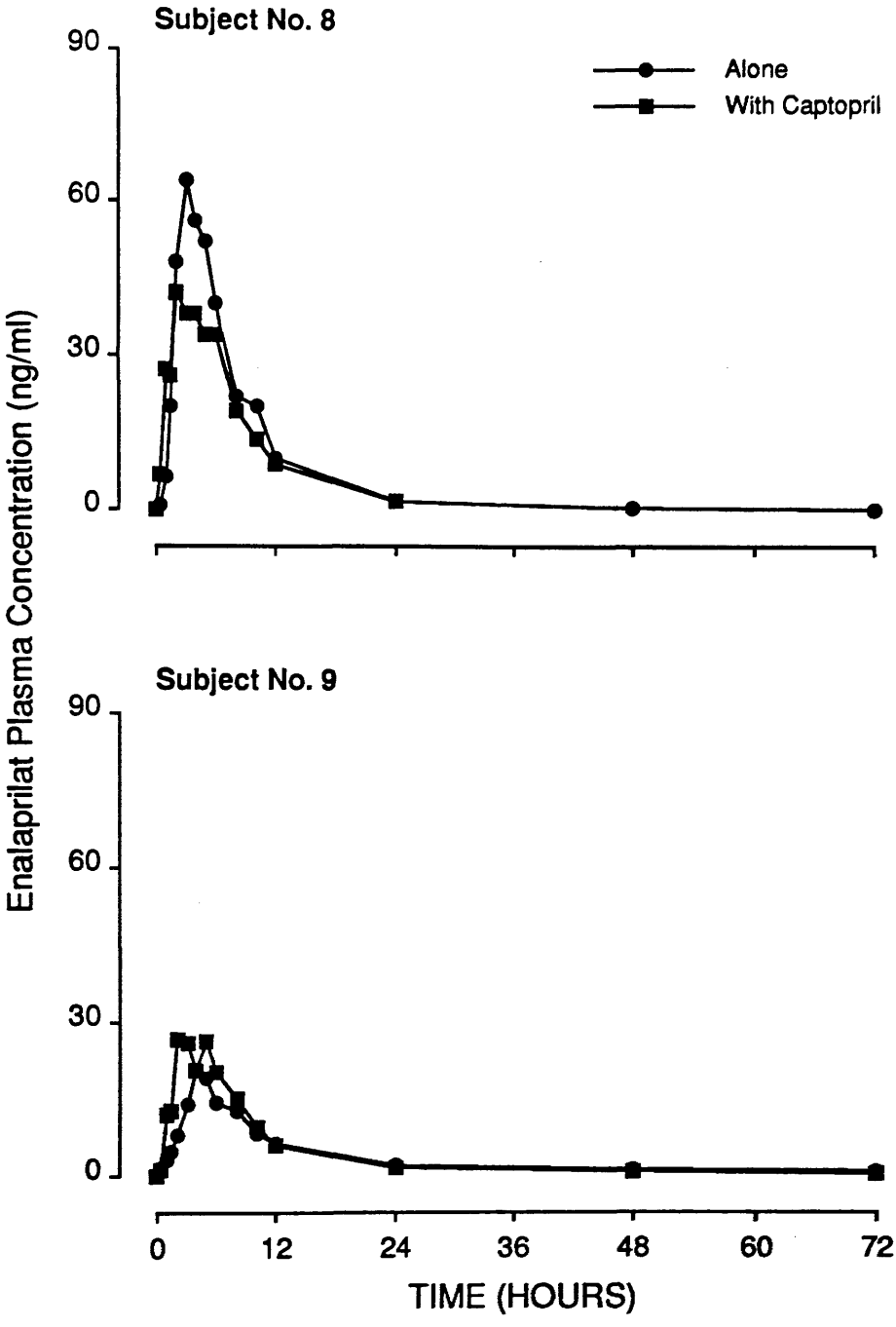


FIGURE 6.6

Enalaprilat Plasma Levels In Subjects 8 And 9

TABLE 6.7

Individual Enalaprilat AUC Data

Subject	Enalaprilat AUC (ng.h/ml) Enalapril Alone	Enalapril plus Captopril
2	412	441
3	533	310
4	432	364
5	476	529
6	562	530
7	440	461
8*	445	357
9	298	297
Mean	450	419
SD	87	97
Mean**	440	413
SD**	86	91

Values are from 0 - ∞ except * which are 0 - 24 hours.

** - Values calculated excluding data for subject no. 8.

The parameters Vc, alpha and K21 from the two compartment model are shown in Table 6.8. Comparison of Vc, alpha and K21 for enalaprilat by paired t-test revealed no significant difference for any of the parameters in the presence of captopril. The calculated terminal half lives for enalaprilat are shown in Table 6.9. Again, the presence of captopril had no effect on the terminal elimination rate for enalaprilat.

6.4 DISCUSSION

The study described in this chapter sought to characterise the pharmacokinetics of enalaprilat in the presence and absence of saturated plasma and tissue binding sites. For analytical reasons, captopril was chosen as the agent to saturate plasma and tissue ACE. Pharmacokinetic analysis of the enalaprilat plasma profiles did not reveal any significant differences in the presence and absence of captopril. However, analysis of the plasma ACE activity data revealed some interesting findings. There are a number of possible explanations for the lack of any observable effect of captopril on enalaprilat's kinetics; Firstly, since binding to ACE is thought to be responsible for the long terminal half life of the ACE inhibitors, captopril would have to be present during the terminal phase, where clearance is due to the dissociation of enalaprilat from ACE, to have an effect. The analytical

TABLE 6.8

Enalaprilat Pharmacokinetic Parameters

Subject	Parameter					
	Vc		Alpha		K21	
	Enalapril Alone	Enalapril Captopril	Enalapril Alone	Enalapril Captopril	Enalapril Alone	Enalapril Captopril
2	167	169	0.18	0.19	0.055	0.061
3	311	516	0.09	0.10	0.048	0.035
4	209	203	0.14	0.19	0.031	0.045
5	101	99	0.29	0.25	0.030	0.022
6	74	72	0.31	0.35	0.017	0.106
7	216	210	0.16	0.16	0.026	0.020
9	398	287	0.15	0.16	0.020	0.077
Mean	211	222	0.19	0.20	0.032	0.052
SD	114	148	0.08	0.08	0.014	0.031

Vc has units of litres.

Alpha has units of h^{-1}.

K21 has units of h^{-1}.

TABLE 6.9

Individual Enalaprilat Terminal Half Lives

Subject	Terminal Half Life	
	Enalapril Alone	Enalapril Plus Captopril
2	14.9	15.4
3	20.5	30.9
4	27.0	20.5
5	29.7	39.6
6	50.6	9.4
7	40.6	65.6
9	59.1	14.1
Mean	34.6	27.9
SD	16.1	19.6

Values have units of h-1.

methodology used to measure captopril levels in the study did not use an internal standard and so the results for total captopril concentration are semi qualitative. However, the mean values are not dissimilar to levels previously reported for total captopril following a single oral dose of 50mg (Hayashi et al, 1985). Free captopril has a short half life of 1.9 hours (Duchin et al, 1982b) and plasma levels are no longer detectable by 6 to 8 hours depending on dose. In contrast, plasma levels of protein conjugated captopril are still measurable at 24 hours following a single oral dose of 50mg (Hayashi et al, 1985). It has been proposed that protein conjugated captopril could act as a storage form of the drug with subsequent release of free captopril (Duchin et al, 1988). Studies have been reported demonstrating adequate blood pressure control with once daily captopril therapy (Schoenberger and Wilson, 1986, Garanin, 1986) which further support the theory that captopril is released from its protein conjugates long after plasma free levels are no longer detectable. Thus, it is not impossible that captopril may be present during the terminal elimination phase for enalaprilat.

Secondly, enalaprilat is a much more potent inhibitor of plasma ACE than captopril (chapter 4). It is possible that captopril may have bound to plasma and tissue ACE binding sites only to be subsequently displaced following the administration of enalapril and its conversion to enalaprilat. However, the analysis of ACE activity,

immediately and one month after sampling following the combination of enalapril and captopril, revealed a significant difference in the two calculated AUC values. This indicated that captopril did cause a significant inhibition of ACE activity and so displacement of captopril by enalaprilat from ACE is unlikely to be the reason for the lack of measurable change in enalaprilat pharmacokinetics.

Finally, there is the possibility of induction of ACE in the volunteers following the twice daily dosing of captopril, 50mg, for five days and subsequent administration of 10mg enalapril. ACE activity has been reported to be induced by treatment with glucocorticoids, macrophage activating factors and following chronic administration of ACE inhibitors (Fyhrquist et al, 1983b). The precise time course of induction of ACE by captopril has not been reported in man. In rat, administration of captopril in drinking water (200 μg/ml) gave rise to a significant increase in serum ACE after one day of treatment (Fyhrquist et al, 1980). The concentration of ACE did not change after a single dose of captopril, 100mg, to eight hypertensive subjects but long term treatment was associated with a gradual rise in ACE concentration over a period of weeks (Boomsma et al, 1981). The ACE activity in hypertensive patients receiving an increasing daily dose of enalapril achieved statistical significance after four days (Fhyrquist et al,

1983b). That induction is occuring in the present study is apparent from Figure 6.6 (b). At 72 hours the ACE activity value following captopril plus enalapril, analysed after one month, was significantly greater than the ACE activity measured 72 hours after the placebo dose. The difference at 48 hours just failed to achieve statistical significance. In addition, the 48 and 72 hour time points for the enalapril plus captopril curve, analysed after one month, were significantly higher than their respective zero hour time point. It may be that ACE activity was induced prior to 48 hours but that the presence of enalaprilat in the plasma masked the increase. Thus, the lack of detectable change in enalaprilat pharmacokinetics could be due to the induction of ACE by the dosing regime employed in the second phase of the study. The resulting increase in the number of ACE binding sites may have negated the effect of captopril's occupancy of original ACE binding sites.

CHAPTER 7

AGE AND THE PHYSIOLOGICALLY RELEVANT CHARACTERISATION OF BENAZEPRILAT PHARMACOKINETICS IN MAN

7.1 INTRODUCTION

7.1.1 Scope Of Chapter

Drugs which inhibit ACE are increasingly used in the management of hypertension and heart failure. Both conditions are particularly common in the elderly. The work presented in this chapter describes a study in which benazepril hydrochloride was administered to young and elderly volunteers. The pharmacokinetics of acute and chronic benazeprilat have been characterised in a physiologically relevant manner and the influence of age examined. In addition, the protein binding of benazeprilat was measured, and again, the effect of age assessed.

7.1.2 Benazepril Hydrochloride And Benazeprilat.

Benazepril hydrochloride is a new ACE inhibitor currently under development. It is a prodrug and after oral administration the ethoxycarbonyl moiety is hydrolysed in the liver to form the active carboxylic acid metabolite, benazeprilat. In normal volunteers benazepril hydrochloride has a high first pass effect and is rapidly and extensively converted to benazeprilat (Kaiser et al, 1989). The elimination of benazepril hydrochloride is virtually complete by four hours post dose. Peak levels of benazeprilat are achieved by one and a half hours post

dose. The elimination of benazeprilat is mainly renal in subjects with normal renal function. Benazeprilat elimination is biphasic, the half lives of elimination are of the order of three and twenty four hours.

7.1.3 Description Of ACE Inhibitor Pharmacokinetics

A characteristic feature of the ACE inhibitors is a long terminal half life of elimination which has been attributed to the saturable binding to, and subsequent dissociation from, ACE itself. In addition to model independent and standard compartmental analysis, the pharmacokinetics of this group of drugs have been described by two novel approaches. Both methods incorporate binding of the inhibitor drug molecules to ACE as part of the model (Francis et al, 1987, Lees et al, 1989). The approach described by Francis et al proposed a model for cilazaprilat, formed after the oral administration of cilazapril, which incorporated binding to plasma ACE only. Furthermore, due to low plasma protein binding of cilazaprilat, non specific binding was considered to be zero. Lees et al (1989) proposed a series of models to describe the pharmacokinetics of perindoprilat administered by intravenous infusion. These models incorporated binding to plasma ACE, binding to tissue ACE or a combination of both binding sites.
The approach proposed by Lees et al (1989) has been

utilised in this chapter. The model has been extended to describe the pharmacokinetics of acute and chronic benazeprilat, formed after the oral administration of benazepril hydrochloride.

7.1.4 Influence Of Age On Drug Disposition

Many physiological changes occur as the human body ages, the influence of these changes on drug disposition are summarised briefly below.

Despite theoretical considerations there is little evidence to suggest that the absorption of drugs is affected by ageing, whilst absorption may be slightly delayed it tends to be just as complete. Conflicting reports are available in the literature as to the effect of age on first pass metabolism. Both reduced and unaltered first pass effects have been reported for the beta-blockers in the elderly (Castleden and George, 1979, and Schneider et al, 1980).

The influence of changes in body composition on volume of distribution, decrease in body water, increase in body fat, depend upon the physico-chemical characteristics of the drug under consideration. Protein binding tends to decrease with age, serum albumin levels fall with advancing years but alpha $_1$-acid glycoprotein levels rise, albeit to a lesser extent.

Glomerular filtration falls by about 10% per decade after the age of forty. Consequently the elimination of renally

cleared drugs is decreased in the elderly.

7.2 METHODS

7.2.1 Study Design

Nine young (9 males) (Subjects 1-9) and nine elderly (7 males, 2 females) (Subjects 10-18) were recruited after routine screening to confirm good health. All subjects were living freely in the community. Written informed consent was obtained from all subjects and the study design was approved by the local Ethical Review Committee. The mean ages (SD) for the young and elderly groups were 26(7) and 71(3)years respectively. Correspondingly weights were 75(11) and 66(11)kg, heights were 176(5) and 168(11)cm and creatinine clearances were 112(31) and 78(25)ml/min respectively. Individual demographic data are shown in Table 7.1.

