METABOLIC INTERACTIONS BETWEEN ALCOHOL, THE LIVER, AND THE GASTROINTESTINAL TRACT

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Dedication

To Marion, Isabel, and Cairns

Summary

This thesis examines the metabolic interactions between alcohol and the liver and gastrointestinal tract. The metabolism of alcohol by the liver and gastrointestinal tract may determine susceptibility to alcohol- related disease, but is also likely to be responsible for alcohol related damage to these organs.

The three introductory chapters review the current understanding of alcohol related gastrointestinal and liver disease and alcohol metabolism. Chapter 1 reviews the epidemiology of alcohol consumption and alcohol related disease, and then discusses in detail the hepatic and gastrointestinal lesions associated with alcohol. Differing patterns of genetic and environmental susceptibility are then discussed, followed by a detailed review of the processes involved in alcohol metabolism, focussing on pathogenic mechanisms related to metabolism (i.e. redox shift, hepatic hypermetabolism, acetaldehyde induced damage, free- radical generation, cytochrome induction, antioxidant, phospholipid and vitamin depletion, endotoxin-mediated damage and mechanisms of hepatic fibrogenesis).

Chapter 2 concentrates on alcohol dehydrogenase, which is quantitatively the most important enzyme involved in alcohol metabolism. The complex nomenclature of alcohol dehydrogenase is discussed. Genetic and environmental influences on hepatic and extra-hepatic alcohol dehydrogenase are discussed. The evidence regarding the controversial role of gastric alcohol dehydrogenase in determining

susceptibility to alcohol related disease, and the genetic and environmental factors that affect its activity, is reviewed in detail. Conventional methods of determining the activity of alcohol dehydrogenase experimentally are discussed.

The complex pharmacokinetics of alcohol are reviewed in detail in Chapter 3. The validity of common assumptions made by researchers examining alcohol metabolism is discussed, and illustrated by using specific examples. This chapter concludes that pharmacokinetic principles based on linear elimination kinetics cannot be applied to studies of alcohol metabolism. The rate of delivery of alcohol to the systemic circulation will partly determine its bioavailability.

Chapter 4 states the aims of this thesis. The first major aim is to determine if a rise in gastric alcohol dehydrogenase activity following *Helicobacter pylori* eradication is associated with any change in the pre-systemic metabolism of alcohol, and to determine to what extent this is influenced by changes in gastric emptying. A secondary aim of this thesis is to develop an improved assay for gastric alcohol dehydrogenase activity. The second major aim of this thesis is to determine if there is any evidence from conventional biochemical liver function tests or plasma markers of hepatic fibrogenesis for hepatic damage following abrupt alcohol withdrawal.

The fifth chapter describes in detail the development of a rapid, colorimetric, semi-automated assay for gastric alcohol dehydrogenase. Use of a coupled enzymatic colorimetric reaction results in an increased sensitivity that has facilitated

replacement of the conventional (time- consuming) homogenisation stage with an incubation stage. Data from experiments designed to determine the optimum assay conditions, and to demonstrate the sensitivity, specificity, and reproducibility of the new method are provided, and the new method is compared with conventional methods. A normal range for gastric alcohol dehydrogenase activity by this new assay method is provided.

In chapter 6, the effect of Helicobacter pylori eradication on alcohol metabolism and gastric alcohol dehydrogenase activity is assessed. There is a marked increase in gastric antral alcohol dehydrogenase activity following *Helicobacter* eradication, although this does not result in a significant change in alcohol first pass metabolism, or the degree of systemic exposure to alcohol. Gastric emptying (assessed by paracetamol absorption) does not change following eradication. Alcohol bioavailability correlates with gastric emptying but not with gastric alcohol dehydrogenase activity.

Evidence for hepatic damage following abrupt alcohol withdrawal in subjects who are alcoholics, but have, at worst, well- compensated liver disease is sought in the experiments described in Chapter 7. Although there is no worsening of hepatic transaminases following alcohol withdrawal when considering the group of subjects as a whole, 32% of subjects did meet pre- defined criteria for worsening of transaminases. Concomitant intake of a small dose of paracetamol during the withdrawal period was associated with a marked transaminase rise. High basal levels, of, and changes following alcohol withdrawal, of plasma levels of

aminoterminal procollagen III peptide suggest that alcoholics may have reduced degradation of collagen whilst still drinking, and may have increased deposition of collagen, or a reduction in hepatic clearance, following alcohol withdrawal. High levels of tissue inhibitor of metalloproteinases- 1 in alcoholics, which did not change following alcohol withdrawal, suggest that there may be inhibition of collagen degradation even in alcoholics who have, at worst, clinically compensated liver disease.

This thesis has three major conclusions. Firstly, it concludes that the colorimetric technique described for assaying gastric alcohol dehydrogenase activity can offer significant advantages over conventional methods. Secondly, although Helicobacter eradication increases gastric alcohol dehydrogenase activity, there is no change in alcohol bioavailability, suggesting that this enzyme plays at most a minor role in determining alcohol bioavailability. Thirdly, the minor changes in hepatic transaminases and markers of fibrosis following alcohol withdrawal may reflect mild liver injury; with perhaps a minor tendency to increased fibrosis or decreased hepatic clearance. The cumulative effect of repeated episodes of abrupt alcohol withdrawal may therefore be clinically significant, although this would require clarification by further studies.

SECTION 1 INTRODUCTION AND AIMS

Chapter 1: The interaction between alcohol and the liver and gastrointestinal tract

1.1 Introduction

Excessive alcohol consumption is a major cause of morbidity and mortality (Lieber, 1995). Approximately 3.5% of the total burden of disease worldwide has been attributed to the effects of alcohol (Murray and Lopez, 1997). The association between the consumption of alcohol and physical disease has been recognised since ancient times (Jellinek, 1976). Andreas Vesalius, the renaissance anatomist, described the association between alcohol and liver cirrhosis in the sixteenth century (Franken and Falk, 1991). Chronic alcohol abuse can predispose to disease in all of the major organ systems. The relationship between alcohol and the liver and gastrointestinal (GI) tract is particularly interesting because these are the major sites both of alcohol related damage and alcohol metabolism. Hepatic and GI alcohol metabolism may therefore be important in determining an individual's overall susceptibility to alcohol related disease.

In this chapter, I will briefly consider the epidemiology of alcohol consumption and disease before discussing the liver and GI tract lesions associated with alcohol. I will then outline the patterns of susceptibility to alcohol induced hepatic and GI damage before discussing in detail the pathogenic mechanisms, which are often linked to alcohol metabolism.

1.2 Epidemiology

Total alcohol consumption fell in most European countries from 1870 until World War II, but subsequently rose again until the 1980's. Over the period 1987- 1998, total alcohol consumption in the UK calculated from legal sales has remained static, at approximately 9.8 litres of pure alcohol per person aged over 15 years per annum. However, certain sub-groups of the U.K. population, notably women and 11-15 year olds of both sexes, have recently shown increased rates of consumption. In 1996, 27% of men and 14% of women in the UK consumed more than 168g and 112g of alcohol per week, respectively (Department of Health, 1999).

Heavy alcohol consumption is linked to an increase in total mortality. However, modest alcohol intake (less than 20-30g per day) is associated with lower all- cause mortality than complete abstinence (Dawson, 2000), (Doll et al., 1994), (Fuchs et al., 1995). This "J-shaped" mortality curve appears to be mainly due to fewer deaths from coronary heart disease, and is more marked in males (Doll et al., 1994) than females (Fuchs et al., 1995). A highly significant positive correlation has been demonstrated between alcohol consumption and death rates from cirrhosis both at a national level (Smart, 1984), (Pequignot, 1960), (Terris, 1967), (Nordstrom, 1987), and in smaller populations. An association between alcohol consumption and deaths from neoplasia, particularly of the upper GI tract has also been observed (Doll et al., 1994), (Fuchs et al., 1995). Fluctuations in per capita consumption of alcohol result in corresponding changes in cirrhosis death rates. Restrictions on alcohol availability are associated with a lower death rate from cirrhosis, for example following

licensing restrictions during World War I in the United Kingdom (Smart, 1984), following wine rationing during World War II in France (Pequignot, 1960), during prohibition in the United States (1919- 1932) (Terris, 1967), and before the end of alcohol rationing in Sweden in 1955 (Nordstrom, 1987).

1.3 Hepatic lesions associated with alcohol

Alcohol excess is associated with a wide spectrum of disease in the liver. Alcoholic liver injury usually progresses from fatty liver (alcoholic steatosis), through alcoholic hepatitis to cirrhosis. All three lesions can coexist, and in some cases, cirrhosis may develop without preceding evidence of alcoholic hepatitis.

1.3.1 Fatty liver

Fatty liver is the most commonly encountered lesion in alcoholic liver disease, occurring in 60-95% of chronic alcoholics (French et al., 1993). It is characterised histologically as an increase in fat deposition within hepatocytes, and is accompanied by an increase in hepatocyte and total liver size. The fat is most often deposited as large, often single, droplets (macrovesicular steatosis), although less commonly smaller droplets may occur (microvesicular steatosis or alcoholic foamy degeneration). Microvesicular steatosis is more often associated with hepatic

mitochondrial damage and may progress more readily to cirrhosis than macrovesicular steatosis (Uchida et al., 1983). Mitochondrial damage is a striking feature of alcoholic liver disease, with giant mitochondria seen in 25% of cases. Fatty liver has been shown to develop within as little as 8 days of heavy drinking (Lieber et al., 1965) and is usually reversible, regressing after approximately one month of abstinence from alcohol.

1.3.2 Alcoholic hepatitis

Estimates of the prevalence of alcoholic hepatitis amongst heavy drinkers vary according to the population studied and the method of diagnosis. In those drinking more than 160g of alcohol daily who come into contact with medical services approximately 30-40% have evidence of alcoholic hepatitis (Lelbach, 1975b). Overall, it is estimated that 10- 35% of those with fatty liver will progress to alcoholic hepatitis after a period of excess alcohol consumption of 15- 20 years (Morgan and Sherlock, 1977). Histologically there is swelling, ballooning, and necrosis of hepatocytes and an inflammatory response that consists predominantly of neutrophils. Mallory's hyaline is an irregular cytoplasmic deposit of eosin- staining material in hepatocytes and is usually considered the hallmark of alcoholic hepatitis, although this is not seen in all cases, and may rarely be seen in liver damage of another cause. Alcoholic hepatitis reverses following abstinence in approximately 30- 40% of cases. However, approximately one third of cases of alcoholic hepatitis

progress to cirrhosis despite abstinence. This is more likely in women, and those with severe alcoholic hepatitis on initial biopsy (Pares et al., 1986).

1.3.3 Cirrhosis

A net increase in the deposition of fibrous tissue in the liver following chronic injury leads to cirrhosis. Fibrous septa link hepatic vein branches, and hepatic vein branches to portal vein branches. The normal liver architecture is lost and regenerative activity in hepatocytes leads to nodule formation. Cirrhosis was traditionally considered to be irreversible, although there are reports of cirrhosis reversal, particularly when is at an early stage (Perez-Tamayo, 1979), (Iredale et al., 1998). Recent insights have suggested mechanisms at a cellular and molecular level by which cirrhosis could reverse or partially regress (Benyon and Iredale, 2000), and these will be discussed in more detail later. Perivenular fibrosis is fibrous thickening around the central hepatic venules and is an early histological marker for the later development of cirrhosis, which may occur without significant evidence of alcoholic hepatitis in the intervening period (Van Waes and Lieber, 1977). Only 10- 20% of heavy drinkers with fatty liver will develop cirrhosis (Sorensen et al., 1984).

Estimates of the level of alcohol consumption required to produce cirrhosis have varied widely in different studies. Older studies tend to estimate the cirrhotogenic threshold more highly. They suggest that ingestion of around 160- 200g of alcohol daily for 20 years will result in 50% of subjects developing cirrhosis, an

approximate cumulative lifetime dose of 600kg of alcohol for men, and 150- 300kg in women (Lelbach, 1975a). Studies that are more recent suggest a threshold as low as 40g of alcohol per day for men and 20g per day for women for cirrhosis (Tuyns and Pequignot, 1984). Alcohol associated liver lesions were not seen with consumption of less than 30g of alcohol per day in the Dionysos study, a large Italian cohort study (Bellentani et al., 1997).

Once the cirrhotogenic threshold has been reached, the risk of cirrhosis does not significantly increase with further alcohol consumption beyond 160g of alcohol per day, suggesting that a critical dose of alcohol has a permissive role in establishing conditions for the development of cirrhosis, which depends on the presence of other factors (Sorensen et al., 1984).

Hepatocellular carcinoma may arise in up to 15% of patients with alcoholic cirrhosis. Cirrhosis, and to a lesser extent alcoholic hepatitis, leads to portal hypertension causing the development of porto-systemic collaterals and shunting with the attendant complications of hepatic encephalopathy, varices, and ascites which are major causes of mortality in advanced liver disease.

1.4 Gastrointestinal lesions associated with alcohol

Alcohol abuse predisposes to a variety of GI tract lesions, although damage to these organs is also unpredictable, despite heavy alcohol intake.

1.4.1 Oesophagus

Alcohol abuse is associated with acute and chronic oesophagitis (Krasner, 1977a). Alcohol can cause direct oesophageal mucosal damage. Acute ingestion of alcohol, especially with food, decreases lower oesophageal sphincter pressure leading to increased gastro- oesophageal reflux (Kaufman and Kaye, 1978). Chronic alcohol ingestion is associated with altered oesophageal motility leading to a decrease in the rate of oesophageal acid clearance, thus precipitating reflux oesophagitis and predisposing to Barrett's oesophagus. The Mallory- Weiss lesion, a linear mucosal tear, is a common traumatic lesion of the gastro-oesophageal junction induced by retching after alcohol binges. There is an increased risk of upper GI tract neoplasia, particularly oropharyngeal and oesophageal carcinoma (a relative risk of 5 in those who drink heavily compared with abstainers (Gronbaek et al., 1998)). There is a synergistic relationship between alcohol and tobacco in the risk of developing these lesions.

1.4.2 Stomach and duodenum

William Beaumont initially recorded that alcohol causes acute haemorrhagic inflammation of the gastric mucosa in 1833 in a series of experiments involving direct visualisation of the mucosa through a gastrostomy (discussed in (Valencia-

Parparcen, 1981)). Alcohol may initiate inflammation through a direct chemical effect, through decreased mucosal resistance to acid, through decreased prostaglandin production (Bode et al., 1996), or by alcohol metabolites such as acetaldehyde that may be produced either by gastric mucosa or by *Helicobacter pylori* (HP) (Roine et al., 1992b). Fermented, but not distilled alcoholic drinks are powerful stimulants of gastric acid secretion (Teyssen et al., 1997). Acute alcoholic gastritis will usually resolve within 72 hours of abstinence. The role of alcohol in contributing to chronic gastritis is less clear. Chronic alcohol abuse was previously though to cause a chronic atrophic gastritis, although the discovery of the role of HP in chronic gastritis has changed this view, as most chronic gastritis in chronic alcoholics will respond to HP eradication but not to abstinence (Uppal et al., 1991).

Alcohol abuse does not predispose to peptic ulceration, except in the presence of alcoholic cirrhosis (Kirk et al., 1980), where the aetiology of peptic ulcer may be different to that in non- cirrhotics. The association between peptic ulcer and HP infection is less marked in cirrhotics, and rates of ulcer healing are lower, than in non- cirrhotics (Tsai, 1998). The net effect of alcohol consumption on gastric motility remains unclear, with studies showing contradictory findings (reviewed in (Bujanda, 2000)).