The study protocol consisted of four study days. The first three days formed a single blind three way crossover design in which single oral doses of benazepril hydrochloride, 10mg, enalapril maleate, 10mg, and placebo were compared in each subject. On the fourth study day all subjects were studied after repeated once daily oral administration of benazepril hydrochloride, 10mg once daily, for eight days. All study days were at least one week apart. Each study day involved timed blood sampling

TABLE 7.1

Individual Demographic Data For Benazeprilat

Pharmacokinetic Study In Man

Subject	Sex	Age (Y)	Weight (Kg)	Height (Cm)	Creatinine Clearance (ml/min)
Young					
1	M	23	75	177	91
2	M	22	70	170	162
3	M	27	102	185	160
4	M	21	62	170	97
5	M	39	73	172	87
6	M	39	71	175	112
7	M	24	73	175	100
8	M	21	69	180	90
9	M	21	76	179	-
Mean		26	75	176	112
SD		7	11	5	31
Elderly					
10	M	76	73	180	74
11	M	73	83	182	110
12	F	74	50	162	44
13	F	74	58	158	80
14	M	66	73	168	74
15	M	70	64	161	98
16	M	73	55	152	44
17	M	69	64	176	72
18	M	68	76	176	104
Mean		71	66	168	78
SD		3	11	11	25

until 72 hours after drug administration. Two extra blood samples were taken at 2 and 6 hours after acute and chronic administration for protein binding determination. Subjects 2-9 then took part in a further study which has been described in detail in chapter 6.

The results reported in this chapter consider the acute and chronic administration of benazepril hydrochloride and the pharmacokinetic analysis thereof. In addition, the analysis of the protein binding determinations is also reported.

7.2.2 Sample Analysis

Plasma concentrations of benazepril and benazeprilat for pharmacokinetic analysis were kindly measured by GC-MS by Ciba Geigy (CRB, Rueil Malmaison, France). The method which has been described in greater detail in chapter 2, section 2.2.5 and chapter 3 (quality control data) is sensitive and specific and determines levels of benazepril and benazeprilat independently. The samples taken for benazeprilat protein binding determination were analysed by IBA, the details of which are given in Chapter 2, section 2.2.7 and chapter 3 (quality control data). The IBA method is specific for any pharmacologically active moiety.

Plasma ACE activity was assayed by HHL, the details of which are given in chapter 2, section 2.2.2 and chapter 3 (quality control data). Plasma ACE activity was

determined following placebo and the acute and chronic doses of benazepril hydrochloride. Plasma ACE activity measurements obtained following the two doses of benazepril hydrochloride were corrected for any changes that occurred following placebo prior to further analysis.

7.2.3 Pharmacokinetic Analysis Of Benazepril And Benazeprilat.

The acute and chronic benazepril data were analysed by non compartmental analysis only, Cmax and Tmax were derived directly from the data. AUC was calculated by the linear trapezoidal rule.

The acute and chronic plasma benazeprilat data were analysed by a variety of techniques. Non compartmental analysis was carried out as described for benazepril. Five standard compartmental models were fitted to the combined acute and chronic data, a one compartment model with first order input (Model A), a one compartment model with zero order input, with and without lag time (Models B and C respectively) and a two compartment model with and without lag time (Models D and E respectively). A further six, non standard or 'binding' models were also fitted to the data. These 'binding' models were based upon a one compartment model with zero order input with either saturable binding in plasma, (Model F), saturable binding in tissue (Model G) or a combination of both binding sites

(Model H). These three binding models were then analysed with an additional parameter to characterise lag time (Models I, J and K respectively). In addition to the standard parameters, Tabs (length of time over which absorption takes place), V/F (oral volume of distribution) and Ke (elimination rate constant), two further parameters that characterise the binding to ACE were also ascertained. Bmax/F is the maximum number of binding sites divided by bioavailability and Au(50)/F is the amount of drug at 50% binding saturation divided by bioavailability. For Models H and K where both plasma and tissue binding were characterised the fraction of binding sites in the tissue was also quantified.

The values for half life of elimination ($t_{1/2}$), oral clearance (CL/F) and the concentration of benazeprilat required to inhibit ACE by 50% (C(50))were calculated from the parameters of the model which best described the data for any one subject.

Three further models were fitted to the combined acute and chronic benazeprilat data. These further three models utilised the plasma ACE activity data in order to better characterise Bmax/F and Au(50)/F. The further models did not consider lag time, just plasma, tissue or both types of binding (Models L, M and N respectively).

Finally, in order to assess any possible changes in Bmax/F and Au(50)/F Models L, M or N were refitted to the separated acute and chronic plasma benazeprilat data, whichever model had best described the combined acute and

chronic data.

The equations for the 'binding' models are derived in the Appendix.

All the standard and 'binding' models were fitted to the data using the statistical package BMDP on an ICL 2976 computer. The derivative free nonlinear regression program PAR was used.

Model selection was based upon the Schwarz and Akaike criteria and a visual inspection of the residual plots and coefficients of variation of the estimated parameters.

A summary of all the models fitted to the data is given in Table 7.2 and illustrated schematically in Figures 7.1 and 7.2.

7.2.4 Protein Binding Of Benazeprilat

The method used to determine the protein binding of benazeprilat was equilibrium dialysis. The method has been described in full detail in chapter 2, section 2.2.9. Two samples were taken at 2 and 6 hours post dose for protein binding determination, for all subjects. The 2 hour sample was timed to coincide with peak plasma benazeprilat levels, the 6 hour sample with lower levels of benazeprilat.

TABLE 7.2

Summary Of Models Fitted To Benazeprilat Data

Model	No. of Compartments	Input (Order)	Lag Time	Binding
A	1	First	No	N/A
B	1	Zero	No	N/A
C	1	Zero	Yes	N/A
D	2	Zero	No	N/A
E	2	Zero	Yes	N/A
F	1	Zero	No	Plasma
G	1	Zero	No	Tissue
H	1	Zero	No	Plasma + Tissue
I	1	Zero	Yes	Plasma
J	1	Zero	Yes	Tissue
K	1	Zero	Yes	Plasma + Tissue
L	1	Zero	No	Plasma
M	1	Zero	No	Tissue
N	1	Zero	No	Plasma + Tissue

Models A to K were fitted to plasma benazeprilat concentration data only, Models L to N were fitted simultaneously to plasma benazeprilat concentration data and plasma percent inhibition of ACE activity data.

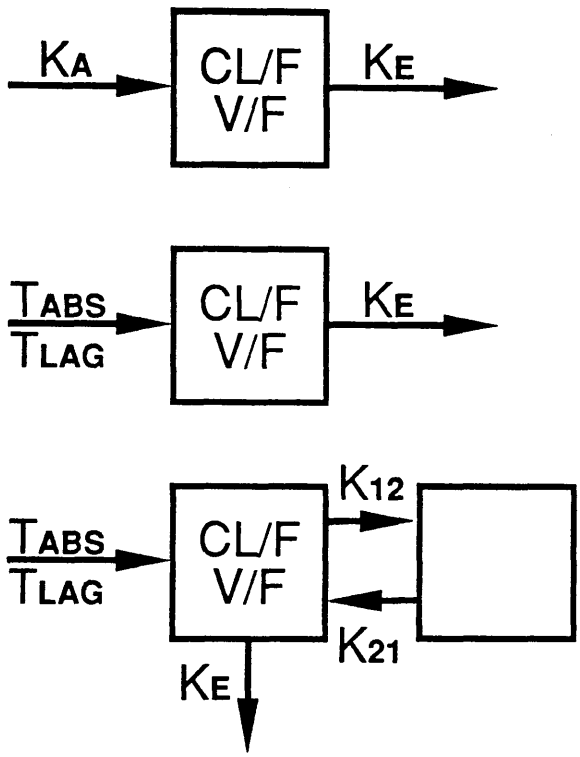


FIGURE 7.1

Diagramatic Representation Of The Standard Models Fitted To The Benazeprilat Data

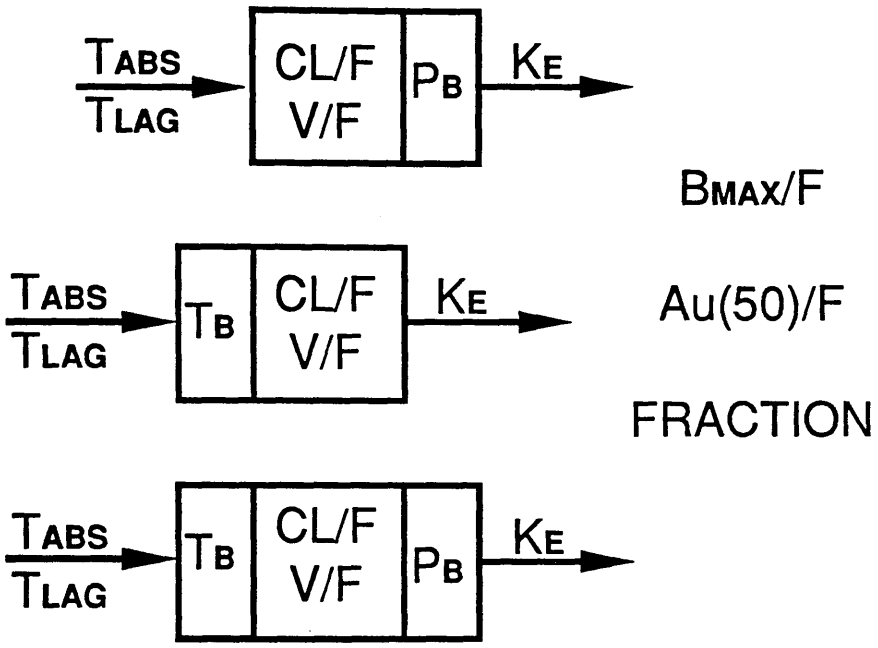


FIGURE 7.2

Diagramatic Representation Of The 'Binding' Models Fitted To The Benazeprilat Data

7.2.5 Statistical Analysis

The Cmax, Tmax and AUC for the benazepril and benazeprilat data, for young and elderly subjects after acute and chronic administration, were compared by ANOVA.

The parameters Bmax/F and Au(50)/F and the calculated values for $t_{1/2}$ and CL/F for benazeprilat, for the young and elderly subjects, were compared by the unpaired t-test.

The parameters Bmax/F and Au(50)/F obtained from fitting the acute and chronic data separately,for the young and old subjects, were analysed by ANOVA. The data were analysed twice, once using unweighted data and once using the reciprocal of the coefficient of variation of the estimated parameter as the weighting function.

The benazeprilat protein binding data for young and old subjects, acute and chronic administration, at 2 and 6 hours post dose was analysed by ANOVA.

7.3 RESULTS

7.3.1 Non Compartmental Analysis Of Benazepril And Benazeprilat.

Plasma benazepril levels quickly reached a peak at 0.5 to 1 hour (median) for both young and elderly volunteers after acute and chronic administration. Levels then declined rapidly and were not detectable beyond 12 hours

post dose. The mean Cmax and AUC values were reduced in the elderly, after both acute and chronic administration although this was not statistically significant. Mean (SD) Cmax values were 114(42.4) and 96.0 (39.7)ng/ml after acute administration and 103(46.5) and 89.5(37.5)ng/ml after chronic administration in young and elderly volunteers respectively. Mean AUC (SD) values were 131(37.8) and 106(36.6)ng.h/ml after acute administration and 117(28.8) and 97.2(35.9) ng.h/ml after chronic administration in young and elderly volunteers respectively. Mean plasma benazepril levels are illustrated graphically in Figure 7.3 and individual AUC, Cmax and Tmax data are presented in Table 7.3.

Conversion of benazepril to benazeprilat was rapid, peak levels of benazeprilat were reached by 3 hours in all subjects. No difference was found in Cmax for benazeprilat between the young and elderly subjects after acute or chronic administration. However, within each age group there was an increase in Cmax after chronic administration although this just failed to achieve statistical significance (p=0.06). Mean (SD) Cmax values were 189(60.2) and 176(45.3)ng/ml after acute administration and 206(39.5) and 197(37.2)ng/ml after chronic administration, in young and elderly volunteers respectively.

A comparison of the AUC data for benazeprilat ($AUC_{0-\infty}$ after acute administration and AUC_{0-24} after chronic

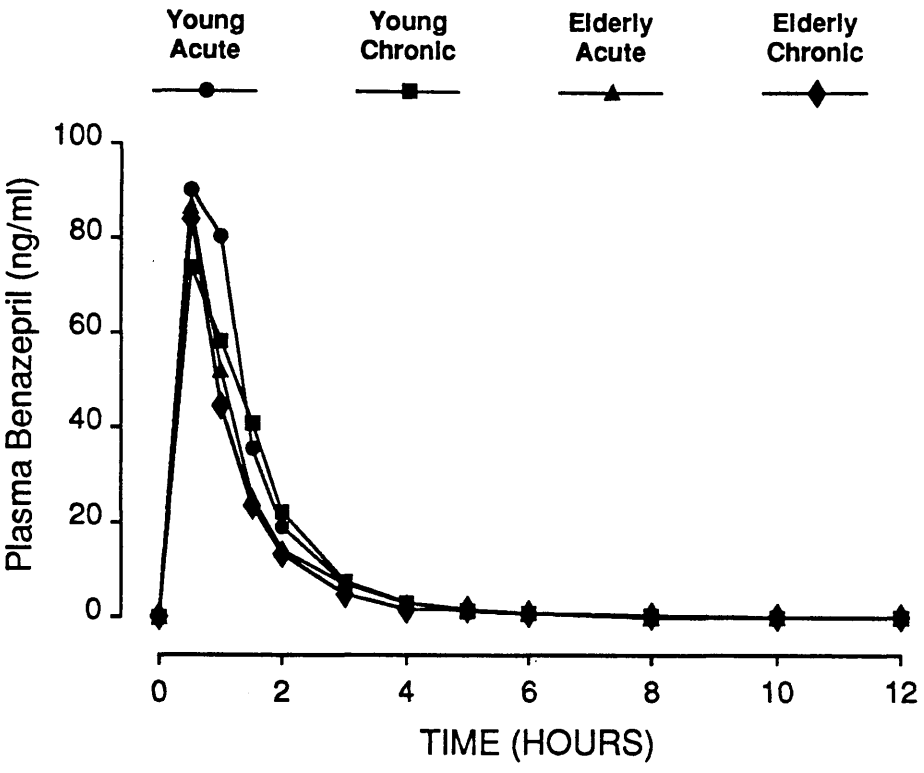


FIGURE 7.3

Mean Plasma Benazepril Levels

TABLE 7.3

Individual Benazepril Tmax, Cmax And AUC Data

Subject	Tmax (h)		Cmax (ng/ml)		AUC (ng.h/ml)	
	Acute	Chronic	Acute	Chronic	Acute	Chronic
Young						
1	1	0.5	115	106	98.0	114
2	0.5	1.5	183	62.3	173	128
3	0.5	1	123	51.2	146	98.0
4	1.5	1	75.4	84.1	177	106
5	0.5	0.5	141	132	149	129
6	0.5	0.5	139	185	145	182
7	0.5	1	43.3	80.6	59.4	92.6
8	1	0.5	133	158	128	121
9	0.5	1.5	77.8	63.9	107	84.7
Mean	0.5	1	114	103	131	117
SD			42.4	46.5	37.8	28.8
Elderly						
10	0.5	0.5	131	135	117	120
11	1	0.5	57.5	60.3	73.6	80.6
12	0.5	0.5	100	59.9	106	50.8
13	0.5	0.5	121	124	116	116
14	0.5	0.5	82.1	82.1	79.7	66.7
15	0.5	0.5	102	88.5	84.9	80.5
16	1.5	1.5	56.0	55.6	139	122
17	0.5	0.5	167	149	176	165
18	0.5	0.5	47.2	50.8	57.6	73.0
Mean	0.5	0.5	96.0	89.5	106	97.2
SD			39.7	37.5	36.6	35.9