The antiseptic properties of alcohol may confer an advantage on alcohol drinkers in terms of lower gastrointestinal infection rates. In the past, wine consumption in particular was advocated for the prevention of infectious disease. Wine is bactericidal for *Salmonella* species in vitro (Marimon et al., 1998). The relative risk

of HP infection is lower in those with greater alcohol consumption (Brenner et al., 1997).

1.4.3 Pancreas

Friedreich first described chronic alcoholic pancreatitis in detail in 1878 (described in (Thiel et al., 1981)). The risk of developing pancreatitis rises with increasing alcohol consumption, and is positively associated with the protein and fat content of the diet in alcoholics (Sarles et al., 1973). Some consider that acute alcoholic pancreatitis is only a single episode that may recur during the course of chronic alcoholic pancreatitis that may be clinically occult. However, isolated acute lesions do occasionally occur after a bout of alcoholism in people who otherwise have a very low alcohol intake (Strum and Spiro, 1971).

Chronic alcohol abuse causes patchy inflammation of pancreatic acini, with resulting areas of necrosis, fibrosis, ductal narrowing, and dilatation. Precipitates of protein and calcium carbonate form in the pancreatic ducts. With progressive fibrosis of there is loss of pancreatic exocrine function, and in severe cases loss of endocrine function. Progressive destruction of the pancreas continues even after abstinence. The risk of pancreatic carcinoma rises with heavy alcohol intake (Burch and Ansari, 1968).

1.4.4 Small intestine, colon, and rectum.

The effects of alcohol on the small and large intestine are less specific and include alterations in gut motility and permeability, altered sodium, micronutrient and water absorption by ATP- dependent transport mechanisms, and altered mucosal protein synthesis (Nakshabendi et al., 1995) leading to diminished brush border enzyme activity (Perlow et al., 1977). Haemorrhagic lesions in duodenal and small bowel villous tips are seen after acute ethanol ingestion. These usually heal within 12- 24 hours (Pirola et al., 1969). The overall consequence of these effects is to cause diarrhoea, promote nutrient deficiencies, and increase specific dietary nutrient requirements.

The data regarding the risk of colorectal carcinoma in alcohol drinkers is unclear, with some studies suggesting an increased relative risk of carcinoma or adenomatous polyps (Cope et al., 1991) whilst others finding no increased risk (Tavani et al., 1998).

1.5 Environmental factors associated with alcohol related damage

From this overview of alcohol- related diseases affecting the liver and GI tract, it can be appreciated that whilst there is an association between the amount of alcohol consumed and physical disease, the onset and severity of illness depends on more than the period and extent of alcohol abuse. I will therefore review the

environmental and genetic factors that are associated with a predisposition to alcohol related disease, before considering in detail the potential mechanisms for these patterns of susceptibility. It is important to distinguish between factors that may predispose to increased alcohol consumption, and others that predispose to increased susceptibility to alcohol- related disease for a given level of alcohol consumption.

1.5.1 Type of alcoholic beverage

In general, it has been assumed that the nature of alcohol consumed (e.g. beer, wine, spirits) was not important in determining susceptibility to alcoholic liver disease. However, the Dionysos study suggested that after adjusting for total consumption, those consuming multiple types of alcoholic beverage might be more at risk than those drinking one type only (Bellentani et al., 1997). Another recent study has found a non- significant trend towards higher lifetime consumption of spirits in alcoholics with liver disease (Dear et al., 2001).

There is evidence that the type of beverage may be important in determining GI tract lesions. The relative risk of upper GI tract (Gronbaek et al., 1998) and colorectal (Anderson et al., 2001) neoplasia in alcoholics is less marked in wine drinkers than in spirit or beer drinkers. In carcinoma of the pancreas, the increased risk with alcohol is largely related to beer consumption (Cuzick and Babiker, 1989). These findings of an attenuation of increased carcinoma risk with wine consumption may

relate to the large quantity of antioxidant polyphenols in wine that have some potential anti-tumour properties.

1.5.2 Pattern of alcohol consumption

The pattern of consumption (intermittent binging versus chronic consumption) may have some importance in liver disease, with those who have a continuous higher consumption (Brunt et al., 1974) and those who consume alcohol outside mealtimes (Bellentani et al., 1997) at more risk of physical disease. This may be related to an increased first pass metabolism of alcohol when it is consumed with food.

In some alcoholics, abrupt alcohol withdrawal will cause delirium tremens, a syndrome of profound cardiovascular and neurological excitability. Severe delirium tremens is associated with a high mortality rate if it is untreated (Adinoff et al., 1988). There is some evidence that abrupt alcohol withdrawal may also predispose to liver damage, particularly in subjects with alcoholic hepatitis (Marshall et al., 1983), (Salum, 1972), (Sabesin et al., 1978), (Helman et al., 1971).

1.5.3 Nutritional factors

Malnutrition often coexists with alcohol related disease. It is often difficult to determine cause and effect when considering the link between alcohol related

disease and nutritional status, although the evidence for a link between malnutrition and disease severity is strong. Prior to the onset of clinically apparent disease, the overall nutritional status of alcoholics is not markedly inferior to non- alcoholics except in the presence of significant social deprivation, although approximately 5% may have some evidence of specific, usually single, nutrient deficiencies (Salaspuro, 1993). However, once there is evidence of physical damage secondary to alcohol, the severity of malnutrition varies directly with disease severity. For example, almost all subjects in studies of alcoholic hepatitis have some evidence of malnutrition, and the severity of malnutrition varied directly with hepatitis severity and mortality (Mendenhall et al., 1984).

Alcoholism may cause primary malnutrition by displacing nutrients in a diet consisting largely of alcohol. Despite being a source of energy (7 Calories per gram), alcohol is often considered nutritionally empty. In animal studies, growth rates are poorer in animals receiving a proportion of their energy from alcohol compared to those on an iso-caloric, non- alcohol diet (Lieber et al., 1965). In alcoholics, the provision of a weight- maintenance diet with supplementary calories in the form of alcohol fails to produce an increase in body weight (Pirola and Lieber, 1972).

Secondary malnutrition can occur due to the effects of alcohol on pancreatic or small bowel function discussed earlier. Acquired nutritional deficiencies may result from altered metabolism or molecular transport causing a non- essential

micronutrient to become essential. An example of this will be discussed later in the context of methionine.

Alcoholic liver disease was once thought to be primarily due to associated malnutrition. Rat studies from the 1940's using a diet with 10- 25% of total calories from alcohol showed that liver lesions developed only if the animals were fed a protein or choline deficient diet (Best et al., 1949). The introduction of a complete liquid diet supplemented with alcohol (the Lieber- DeCarli diet) allowed higher alcohol intakes of around 36% of total calories to be achieved and this was associated with development of fatty liver in nutritionally replete rats (Lieber et al., 1965). In the same paper, the authors report that administration of alcohol under controlled conditions to otherwise abstinent former alcoholics resulted in the development of fatty liver. More severe liver lesions can be reproduced in rats by prolonged intra-gastric infusions of ethanol (French, 2001).

It has not been possible to reproduce the exact range of hepatic lesions in animals that are seen in alcoholic liver disease in man. However, studies using the Lieber-DeCarli diet in baboons have shown that lesions very similar to human alcoholic cirrhosis can occur with chronic alcohol excess and a nutritionally adequate diet (Lieber and DeCarli, 1974).

The risk of alcoholic liver disease rises with increasing dietary fat (reviewed by (French, 1993)), and there is an association between obesity and alcoholic steatosis, hepatitis, and cirrhosis (Naveau et al., 1997). Polyunsaturated fat may be more

important than saturated fat in determining the susceptibility to liver disease. Polyunsaturated fatty acids (PUFA) are more prone to peroxidation than saturated fats and induce alcohol metabolising enzymes (Wade et al., 1985). Peroxidative damage is also more likely to occur in those with deficient diets due to impaired antioxidant defences.

The importance of malnutrition in physical disease outside the liver, e.g. thiamine deficiency and Wernicke's encephalopathy, is well established.

1.5.4 Hepatic and gastrointestinal infection

Co-exiting liver disease as a result of chronic viral hepatitis may influence the progression of alcoholic liver injury. The prevalence of serum antibodies suggesting previous hepatitis B exposure in patients with alcoholic cirrhosis was estimated at approximately 30% in Scotland in 1981 (Mills et al., 1981), and 30% in the United states in 1991 (Mendenhall et al., 1991). The widespread vaccination for hepatitis B may have changed the prevalence of this infection. Alcoholics with hepatitis B tend to present with cirrhosis at a younger age (Ohnishi et al., 1982), although other studies suggest that markers of hepatitis B infection are unrelated to the severity of liver injury (Mendenhall et al., 1991). However, the same study, and an Italian study (Loguercio et al., 2000), found that hepatitis C infection was associated with more severe liver disease in alcoholics. The prevalence of hepatitis C in alcoholics varies widely, being nil in a West of Scotland study (Bird et al., 1995), and approximately

5% in a male US population (Mendenhall et al., 1991). Alcohol abuse is associated with higher levels of hepatitis C virus RNA, and with a decreased response rate to interferon therapy (Loguercio et al., 2000).

Gastrointestinal bacteria are capable of metabolising alcohol, which may promote GI damage or carcinogenesis. Intestinal bacteria may also directly contribute to liver damage by increased bacterial translocation. It has been suggested that urea metabolism to ammonia by HP may increase the risk of hepatic encephalopathy in alcoholic liver disease (Mendenhall et al., 1993), although this has not been confirmed by later studies (Calvert et al., 2001).

1.6 Genetic factors associated with alcohol related damage

1.6.1 Family and twin studies

Almost all family studies have shown higher rates of alcoholism among relatives of alcoholics than in the general population (Jenkins and Thomas, 1981). In an attempt to separate common genetic from environmental factors in families, studies in mono- and di- zygotic twins have been performed. Monozygotic twins shown a higher concordance rate for alcoholism and alcoholic cirrhosis compared to di-zygotic twins (Hrubec and Omenn, 1981). Twin studies also suggest a high degree of genetic influence on ethanol elimination rate and susceptibility to alcohol intoxication (Kopun and Propping, 1977).

1.6.2 Gender

The prevalence of alcoholic cirrhosis is higher in males than females, being estimated in Scotland at 2.3:1 in 1981 (Hislop, 1981). However, the male: female ratio of problem drinking is higher still, being estimated at 5- 10, although there has been a disproportionate rise recently in alcohol consumption by females (Department of Health, 1999). Differences in susceptibility between the sexes are also likely to be culturally determined in part.

Females are generally considered more susceptible to the pathogenic effects of alcohol than males (Naveau et al., 1997). The cirrhotogenic threshold probably occurs at a lower alcohol dose in females (Norton et al., 1987). Women appear to develop liver disease more rapidly and at the time of presentation their liver disease is more advanced than men (Ashley et al., 1977), (Morgan and Sherlock, 1977), although this may be partly related to a lower index of suspicion for alcohol- related disease in women. As noted earlier, liver disease is more likely to progress following abstinence in women (Pares et al., 1986).

Females have a lower body weight and increased percentage body fat resulting in a higher peak blood alcohol concentration following ingestion of the same oral ethanol dose. There are notable sex differences in hepatic and gastrointestinal metabolism of alcohol and in the response to inflammation that may influence

susceptibility. Auto antibodies are found more frequently in female patients with alcoholic liver disease (Krasner et al., 1977b).

1.6.3 Ethnicity

Different ethnic groups have differing susceptibilities to alcohol- related disease. This is likely to be due to a combination of genetic, environmental, and cultural factors. Alcoholism and alcohol- related disease is less frequent in Oriental populations, probably because of differences in acetaldehyde metabolism. African – Americans have a higher risk of upper GI tract neoplasia than white Americans after standardizing for alcohol and tobacco exposure (Brown et al., 1994).

Alcohol dehydrogenase isoenzyme distribution shows marked ethnic variation, and this is discussed in more detail in chapter 2. Native Americans and Eskimos have a high prevalence of alcoholism and alcoholic liver disease. Alcoholic hepatitis is associated with a higher mortality in Native Americans than Hispanics, Caucasians, or African-Americans (Rex et al., 1985). Whilst some studies have suggested an increased alcohol metabolic rate in Orientals, Hispanics, Native Americans and Eskimos compared to Caucasians, other studies have found little significant difference, and stress that the intra- ethnic variability in the alcohol metabolic rate is greater than that observed between ethnic groups (Bennion and Li, 1976), (Reed, 1978).

1.6.4 Genetic studies

Family studies suggest that susceptibility to alcohol related disease is multifactorial, being partly environmental and partly polygenic. When considering specific genes determining susceptibility to alcohol related injury, the obvious candidates for study are those involved in regulation or expression of alcohol metabolising enzymes. However, the hereditary component of alcohol related disease suggested by family studies cannot be explained by the known differences in alcohol metabolising genes alone (Whitfield, 1997).

The pathological response to alcohol in many organs, particularly the liver, appears to be mediated by inflammation, and hence the potential for genetically determined differences in the inflammatory response have been explored. The human leukocyte antigen (HLA) loci have been recognised for decades, and these were therefore the subjects of many early studies on the genetics of alcohol related disease. Early reports identified many possible HLA associations with alcoholic liver disease, for example, HLA B8, B13 and B40. However, subsequent studies (Mills et al., 1988) and meta- analyses have identified no clear association, and it is likely that earlier studies were flawed.

Despite the association of alcoholism with iron overload, heterozygosity for the haemochromatosis gene does not appear to increase the risk of alcohol- related liver damage (Grove et al., 1998).

Recent advances in genetic techniques have allowed studies that focus on individual genes associated with inflammatory cytokines, which will be discussed in more detail later in this chapter.

1.7 Alcohol metabolism

Alcohol is both water and lipid soluble and is therefore capable of permeating all tissues. The body has no mechanism for storing or partitioning alcohol, and therefore any ingested alcohol has to be metabolised or excreted unchanged. The acute effects of alcohol on the central nervous system are probably mediated by alteration in the fluidity of cell membranes. However, the major site of alcohol related damage is in the tissues where alcohol is metabolised, and hence it is important to consider how metabolism may cause damage. However, metabolism may also have a protective role by limiting the exposure of certain organs to alcohol. At concentrations encountered in social drinking, approximately 1% of alcohol is eliminated from the body via the lungs, kidneys, and skin without being metabolised (Holford, 1987).

1.7.1 Alcohol dehydrogenase

Alcohol dehydrogenase (ADH) is a zinc- dependent oxido- reductase metallo-enzyme that is responsible for the metabolism of the majority (90%) of ethanol reaching the systemic circulation at low concentrations in normal subjects. There are multiple molecular forms of ADH with differing kinetic properties and distributions. This is discussed in more detail in chapter 2. ADH is relatively substrate non-specific, being able to metabolise a variety of primary and non- primary alcohols (including methanol, ethylene glycol and retinol, sterols and aldehydes) (Kim et al., 1992), (Raskin et al., 1976).

The vast majority of alcohol dehydrogenase (80- 90% of total activity) is present in the liver in hepatocyte cytosol. ADH is also found throughout the gastro- intestinal tract particularly in the oesophagus and stomach (Pestalozzi et al., 1983), (Buhler and Wartburg, 1982), (Yin et al., 1997). The equation for the metabolism of alcohol to acetaldehyde by ADH is shown in Figure 1.1a. Nicotinamide adenine dinucleotide (NAD) is required as a cofactor by ADH. The equation for the second metabolic step, the metabolism of acetaldehyde to acetate by acetaldehyde dehydrogenase, is shown in Figure 1.1b. The metabolism of acetate by the enzymes of the citric acid cycle to carbon dioxide and water takes place mainly in the peripheral tissues.

a) $CH_3\text{-}CH_2\text{-}OH + NAD^+ \rightarrow CH_3\text{-}CHO + NADH + H^+$

Alcohol oxidation by alcohol dehydrogenase

b) $CH_3\text{-}CHO + NAD^+ + H_2O \rightarrow CH_3\text{-}COO^- + NADH + 2H^+$

Acetaldehyde oxidation by acetaldehyde dehydrogenase

c) $CH_3\text{-}CH_2\text{-}OH + NADPH + H^+ + 2O_2 \rightarrow 2H_2O_2 + CH_3\text{-}CHO + NADP^+$

Alcohol oxidation by microsomal ethanol oxidising system (CYP2E1)

d) $CH_3\text{-}CH_2\text{-}OH + H_2O_2 \rightarrow CH_3\text{-}CHO + 2H_2O$

Alcohol oxidation by catalase

e) $R\text{-}COOH + CH_3\text{-}CH_2\text{-}OH \rightarrow R\text{-}CO\text{-}O\text{-}CH_2\text{-}CH_3 + H_2O$

Non-oxidative metabolism of alcohol by fatty acid ethyl esterase

Figure 1.1 Chemical equations related to alcohol metabolism

Ethanol: $CH_3\text{-}CH_2\text{-}OH$, Acetaldehyde: $CH_3\text{-}CHO$, Acetate: $CH_3\text{-}COO^-$, Nicotinamide adenine dinucleotide: NAD^+, Nicotinamide adenine dinucleotide phosphate: $NADP^+$, Hydrogen peroxide: H_2O_2, R-COOH: Fatty Acid. (Gillham et al., 1997)

The presence of ADH in the gastrointestinal tract has led to interest in the role of the GI tract, and the stomach in particular, in the pre- systemic metabolism of ethanol. Alcohol is absorbed poorly by the stomach, and much more rapidly by the jejunum. Theoretically, those with high activity of gastric ADH (GADH) may be resistant to the effects of alcohol, as a greater amount will be metabolised prior to entry into the portal system and thereafter the systemic circulation. The rate of gastric emptying will determine the exposure time of GADH to ingested alcohol as well as influencing the rate of delivery of absorbed alcohol to the liver. Individuals who have undergone gastrectomy show a decreased pre-systemic metabolism of ethanol (Caballeria et al., 1989b), (Frezza et al., 1997). The various genetic and environmental factors that affect ADH activity are discussed in detail in Chapter 2. The contribution of the stomach to the pre-systemic metabolism of alcohol remains controversial (Gentry et al., 1994a), (Gentry et al., 1994b), (Levitt, 1994a).

Hepatic ADH-dependent alcohol metabolism reaches a maximal rate at low levels of alcohol ingestion that are below those encountered in normal social drinking. The rate of alcohol elimination in non- alcoholics, which is mainly ADH dependent, is approximately 0.1g ethanol/ kg body weight/ hour (Holford, 1987). The factor determining the rate of maximal ADH- dependent ethanol metabolism is the availability of NAD, rather than the amount of ADH present. In other words, ADH is not fully saturated when the maximal rate is reached. The availability of NAD is dependent on the ability of mitochondria to indirectly regenerate NAD from NADH, its reduced form, via the electron transport chain, a process that ultimately requires

oxygen. The rate of alcohol metabolism in healthy individuals is directly related to liver cell mass. Some studies have suggested that because adult males and females tend to have a liver of the same size, their overall alcohol elimination rates are the same. However, when this is expressed as a proportion of lean body mass, females have a higher elimination rate as they have a greater liver mass per unit of lean body mass (Kwo et al., 1998). However, there is still controversy regarding the sex difference in the rate of alcohol metabolism, with others suggesting that the female alcohol elimination rate is faster in studies which adjusted the alcohol dose to give the same peak blood alcohol concentration as males, and expressing the rate independent of body weight (Thomasson et al., 1995).

A modest increase in rate of alcohol elimination by ADH can be achieved by co-administration of large amounts of fructose (Brown et al., 1972). This probably results from an increased rate of re-oxidation of NADH by the products of fructose metabolism.

Ingestion of large quantities of alcohol decreases the ratio of NAD: NADH and pyruvate: lactate, the so- called "redox state" of the liver, which profoundly affects the other metabolic functions of the liver. Gluconeogenesis is reduced which may cause hypoglycaemia. Substrate utilization by the Krebs' cycle and beta- oxidation and export of fatty acids is reduced, leading to triglyceride accumulation and fatty liver. Higher oestrogen levels in females also have an inhibitory effect on beta-oxidation of fatty acids, which may partly account for their accelerated development of alcoholic fatty liver.

The rate of hepatic clearance of some drugs by pathways that are also dependent on NAD (e.g. glucuronidation) is reduced (Lieber, 1994). Excess NADH has a pro-oxidant effect by increasing the synthesis of purines and shifting their metabolism from xanthine dehydrogenase to xanthine oxidase (Kato et al., 1990) and by promoting the liberation of iron from ferritin.

1.7.2 Hepatic hypermetabolism

Following acute alcohol ingestion, it is estimated that approximately 40% of liver oxygen consumption in the non- alcoholic is used in the metabolism of ethanol to acetate (Greenway and Lautt, 1990). After chronic alcohol administration, the oxygen demand of the liver rises (Souda et al., 1996), probably as a result of enzyme induction. There is a rise in liver blood flow, mainly due to increased portal vein flow, after alcohol administration, both intravenously (Mendelhoff, 1954), (Silva et al., 1994) and orally (Piano et al., 1991), (Kogire et al., 1992), (Orrego and Carmichael, 1992). This increased blood flow may not be sufficient to meet the oxygen demands of the liver following chronic ethanol consumption (Fukui et al., 1990), and is shunted away from the liver by collaterals when cirrhosis is present (Luca et al., 1997). Liver blood flow falls rapidly on alcohol withdrawal, whilst hepatic oxygen demand remains elevated for approximately 30 days thereafter (Israel and Orrego, 1984).

The liver lobule is perfused from the portal vein and hepatic artery branches in the portal tracts towards the central venules, with the lowest oxygen tension therefore arising in the perivenular areas (zone 3). This is the area of most prominent liver damage in alcoholic liver disease. Hence, alcoholics may be prone to ischaemic liver injury (Kasahara et al., 1988), especially in situations that further decrease hepatic oxygen delivery, e.g. anaemia, acute alcohol withdrawal (Hadengue et al., 1988), (Hadengue et al., 1994), and hypotension.

1.7.3 Acetaldehyde

Acetaldehyde, the first product of alcohol metabolism, is highly reactive and consequently can promote damage by bonding with other molecules. Hepatic acetaldehyde dehydrogenase (ALDH), metabolises over 90% of acetaldehyde. It has multiple molecular forms, of which the Class 1 and 2 isoenzymes, coded for by ALDH1 and ALDH2 loci respectively, are involved to a significant extent in acetaldehyde oxidation. ALDH2 is located in mitochondria. It has a relatively low Michaelis constant (K_m) (i.e. a high substrate affinity) and a high maximum reaction velocity (V_{max}), and is responsible for the vast majority of hepatic acetaldehyde metabolism (Bosron et al., 1993). Under normal circumstances, significant concentrations of acetaldehyde can only be found in the liver and colon. In alcoholics, higher levels of acetaldehyde are detectable in the blood after alcohol ingestion than in non- alcoholics (Peterson and Polizzi, 1987). This is probably as a

consequence of hepatocyte and mitochondrial damage because levels return to normal on re-administering alcohol after a prolonged period of abstinence (Di Padova et al., 1987b).

Bonding of acetaldehyde with protein results in the formation of acetaldehyde-protein adducts (for example with microsomal proteins, collagen, albumin, haemoglobin, lipoproteins, and the tubulin of microtubules (reviewed by (Lieber, 1994)). These adducts may alter the biological function of proteins, or may impair protein excretion. Impaired excretion may account for the ballooning of hepatocytes observed histologically, as this is associated with increased hepatocyte protein content (Tuma and Sorrell, 1988). These adducts are also antigenic. Antibodies directed against adducts are found in the blood of alcoholic patients (Niemela et al., 1987). Inoculation of ethanol fed animals with sera from humans with alcoholic cirrhosis causes hepatocyte cytotoxicity mediated by antibodies to acetaldehyde-protein adducts (Clot et al., 1997), (Israel, 1997).

Acetaldehyde that is not metabolised by ALDH can be converted to acetate by xanthine oxidase. This will promote free- radical mediated peroxidation of lipids that can also occur from acetaldehyde directly.

The mitochondria are likely to be particularly susceptible to damage as they are a major site of acetaldehyde metabolism and therefore a target for direct or free radical induced peroxidation of lipids, protein, and DNA. Mitochondrial DNA is more sensitive to damage as it lacks the protection of histone and non- histone

proteins associated with nuclear DNA, and it has a relatively limited ability for self-repair.

High blood concentrations of acetaldehyde (higher than those usually observed in chronic alcoholics) may occur following acute alcohol ingestion because of low ALDH activity. This has been termed the acute aldehyde syndrome. This high acetaldehyde level produces aversive symptoms clinically, with flushing, nausea, vomiting, and dizziness. The unpleasant nature of these symptoms forms the rationale for the use of disulfiram, an acetaldehyde inhibitor often prescribed in an attempt to enforce abstinence. Polymorphisms of the mitochondrial form of ALDH are recognised. The common allele in Western populations is ALDH 2*1, with the common genotype denoted 2*1/2*1. The 2*2 polymorphism results in very low mitochondrial ALDH activity. Populations of Mongoloid descent (Chinese, Japanese, Taiwanese, etc) commonly have the 2*2 allele, with approximately 50% having reduced ALDH activity. Those who are 2*2 homozygous, with almost no mitochondrial ALDH activity, experience the symptoms of acute aldehism after alcohol ingestion. Alcoholism is therefore much less common in Oriental populations. Heterozygosity (i.e. ALDH 2*1/2*2) is associated with a higher incidence of damage in alcohol metabolising organs (Agarwal, 1997), and this is probably because of higher acetaldehyde levels than in 2*1 homozygotes, but a less marked flushing response than in 2*2 homozygotes.

There is a relative imbalance in the activities of alcohol and acetaldehyde dehydrogenase (ALDH) in the oesophagus, and to a lesser extent the colon, with a

higher relative activity of alcohol dehydrogenase (Yin et al., 1997). This will favour increased local production of acetaldehyde, and is one proposed mechanism by which alcohol may predispose to malignancy at these sites.

Many gastro- intestinal bacteria, including HP (Kaihovaara et al., 1994), contain alcohol dehydrogenase. In the presence of alcohol, either from ingestion into the upper GI tract, or by back diffusion of absorbed alcohol into the lumen, these bacteria may produce acetaldehyde. These may cause local tissue damage or promote carcinogenesis, especially in areas of relatively low ALDH activity (Seitz et al., 1990). An association with the ALDH 2*2 allele and oesophageal carcinoma has been described (Hori et al., 1997), (also reviewed by (Agarwal, 1997)). The bacterio-colonic pathway for ethanol oxidation may also account in part for the lost calories associated with a diet high in alcohol. Higher acetaldehyde levels have been found in the colon than in the liver after acute alcohol ingestion (Jokelainen et al., 1996).

1.7.4 Microsomal ethanol metabolism

The second major pathway of hepatic ethanol metabolism is via the cytochrome-dependent microsomal ethanol oxidising system, in particular the cytochrome p450 2E1 (CYP2E1) enzyme sub-fraction (Lasker et al., 1987), but also to a lesser extent the CYP1A2 and CYP3A4 fractions (Figure 1.1c). These are a series of low-specificity oxidases found in the endoplasmic reticulum that are largely involved in

metabolism of drugs and other foreign substances (xenobiotics). They are inducible on chronic exposure to substrates including alcohol, some drugs (particularly anticonvulsants), and polyunsaturated fatty acids (Wade et al., 1985). In chronic alcoholics, they account for the increased rate of alcohol metabolism that is frequently observed. They may account for up to 50% of the metabolism of ethanol in chronic alcoholics, but usually contribute less than 10% in moderate drinkers who are not taking other medications causing enzyme induction. They contribute most to alcohol metabolism at high blood levels, because they have a higher K_m than ADH. They require NADPH rather than NAD as a cofactor, and generate hydrogen peroxide (H_20_2) along with acetaldehyde, with the formation of free radical intermediaries (via the Fenton reaction). ATP is not generated by alcohol oxidation by this route, and hence this form of metabolism is energy wasting and will contribute to the lost calories of diets high in alcohol.

Induction of microsomal enzymes by ethanol may lead to a shift in metabolism of other substrates, particularly xenobiotics, resulting in increased production of atypical and often toxic metabolites by microsomal oxidation. The clearest example of this is the increased susceptibility of alcoholics to paracetamol toxicity. Paracetamol is normally metabolised by glucuronidation or sulphation and is then excreted by the kidneys. In the presence of high paracetamol levels, (e.g. following overdose) it undergoes cytochrome- dependent oxidation to N-acetyl-benzo-quinone-imine. This precipitates hepatocyte death by inducing free radical damage and covalent bonding to structural proteins unless a glutathione- dependent

metabolic pathway inactivates it. The toxic threshold of paracetamol is reduced in alcoholics because of the induction of microsomal enzymes.

The period immediately following alcohol withdrawal after chronic drinking may be a particularly sensitive time for damage from xenobiotic metabolism, because the microsomal enzymes will not be saturated with ethanol, but remain induced and therefore have a higher activity.

Further evidence that the microsomal enzymes contribute significantly to alcohol-mediated damage comes from the observation of the pattern of injury in alcoholic liver disease, and studies of CYP2E1 inhibitors. As discussed earlier, the majority of damage occurs in zone 3, which is particularly prone to hypoxia. This is also the site where the CYP2E1 enzymes are located. Animal studies have shown increased acetaldehyde adduct formation in zone 3, and studies using CYP2E1 inhibitors (Morimoto et al., 1995) have shown attenuation of alcoholic liver injury in zone 3.

1.7.5 Other forms of alcohol metabolism

Alcohol can also be metabolised by catalase in the presence of hydrogen peroxide (Figure 1.1d). However, under physiological conditions catalase appears to play no major role in ethanol metabolism.

After acute alcohol ingestion the concentration of fatty acid ethyl esters (FAEE), formed by non- oxidative metabolism (Figure 1.1e), rises in the pancreas, liver, heart and adipose tissue (Laposata and Lange, 1986). Adipose tissue has the highest concentration of FAEE, followed by the pancreas. The activity of fatty acid ethyl esterase is much higher, and the activity of ADH much lower, in the pancreas compared to the liver. In animal studies, FAEE activate inflammation and collagen deposition in vitro (Lugea et al., 2001). Non- oxidative alcohol metabolism may therefore be more important than oxidative metabolism in the causation of alcoholic pancreatitis.

1.8 Oxidative stress and antioxidant defences

As discussed earlier, alcohol metabolism promotes oxidative damage by a variety of means. Increased NADH and acetaldehyde increases xanthine oxidase activity with the production of superoxide ($O_2^{\cdot-}$). Acetaldehyde can form peroxides directly. Induction of cytochrome dependent enzymes leads to free radical intermediaries, and the generation of hydrogen peroxide. Hydrogen peroxide may react to form further free radicals, or may be removed by glutathione peroxidase, which consumes hepatic reduced glutathione.