Mean data shown for Tmax are median values.

administration) revealed no significant difference between acute and chronic administration. However, although the AUC data were increased in the elderly, when compared to the young volunteers, after both acute and chronic administration, this just failed to achieve statistical significance (p=0.07). Mean AUC (SD) values were 1106(319) and 1486(530)ng.h/ml after acute administration and 1097(253) and 1345(407)ng.h/ml after chronic administration for young and elderly volunteers respectively.

Mean plasma benazeprilat levels are illustrated graphically in Figure 7.4 and individual AUC, Cmax and Tmax data are shown Table 7.4.

7.3.2 Compartmental Analysis Of Benazeprilat.

Of the five standard models fitted to the combined acute and chronic plasma benazeprilat data (Models A-E) ten of the volunteers (5 young and 5 elderly) were best described by a two compartment model with zero order input and lag time (Model E). Four volunteers were best described by a one compartment model with zero order input and lag time (Model C). A further three volunteers were best described by a one compartment model with zero order input (Model B) and the remaining volunteer was best described by a two compartment model with zero order input (Model D).

A further six models were then fitted to the combined acute and chronic plasma benazeprilat data (Models F-K).

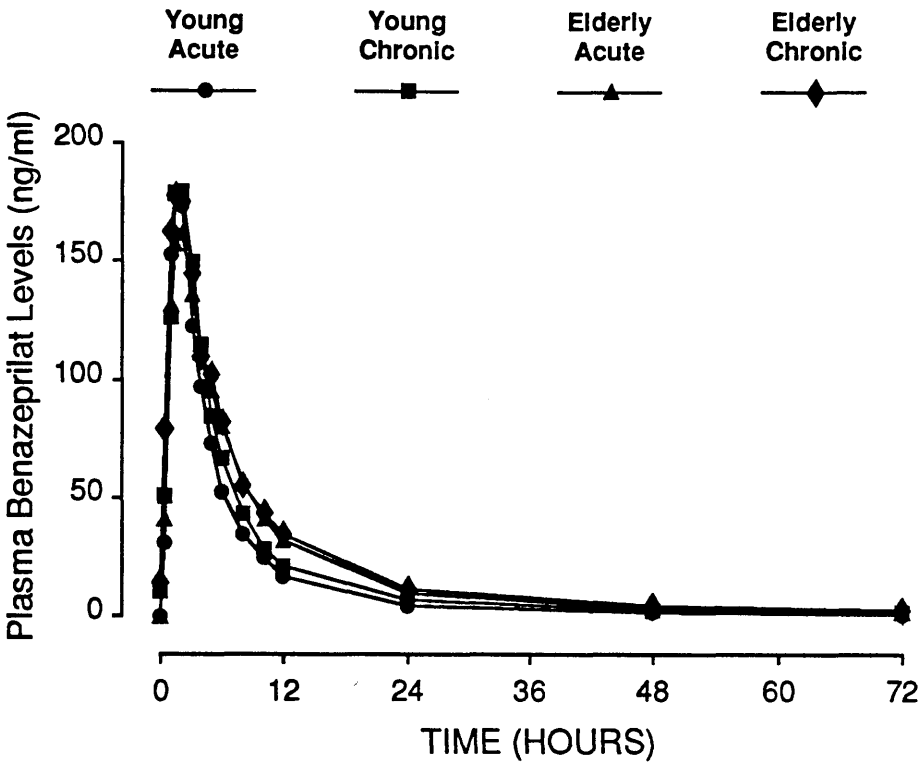


FIGURE 7.4

Mean Plasma Benazeprilat Levels

TABLE 7.4

Individual Benazeprilat Tmax, Cmax And AUC Data

Subject	Tmax (h)		Cmax (ng/ml)		AUC (ng.h/ml)	
	Acute	Chronic	Acute	Chronic	Acute	Chronic
Young						
1	2	1.5	151	197	861	912
2	1	3	294	198	1677	1289
3	1.5	3	107	166	916	1282
4	2	1.5	174	152	1109	907
5	1.5	1.5	158	231	902	1038
6	1	1.5	275	289	1565	1594
7	1.5	2	177	203	858	840
8	1.5	1	160	196	847	869
9	2	2	203	219	1221	1139
Mean	1.5	1.5	189	206	1106	1097
SD			60.2	39.5	319	253
Elderly						
10	1.0	1	131	202	1179	1169
11	2	1.5	139	162	984	974
12	1.5	1	187	145	1845	1193
13	2	1.5	188	248	1425	1389
14	1	1.5	202	218	1560	1505
15	1.5	1	173	159	1016	792
16	3	3	256	242	2564	2178
17	1.5	1.5	204	215	1828	1657
18	1.5	2	106	181	977	1252
Mean	1.5	1.5	176	197	1486	1345
SD			45.3	37.2	530	407

Mean data shown for Tmax are median values.

These models, based upon a one compartment model with zero order input, incorporated either saturable binding to plasma, tissue or plasma and tissue constituents. In addition, the presence and absence of a parameter to characterise lag time was also considered. The best model to describe the data was then assessed across Models A to K. Twelve of the volunteers were best described by Model G (5 young and 7 elderly) which incorporated binding to tissue constituents only. A further three volunteers were best described by Model H which included binding to both plasma and tissue constituents (2 young and 1 elderly). One volunteer was best described by Model F, plasma binding only. The two remaining volunteers were best described by standard models, a one compartment model with zero order input (Model B) and a two compartment with zero order input and lag time (Model E).

Of all the volunteers best described by the 'binding' models the addition of a parameter to characterise lag time did not improve the fit of the model to the data.

The calculated parameters $t_{1/2}$ and CL/F as well as V/F, Bmax/F and Au(50)/F, and the model from which they were derived, are shown in Table 7.5. For all volunteers, except volunteers 2 and 3 (who were best described by standard models), $t_{1/2}$, CL/F and V/F represent free drug only. For volunteers 2 and 3 the data represent total drug. There was no significant difference between young and elderly for any of the parameters shown.

TABLE 7.5

Benazeprilat Pharmacokinetic Parameters Derived From Models A to K, Whichever Best Described The Data For Any One Subject.

Subject	Model	t1/2 (h)	CL/F (ml/min)	Bmax/F (μg)	Au(50)/F (μg)	V/F (l)
Young						
1	G	2.39	163	3359	30.3	33.7
2	B	3.15	132	-	-	35.9
3	E	5.33	136	-	-	63.0
4	G	1.28	165	3620	318	18.3
5	H	1.92	172	3095	6.0	28.6
6	G	2.39	95.7	2226	455	19.8
7	G	0.91	193	4273	411	15.2
8	H	1.87	199	3395	10.5	32.3
9	G	0.72	138	5418	509	8.6
Mean		2.22	155	3627	249	28.4
SD		1.40	32.7	1000	225	16.0
Elderly						
10	G	3.46	124	2940	366	37.1
11	G	2.24	154	3552	348	29.9
12	G	4.62	112	1912	213	44.8
13	G	2.39	105	3343	270	21.8
14	G	3.47	99.7	2093	363	29.9
15	F	2.10	228	358	21.7	41.5
16	G	3.01	67.1	2345	457	17.5
17	H	1.98	192	4498	585	33.0
18	G	3.01	126	4940	249	32.9
Mean		2.92	134	2887	319	32.0
SD		0.85	49.7	1402	159	8.7

The mean (SD) percent coefficients of variation for the estimation of Bmax/F and Au(50)/F were 31 (22) and 85 (91)% respectively. The high coefficients of variation indicate poor estimation of these parameters. In order to try and improve the estimation of Bmax/F and Au(50)/F the 'binding' Models L-N were fitted to the combined acute and chronic plasma benazeprilat data simultaneously with the combined acute and chronic plasma percent inhibition of ACE activity data. The addition of a parameter to describe lag time was not considered since this parameter had not previously helped to describe the fit of the 'binding' models to the data. The mean (SD) coefficients of variation for Bmax/F and Au(50)/F, for whichever model (L, M or N) best described the data, were 22 (8) and 28 (12)%. Thus, the simultaneous fitting of plasma benazeprilat and percent inhibition of ACE data considerably improved the precision in estimation of the binding parameters. One volunteer was best described by the model with plasma binding only (Model L), fifteen volunteers were best described by the model with tissue binding only (Model M) and the two remaining volunteers were best described by the model with both plasma and tissue binding (Model N). It was not possible to compare the fit of Models L, M or N to the data with the fit to Models A to K since the inclusion of the percent inhibition of ACE data increased the total sum of squares and the number of degrees of freedom for the data.

The parameters $t_{1/2}$ and Cl/F were recalculated and together

The parameters $t_{1/2}$ and Cl/F were recalculated and together with V/F, Bmax/F and Au(50)/F, from whichever model (L, M or N) best described the data are shown in Table 7.6. Mean (SD) CL/F (free drug) was 146(27.8) and 123(28.8)ml/min for young and elderly volunteers. Mean (SD) $t_{1/2}$ (free drug) was 2.09(1.05) and 2.97(0.86)hours for young and elderly volunteers respectively. Mean (SD) V/F (free drug) was 25.2(10.4) and 31.2(10.4)litres for young and elderly volunteers respectively. The mean (SD) values for the binding parameters Bmax/F and Au(50)/F were 3250(1046) and 2824(1480)μg and 242(125) and 252 (240)μg for young and elderly volunteers respectively. Again no significant difference was found between the young and elderly although in the case of $t_{1/2}$ the difference only just failed to achieve significance ($p=0.07$).

The mean C(50) value calculated from the parameters from models L-N, whichever best described the data for any one subject was 8.89 (5.84) ng/ml.

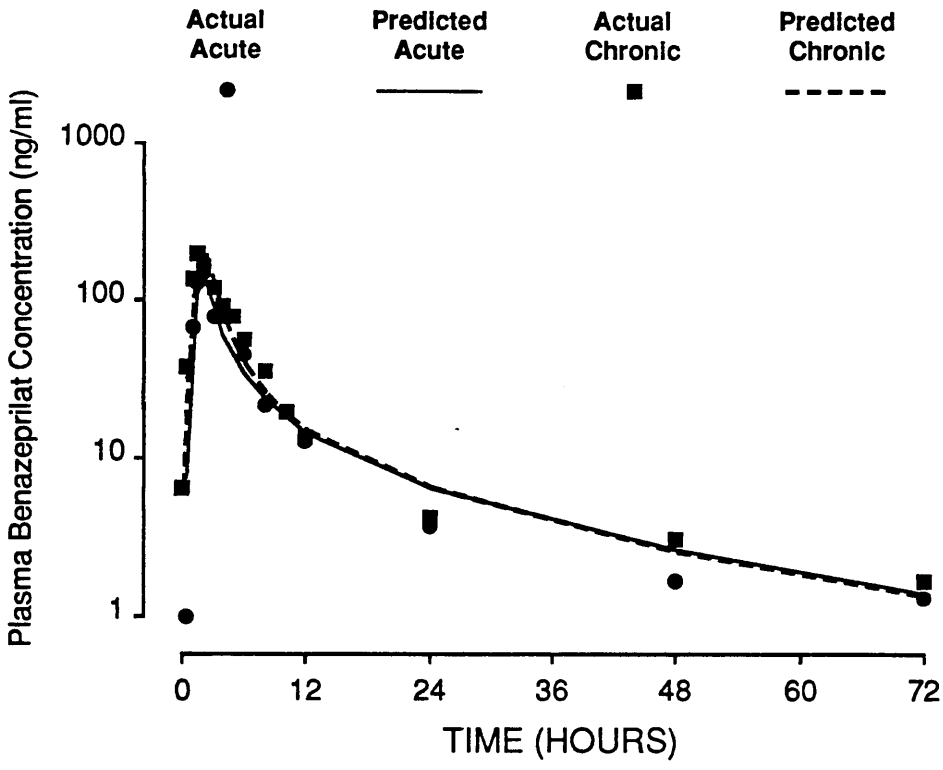
The actual and predicted data for the best model to describe the data for each subject are shown in Figures 7.5 to 7.22.

All the models described so far were fitted to the combined acute and chronic data. The final analysis refitted either Model L, M or N (whichever previously best described the data) to the separated acute and chronic data. The values for Bmax/F and Au(50)/F were then

TABLE 7.6

Benazeprilat Pharmacokinetic Parameters Derived From Models L to N, Whichever Best Described The Data For Any One Subject.