Increased oxidative stress results in membrane damage with loss of structure and function, and DNA damage that can cause mutagenesis, loss of function, and cell death. Free radical oxidation of protein results in similar effects to those described

for acetaldehyde- protein adducts. An increase in free radical generation will also lead to depletion of intra-hepatic antioxidant defences. Alcoholics have reduced levels of hepatic glutathione, and increased levels of molecules derived from lipid peroxidation (Clot et al., 1994). There is evidence of diminished levels of vitamins A, C (Beattie and Sherlock, 1976), E, and selenium, in alcoholic liver disease (reviewed by (Lieber, 1994)). Antioxidant depletion is exacerbated by malnutrition. In animal studies, dietary deficiency of antioxidant micronutrients is associated with increased hepatic lipid peroxidation (Kawase et al., 1989). Chronic alcoholism also increases the liberation of iron from ferritin and promotes hepatocyte iron uptake.

A polymorphism of the superoxide dismutase gene has recently been described in association with an increased incidence of hepatic lesions, although the functional significance of this polymorphism is not known at present (Degoul et al., 2001).

1.9 Glutathione and phosphatidylcholine

Glutathione can be synthesised from cysteine, which in turn can be synthesised from methionine, an essential amino acid. Alcoholics appear to have impaired methionine utilization, with depletion of the active methionine metabolite S- adenosyl methionine (SAM) due to impaired SAM synthetase activity. SAM is also required for transmethylation reactions and production of phosphatidylcholine from phosphatidylethanolamine. Depletion of SAM may therefore affect DNA synthesis and membrane lipid constituents of the cell and mitochondrion. A study of SAM

supplementation has shown a promising reduction in mortality in alcoholics with Child's group A and B cirrhosis (Mato et al., 2000).

Alcoholism decreases liver phospholipid and phosphatidylcholine. An additional acquired enzyme defect of phospholipid methyltransferase means that synthesis of phosphatidylcholine may still be impaired in alcoholics following SAM supplementation. Phosphatidylcholine supplementation corrects the hepatic deficit and protects against fibrosis and cirrhosis in the alcoholic baboon model (Lieber et al., 1994).

1.10 Hepatic inflammation and endotoxin mediated injury

In contrast to the proposed barrier role of the stomach to the effects of alcohol on the liver and systemic organs, the lower GI tract may provide the stimulus for hepatic inflammation in susceptible alcoholics. Translocation of colonic bacteria may be responsible for initiating or perpetuating the inflammatory response in alcoholic hepatitis. Increased intestinal permeability to macromolecules and increased circulating levels of endotoxin have been demonstrated in alcoholics, with correlation between the severity of liver disease and the degree of increased permeability (Parlesak et al., 2000). In animal models, inoculation of gut derived endotoxin (lipopolysaccharide, (LPS)) augments alcohol induced liver injury, and gut sterilization decreases alcohol induced hepatic damage (Enomoto et al., 2001). The inflammatory effects of LPS appear to be mediated by Kupffer cells that are

activated via the LPS receptor (CD14). Chronic alcohol exposure increases the sensitivity of Kupffer cells to LPS (Enomoto et al., 2001). Activated Kupffer cells induce further inflammation by secretion of inflammatory mediators. Tumour necrosis factor alpha (TNF alpha) appears to be of major importance in promoting hepatic inflammation. The severity of hepatic inflammation correlates with levels of pro-inflammatory cytokine such as TNF alpha and interleukin 1. In animal models, females appear more susceptible than males to alcohol and LPS mediated hepatic injury. This may be because of more pronounced sensitisation of Kupffer cells by alcohol in females, or because of a greater increase in intestinal permeability (Iimuro et al., 1997).

Several genetic polymorphisms associated with cytokines that regulate the inflammatory response have been identified. A rationale for the association of these polymorphisms with alcoholic liver disease can be proposed in some cases where the functional significance of the polymorphisms is understood, and the associated polymorphism will tend to exaggerate the inflammatory response. This is the case with the interleukin- 10 (IL-10) 627*A polymorphism which is associated with decreased IL-10 expression. It will favour an increased inflammatory response, and is found more frequently in alcoholics with severe liver disease than those with mild or no liver disease (Grove et al., 2000). However, in other cases the functional significance of certain polymorphisms associated with alcoholic liver disease is less well understood, for example the TNF alpha promoter polymorphism G-283A, was

identified in one study to be more common in alcoholic steatohepatitis (Grove et al., 1997), although its functional significance is controversial (Bayley et al., 2001).

1.11 Mechanisms and markers of alcohol- induced fibrosis

The cirrhotic liver contains approximately six times the amount of collagen (mainly types I and III) of normal liver (Rojkind and Martinez-Palomo, 1976). Extracellular matrix (ECM) was formerly considered almost inactive metabolically, and apart from some isolated reports (Perez-Tamayo, 1979), fibrosis was previously thought to be irreversible. Fibrosis is now recognised to be a dynamic process, involving an imbalance between the ongoing processes of ECM synthesis and degradation, and is now considered at least partly reversible, particularly for hepatic fibrosis of non-alcoholic aetiology.

The fundamentally important cell in hepatic fibrosis is the hepatic stellate cell (HSC) (also known as the Ito cell or hepatic lipocyte), which is normally present in the space of Disse. The quiescent HSC can become activated by a number of stimuli, in particular transforming growth factor beta (derived from Kupffer cells and also from HSC themselves), but also other inflammatory cytokines, absence of normal ECM, and other factors that are yet to be characterised fully. Activated HSC proliferate and

undergo change, with loss of retinyl ester stores, and assume a myofibroblast phenotype, secreting both collagenous and non-collagenous ECM.

A family of zinc- containing proteinases termed matrix metalloproteinases (MMP) degrades ECM proteins. There are 3 broad groups of MMP with differing substrate specificities: collagenases, gelatinases and stromelysins. Interstitial collagenase, also called MMP1, degrades fibrillar collagen. The activity of the MMP are regulated in part by endogenous inhibitors, termed tissue inhibitor of metalloproteinases (TIMP), of which there are four types identified at present. HSC secrete MMP1-3, TIMP1 and TIMP2 (Iredale et al., 1992) and are hence capable of synthesising, degrading and inhibiting the degradation of matrix.

The activity of hepatic collagenase decreases as hepatic fibrosis increases in severity (Maruyama et al., 1982). Fibrotic human liver has increased TIMP1, TIMP2, and MMP2 mRNA compared with normal liver (Benyon et al., 1996). MMP1 mRNA does not increase significantly in fibrosis (Milani et al., 1994). These findings suggest that inhibition of collagen degradation is the major factor in determining the net collagen increase in hepatic fibrosis. Hepatic fibrosis correlates well with hepatic and plasma levels of TIMP1 in a range of liver diseases (Murawaki et al., 1997).

Animal studies of experimentally induced fibrosis have suggested that HSC apoptosis may be the key event in regression of fibrosis after removal of the toxic insult (Iredale et al., 1998).

The gold standard at present for the assessment of hepatic fibrosis is liver biopsy. A serum marker of fibrosis would, however, avoid the risks and morbidity associated with this procedure, and may provide information about the mechanisms of fibrogenesis in addition to the "snapshot" of fibrosis provided by biopsy. Markers that have been evaluated in alcoholic liver disease include collagen precursor molecules, such as aminoterminal procollagen III peptide (PIIINP) (Rohde et al., 1979), molecules derived from synthesised matrix, e.g. type IV collagen and laminin (Hirayama et al., 1996), (Tsutsumi et al., 1996), and ECM regulatory molecules, e.g. MMP and TIMP1 (Benyon et al., 1996), (Murawaki et al., 1997). The collagen precursor molecules (e.g. PIIINP) tend to reflect fibrogenesis rather than the extent of fibrosis (Rojkind, 1984). Markers derived from matrix (e.g. 7S component of type IV collagen) tend to reflect the extent of fibrosis as they are derived from established rather than new ECM (Hirayama et al., 1996). Levels of all markers tend to be higher in alcoholic rather than non- alcoholic liver disease (Hirayama et al., 1996), and tend to fall several weeks after cessation of alcohol consumption (Tsutsumi et al., 1996). Factors other than fibrosis, e.g. changes in hepatic blood flow, may affect marker levels (Smedsrod, 1988).

The markers described tend to correlate weakly but significantly with hepatic fibrosis, limiting their usefulness in standard clinical practice (Chossegros, 1995). Direct comparison of markers suggests that type IV collagen may be slightly superior in differentiating the degree of hepatic fibrosis (Tsutsumi et al., 1996).

However, the precursor and regulatory molecules may be more useful in studies concerned with mechanisms of increased fibrosis in response to specific stimuli.

Chapter 2: Characteristics of alcohol dehydrogenase

2.1 Introduction

Alcohol dehydrogenase (ADH) is found in almost all living organisms and is the major enzyme of alcohol metabolism in mammals, including humans. ADH is composed of a complex family of enzymes of different classes, with differing distributions, substrate preferences, and enzyme kinetics. Although ethanol is often the most abundant alcohol available for metabolism, it is very unlikely that it is the intended natural substrate for all the ADH family. It is improbable that many different ADH genes would be retained over millions of years solely to protect against ethanol- related risks (Lands, 1998). The ingestion of ethanol may therefore divert ADH subtypes from reacting with their customary physiological substrates. This chapter outlines some of the characteristics of ADH, with emphasis on aspects which may determine susceptibility to alcohol related disease or alcoholism. I will also discuss briefly the various methods of identifying and assaying ADH experimentally.

2.2 Nomenclature

The diversity of ADH enzymes arises from ancient gene duplication followed by successive functional mutations. A nomenclature based on class, isozyme and allelozyme, which takes account of this diversity, is now recommended. Human ADH is a dimeric, zinc- containing metalloenzyme with subunits of mass of approximately 40KDaltons. Both homo- and heterodimeric combinations are possible but by definition, functional dimers must arise from within the same class (Jornvall and Hoog, 1995). The grouping of protein subunits by class is shown in Table 2.1. For example, the dimers $\alpha\alpha$, $\sigma\sigma$, $\alpha\beta_1$, $\beta_1\beta_2$, $\beta_1\gamma_2$, are possible functional combinations, whereas $\alpha\sigma$, $\pi\beta_1$, $\chi\beta_2$, $\chi\pi$, are not. Substrate and inhibitor kinetics differ more markedly between different classes than between isozymes. There is a greater degree of amino acid homology between isozymes (around 90%) than between classes (around 40%). These differences are due to differing lengths of time of genetic divergence, with isozymes having diverged more recently than classes. This is summarized graphically in Figure 2.1.

The nomenclature of ADH can be confusing. The terms class and isozyme have, on occasion, been used interchangeably. ADH classes and genes have been named numerically in order of discovery, resulting in a non- consecutive ordering of genes and their products as a consequence of discovery of genes and their associated proteins at different times. To improve clarity of nomenclature, renumbering of some of the ADH genes has been suggested (Jornvall and Hoog, 1995).

Figure 2.1 Phylogeny of alcohol dehydrogenase

Lower Homology — Higher Homology

Ancient			More recent
Classes		Isozymes	Allelozymes
Class III	Class II, Class IV, Class I	α, β, γ	$\beta_1, \beta_2, \beta_3$

This illustrates the increasing diversity of ADH with time. The time span shown represents many millions of years. It has been estimated that Class I emerged as a distinct class 75 million years ago, with class I isozymes emerging 50 million years ago (Stone et al., 1993). Enzyme classes or sub- classes emerged more recently (toward the right side of the line) and tend to have greater similarity (higher amino acid homology) than those emerging in the more distant past (towards the left side of the line).

Table 2.1 Characteristics of alcohol dehydrogenase by class

Class	Protein subunit/ Isozyme	Gene	Location	Allelozyme (if present)	K_m	Relative activity	Degree of Pyrazole Inhibition	Ethnic Diversity
I	α	ADH1	Liver		4.2	1	High	
	β	ADH2*1	Liver	β_1	0.05	0.4		90% of Caucasians are homozygotes
		ADH2*2	Lung	β_2	0.9	14		Fq 50% in Orientals
		ADH2*3	Kidney	β_3	36	13		Fq 25% in African-Americans
	γ	ADH3*1	Liver, Kidney, Stomach, SI, LI	γ_1	1	3.6		3*1 allelic Fq: Caucasians 50%, Orientals 90%
		ADH3*2		γ_2	<1	1.4		
II	π	ADH4	Liver only		30	0.8	Lower	
III	χ	ADH5	Most tissues	χ_1 & χ_2	NS	NS	Lowest	Shows least diversity
IV	σ (or μ)	ADH6 (or 7)	UGIT, Not Liver		40	Highest of all ADH	High	Absent in 70% of Orientals
V		ADH7 (or 6)	Liver, stomach	Unknown				
VI		ADH8	Animal liver ? human also					

K_m= Michaelis constant (mM). Relative activity= ratio of maximum reaction velocity (V_{max}) compared to ADH1, for ethanol as substrate, assuming homodimeric enzyme. NS= Not saturatable by ethanol. Revised gene numbering of Class IV and V has been recommended (Jornvall and Hoog, 1995), old numbering system in brackets. UGIT= upper gastrointestinal tract (mouth, oesophagus, stomach). SI= small intestine, LI= large intestine. "?"= may have been identified. Fq= allelic frequency. Data in this table compiled from (Jornvall and Hoog, 1995), (Persson, 1997), (Seitz and Oneta, 1998), (Chrostek and Szmitkowski, 1996), (Wierzchowski et al., 1992), (Yin et al., 1997), (Stone et al., 1993), (Thomasson et al., 1995), (Bosron et al., 1993), (Lands, 1998), (Moreno et al., 1994)

2.3 Class characteristics of alcohol dehydrogenase

These are outlined in Table 2.1. Class I ADH is abundant in the liver and quantitatively has the greatest contribution to hepatic ADH metabolism. It is considered the "classical" human ADH. The γ subunits may also be important in gastric alcohol metabolism, and the metabolism of 3β & 17 β hydroxysteroids. Orientals metabolise alcohol faster than Caucasians, and this is likely to be due to the higher prevalence of the higher activity ADH 2*2 allele in oriental populations.

The class II subunit was originally denoted π for "pyrazole resistance" as it was found to be relatively insensitive to pyrazole inhibition compared to class I. However, it is still more sensitive to pyrazole than class III ADH. It has not been identified outside the liver. Its endogenous substrates may be steroids and bile acids.

Class III ADH is now recognized as the same enzyme as glutathione- dependent formaldehyde dehydrogenase. An enzyme similar to Class III ADH is present in almost all species, including prokaryotes. Vertebrate class III ADH shows a remarkable degree of structural consistency between species. It is considered the "ancestral" ADH. It is unsaturatable by ethanol, and its primary function is probably unrelated to metabolism of ethanol, to which it contributes little quantitatively. It is the only ADH found in brain, testis, and placenta. It can

metabolise retinol (as can class IV ADH) (Kim et al., 1992), and leukotriene derivatives.

Class IV ADH (subunit originally denoted σ for "stomach", or μ) is found only in the upper GI tract. It has the highest activity for ethanol metabolism of any ADH class, and does not display substrate inhibition at high ethanol concentrations (Stone et al., 1993). This may be important in ethanol metabolism by the stomach as the gastric luminal ethanol concentration which is easily achievable during social alcohol consumption (around 0.5- 1M) is much higher than that found in the liver. The absence of this enzyme in 70% of Orientals may account for their overall lower gastric ADH metabolism of ethanol, particularly as γ ADH shows substrate inhibition.

2.4 The role of alcohol dehydrogenase as a determinant of alcoholism and alcohol related disease

The rate of alcohol elimination can vary as much as 2- 3 fold between individuals. It has been estimated that 50% of the variability in alcohol metabolism is genetically determined (Bosron et al., 1993), and that polymorphisms of ADH may account for a substantial proportion of this. However, as discussed in Chapter 1, the availability of reduced NAD is a major limiting factor in the overall alcohol metabolic rate at reasonable alcohol concentrations. In theory, we might expect higher activity ADH allelozymes to increase the rate of acetaldehyde generation, and therefore be associated with

lower rates of alcoholism due to the aversive nature of acetaldehyde, particularly in individuals who have the mutant inactive polymorphism for acetaldehyde dehydrogenase, ALDH2*2 (discussed in chapter 1). However, we might also expect those with high activity ADH allelozymes who become alcoholics to be more prone to acetaldehyde- mediated damage, although this might be offset by a lessening of damage due to alcohol directly, as this is metabolised faster.

The association of Class I β and γ allelozymes and alcoholism and alcoholic liver disease has been studied extensively. A meta- analysis of these studies (Whitfield, 1997) confirms the theoretical expectations, with the lower activity ADH2*1 allele associated with an increased risk of alcoholism (common odds ratio 2.8). There is a progressive increase in risk from lower risk ADH2*2 homozygotes, medium risk ADH2*1/2*2 heterozygotes, to higher risk ADH2*1 homozygotes. These positive findings have only been observed in Oriental populations, although the low frequency of the ADH 2*2 allele in Caucasians means that studies in this population were underpowered, with wide confidence intervals. The ADH 2*1 allele is associated with a reduced tendency to alcoholic liver disease in Oriental populations (common odds ratio 0.56), again confirming theoretical expectations. The findings are again inconclusive in Caucasians. These associations with alcoholic liver disease and alcoholism were not conditional on the status of the acetaldehyde dehydrogenase allele.

The higher activity ADH 3*1 polymorphism is associated with a decreased risk of alcoholism in Orientals, but not Caucasians. However, this positive finding

may be related to linkage disequilibrium between the ADH 2*1 and ADH 3*2 alleles in Orientals.

The ADH 2*3 allele is usually observed only in populations of African decent. It was found in 25% of an African- American population, and its presence significantly influences the rate of alcohol metabolism, although it is not associated with an altered risk of alcoholism (Thomasson et al., 1995).

In contrast to the findings with liver disease, the lower activity ADH 2*1 allele is associated with an increased risk of carcinoma of the oesophagus (Hori et al., 1997). The risk is highest in 2*1 homozygotes, intermediate in heterozygotes, and least in 2*2 homozygotes. This risk is independent of, but synergistic with, the effect of ALDH allele status, which was discussed in Chapter 1.

2.5 Factors influencing alcohol dehydrogenase activity

Genetic and environmental factors other than variation in class and subclass can influence ADH activity.

2.5.1 Age

Gastric ADH activity has been shown by some, but not all, researchers to decrease with advancing age (Pozzato et al., 1995), (Moreno et al., 1994),

(Matsumoto et al., 2001). This was not observed by Yin et al., 1997 in a study of subjects of Oriental origin.

2.5.2 Gender and ethnicity

Young and middle- aged females have lower GADH activity compared to males, although with advanced age the sex difference tends to diminish (Frezza et al., 1990), (Seitz et al., 1993), (Baraona et al., 2001).

There have been marked differences in ADH activity observed in different racial groups (Dohmen et al., 1996), reflecting the differences in amount of class IV ADH, and class I genetic polymorphisms. These ethnic and gender differences have been suggested by some (Frezza et al., 1990), (Baraona et al., 2001) (Dohmen et al., 1996) to contribute to in lower first pass metabolism, possibly causing an increased susceptibility to the effects of alcohol, although this remains controversial (Gentry et al., 1994a), (Levitt, 1994a), (Gentry et al., 1994b).

2.5.3 Alcoholism

The activity of hepatic ADH decreases in alcoholic liver disease (Vidal et al., 1990), (Panes et al., 1989). This is related to the severity of the underlying liver disease, rather than the amount of alcohol consumed, and is therefore likely to be a direct consequence of hepatic (in particular mitochondrial) damage. Gastric

ADH activity is also decreased in alcoholism, most likely as a consequence of alcoholic gastritis (Seitz et al., 1993).

2.5.4 *Helicobacter pylori*

Helicobacter pylori (HP) infection is associated with reduced gastric ADH activity (Salmela et al., 1994), (Thuluvath et al., 1994), (Gupta et al., 1994), (Simanowski et al., 1998), (Roine et al., 1995), (Kechagias et al., 2001), (Matsumoto et al., 2001), despite the presence of a native ADH within HP (Kaihovaara et al., 1994), (Salmela et al., 1993). This is likely to be secondary to the gastritis that results from infection (Pedrosa et al., 1996), (Brown et al., 1995). The attenuation in activity is mainly antral, in keeping with the usual distribution of HP infection. Eradication of HP is associated with a rise in gastric ADH activity (Gupta et al., 1994), (Kechagias et al., 2001), (Simanowski et al., 1998).

2.5.5 Alcohol dehydrogenase inhibitors

Methylpyrazole is regarded as the classical inhibitor of ADH. It has recently been shown to be at least as effective as ethanol in the prevention of formaldehyde toxicity resulting from ADH metabolism in methanol poisoning (Brent et al., 2001). Imidazole derivatives have long been recognized as inhibitors of horse ADH, and certain drugs with substituted imidazole groups,

such as Cimetidine, can inhibit ADH. Multiple studies have addressed the interaction between H_2 receptor antagonists and ADH. Cimetidine competitively inhibits γ and β_2 subunits, and non- competitively inhibits π subunits in vitro (Stone et al., 1995). Some studies have shown that Cimetidine therapy can produce an increase in the peak blood alcohol concentration, the area under the curve for blood alcohol concentration versus time, and the total dose of alcohol absorbed (Palmer et al., 1991), (Caballeria et al., 1989a), (Hernandez-Munoz et al., 1990). The dose of Cimetidine required for inhibition is 100 times greater for hepatic than gastric ADH (Caballeria, 1991). An increase in peak blood ethanol concentration following Cimetidine is only apparent with oral rather than intravenous administration (Hernandez-Munoz et al., 1990), suggesting that the effect is on gastric rather than hepatic ADH in vivo. The first pass metabolism of alcohol is also influenced by the rate of gastric emptying (Dziekan et al., 1997), (Oneta et al., 1998), (Kechagias et al., 1998), (Levitt et al., 1997), (Pedrosa et al., 1996), which may provide another explanation for the observed effects of H_2 antagonists. Cimetidine does not appear to influence gastric emptying, but the increase in blood alcohol level observed with Ranitidine may be related to its effect on gastric motility (Amir et al., 1996).

Studies addressing the effects of other H_2 antagonists have shown no effect of Famotidine (Mallat et al., 1994), (Casini et al., 1994), and conflicting results for Ranitidine and Nizatidine (reviewed by (Gugler, 1994), (Frezza et al., 1992), (Baraona et al., 1994), and (Caballeria, 1991)). Aspirin has also been shown to have a minor inhibitory effect on gastric ADH (Gentry et al., 1999), (Roine et al.,

1990). Proton pump inhibitors do not appear to affect gastric ADH (Battiston et al., 1997), (Pozzato et al., 1994), (Roine et al., 1992a).

2.6 Experimental detection of alcohol dehydrogenase

Methods of detection of ADH in tissue extracts can be divided into two broad groups depending on whether they detect the presence of ADH protein, or detect the enzymatic activity of ADH. Ultraviolet spectrophotometry has been used for over 50 years to detect ADH activity (Theorell and Chance, 1951). The generation of NADH from NAD by ADH in the presence of ethanol results in a change in the NADH absorption peak at 340nm. Although this technique is simple, it lacks sensitivity and specificity, particularly for crude tissue homogenates, which may contain other NAD- dependent oxido- reductase enzymes, which can also generate NADH.

Coupling of the ADH- dependent ethanol oxidation with the reduction of a coloured substrate (or product) by NADH can improve sensitivity, but not necessarily specificity (for example, nitroblue tetrazolium (Fibla and Gonzalez-Duarte, 1993)). The green coloured aldehyde N, N- dimethyl-4- nitrosoaniline (NDMA) is a special example of coupling. NDMA is a substrate of ADH, and hence the coupling reaction involves the enzymatic action of ADH directly, as well as being dependent of NADH generated by oxidation of ethanol (Dunn and Bernhard, 1971). It therefore offers advantages in terms of both sensitivity and specificity. It has been used to assay ADH activity in serum samples (Skursky et

al., 1979), (Chrostek and Szmitkowski, 1996), (Chrostek and Szmitkowski, 1997).

The synthesis of specific anti- ADH antibodies has allowed detection of ADH protein, but not activity, facilitating both immunohistochemical (Pestalozzi et al., 1983) and quantitative analysis (Buhler and Wartburg, 1982) of specimens from the liver and GI tract.

Various methods have been used in an attempt to identify the activity of specific classes or sub-classes of ADH. Isoelectric focussing has been used to distinguish between allelozymes (Yin et al., 1997). Class- specific fluorogenic substrates have been identified which ADH can catalyse in the absence of alcohol (Wierzchowski et al., 1992). These are useful for identifying differences in activities of class I or class II ADH in different groups. However, as their reaction kinetics differ markedly from ethanol, they are of little use in assessing the overall contributions of individual classes to ADH activity, and their total activities differ markedly from total ADH activity assessed by other means (Chrostek and Szmitkowski, 1995), (Chrostek et al., 1994), (Chrostek and Szmitkowski, 1996), (Chrostek and Szmitkowski, 1997).

Chapter 3: Pharmacokinetics of alcohol

3.1 Introduction

The pharmacokinetics of alcohol in humans are complex, and in the past have often been misunderstood by researchers interested in alcohol metabolism. In this chapter, I will review the current understanding of alcohol absorption, distribution and elimination, and various pharmacokinetic models that are in use. A typical blood alcohol concentration (BAC) versus time curve, with the phases attributable to absorption and distribution, and to elimination, is shown in Figure 3.1.

3.2 Alcohol elimination

One of the first descriptions of alcohol elimination in humans, by Mellanby in 1919, suggested that the elimination rate could be considered, for practical purposes, to be constant, and independent of BAC (Mellanby, 1919). It is now understood that in healthy individuals, alcohol metabolising enzymes begin to become saturated at relatively low BAC, which is easily attainable during social drinking (around 7- 20 mg/dl, (i.e. 1.5- 4.3 mmol/l)) (Holford, 1987). Therefore, as BAC increases, the rate of ethanol elimination will not increase in direct proportion. At high BAC, a change in BAC results in little corresponding change in the alcohol elimination rate. Displaying this information graphically gives rise to what has been described as a "Hockey Stick" appearance of the elimination portion of the BAC versus time curve, with a relatively straight line

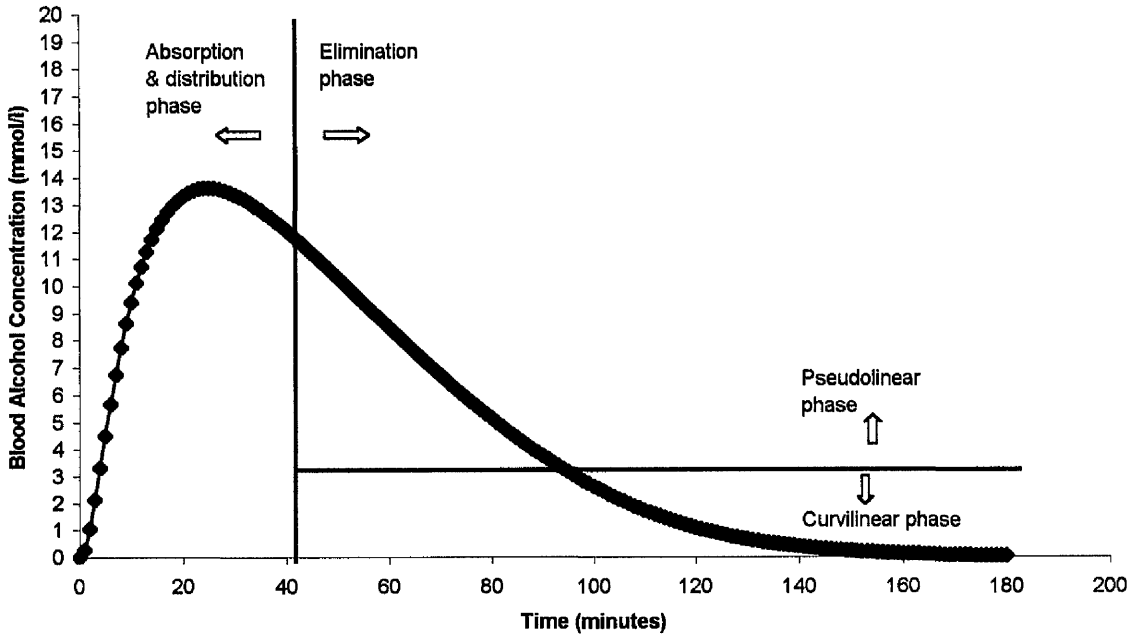

Figure 3.1 An ideal blood alcohol concentration versus time curve

The vertical line shows the division of the curve into a phase dependent mainly on absorption and distribution, and a phase dependent solely on elimination. The horizontal line shows the division of the elimination part of the curve into a pseudo-linear phase (where alcohol elimination appears to be almost constant), and a curvilinear phase, which typically occurs at blood alcohol concentrations of around 1.5- 4.3 mmol/l.

"pseudolinear" portion of the BAC versus time curve at higher BAC, and a concave- upward "curvilinear" portion at lower concentrations (Figure 3.1). Viewed simplistically, this curve would suggest a combination of zero- order elimination kinetics at high BAC, and first- order elimination kinetics (like most common therapeutic drugs) at lower BAC.

The Michaelis- Menten equation (Appendix A.2, Equation 3) is the classical biochemical equation describing the relationship between enzyme activity. If it is assumed that all the alcohol eliminating enzymes can be considered as acting as a "single" enzyme (which is valid at all but very high BAC), then the Michaelis-Menten equation and the equation for the "Hockey Stick" curve should be related. This is indeed the case, and integration of the Michaelis- Menten equation produces an equation for a line with a "Hockey Stick" appearance (Lundquist and Wolthers, 1958) (the integrated form is also given in Appendix A.3, Equation 6). This equation has been used by a number of researchers to estimate alcohol elimination rate (Amir et al., 1996), (Lin et al., 1976), (Roine et al., 1991), (Gentry et al., 1999), (Lim et al., 1993), (Di Padova et al., 1992), (Levitt and Levitt, 1994b), (Levitt et al., 1997), (Baraona et al., 2001), and to estimate population means for the parameters K_m and V_{max} (Pieters et al., 1990), (Lundquist and Wolthers, 1958), (Wagner et al., 1989).

A simpler method of estimating the alcohol elimination rate is to measure the gradient of the pseudo-linear portion of the BAC versus time curve after the absorption and distribution phase is complete, and consider the curvilinear phase to be so small as to be inconsequential. The equation for this model (which is

essentially the same as Mellanby's original description) of alcohol elimination was proposed in 1932 by Widmark, and is still used frequently today, in both research (Thomasson et al., 1995) and forensic applications.

However, the inaccuracies in this model has led to suggestions that this equation should no longer be used, particularly when trying to estimate the alcohol elimination rate of particular populations (Wilkinson, 1980). The inaccuracy due to the non- linear portion of the curve may be greater in subjects who have a higher saturation threshold, for example, chronic alcoholics who have induction of CYP2E1 enzymes.

All of these models of elimination make certain assumptions. They assume that, for practical purposes, we can consider alcohol to be eliminated uniformly and simultaneously from the body compartment in which it distributes, i.e. we are considering the body as a single compartment. In reality, elimination will only take place in the body water compartment found within the liver (the contribution of hepatic and renal excretion of alcohol is negligible at all but highly toxic BAC), but the rapid equilibration of differences in alcohol concentration in body water makes violation of this assumption unimportant.