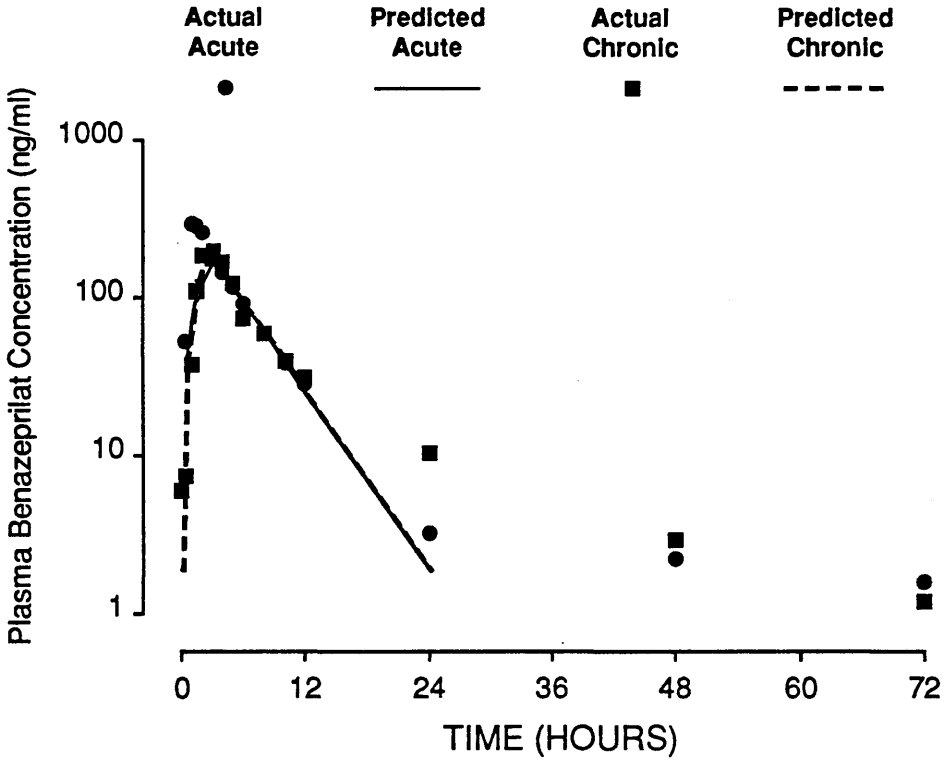
Subject	Model	t1/2 (h)	CL/F (ml/min)	Bmax/F (μg)	Au(50)/F (μg)	V/F (l)
Young						
1	M	1.28	167	5119	180	18.6
2	M	2.04	124	2655	222	21.8
3	M	4.62	131	2611	412	52.3
4	M	1.65	167	3002	177	23.8
5	M	1.65	145	3933	261	20.7
6	M	2.77	94.6	1587	115	22.7
7	M	1.31	186	3480	181	21.1
8	M	1.78	163	4170	144	25.1
9	M	1.69	139	2692	484	20.4
Mean		2.09	146	3250	242	25.2
SD		1.05	27.8	1046	125	10.4
Elderly						
10	N	3.15	158	2499	96.7	43.1
11	M	2.39	155	3280	242	32.1
12	M	4.62	114	1905	154	45.5
13	M	2.57	103	3211	187	22.9
14	L	3.46	124	348	49.2	36.4
15	M	1.65	145	3949	265	20.7
16	M	2.57	65.2	3531	265	14.5
17	N	3.65	115	1376	53.1	36.4
18	M	2.67	128	5317	407	29.6
Mean		2.97	123	2824	252	31.2
SD		0.86	28.8	1480	240	10.4



Data predicted by a one compartment model with tissue binding

FIGURE 7.5

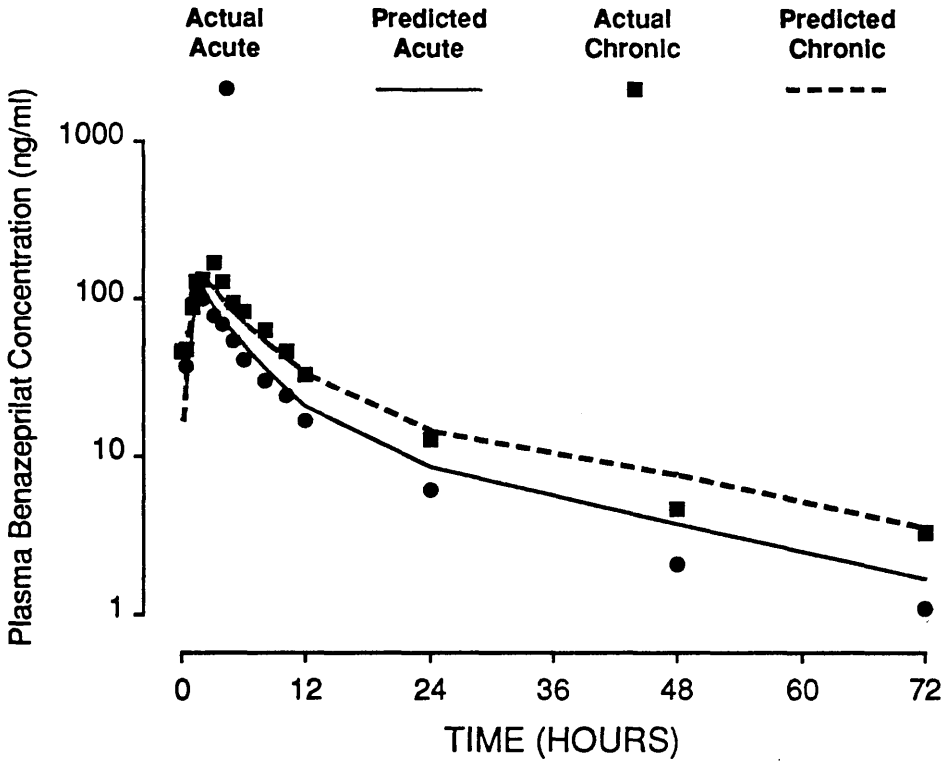
Actual And Predicted Benazeprilat Data For Subject No. 1



Data predicted by a one compartment model

FIGURE 7.6

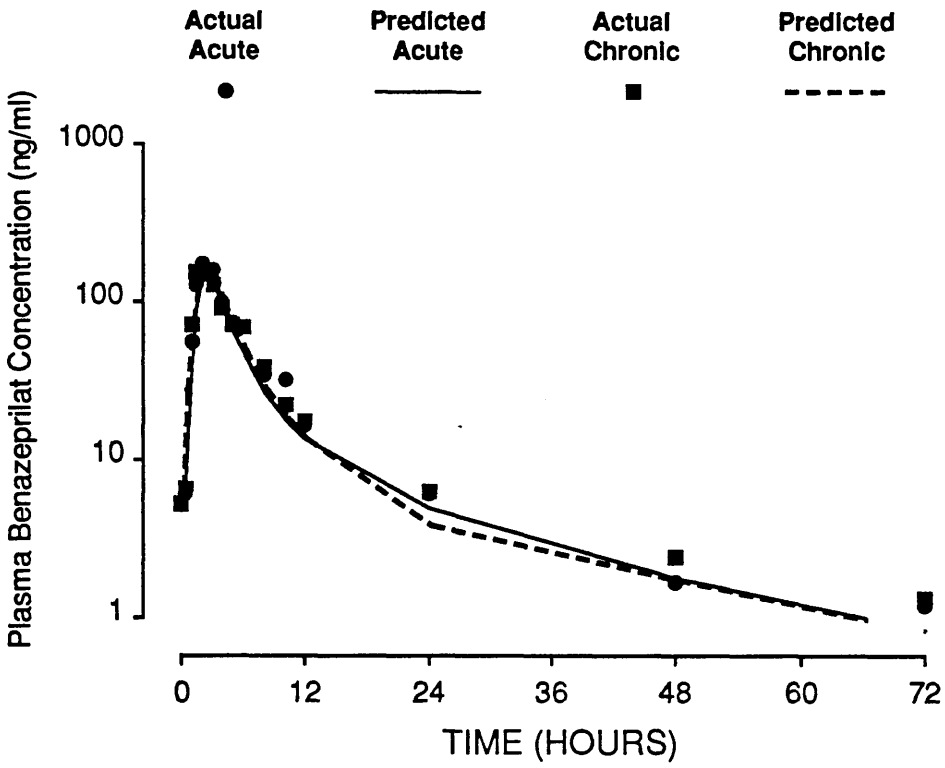
Actual And Predicted Benazeprilat Data For Subject No. 2



Data predicted by a two compartment model with lag time

FIGURE 7.7

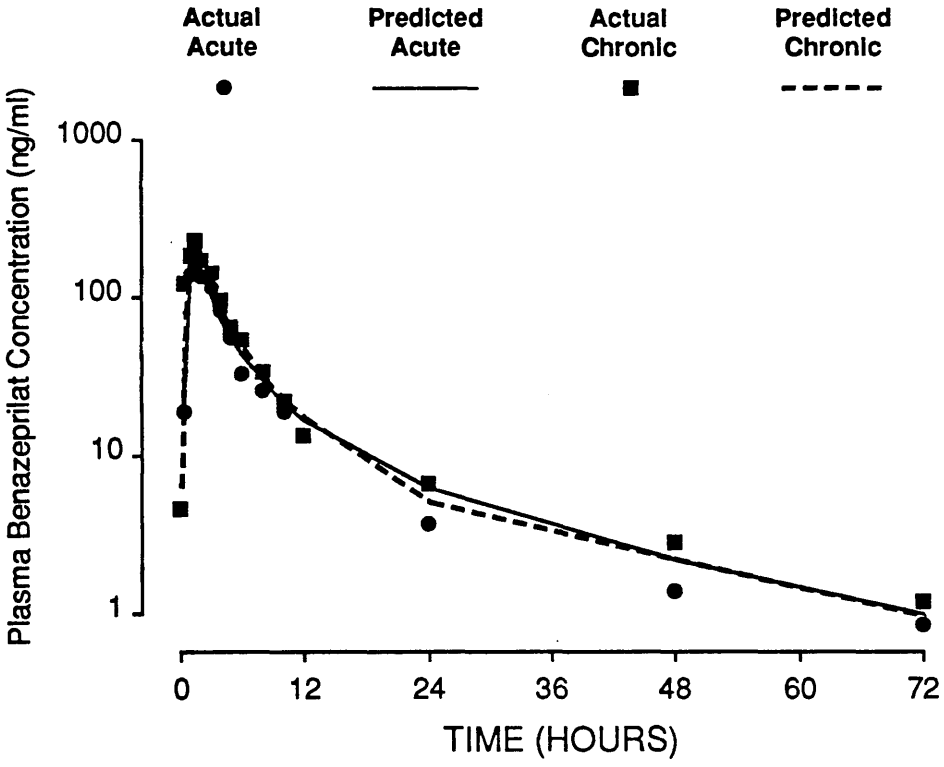
Actual And Predicted Benazeprilat Data For Subject No. 3



Data predicted by a one compartment model with tissue binding

FIGURE 7.8

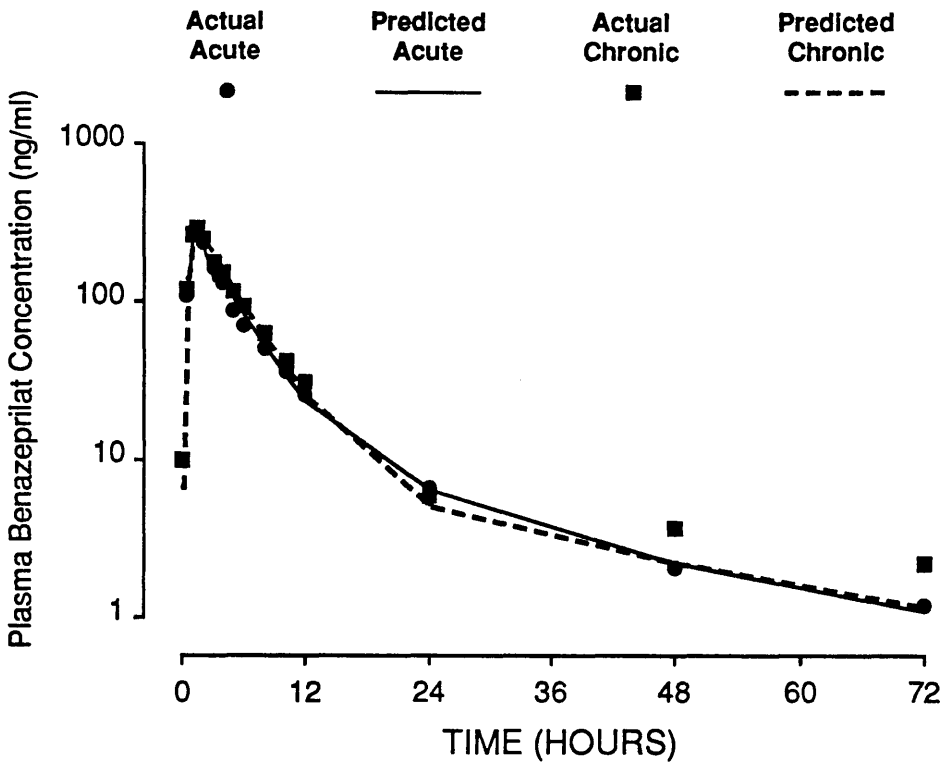
Actual And Predicted Benazeprilat Data For Subject No. 4