A number of assumptions that are commonly made when dealing with first-order elimination kinetics will not apply when considering alcohol. The assumption that is perhaps applied wrongly most often is that the total dose of alcohol absorbed is proportional to the area under the curve (AUC). In non-linear kinetic systems, a change in the rate of administration will change the AUC, even without a change in dose, whilst a small increase in dose will result

in a disproportionate rise in AUC. This is illustrated in Figures 3.2 and 3.3, using a pharmacokinetic model developed on Microsoft Excel, with Michaelis- Menten elimination kinetics. Many researchers have wrongly assumed that AUC and total dose absorbed are proportional (Simanowski et al., 1998), (Caballeria et al., 1989b), (Frezza et al., 1990), (Dziekan et al., 1997), (Kechagias et al., 1998), (Oneta et al., 1998), (Pedrosa et al., 1996), (Mallat et al., 1994), (Pozzato et al., 1994), (Ammon et al., 1996), (Roine et al., 1990), (Battiston et al., 1997), (Casini et al., 1994). However, the AUC is a good indicator of the duration and extent of systemic alcohol exposure, and hence it can still provide useful information about the potential for alcohol related damage (Thomasson et al., 1995). The total dose of alcohol absorbed will be proportional to the area under the curve of elimination rate versus time, rather than the area under the curve of concentration versus time, and can thus be estimated by integration of the Michaelis- Menten equation (the proof is given in Appendix A.4, Equation 7) (Gentry et al., 1992), (Lin et al., 1976).

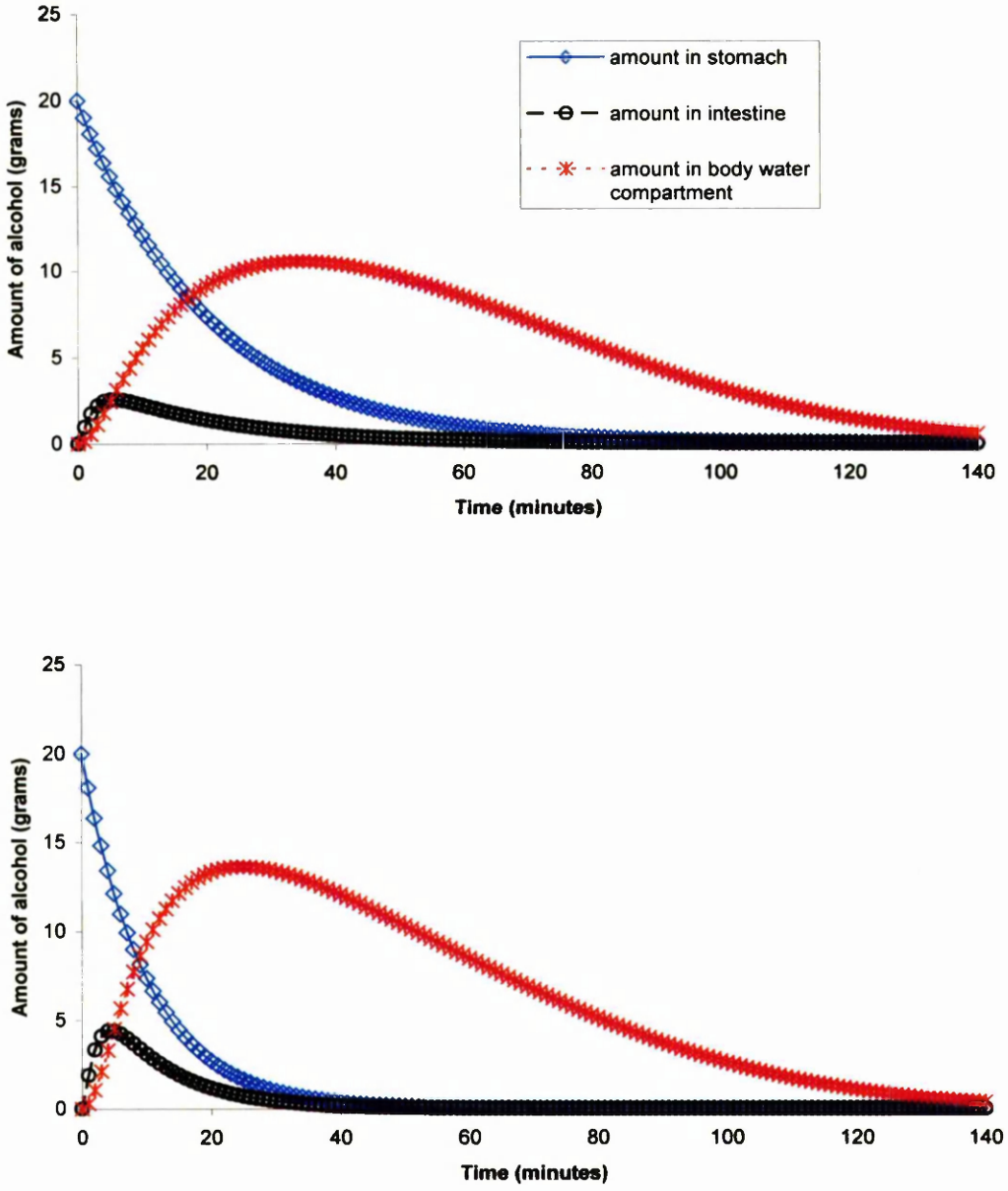

Figure 3.2 A sample pharmacokinetic model for alcohol absorption, distribution, and elimination- effect of change in gastric emptying.

These graphs illustrate a model of the delivery of alcohol from the stomach to small intestine, and its subsequent absorption and elimination. Note the amount of alcohol in various compartments, rather than the concentration of alcohol, is displayed (to allow visualization of the 3 compartments on one pair of axes). In this model, there is no gastric lag phase, ingestion is in the form of a single gastric bolus, and gastric emptying and intestinal absorption are first order. Alcohol elimination is by Michaelis- Menten kinetics. For simplicity, bioavailability is assumed to be 100%. Distribution is instantaneous in a single body water compartment. These graphs illustrate that a doubling of the rate constant for gastric emptying (lower graph) results in a 30% greater peak "alcohol amount in body water", and a 12% increase in the area under the "alcohol amount in body water" versus time curve, despite the overall alcohol dose remaining the same.

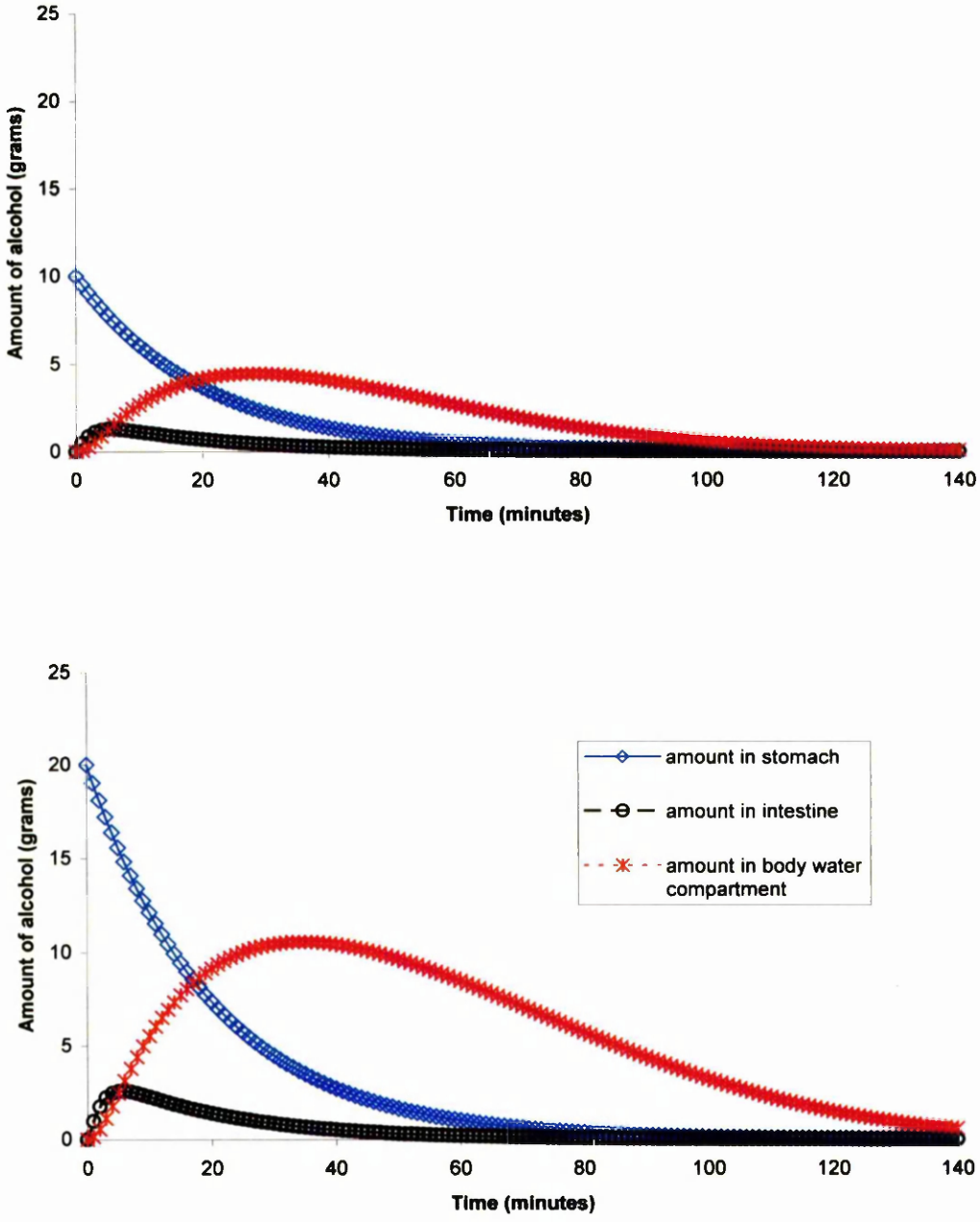

Figure 3.3 A sample pharmacokinetic model for alcohol absorption, distribution and elimination- effect of change in alcohol dose.

The pharmacokinetic model is described in detail in Figure 3.2. These graphs illustrate that a doubling of the dose of alcohol administered results in a disproportionate increase (almost three times) in the area under the curve of "alcohol amount in body water" versus time curve. The rate constant for gastric emptying is the same in both graphs.

3.3 Alcohol ingestion, absorption and distribution

Prior to the linear phase of alcohol elimination, the processes of ingestion, absorption, and distribution will describe the BAC versus time curve. Various models have been described to explain this phase of the curve.

3.3.1 Ingestion

Alcohol is often delivered as an oral bolus under experimental conditions, although it may more accurately reflect social drinking to describe the ingestion of alcohol as a zero- order (continuous rate) process during the period of consumption.

3.3.2 Absorption

Various models have been applied to describe alcohol absorption. Although some alcohol is absorbed from the stomach (Levitt et al., 1997), this occurs slowly relative to absorption from the jejunum. Hence, a model is often used which describes separate components for gastric emptying and intestinal absorption. The delay in gastric emptying is then usually represented either as a lag phase, i.e. a period of time during which no absorption takes place, or as a phase described by first order principles, i.e. the rate of delivery of alcohol from the stomach is directly related to the concentration of alcohol in the stomach, and

occurs continuously. However, there is evidence that in the fed state, high concentrations of alcohol (16% weight/ volume or higher) may retard gastric emptying disproportionately (Roine et al., 1991), and a first order model may therefore be overly simplistic. Once alcohol has entered the jejunum, absorption is regarded as a first order process, in common with most other ingested substances, and is much more rapid than alcohol absorption from the stomach (Levitt et al., 1997), (Lim et al., 1993). All of the alcohol reaching the lumen of the small intestine is usually absorbed (Lim et al., 1993). Ignoring first pass metabolism for the present, it can be seen that the rate of increase in the BAC versus time curve, and the magnitude of the peak BAC will therefore increase directly with the rate of delivery of alcohol to the small intestine, which, in turn, will be dependent on the rate of gastric emptying. This implies that even if gastric alcohol dehydrogenase activity (GADH) were negligible, the stomach would still have a crucial role in determining BAC (and, given the non- linear nature of elimination, also the AUC) solely as result of its function as a reservoir. Figure 3.2 illustrates the effect of changes in the rate of gastric emptying.

3.3.3 Distribution

Alcohol is capable of permeating all tissues, and is not stored in specific sites, but tends to distribute itself mainly in body water, and hence its volume of distribution usually reflects total body water closely. This is approximately 40 litres for a 70kg man aged 40. In single compartment models, distribution is usually assumed to be a rapid and uniform process throughout the body water

compartment. However, if we are using the declining part of the BAC versus time curve to estimate alcohol elimination rate (either by Michaelis- Menten kinetics, or Widmark's method), the part of the curve that immediately follows the peak should be avoided, as its gradient is partly determined by distribution (and it will hence be steeper), which in reality is not instantaneous.

3.4 Overall bioavailability

The amount of an ingested drug reaching the systemic circulation is rarely equal to the amount ingested. This may be due to a combination of incomplete absorption and hepatic metabolism via the portal circulation, prior to entry of the ingested substance into the systemic circulation. Incomplete absorption from the GI tract may be related to the ingested substance being metabolised by the GI tract, bound in the GI lumen, or having a limited ability to cross the GI mucosa.

When alcohol is administered orally, it is incompletely delivered to the systemic circulation (Julkunen et al., 1985), (Di Padova et al., 1987a). Alcohol is not bound in the lumen of the GI tract, and it crosses mucosal surfaces readily, therefore incomplete systemic delivery is likely to be related to either metabolism in GI tract (by GADH) or to hepatic first pass metabolism.

Quantitatively, overall first pass metabolism is defined as the amount of a substance administered by the oral route minus the amount of substance reaching the systemic circulation. For substances obeying linear pharmacokinetic principles, this is proportional to the AUC following IV administration (AUC_{IV})

minus AUC following oral administration (AUC_O). Bioavailability is expressed as a ratio of substance entering the systemic circulation: substance ingested. In first order systems this can be calculated from AUC_O divided by AUC_{IV}. Overall bioavailability can also be derived from F x f, where f= fraction crossing GI tract, and F=hepatic bioavailability. In a system with first order hepatic elimination, hepatic bioavailability is usually a constant. However, with zero order kinetics, the hepatic bioavailability will rise as the delivery of the ingested substance to the liver increases, because of saturation of hepatic metabolism. The equation relating hepatic (and overall) bioavailability to portal vein concentration is given in Appendix A.5, Equation 10. Hence, if the rate of alcohol absorption increases, there will be a rise in overall bioavailability (Levitt and Levitt, 1994b), (Holford, 1987). Therefore, we can again appreciate the importance of the reservoir function of the stomach. It can influence overall bioavailability regardless of the activity of gastric ADH. In addition, a prolonged gastric emptying time will increase the exposure of gastric luminal alcohol to GADH.

Some authors have demonstrated the importance of gastric emptying in influencing overall bioavailability (although not all have realised that differences could be explained from non- linear pharmacokinetics principles alone) (Oneta et al., 1998), (Pedrosa et al., 1996), (Amir et al., 1996), (Kechagias et al., 1998), (Dziekan et al., 1997), (Levitt et al., 1997). The bioavailability of alcohol can vary markedly both within and between individuals, and is strongly influenced by the ingestion of solid food. In the fasting state, the bioavailability of alcohol is often 90% or greater, whilst in the fed state, this can fall to around 50- 60% (Lin et al., 1976). Despite the strong influence of gastric emptying, many researchers

have not taken account of it when attempting to assess the contribution of gastric ADH to overall bioavailability. Oneta et. al. recently suggested that "knowledge of gastric emptying time is a major pre-requisite for the study of first pass metabolism of alcohol in humans" (Oneta et al., 1998). Figure 3.4 is a flowchart illustrating the complex interactions that determine the systemic bioavailability of alcohol.

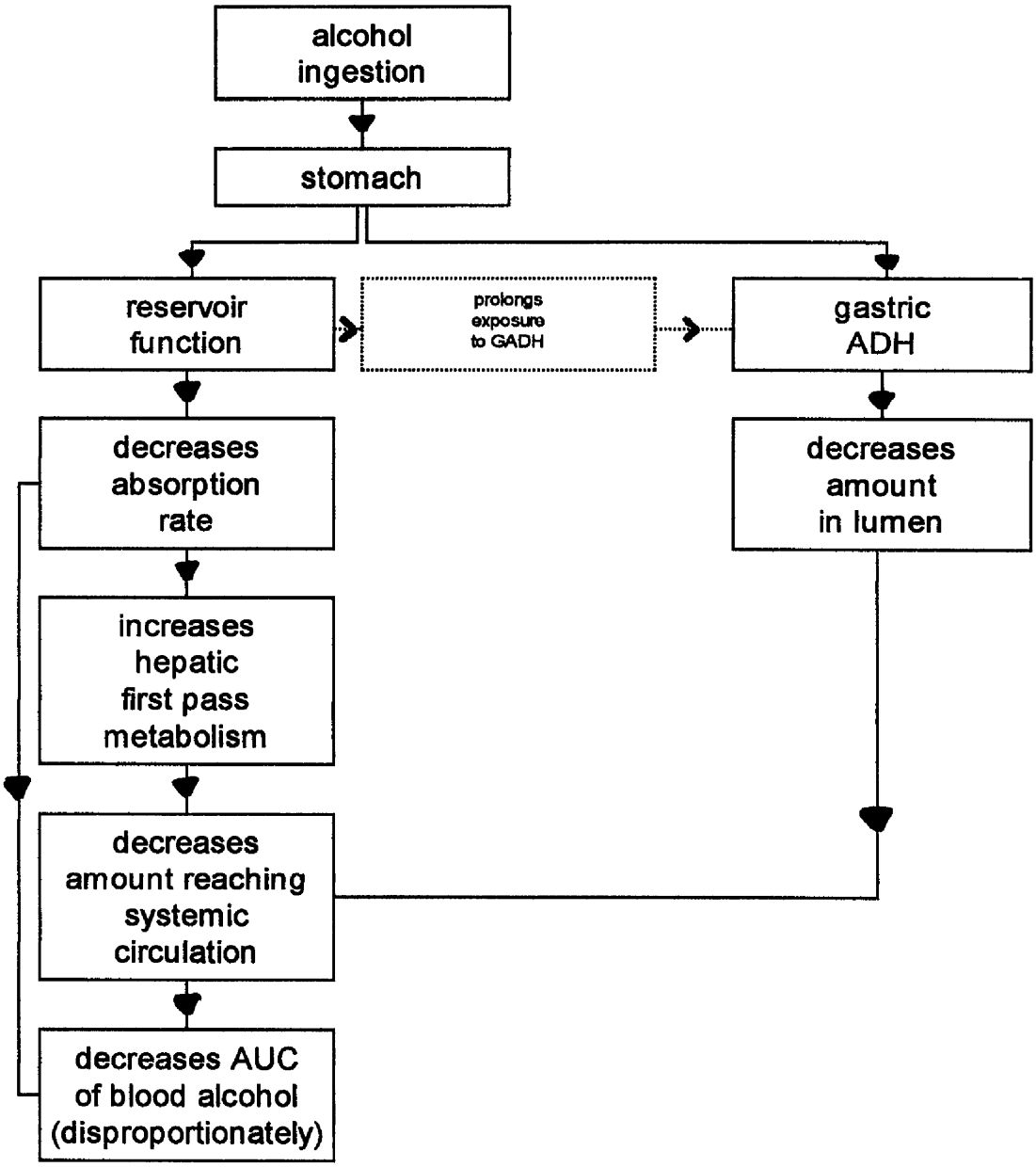

Figure 3.4 Flowchart illustrating the role of the stomach in alcohol first pass metabolism

Chapter 4: Aims of thesis

The principal aim of this thesis is to explore the relationship between alcohol metabolism and potential mechanisms of, or susceptibility to, alcohol related damage. Two main hypotheses are considered.

The first hypothesis considered is that susceptibility to alcohol- related damage may partly be determined by gastric alcohol dehydrogenase (ADH). Gastric ADH can metabolise alcohol before it reaches the systemic circulation. Hence, subjects with higher levels of gastric ADH may absorb less alcohol, and may therefore be less susceptible to the effects of alcohol. However, the clinical relevance of gastric ADH remains controversial. Reports have suggested that *Helicobacter pylori* eradication has been associated with a rise in gastric ADH activity. This thesis therefore studies whether *Helicobacter pylori* eradication is associated with a significant rise in gastric ADH, and whether this is associated with a change in pre- systemic alcohol metabolism. A rise in gastric ADH activity associated with an increase in pre- systemic alcohol metabolism would support the hypothesis, and might provide a useful basis for further studies that could attempt to modify an individual's alcohol metabolism by *Helicobacter pylori* eradication. A rise in gastric ADH activity without an associated increase in pre- systemic metabolism would suggest that gastric ADH does not play a significant role in alcohol metabolism.

To study the effects of *Helicobacter pylori* eradication on gastric ADH activity it is necessary to have a robust assay for gastric ADH. The secondary aim of this thesis was to develop an assay which would improve upon existing ADH assays, in that it would be able to detect reliably the low levels of activity found in standard endoscopic gastric biopsies following a minimal degree of tissue preparation, and which would use semi- automated, but readily available, laboratory equipment where possible. This thesis aims to ensure that the assay method is optimised, robust, and validated.

The second hypothesis considered is that abrupt alcohol withdrawal may induce hepatic damage. The hypermetabolic response of the liver to chronic alcohol excess induces a relative hepatic hypoxia. Abrupt alcohol withdrawal may worsen this hypoxia by reducing hepatic blood flow, resulting in ischaemic damage. The induction of CYP450 enzymes by chronic alcohol excess may result in hepatic damage caused by the metabolic products of these enzymes. During alcohol withdrawal, these enzymes remain induced but are no longer saturated by alcohol, increasing their ability to cause damage at that time, particularly by metabolism of certain drugs. This thesis studies markers of hepatic damage (hepatic transaminases) and markers of hepatic fibrogenesis (amino-terminal procollagen III peptide and tissue inhibitor of metalloproteinase 1) during the period of alcohol withdrawal, to determine if there is evidence for hepatic damage during this period.

SECTION 2 STUDIES ON GASTRIC ALCOHOL DEHYDROGENASE AND PRE-SYSTEMIC METABOLISM OF ALCOHOL

Chapter 5: Development of a sensitive, colorimetric, semi- automated assay for alcohol dehydrogenase in endoscopic gastric biopsies.

5.1 Introduction

Alcohol dehydrogenase (ADH) is present throughout the gastro- intestinal (GI) tract (Pestalozzi et al., 1983), with a relatively high activity in the stomach (Buhler and Wartburg, 1982), (Yin et al., 1997). It may contribute to the first pass metabolism of alcohol (Pozzato et al., 1995), (Gentry et al., 1994a), (Hernandez-Munoz et al., 1990), (Frezza et al., 1990), (Roine et al., 1990), (Haber et al., 1996), (Baraona et al., 2001), and it may be important in carcinogenesis in the GI tract (Seitz et al., 1990), (Hori et al., 1997). This enzyme is therefore currently the subject of much research interest. Although ADH is easily assayed using conventional ultraviolet (UV) spectrophotometry, this method is non- specific and it lacks sensitivity for the small amounts of ADH found in standard endoscopic biopsies. The standard method of tissue preparation, namely homogenisation, can be time consuming, particularly when dealing with a large number of samples, and can be a source of analytical variability, due to non- uniform homogenisation techniques.

The strongly coloured cyclical aldehyde- like substrate N, N-dimethyl-4-nitrosoaniline (NDMA) (Figure 5.1) can be reduced to a colourless form in the presence of ADH and an alcoholic substrate (Dunn and Bernhard, 1971).

Preliminary reports in serum have suggested this may offer advantages in sensitivity and specificity over traditional ultraviolet spectrophotometry (Skursky et al., 1979), (Chrostek and Szmitkowski, 1996), (Chrostek and Szmitkowski, 1997). In our laboratory, previous workers have successfully assayed prostaglandins, leukotrienes, histamine, and cyclic adenosine mono-phosphate in gastric biopsies by using an incubation technique, rather than homogenisation (Taha et al., 1991).

The aims of this study are threefold. Firstly, to develop, optimise, and validate a gastric ADH (GADH) assay with improved sensitivity and specificity by using NDMA to detect GADH activity in standard endoscopic gastric biopsy samples. Secondly, it is hoped that an increase in sensitivity may allow reliable ADH detection in samples prepared by incubation rather than homogenisation. Thirdly, by semi- automation using 96-well micro-titre plate technology, it is hoped that a large number of biopsies can be rapidly processed.

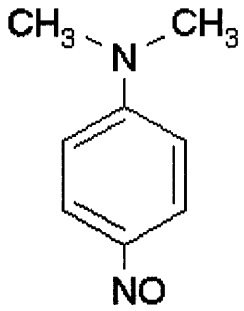

Figure 5.1 Molecular structure of N, N-dimethyl-4-nitrosoaniline

5.2 Patients, materials, and equipment

5.2.1 Patients

After giving informed consent, one hundred and six subjects undergoing upper gastrointestinal endoscopy for investigation of dyspepsia, dysphagia, or weight loss had paired gastric biopsies taken using standard endoscopic biopsy forceps (Olympus FB3K) from the antrum and body of the stomach for ADH determination. Further biopsies were taken from the antrum and body for standard histological analysis, and a rapid urease (CLO^{TM}) test for *Helicobacter pylori* (HP) was taken from the gastric antrum. The biopsy material from fifty of these subjects (both males and females) was used to optimise the assay. In some cases, biopsy material from different subjects was pooled to minimise individual variation. Biopsy material from the remaining fifty- six male Caucasian subjects (age 22- 72, median 40) was used to provide a reference range for GADH using this assay. The endoscopic diagnoses of the subjects whose biopsies were used to provide a reference range are summarised in Table 5.1. Subjects with a malignant diagnosis, and those taking any medication within 2 weeks of the endoscopy which is suspected of interacting with GADH (including H_2 receptor antagonists, proton pump inhibitors, aspirin, and other anti- ulcer therapy with the exception of simple antacids), were excluded from the study. The hospital ethics committee granted ethical approval for this study.

5.2.2 Materials and equipment

The following reagents were obtained from Sigma- Aldrich Ltd. (Dorset, UK): β nicotinamide adenine dinucleotide (NAD); 4-methyl pyrazole (4MP); glutamate dehydrogenase; acetaldehyde dehydrogenase; ethyl alcohol (ethanol); n-butanol; n-propanol; NDMA; Roswell Park Memorial Institute tissue culture medium, number 1640 (RPMI 1640), with added L-glutamine (final concentration 3 mM); lactate dehydrogenase; alcohol dehydrogenase from baker's yeast (yeast ADH); alcohol dehydrogenase from horse liver. A commercial assay was used to determine the protein content of the processed biopsy specimens (Micro protein determination, phenol reagent method, Sigma, UK, catalogue no: 690-A), a variation of Lowry's method (Lowry et al., 1951).

Table 5.1 Endoscopic diagnosis in fifty- six subjects in whom biopsy material was used to provide a reference range for gastric alcohol dehydrogenase

Diagnosis	Number	Percentage
Normal Endoscopy	12	21.4
Gastritis	13	23.2
Oesophagitis	13	23.2
Duodenitis	10	17.8
Gastric Ulcer	3	5.4
Duodenal Ulcer	7	12.5
Duodenal scarring	7	12.5
Barrett's oesophagus	1	1.8

All subjects were being investigated for dyspepsia, dysphagia, or weight loss. Note that some subjects have more than one endoscopic diagnosis. The *Helicobacter pylori* status was: 37 positive, 19 negative

In addition, a suspension of cultured HP was obtained. This was prepared by Dr Q Zhang, using the methods described in his PhD thesis (Zhang, 1997). In summary, this technique involved primary culture of HP obtained from a gastric biopsy on Colombia blood agar plates in a micro-aerobic environment at 37°C. The cultured HP were then suspended in phosphate buffered saline (PBS), pH 7.4, to an approximate concentration of 50 million organisms per millilitre, as determined by spectrophotometry (an optical density of 0.45 at 620nm).

Polypropylene cryogenic vials (volume: two millilitres) were used to store gastric biopsies whilst frozen, and polystyrene twenty-four well tissue culture cluster plates were used for incubation of the gastric biopsies (both obtained from Corning Costar Corp., Cambridge, Massachusetts, USA). Polystyrene ninety- six well microtitre plates (Iwaki brand) were obtained from Asahi Techno Glass Inc, Japan.

The ninety- six well plates were read using a Dynatech MR5000 automated microtitre plate reader, with 450nm and 620nm filters (Dynatech Labs Ltd., Billinghurst, West Sussex, UK). The plate reader was linked to a personal computer to allow computerised data collection and analysis using software prepared by the author for Microsoft Excel version 9.0 (Microsoft Corp., Seattle, Washington, USA).

A Beckman DU-70 spectrophotometer was used for conventional UV spectrophotometry (Beckman Coulter Ltd., High Wycombe, Buckinghamshire, UK).

5.3 Methods

5.3.1 Final assay method

The following paragraphs describe the optimised method for the ADH assay. The incubation times, substrate concentrations, pH, etc. were varied during the optimisation experiments that are described in detail in section 5.3.2. Values close to the optimum concentration were chosen in each case for the final assay except for ethanol and pH. An ethanol concentration of 750mM ethanol was chosen because this is within the range of the intra-gastric concentrations of alcohol found in social alcohol drinking. A pH value of 7.4 was chosen to reflect gastric mucosal cell cytosolic pH.

Immediately after they were obtained at endoscopy, gastric mucosal biopsies used for the determination of GADH were placed individually into empty cryogenic vials and frozen in liquid nitrogen. These were transferred to a freezer for storage at –70° C within 3 hours of being obtained. Biopsies were defrosted when required for batch processing and then weighed prior to incubation or homogenisation.

The biopsies that were incubated were placed in covered tissue culture plates for 24 hours in 1000ul of RPMI 1640 in an atmosphere of 5% carbon dioxide, 75% nitrogen and 20% oxygen at 37^0C. The cell culture fluid was then retrieved and used for further analysis.

A stock solution of NDMA 20mM was prepared in phosphate buffered saline (PBS) (pH 7.4). This solution remained stable for several weeks when sealed and stored in darkness at 3°C. Phosphate buffered saline (pH 7.4) was used to dissolve the contents of a fresh 10mg pre-weighed vial of NAD, which was then mixed with the stock NDMA. Two hundred microlitres of this solution was added to each well of the 96- well microtitre plate, containing 50ul of the biopsy homogenate or incubation fluid. After five minutes, the reaction was started with the addition of ethanol, to give a final volume of 267ul.The final concentrations were: NAD (187uM), NDMA (100uM), and ethanol (750mM).

Samples were analysed in duplicate in adjacent columns, and duplicates were paired with matched duplicate samples containing the ADH inhibitor 4MP (18.7mM). The 4MP concentration was optimised so that it inhibited any significant reaction observed with the biopsy samples. A sample layout of the 96- well plate is shown in Figure 5.2. Analysis was carried out at 20^OC.