Data predicted by a one compartment model with plasma and tissue binding

FIGURE 7.9

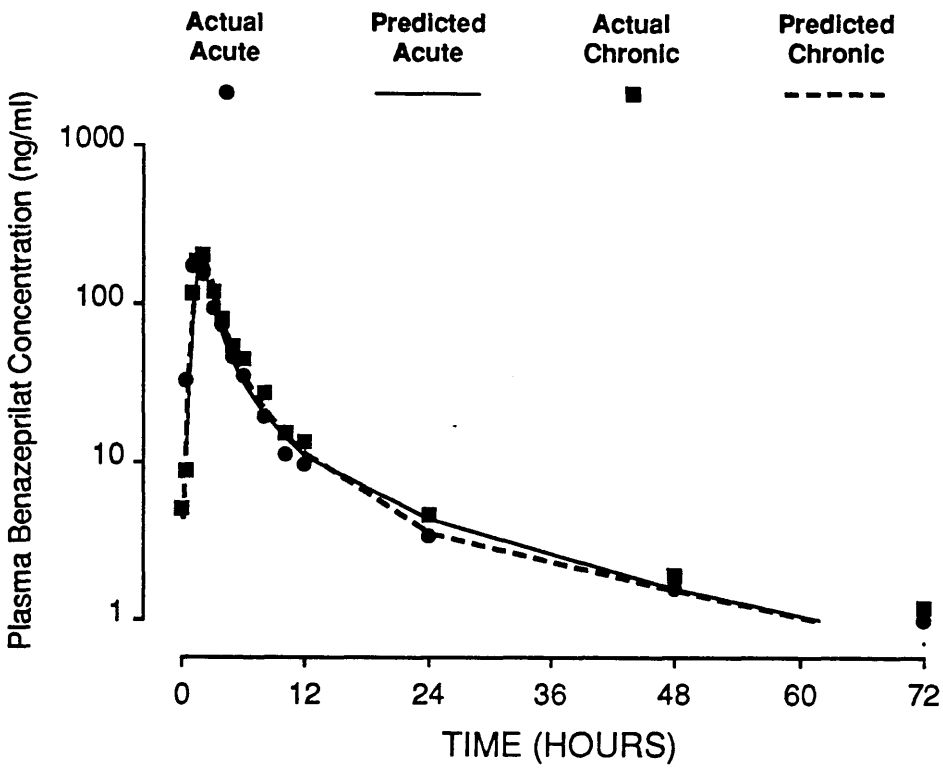
Actual And Predicted Benazeprilat Data For Subject No. 5



Data predicted by a one compartment model with tissue binding

FIGURE 7.10

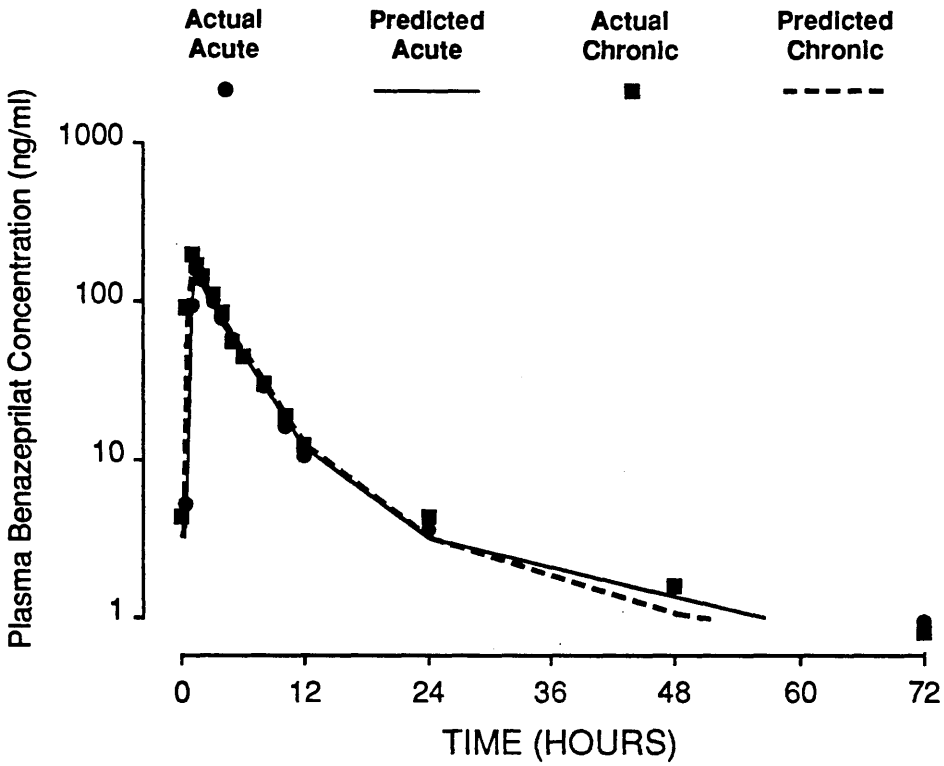
Actual And Predicted Benazeprilat Data For Subject No. 6



Data predicted by a one compartment model with tissue binding

FIGURE 7.11

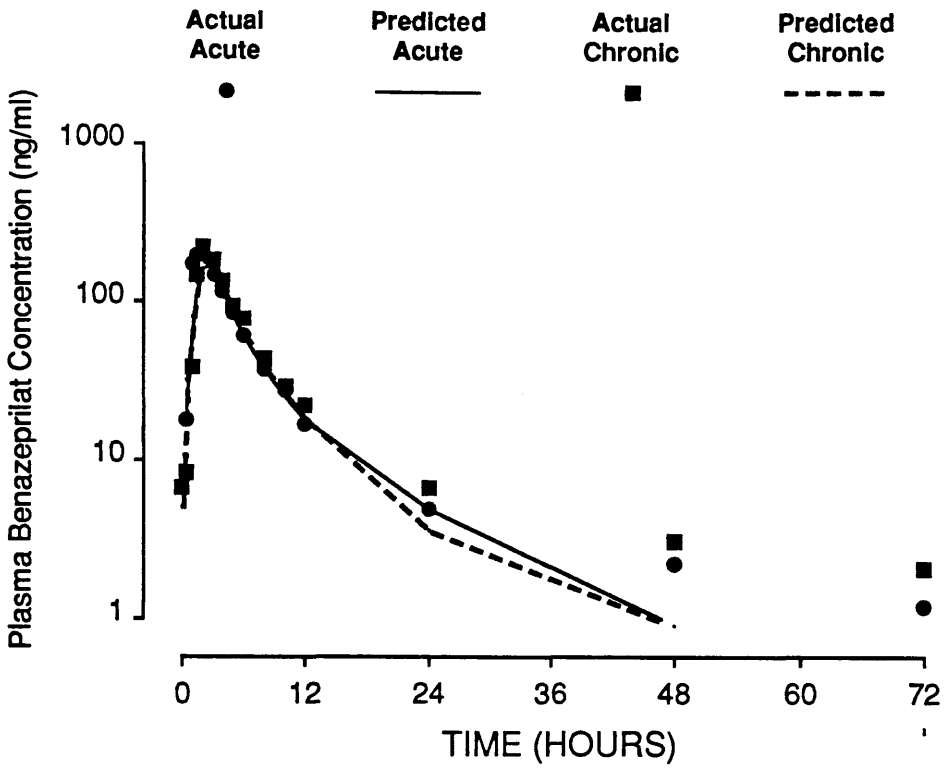
Actual And Predicted Benazeprilat Data For Subject No. 7



Data predicted by a one compartment model with plasma and tissue binding

FIGURE 7.12

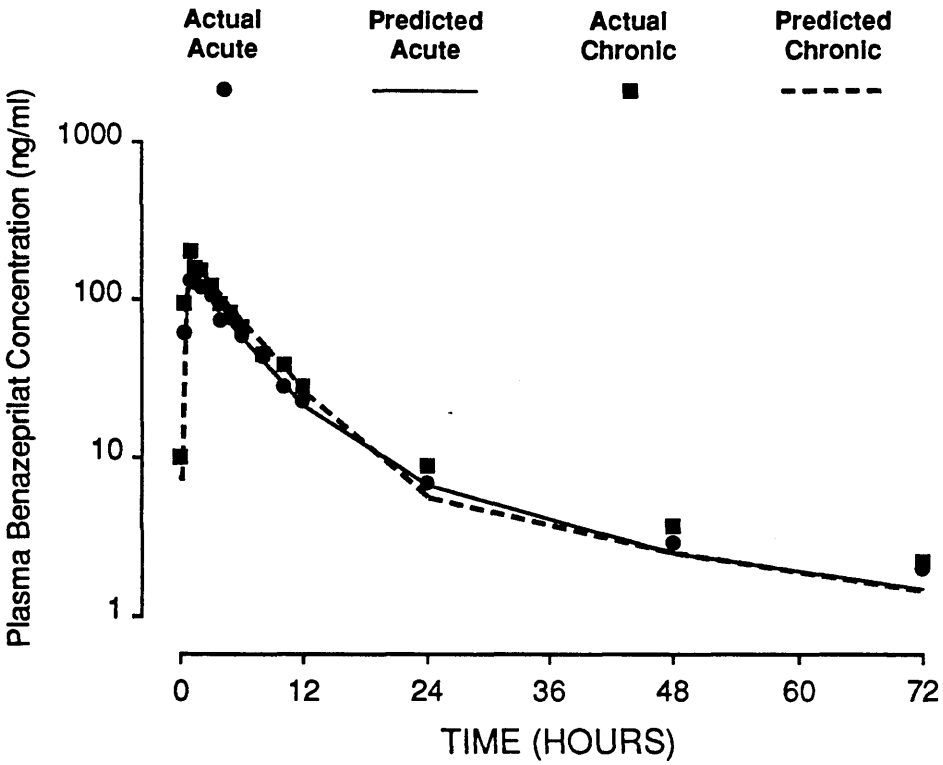
Actual And Predicted Benazeprilat Data For Subject No. 8



Data predicted by a one compartment model with tissue binding

FIGURE 7.13

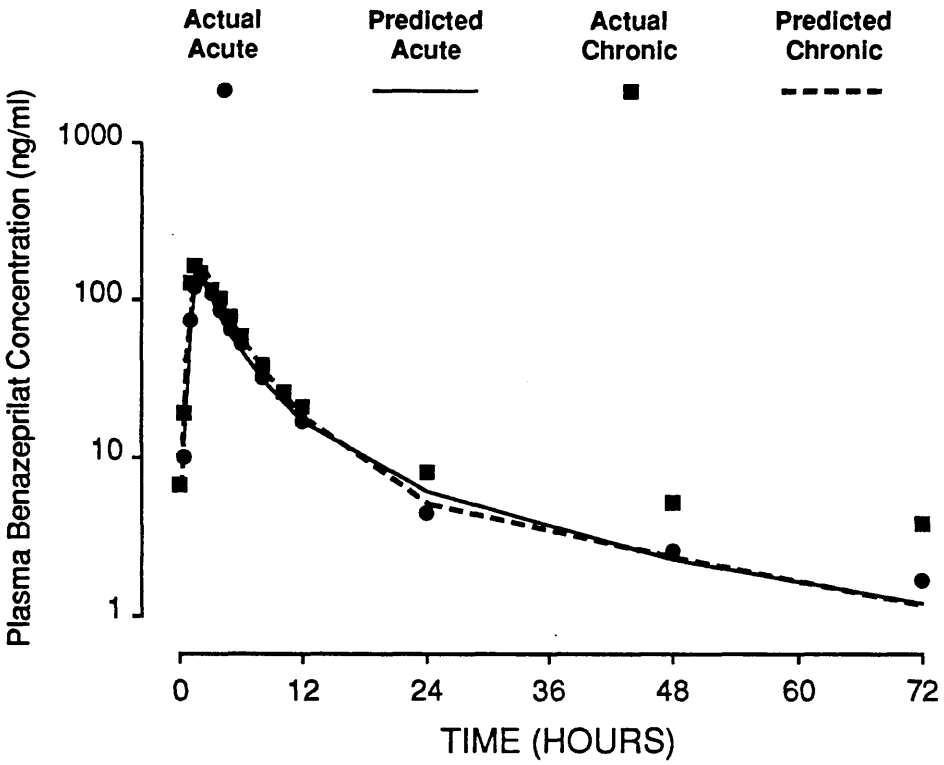
Actual And Predicted Benazeprilat Data For Subject No. 9



Data predicted by a one compartment model with tissue binding

FIGURE 7.14

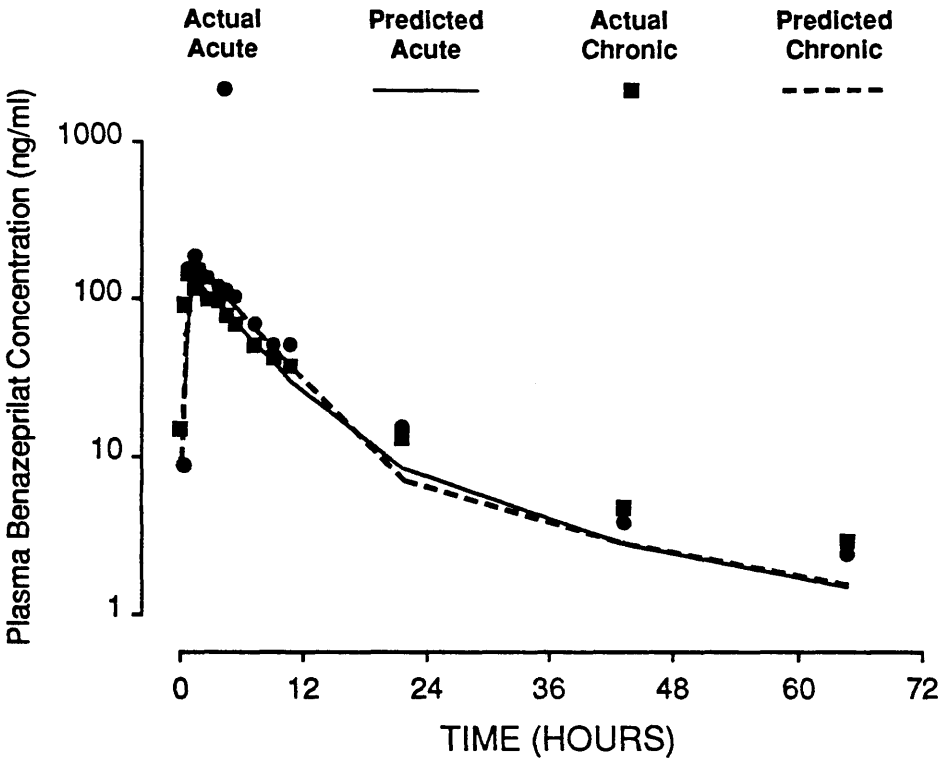
Actual And Predicted Benazeprilat Data For Subject No. 10



Data predicted by a one compartment model with tissue binding

FIGURE 7.15

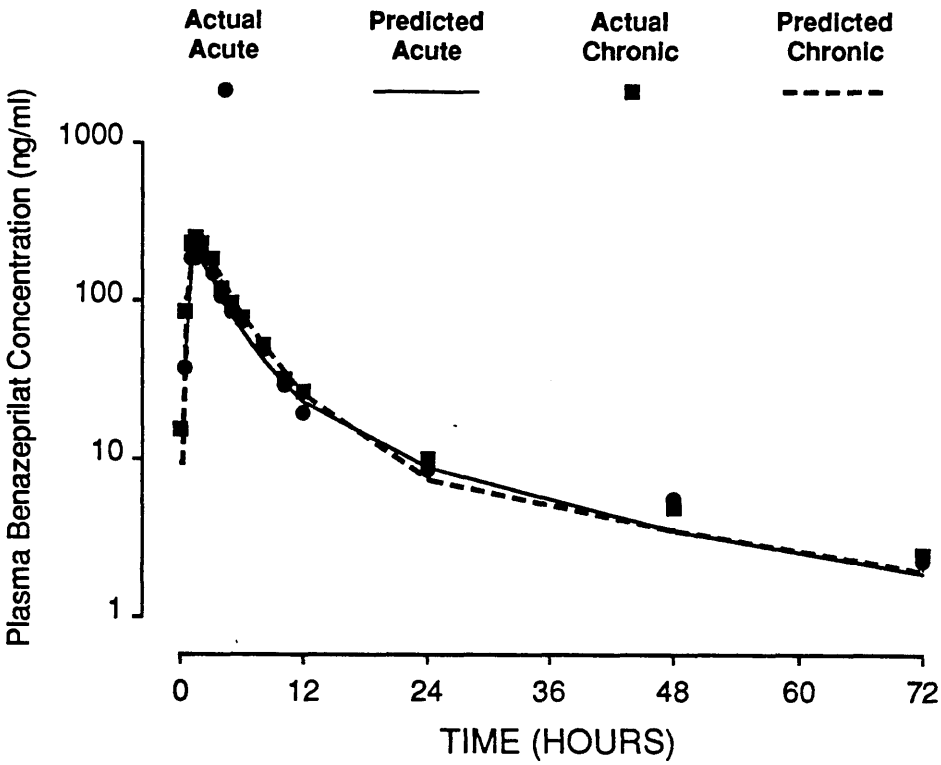
Actual And Predicted Benazeprilat Data For Subject No. 11



Data predicted by a one compartment model with tissue binding

FIGURE 7.16

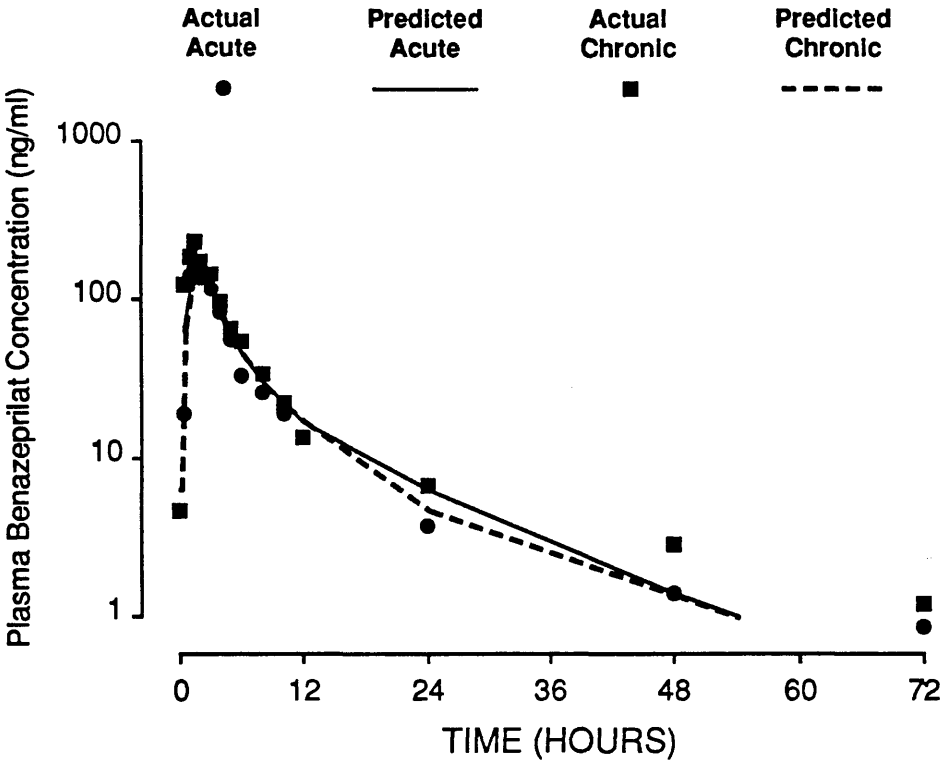
Actual And Predicted Benazeprilat Data For Subject No. 12



Data predicted by a one compartment model with tissue binding

FIGURE 7.17

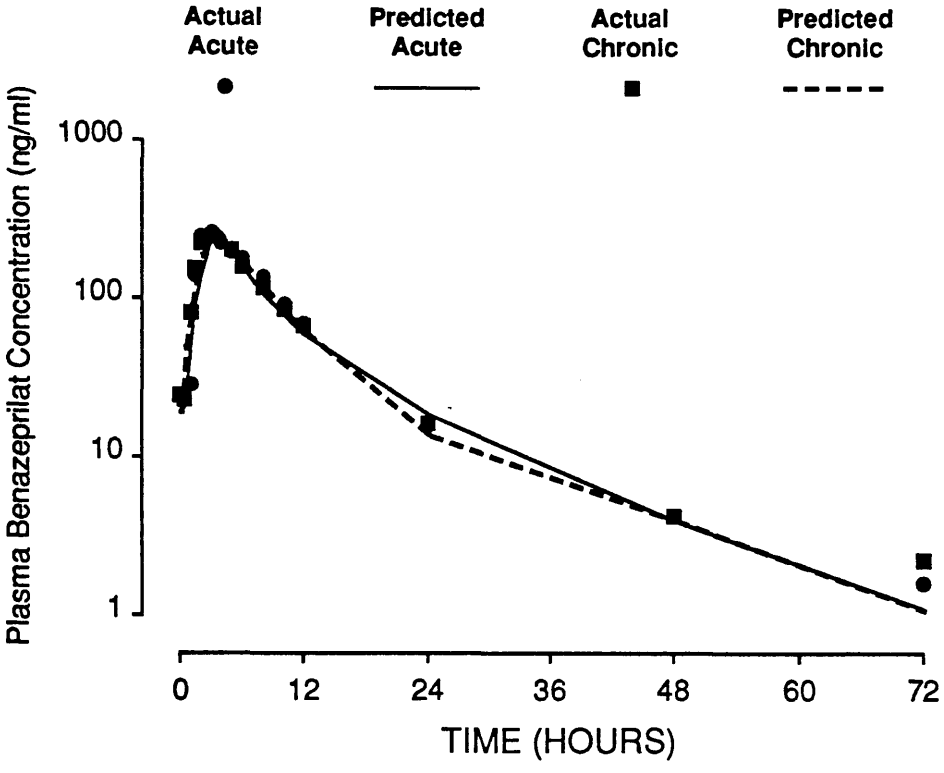
Actual And Predicted Benazeprilat Data For Subject No. 13



Data predicted by a one compartment model with plasma binding

FIGURE 7.19

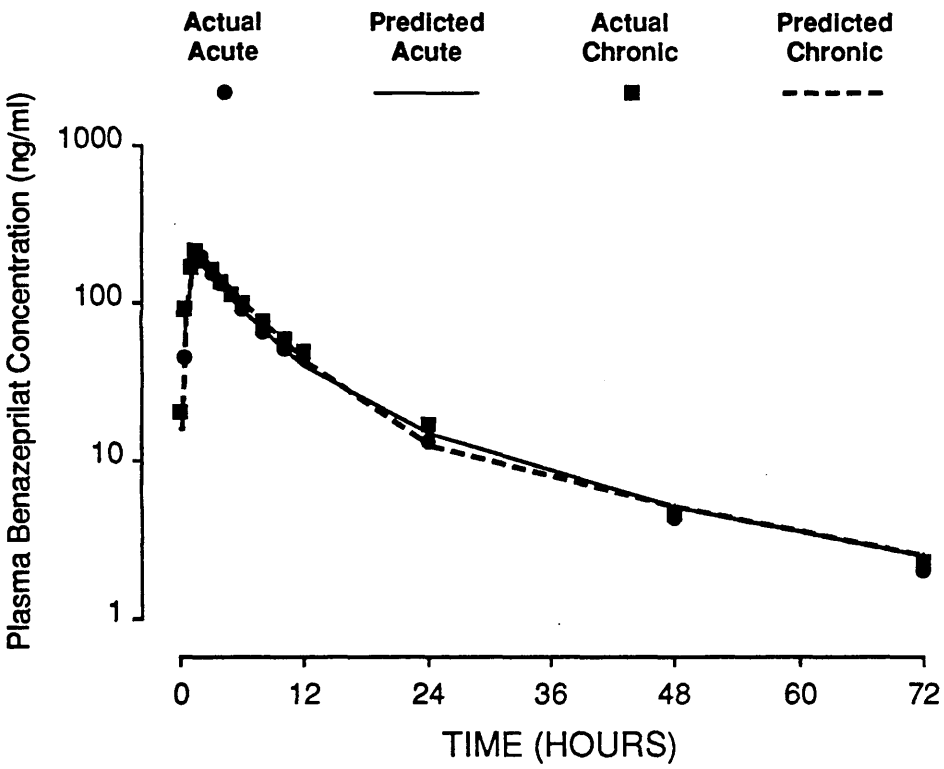
Actual And Predicted Benazeprilat Data For Subject No. 15



Data predicted by a one compartment model with tissue binding

FIGURE 7.20

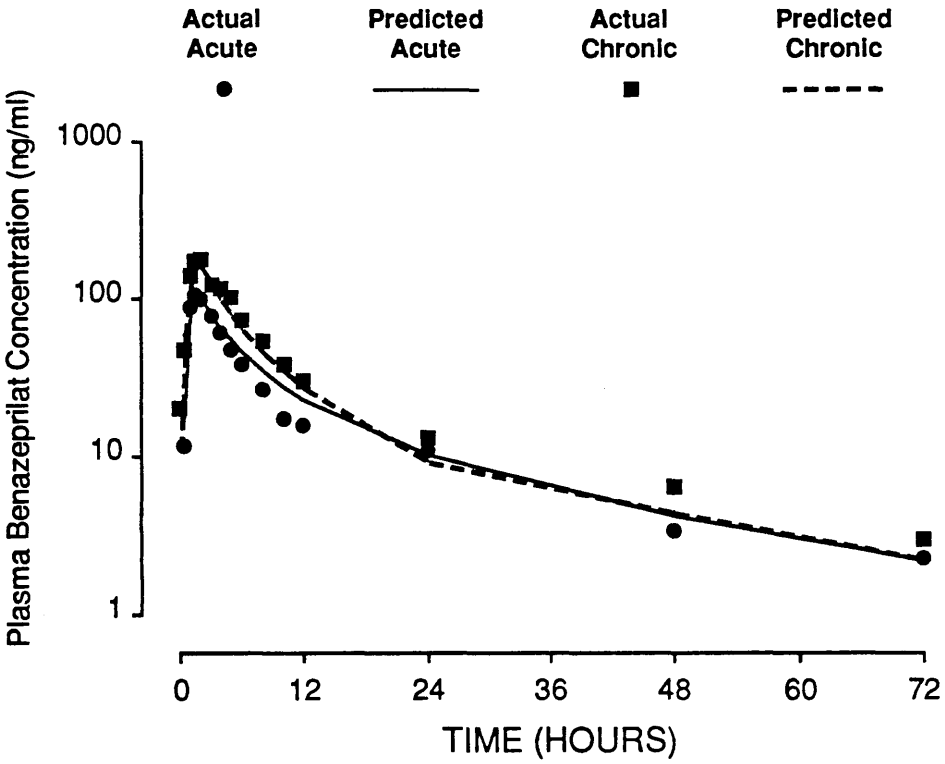
Actual And Predicted Benazeprilat Data For Subject No. 16



Data predicted by a one compartment model with plasma and tissue binding

FIGURE 7.21

Actual And Predicted Benazeprilat Data For Subject No. 17



Data predicted by a one compartment model with tissue binding

FIGURE 7.22

Actual And Predicted Benazeprilat Data For Subject No. 18

and elderly subjects. No significant effect of age was found. The mean (SD) values for Bmax/F and Au(50)/F for all subjects were 2986(1104) and 4622(4115) μg and 170(131) and 264(218) μg for acute and chronic administration respectively. Although both Bmax/F and Au(50)/F were increased after chronic administration, this did not achieve statistical significance, irrespective of whether the weighted or unweighted data were used (p=0.17 and p=0.13 for Bmax/F and Au(50)/F respectively for the unweighted data analysis). Individual data for Bmax/F and Au(50)/F after acute and chronic administration are shown in Table 7.7.

7.3.3 Protein Binding.

The protein binding of benazeprilat was measured at 2 and 6 hours post dose for young and elderly volunteers after acute and chronic administration. The data were analysed by repeated measures ANOVA. There was one data point far outwith the range of the data for the remaining subjects, subject no. 11, 2h after acute administration. The amount of drug bound for this point was found to be 76.2% which was confirmed by repetition. However, the point was omitted since inclusion rendered the data non normal and invalidated the assumption of homoscedasticity.

No effect due to acute or chronic administration was found. There was a significant effect of age on binding (p=0.02), however, there was an age time interaction and

TABLE 7.7

The Individual Values For Bmax/F and Au(50)/F Derived From Separate Fitting Of Benazeprilat Acute And Chronic Data.

Subject	Bmax/F (μg)		Au(50)/F (μg)	
	Acute	Chronic	Acute	Chronic
Young				
1	4762	2754	53.6	472
2	2620	5310	175	114
3	2130	2966	206	617
4	2731	5778	119	152
5	3742	1873	136	487
6	1948	2596	145	151
7	3230	5118	131	115
8	4136	2452	82.5	154
9	2400	5615	549	223
Mean	3078	3774	177	276
SD	961	1664	146	194
Elderly				
10	1552	3891	77.6	110
11	3004	19216	396	44.4
12	4239	4897	99.0	383
13	3529	5969	246	134
14	307	335	38.0	48.4
15	3053	1701	86.0	182
16	3101	7392	288	715
17	2825	1151	76.8	63.2
18	4444	4191	153	590
Mean	2895	5416	162	252
SD	1284	5663	121	251
Young + Elderly				
Mean	2986	4622	170	264
SD	1104	4115	131	218

so binding had to be assessed at the individual time points. A significant difference was found only at 6 hours (Bonferroni 95% confidence interval 1.46 to 4.48) and not at 2 hours (Bonferroni 95% confidence interval -0.32 to 2.78). The mean protein binding for benazeprilat was 91.5(1.56) (n=18) and 90.3(1.83)% (n=17) at 2 hours post dose and 92.2(1.29) and 89.2(2.40)% (both n=18) at 6 hours post dose for the young and elderly subjects respectively. No relationship was found between the degree of protein binding and the plasma concentration of benazeprilat. The range of benazeprilat concentrations observed in the protein binding samples was 46.7 to 495ng/ml.

7.4 DISCUSSION

A long terminal half life of elimination has important pharmacological implications in terms of drug accumulation and possible toxicity. The long terminal half life of the ACE inhibitors makes little contribution to the accumulation of these drugs (Till et al, 1984 and Nussberger et al, 1987). The characterisation of a value for the effective half life of accumulation for enalaprilat (Till et al, 1984), which was consistent with the observed accumulation of the drug, approximated the half life of the phase immediately preceeding the terminal phase. The proposal that the long terminal phase of

elimination is due to the dissociation of these drugs from ACE is supported by the loss of dose linearity during the terminal phase (Till et al, 1982 and Meredith et al, 1989).

Two different approaches have been used to characterise the pharmacokinetics of these drugs using models which incorporate saturable binding. The model proposed by Francis et al (1987) has been used to describe the pharmacokinetics of cilazaprilat after different oral doses of cilazapril (Francis et al, 1987 and Meredith et al, 1989). The model incorporates binding to plasma ACE only. Furthermore, due to the low plasma protein binding of cilazaprilat, the nonspecific binding of cilazaprilat was assumed to be zero. Benazeprilat is a highly protein bound drug (approximately 90%) so this assumption cannot be made. Hence, it was not possible to use the approach described by Francis for the analysis of benazeprilat.

The second method, described by Lees et al (1989) was used to characterise the pharmacokinetics of perindoprilat after intravenous infusion. Models that described saturable binding in plasma, tissue or both plasma and tissue were proposed. It was the approach put forward by Lees that has been extended and used to describe the pharmacokinetics of benazeprilat after oral, acute and chronic, administration of benazepril hydrochloride.

The combined acute and chronic plasma benazeprilat data were fitted to a range of standard and 'binding' models. The vast majority of the data were best described by a one

compartment model with zero order input and tissue binding. Of the volunteers who were best described by the model which incorporated both plasma and tissue binding, the majority of the binding took place in the tissues. This is consistent with the results reported by Lees et al (1989) where the amount of binding of perindoprilat in the tissue was 250% greater than that in the plasma. The characterisation of tissue as the major site of binding is not unexpected. It is now widely recognised that the pharmacodynamic actions of the ACE inhibitors are as a consequence of inhibition of tissue ACE (Unger et al, 1985, Unger et al, 1987, Dzau, 1988, Swales and Samani, 1989 and Ganten et al, 1989).

The calculated parameter $t_{1/2}$ represents free drug only (characterised in Models L to N). The value of approximately 3.0 hours is consistent with that reported for the initial half life of elimination for benazeprilat after oral administration of benazepril hydrochloride to normal volunteers (Kaiser et al, 1989). This lends further support to the argument that the long terminal half life of elimination of ACE inhibitors is due to the slow dissociation of the drug from ACE.

The calculated mean (SD) value for the C(50) of benazeprilat of 8.89 (5.84) ng/ml is similar to the in vitro value obtained for benazeprilat of 5.14 (1.08) ng/ml (Chapter 4).

The accumulation characteristics of benazeprilat after

repeated administration have also been characterised by use of the 'binding' models used to describe the data. The 'binding' model which best described the data for any one subject predicted very little accumulation of the drug after chronic administration. The predicted plasma levels fell to the same level as those predicted for acute administration. This was not the case for the best standard model where the predicted plasma levels after chronic administration were higher and declined in a parallel fashion to those predicted for acute administration.

The phenomenon of induction of ACE by administration of ACE inhibitors has been well documented (Fyhrquist et al, 1983a and Boomsma et al, 1981) although there appears to be little clinical significance of the phenomenon. The effect of induction of ACE synthesis was studied by examining the parameters Bmax/F and Au(50)/F obtained after analysis of the separated acute and chronic data. Both parameters increased after chronic administration although this did not achieve statistical significance, suggesting that limited induction of ACE synthesis may have occurred. The lack of a significant change may be due to the possibility that the dosage regime employed in the present study was insufficient to induce ACE, or, that a change in bioavailability occurred with chronic administration. The latter could have masked any changes in the binding parameters. The results obtained for the protein binding analysis of benazeprilat also did not

change between acute and chronic administration. It can be concluded therefore, that, in the absence of any changes in bioavailability, once daily dosing with 10mg benazepril hydrochloride is insufficient to cause a significant increase in the amount of ACE.

Two different analytical techniques were used to measure benazeprilat concentrations within this chapter. The GC-MS method used to measure plasma benazeprilat levels is specific for benazeprilat only. The IBA method used to determine the protein binding of benazeprilat is specific for any pharmacologically active moiety. Thus, the protein binding results may not be specifically for benazeprilat but could also include any other active molecules that bound to plasma proteins. Glucuronides of benazeprilat have been detected in considerable amounts in urine after oral administration of benazepril hydrochloride (Waldmeier et al, 1990). However, the result obtained in this chapter for the amount of drug bound to plasma protein is slightly lower than that previously reported (95%, Dieterle et al, 1989) and so the results found in the present study may reflect the binding of benazeprilat only. The high degree of protein binding and low reported value for volume of distribution at steady state (8.7 litres, Dieterle et al, 1989) for benazeprilat may also have implications in explaining the long terminal half life previously reported for this compound. ACE inhibitors bind to ACE by a two step

mechanism.

The inhibitor (I) rapidly binds to the enzyme (E) to form an initial enzyme inhibitor (EI) complex which then undergoes a slow isomerisation to form a tightly bound complex (EI^*) which has a very small dissociation constant (Bünning 1987). Ahnfelt et al (1989) demonstrated that highly protein drugs with low volumes of distribution that undergo a two stage binding process, similar to that described for the ACE inhibitors, exhibit extremely long terminal half lives of elimination. The long half lives were inversely related to the dissociation constant for the tightly bound enzyme inhibitor complex.

The influence of age on the pharmacokinetics of benazeprilat was also examined in the present study. Initially it was thought that ACE inhibitors were unlikely to be of clinical benefit in elderly hypertensives since PRA tends to fall with increasing age (Meade et al, 1983). However, it was shown that the fall in blood pressure was not consistently related to the pretreatment PRA (Gavras et al, 1981) thus belying the reason for not using ACE inhibitors in elderly hypertensives.

Benazeprilat is mainly eliminated by the renal route but some degree of hepatic elimination does occur (Kaiser et al, 1989). A decrease in renal clearance in the elderly has been reported to contribute to the enhanced intensity

and duration of action of the ACE inhibitors (Lees et al, 1988). The creatinine clearances of the elderly volunteers in the present study were significantly lower ($p=0.02$) than those of the young volunteers, but they did not approach the value of 30ml/min, below which, for enalapril, dosage adjustment has been reported as necessary (Kelly et al, 1986).

No significant effect was found for the effect of age on any of the pharmacokinetic parameters reported for the present study although in the case of benazeprilat Cmax, AUC and $t_{1/2}$ (free drug), this just failed to achieve statistical significance. The non significant increases in these data are likely to be caused by an age dependent decline in renal function.

In conclusion, physiologically relevant models have been used to describe the benazeprilat data. The models have characterised the binding of the drug to ACE and have given a much shorter, clinically realistic estimate of the terminal half life of elimination which is in keeping with the accumulation characteristics of the drug. No significant differences were found between the young and elderly for the parameters calculated and this suggests that, on pharmacokinetic grounds, dosage adjustment will not be required. It must be appreciated that where more profound degrees of renal impairment are present in diseased elderly patients, this may not hold true.

CHAPTER 8

GENERAL DISCUSSION

In the late 1960's, ACE was a poorly defined peptidase of little interest. Since then, extensive study of the active site of the enzyme has enabled potent and specific inhibitors of ACE to be developed as antihypertensive drugs. The ACE inhibitors are now also being successfully used in the treatment of cardiac failure, and in the future new therapeutic indications may be defined. The work presented in this thesis has been concerned with the evaluation of the disposition characteristics of the ACE inhibitors and their concentration effect relationships, with a view to optimising therapy with existing ACE inhibitors and discerning desirable characteristics in a new ACE inhibitor.

Before the pharmacokinetics of any novel compound can be characterised, accurate, precise, sensitive and specific assay methodology must be available. In the case of the ACE inhibitors, in addition to methods to quantify plasma drug levels, assay techniques were also required to measure ACE activity. Where more than one method is being used for the same measurement, a comparison of methods is essential to ensure the compatibility of results. Three groups of methods have been compared within this thesis, two different methods to measure ACE activity, three different methods to measure plasma enalaprilat levels and three different methods to measure plasma benazeprilat levels. The statistical approach adopted to assess the methods compared both the means and the variability within the methods. The two methods used to measure ACE activity

produced results that were in good agreement with each other. This was not the case for determination of levels of enalaprilat or benazeprilat. In the case of enalaprilat no further metabolism has been reported, therefore, all analytical techniques, whether specific for enalaprilat or any pharmacologically active moiety, should give the same result. One method gave consistently lower results, the method which relies on the displacement of the binding of radiolabelled MK-351A. No obvious reason could be found for this. In contrast, benazeprilat has been reported to be metabolised further, glucuronides of both benazepril and benazeprilat have been identified in human urine after an oral dose of benazepril hydrochloride (Waldmeier et al, 1990). Glucuronides have also been found in baboon urine, however they were reported to be unstable (Waldmeier and Schmid, 1989). It could be that possible instability of the human glucuronides may have contributed to the discrepancy between the three methods to measure plasma benazeprilat. The results found for the three groups of assay methods highlight the need to know the specificity of the technique being used.

The definition of ACE inhibition is dependent upon the methodology used, different results are obtained when alternative substrates or different substrate conditions are utilised (Nussberger et al, 1989). It is not possible to compare the efficacy of the ACE inhibitors when identical assay techniques have not been applied. Within

this thesis all measurements of ACE activity were based on the formation and quantification of hippuric acid from the synthetic substrate Hip-His-Leu.

The concentration effect relationship of two different series of ACE inhibitors was assessed in vitro, within plasma from individual rabbits and human volunteers. The potency of the different drugs was assessed by comparing the C(50) values, the concentration of drug required to inhibit ACE activity by 50%. Significant differences were found for individual drugs between rabbit and man, also, variations were found in the rank order of potency of the series of compounds between rabbit and man. The differences in C(50) in rabbit and man reflect that different doses would be needed to obtain the same degree of ACE inhibition and, presumptively, other associated pharmacological effects.

The comparison of the in vivo/in vitro relationship for perindoprilat and quinaprilat in plasma from individual subjects was also investigated. For both compounds the in vivo potency was significantly lower than that characterised in vitro, indicating greater inhibition of ACE in vivo.

After the introduction of captopril and enalapril onto the market, the therapeutic dose range for both drugs was reduced. Since the therapeutic range for any drug is initially characterised in animals, the differences in potency found between rabbit and man may be one of the factors that contributed to the post marketing dosage

reduction. The discrepancy between the in vivo and in vitro values may also help to explain the reduction in recommended doses for captopril and enalapril. Any calculations based upon less potent in vitro values would have overestimated the amount of drug needed to elicit the same response in vivo.

Further in vitro studies were carried out that may provide an explanation for an in vivo hysteresis observed with perindoprilat. Lees (1986) carried out two in vivo studies to characterise the pharmacokinetics of perindoprilat. Firstly, after oral administration of perindopril and secondly, after intravenous administration of perindoprilat. Following oral administration of parent compound, when the plasma perindoprilat concentrations were plotted against percent inhibition of ACE activity, a hysteresis loop was observed. The hysteresis loop was not observed after intravenous administration of the active metabolite. The presence of hysteresis after an oral but not after an intravenous dose of a compound is unusual. Conventional thinking would predict the presence of hysteresis after intravenous administration, where the appearance of effect lags behind the immediate peak plasma levels of the compound. Hysteresis would not be predicted after oral administration where both peak effect and peak plasma levels are delayed after administration. Hysteresis was also not observed after oral administration of enalapril or benazepril.

Many of the new ACE inhibitors currently under development are prodrugs with very low activity, these prodrugs require deesterification in the liver to form the active metabolite. Since the hypotensive action of the ACE inhibitor prodrugs is a consequence of the inhibition of ACE by the active metabolite, the in vitro studies were carried out to study the effect of the parent compound on the efficacy of the metabolite. The in vitro dose response curves for the active metabolites were characterised in the presence and absence of parent compound. In man, enalapril caused a small decrease in the potency of enalaprilat, manifested by a 21% increase in the C(50) value. Also in man, perindopril caused a large, significant decrease in the potency of perindoprilat, the C(50) value for perindoprilat increased by 109%. No effect was observed of benazepril on benazeprilat. Thus, it may be speculated that the decrease in potency for perindoprilat may be due to steric hindrance caused by the parent compound binding on or near the active site of ACE. This affords a possible explanation of the hysteresis seen after the oral administration of perindopril but not after the intravenous administration of perindoprilat.

Severe problems with first dose hypotension have been observed after the administration of enalapril (Webster et al, 1985). Unpublished anecdotal evidence suggests that there may be a reduced incidence of first dose hypotension following administration of perindopril. Thus, it appears

that there are differential effects between different ACE inhibitors with respect to first dose hypotension. The differential may be related to the different affinities of the various parent compounds for the ACE molecule. It may be interesting to consider whether more potent steric inhibitors of ACE binding could be of benefit in the treatment of patients with profound first dose hypotension or ACE inhibitor poisoning.

With the exception of captopril, it is a characteristic feature of the ACE inhibitors that they have a long terminal half life of elimination. Values in excess of 30 hours have been reported (Meredith et al, 1989, Hockings et al, 1986). A long terminal half life of elimination usually bears the risk of accumulation of drug and possible subsequent side effects. This is not the case for the ACE inhibitors, little accumulation has been observed for this group of drugs (Till et al, 1984, Nussberger et al, 1987). Till and co-workers proposed that the prolonged elimination is due to binding to, and subsequent dissociation from, ACE itself. Thus, there is a 'one-time' contribution of binding to accumulation.

Two practical approaches have been explored to examine the disposition characteristics of these drugs, especially the contribution of binding to ACE to the terminal elimination phase of the ACE inhibitors. Firstly, the pharmacokinetics of intravenous radiolabelled spiraprilat were assessed in the presence and absence of saturated ACE

binding sites in rabbit. The ACE binding sites were saturated by predosing the animals with unlabelled spiraprilat. No change in the terminal elimination half life or clearance was found. However, since the value for the terminal half life was found to be only of the order of 2.5 hours, coupled with the previously observed variations between man and rabbit for the in vitro parameters calculated, it was concluded that the rabbit was not a good model for the disposition of ACE inhibitors in man. Secondly, the pharmacokinetics of enalaprilat were characterised in the presence and absence of saturated ACE binding sites in man. In this instance captopril was chosen as the agent to saturate the binding sites. Again no change was detected in enalaprilat pharmacokinetics, although the observation of induction of ACE may have negated the effect of captopril's occupancy of original ACE binding sites. These results are in agreement with those published by Waldmeier et al (1990) who investigated the disposition of radiolabelled benazepril hydrochloride after single and repeated oral doses. No change was found in the radiolabelled benazeprilat pharmacokinetics, the authors do not report whether induction of ACE had occurred to confound their results.

A third, mathematical approach was explored to characterise the binding of ACE inhibitors to ACE. The pharmacokinetics of benazeprilat, formed after the oral administration of acute and chronic benazepril

hydrochloride, were described using extended versions of the models proposed by Lees et al (1989) to describe the pharmacokinetics of perindoprilat infusions. The models incorporate saturable binding in plasma, in tissue or in both types of binding site. When the combined acute and chronic plasma benazeprilat data were fitted simultaneously with the plasma percent inhibition of ACE data, over 80% of the volunteers were best described by a one compartment model with zero order input and tissue binding only. One of the assumptions made by the model is that there is instant access to the tissue binding sites from the plasma. In a physiological sense tissue sites could be considered to be on the surface of the vascular endothelium. The value for the volume of distribution divided by bioavailability, characterised by the model, was of the order of 30 litres. No absolute data have been published on the bioavailability of benazeprilat, however, Waldmeier et al, (1990), have stated that at least 37% of a 20mg dose of benazepril is absorbed. Thus the actual value for the volume of distribution would be approximately 11 litres, close to the value for the extracellular fluid volume. Since the drug is not extensively distributed outwith the plasma, the assumption made by the model that the drug has instant access to tissue binding sites is not invalidated by benazeprilat. The value for the half life of elimination of free drug obtained from the model was of the order of 2.5 hours.

This is consistant with the initial half life value of 3 hours characterised in normal volunteers (Kaiser et al, 1989) and is in keeping with the accumulation characteristics of the drug. The effective half life of accumulation for enalaprilat, proposed by Till et al, (1984) also closely approximated the half life of the phase immediately preceeding the terminal phase.

The models used quantified the binding site capacity available and also the amount of drug required to inhibit binding by 50%. Whilst the values for these parameters are complicated by the unknown bioavailability, they do give an estimate of the total amount of ACE available for binding, and hence to produce an effect.

In conclusion, the practical approaches were unable to characterise the contribution of binding to ACE to drug pharmacokinetics. However the models used to describe the benazeprilat data have characterised the binding of the drug to ACE and have given a physiologically appropriate value for the half life of elimination.

One of the major aspects of ACE inhibition is the clinical dissociation between the duration of antihypertensive activity and the time course of plasma ACE inhibition. This can be accounted for by the wide consensus of opinion that the major site of action of the ACE inhibitors is in the tissues. The existence of many localised RAS's in different tissues has been demonstrated, including widespread distribution within blood vessels. Some investigators have raised the interesting possibility that

the structure of ACE may vary in different organs (Sakharov et al, 1988). Other investigators have suggested that it may be possible to design inhibitors more specific for one tissue than another (Johnston et al, 1989). Cushman et al (1987) proposed that tissue bioavailability may be more important than a differing ability to bind to the active site of ACE from different tissues. Low concentrations of esterase activity have been found in a variety of tissues (Larmour et al, 1985). It is not yet known if these tissues have the capacity to significantly convert prodrugs to active metabolites.

The physicochemical characteristics of the new ACE inhibitors under development are likely to be of importance in the pharmacological differentiation between these drugs. Chemical structure and lipophilicity will be major determinants of the penetration of the drug into the various tissue sites of action, the duration of action and the route of elimination. It may be that ACE inhibitors cleared by non renal mechanisms will offer an important alternative in the therapy of hypertension and cardiac failure.

APPENDIX

DERIVATION OF EQUATIONS FOR THE 'BINDING' MODELS

All the 'binding' models were based on a one compartment model with zero order input. The total amount of drug present in the body was unknown, due to the unknown bioavailability of benazeprilat. The dose of benazepril hydrochloride administered was 10mg which is equivalent to 8600/F μg of benazeprilat in the body. Benazeprilat was assumed to be present in bound and unbound states, and that binding was a saturable phenomenon. Blood withdrawn from the compartment contained unbound benazeprilat plus any drug bound to circulating binding sites. Benazeprilat bound to tissue binding sites was not available for sampling. It was also assumed that benazeprilat had instant access to the tissue binding sites, thus these sites could be considered to be on the surface of the vascular endothelium. The concentration of benazeprilat in any plasma sample was the sum of the unbound and circulating bound drug, divided by the volume of the compartment (V). Elimination of benazeprilat was assumed to occur as a first order function of the unbound drug. The volume of the compartment is constant but since amount of total benazeprilat measured in a sample is dependent on the dose, due to the saturable binding, Vss is also dependent on the dose.

The rate of change of total drug in the body can be described by

$$dA/dt = \frac{8600}{Tabs.F} - Au.Ke \qquad (1)$$

where Tabs is the duration of absorption

Au is the amount of free drug

Ke is the elimination constant for free drug

F is the bioavailability.

Binding was assumed to be saturable and to be described by a Michaelis- Menten equation, thus total benazeprilat in the body could be described in terms of free and bound benazeprilat.

Since $$A = Au + \frac{Bmax.Au}{Au50 + Au} \qquad (2)$$

where A is the total drug in the body

Bmax is the maximum number of binding sites

Au50 is the affinity constant for binding.

It should be noted that the parameters Bmax and Au50 are really Bmax/F and Au50/F since they are also affected by the unknown bioavailability, however the parameters are presented just as Bmax and Au50 in the following equations for clarity.

Therefore $$A = \frac{Bmax.Au + Au.(Au50 + Au)}{Au50 + Au}$$

$$A\,(Au50 + Au) = Bmax.Au + Au.(Au50 + Au)$$

$$A.Au50 + A.Au = Bmax.Au + Au.Au50 + Au^2$$

And $$Au^2 + (Bmax + Au50 - A).Au - A.Au50 = 0 \qquad (3)$$

This is simply $aAu^2 + bAu + c = 0$ (4)

Equation (4) can be solved using

$$Au = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a} \quad (5)$$

Thus

$$Au = \frac{-(Bmax + Au50 - A) + \sqrt{(Bmax + Au50 - A)^2 + 4(A.Au50)}}{2}$$

which is equation (6).

Equations (1) and (6) were combined to describe the rate of change of total benazeprilat in the body, dA/dt.

$$dA/dt = \frac{8600}{tabs.F} - 0.5*Ke\ [-Bmax - Au50 + A\ \{(Bmax + Au50 - A)^2 + 4*A*Au50\}^{0.5}\ ] \quad (7)$$

The combined equation was used by the function subroutine in BMD-PAR as described in the manual for the software (Ralston, 1983) and was the same for all the 'binding' models described in chapter 7.

The equations derived so far have been for amounts of drug rather than concentration. The dose dependency of Vss makes this essential. BMD-PAR rescaled the estimates of A for each time of interest. This was done differently for each of the 'binding' models.

Models F, I and L assume that all the benazeprilat was available for sampling (ie. no drug was bound to tissue binding sites) thus the estimate of concentration was

$$A / V \quad (8)$$

Models G, J and M assumed that only unbound benazeprilat was available for sampling (ie. benazeprilat bound to tissue binding sites only). This is equivalent to Au / V but since Au was not known it had to be expressed in terms of A

$$0.5 [-Bmax - Au50 + A + \{(Bmax + Au50 - A)^{2} + 4 * A * Au50\}^{0.5}] / V \quad (9)$$

Models H, K and N assumed that benazeprilat bound to plasma and tissue binding sites. Bmax was scaled by the fraction of binding which took place in the tissues, f.

$$\text{Tissue binding} = f * Bmax \quad (10)$$

The total concentration of benazeprilat measured in plasma was equal to unbound plus plasma bound drug,

$$0.5 [-f*Bmax - Au50 + A + \{(f*Bmax + Au50 - A)^{2} + 4 * A * Au50\}^{0.5} / V \quad (11)$$

This is equivalent to total drug minus tissue bound drug

$$A - f*Bmax * Au / (Au50 + Au) \quad (12)$$

The fortran subroutine for Model H is shown below to demonstrate how the function was applied in practise.

MODEL H

```
      CALL DEFUN(F,P,X,N,KASE,NVAR,IPASS,XLOSS)
      RETURN
      END
      SUBROUTINE DIFEQ(F,P,X,N,KASE,NVAR,NPAR,IPASS,XLOSS)
      IMPLICIT REAL *8(A-H, O-Z)
      COMMON/DECON/Z(10),DZ(10),T,NEQN,IGO,IT,NEW
      DIMENSION X(NVAR),P(NPAR)
      GOTO (100,200,300),IGO
100   CONTINUE
      IT=1
      NEQN=1
      Z(1)=0.0
      TABS=P(1)
      RETURN
200   CONTINUE
      IF(T.LE.TABS)DZ(1)=8600/P(1))-0.5*P(2)*(-P(4)-P(5)+
     +Z(1)+((P(4)+P(5)-Z(1))**2+4*Z(2)*P(5))**0.5)
      IF(T.GT.TABS)DZ(1)=-0.5*P(2)*(-P(4)-P(5)+
     +Z(1)+((P(4)+P(5)-Z(1))**2+4*Z(1)*P(5))**0.5
      RETURN
300   CONTINUE
      F=0.5*(-(P(6)*P(4))-P(5)+Z(1)+(((P(6)*P(4))+
     +P(5)-Z(1))**2+4*Z(1)*P(5))**0.5/P(3)
      IF(X(3).LT..5)RETURN
      TABS=X(1)+P(1)
      NEW=0
```

Where
P(1) = Tabs
P(2) = Ke
P(3) = V/F
P(4) = Bmax/F
P(5) = Au(50) (C(50) = Au50 / V)
P(6) = f.

The ACE activity data in models L, M and N were fitted simultaneously to the equation

$$\% \text{ ACE Inhibition} = \frac{P(7) * Au}{P(5) + Au}$$

where P(7) is the maximum % inhibition of ACE activity and Au is as defined in equation (6).

PRESENTATIONS AND PUBLICATIONS

PRESENTATIONS

Harrigan, J.R., Hughes, D.M. & Meredith, P.A. Characterising Inter-Species Differences In ACE Inhibition. Presented To The Caledonian Clinical Pharmacology Group, Dundee, 07 October 1988.

Harrigan, J.R., Hughes, D.M. & Meredith, P.A. Characterising Inter-Species Differences In ACE Inhibition. Presented To The British Pharmacological Society, London, 20 December 1988.

Harrigan, J.R., Hughes, D.M., Meredith, P.A. & Reid, J.L. Characterising The Effect Of Parent Compound On The In Vitro Potency Of The Metabolite Of Five ACE Inhibitors. Presented To The IV World Conference On Clinical Pharmacology & Therapeutics, Mannheim-Heidelberg, 25 July 1989.

Harrigan, J.R., Hughes, D.M. & Meredith, P.A. A Possible In Vitro Explanation For The In Vivo Hysteresis Seen With Perindopril. Presented To The Drug Metabolism Drug Discussion Group, Bath, 30 September 1989.

Harrigan, J.R., Lees, K.R. & Meredith, P.A. Age And The Physiologically Realistic Characterisation Of Benazeprilat Pharmacokinetics. Presented To The British Pharmacological Society, 03 January 1990.

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Harrigan, J.R., Hughes, D.M. & Meredith, P.A. (1989). Characterising Inter-Species Differences In ACE Inhibition. British Journal Of Clinical Pharmacology, **27**, 656P - 657P.

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