The absorbance of each well at 450nm was measured, using a reference value at 620nm. Readings were taken at the following times: -5, -3, -1, 0, 1, 2, 3, 4, 5, 7, 10, 15, 20, 30, and 40 minutes. GADH activity was calculated using a kinetic plot of absorbance change vs. time determining activity from the initial linear portion (Figure 5.3).

	1	2	3	4	5	6	7	8	9	10	11	12
A	NDMA standard	Duplicate of preceding column	90m	Duplicate of preceding column	90m + 4MP	Duplicate of preceding column	92m	Duplicate of preceding column	92m + 4MP	Duplicate of preceding column	94m	Duplicate of preceding column
B	NDMA standard/2		90n		90n + 4MP		92n		92n + 4MP		94n	
C	NDMA standard/4		90o		90o + 4MP		92o		92o + 4MP		94o	
D	NDMA standard/8		90p		90p + 4MP		92p		92p + 4MP		94p	
E	NDMA standard/16		91m		91m + 4MP		93m		93m + 4MP		94m + 4MP	
F	NDMA standard/32		91n		91n + 4MP		93n		93n + 4MP		94n + 4MP	
G	NDMA standard/64		91o		91o + 4MP		93o		93o + 4MP		94o + 4MP	
H	NDMA standard/128		91p		91p + 4MP		93p		93p + 4MP		94p + 4MP	

Figure 5.2 Sample layout of a 96-well microtitre plate

The 96-well plate has 12 columns (1-12) and eight rows (A-H). This figure illustrates the layout for a typical plate. The first 2 rows contain serial dilutions of NDMA (highest concentration 150uM), of the same volume as the reaction mixture. Five subjects (numbers 90-94) each had 4 biopsies taken (denoted m-p). Each biopsy is run in duplicate (for the first 4 samples, these are in adjacent columns), and each "active" duplicate pair are run against a "blank" duplicate pair which contain the same reaction mixture as the active wells, but in addition contain 4-methylpyrazole (4MP), the ADH inhibitor.

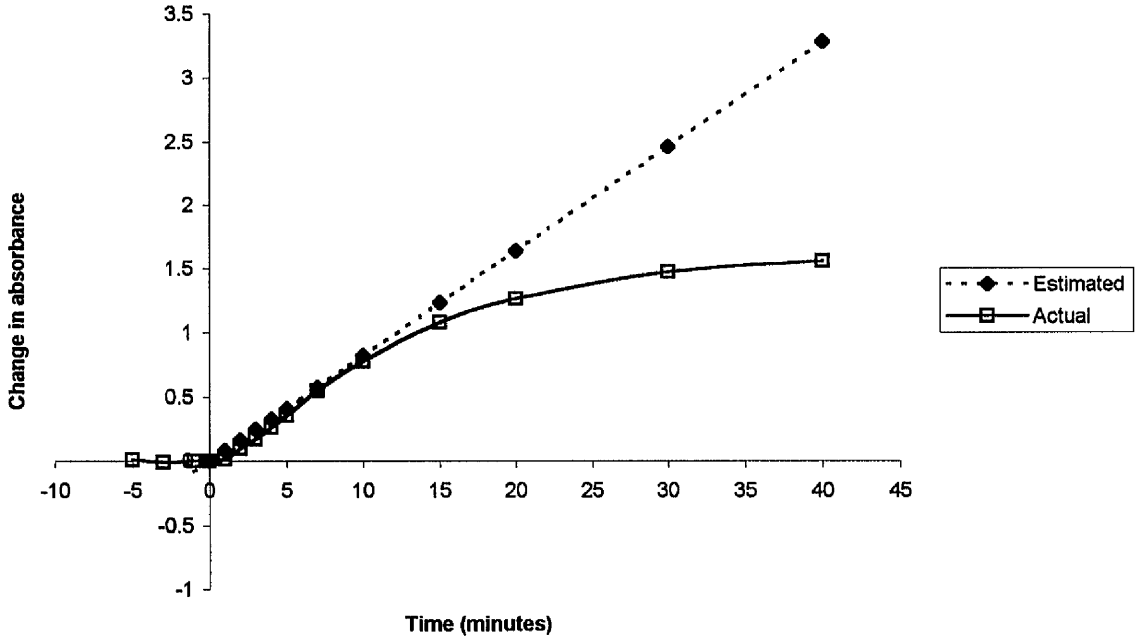

Figure 5.3 Calculation of gastric alcohol dehydrogenase activity from gradient of initial absorbance change versus time

This graph illustrates how enzyme activity was estimated from the gradient of the initial linear portion of the curve. In this example, the enzyme activity decreases after 10 minutes, probably because of depletion of substrate and accumulation of product. This example also illustrates that there is no appreciable enzyme activity prior to the addition of substrate at time zero.

Visual verification of the linearity of the initial portion of the curve was determined for every sample by inspection of graphs generated using Microsoft Excel. Protein concentration of the homogenate or incubation fluid was determined using the commercial assay, which was performed by Mr A Fletcher (Department of Human Nutrition, University of Glasgow).

The equation relating gradient (i.e. change in absorbance over time, $\Delta A/\Delta T$) to enzyme activity is derived from the Beer- Lambert law, $A=\varepsilon \cdot C \cdot l$, where A= absorbance, ε= molar absorptivity coefficient, C = concentration, and l= path length. The published molar absorptivity coefficient of NDMA is $35400 M^{-1} cm^{-1}$ at 440nm. By analysing serial dilutions of known concentration of NDMA in our 96- well plates (see results), we have determined that $\varepsilon \cdot l$ for this assay at 450nm is $16.12 mM^{-1}$. Enzyme activity was defined in the standard fashion, i.e. one unit of enzyme activity is equal to one micromole of product formed per minute. For alcohol oxidation by GADH, this is equivalent to one micromole of substrate consumed (Chapter 1, Figure 1.1a). Enzyme activity was expressed in milliunits per mg protein (umol substrate consumed $\cdot$ min^{-1} $\cdot$ mg protein $^{-1} \cdot 10^3$).

Hence the equation is:

$$\text{activity (mU/mg protein)} = \left(\frac{\Delta A}{\Delta t}\right) \cdot \left(\frac{0.267}{16.12}\right) \cdot \left(\frac{1000}{\text{protein content}}\right)$$

$$= \left(\frac{\Delta A}{\Delta t}\right) \cdot \left(\frac{16.56}{\text{protein content}}\right)$$

The ADH activity in the gastric antrum and body was determined from the mean of the activities from the pair of biopsies taken from each site.

5.3.2 Optimisation of assay

Unless otherwise stated, the reaction conditions are the same as those for the final assay, with the exception of the parameter being optimised.

5.3.2.1 N, N-dimethyl-4-nitrosoaniline concentration

Unlike a conventional spectrophotometer cuvette, the length of the fluid path through which the light beam passes in the microtitre plate reader will vary with the volume of fluid in each well. It is therefore necessary to determine an accurate estimate of the product of path length and molar absorptivity coefficient, and to ensure the relationship between concentration and absorption is reproducible over a wide range of NDMA concentrations. The optimum initial concentration of NDMA should correspond to a relatively high absorbance reading, but still be within the absorbance range that can be reliably estimated by the plate reader.

The absorbance readings at 450nm of serial dilutions of NDMA were determined. The volume of solution in each well was the same as the final reaction volume (267 microlitres).

5.3.2.2 Serial dilution of biopsy material

To determine that a linear relationship exists between enzyme activity in the biopsy specimens and absorbance change, the assay was performed using serial dilutions of pooled incubated biopsy specimens. The diluting agent used was RPMI 1640. Each subsequent dilution halved the concentration of biopsy material in the reaction well.

5.3.2.3 Substrate concentration

Ethanol may not be the naturally intended substrate of GADH. Different ADH isozymes have differing affinities (as described by their Michaelis constant) for ethanol, and substrate inhibition by ethanol has been described for some isozymes of ADH. The GADH activity in gastric biopsies was therefore determined using a range of concentrations of several primary alcohols as substrate. The alcohols used were: ethanol, n-butanol and n-propanol at concentrations of 5, 10, 50, 100, 250 and 500 mM. In addition, enzyme activity was also determined at higher concentrations of ethanol (1, 2 and 3M).

The optimum concentration of NAD was determined using ethanol as a substrate with NAD concentrations of 100, 200, 500, 1000 and 2000 mM. Pooled biopsy specimens were used in all cases.

5.3.2.4 pH

To assess the optimum pH, quantities of PBS were prepared to pH 3.5, 5.4, 6.0, 6.45, 7.4, 8.0, and 9.7. These were used to prepare the reaction mixture, and pooled biopsies were analysed using ethanol as substrate.

5.3.2.5 Homogenisation versus incubation

In ten subjects, four biopsies were obtained as close together as possible in the gastric antrum, and were frozen in the usual manner. Two of these biopsies were processed by incubation, and the remaining two were homogenised. The biopsies that were homogenised were placed individually in a glass Potter- Elvehjem type homogeniser with 500ul of RPMI. The biopsy tissue was manually disrupted in this homogeniser for 5 minutes. A further 500ul of RPMI was added, removing any debris from the homogeniser pestle. The specimens were then centrifuged for 5 minutes at 980'g. The specimen supernatant was used for further analysis. This is similar to the homogenisation techniques employed in conventional ADH assays (Thuluvath et al., 1994), (Hernandez-Munoz et al., 1990), (Simanowski et

al., 1998). The activity of each pair of incubated gastric biopsies was then compared with the corresponding pair that had been homogenised.

5.3.2.6 Incubation time

Biopsies from ten subjects were incubated in the standard manner. However, 22ul of incubation fluid was removed after 6, 12, 24, 36, and 48 hours of incubation. The fluid obtained from these biopsies at these specific time points was pooled, and later analysed to determine if there was a difference in activity with differing incubation times.

5.3.2.7 Comparison with other oxido- reductase enzymes

Oxido-reductase enzyme assays that employ conventional UV spectrophotometry, based on the absorbance band at 340nm (due to the presence of NAD in reduced form (NADH)), often have problems related to specificity. A significant background rate of NADH production is often observed in the absence of substrate (Zahlten et al., 1980), a phenomenon which has been termed "nothing dehydrogenase". Therefore, a number of other commercially available oxido- reductase enzymes were assayed to check the specificity of the assay. These enzymes were dissolved in PBS pH 7.4, and 50ul of either enzyme solution or pooled gastric biopsy incubation fluid was added to the reaction mixture. The amount of non- GADH oxido-reductase enzyme used for each well

was in excess of the amount of GADH normally detected, to increase the likelihood of detecting any small influence of these enzymes on the assay.

The amount of enzyme in each well was as follows: 2500mU acetaldehyde dehydrogenase, 2900mU lactate dehydrogenase, 3000mU glutamate dehydrogenase. The approximate GADH activity of the pooled gastric biopsy specimens used for this experiment was 1mU per well.

5.3.2.8 Horse liver alcohol dehydrogenase- comparison with conventional method

To provide an external standard, a commercial preparation of horse liver ADH was also assayed both by the conventional spectrophotometric method of Dalziel (Dalziel, 1957), and the NDMA method. Serial dilutions of horse ADH were used to provide a range of activities from 10mU to 0.005mU.

To perform the ADH assay by Dalziel's method, the absorbance change at 340nm was determined for a 3ml solution of glycine sodium hydroxide buffer (pH 10.0) containing NAD (final concentration 750microM), ethanol (final concentration 750mM), and horse ADH. To determine the enzyme activity, the absorbance change in a "blank" cuvette (containing the same reaction mixture with the addition of 4MP, final concentration 16.7 mM) was subtracted from the absorbance change in the "active" cuvette.

5.3.2.9 Analysis of prokaryotic alcohol dehydrogenase

Fifty microlitres of PBS, pH 7.4 containing either HP suspension (approximately 2.5 million organisms) or yeast ADH (final activity of 3000mU per well, as determined by manufacturers' assay) was used instead of biopsy material, and the assay was performed in the standard manner. It became apparent that there was a significant change in absorbance in the "blank" wells in addition to the "active" wells, and therefore the absorbance change in each pair of wells was analysed separately. A range of concentrations of 4MP (18.7, 37.5, 75 and 150mM) was used in the blank wells of the HP samples to determine the concentration at which 4MP inhibited HP ADH activity.

5.4 Statistics

Data were analysed using Student's t-test, paired or unpaired where appropriate, after correction by square root or logarithmic transformation where required. Least squares linear regression was used to calculate the gradient of the initial portion of the optical density versus time curve, and to examine linear relationships between the data. Multiple regression was used to correct for potential confounding factors when assessing the effect of HP status, age and the duration of freezing (Tabachnick and Fidell, 1996). One- way analysis of variance was used to calculate coefficient of variation, using the method described by Bland (Bland, 1995). Correlations were performed using Pearson's product moment correlation for normally distributed data, and Spearman's rank

correlation for non- parametric data. Comparison of two methods of measurement was performed using a Bland and Altman plot (Bland and Altman, 1986). All tests were 2-tailed, and statistical significance was taken as $p<0.05$. Statistical analyses were performed using SPSS version 9.0 (SPSS Inc, Chicago, Illinois, USA) and Minitab version 11.2 (Minitab Inc, Philadelphia, Pennsylvania, USA).

5.5 Results

5.5.1 Optimisation experiments

There was a linear relationship between absorbance and NDMA concentration: $R^2=0.997$, $p<0.001$. This is shown graphically in Figure 5.4. The product of path length and molar absorptivity coefficient corresponds to the gradient of this regression equation, i.e. $16.12mM^{-1}$.

There was a linear relationship between absorbance change and concentration of biopsy material determined by serial dilution. $R^2=0.998$, $p<0.001$ (Figure 5.5).

Within the range of alcoholic substrate concentrations 10- 500mM, GADH activity was greatest with propanol, followed by butanol then ethanol (Figure 5.6). The enzyme activity with all 3 alcoholic substrates was relatively constant between 250 and 500mM. Activity increased in a non- linear fashion with ethanol concentrations above 500mM, with a relative plateau between 500mM-

1M and 1M- 2M (Figure 5.6). The optimum NAD concentration was 200microM (Figure 5.7). The optimum pH was 6.45 (Figure 5.8).

The enzyme activity observed after incubation was almost identical to that observed after homogenisation. There was a strong linear relationship between the mean activity of biopsy pairs from the same site, in the same subject, prepared by homogenisation and incubation ($r=0.89$, $p<0.001$, $R^2=0.79$) (Figure 5.9). Analysis of the Bland and Altman plot (Figure 5.10) suggests there may be a non- significant trend towards over-estimation of the enzyme activity when using the incubation method in samples of high activity.

The GADH activity was only minimally influenced by the duration of incubation, with longer incubation times being associated with a higher activity (GADH activity at 6, 12, 24, 36 and 48 hours was 21.85, 21.60, 23.30, 23.45, and 25.40 mU/mg protein, respectively. (Figure 5.11).

The activity of other oxido- reductase enzymes measured in our assay contributed to less than 2% of the ADH activity measured (Figure 5.12).

One unit of ADH activity measured by the NDMA method is equivalent to 0.56 units by Dalziel's method, $R^2=0.995$ (Figure 5.13).

The activity of yeast ADH measured by the NDMA method was much lower than the manufacturer's estimated activity (0.22mU per well by NDMA versus 3000mU per well from manufacturer's estimate). The activity of HP ADH by the NDMA method was 0.19mU per million organisms. Significant inhibition of HP

ADH by 4MP was observed at a concentration of 4MP of 75mM. Almost complete inhibition was obtained at 150mM (Figure 5.14).

5.5.2 Results obtained with final assay method

Our mean biopsy weight was 7.8mg. There was a strong linear relationship between the enzyme activity expressed as per mg of biopsy weight and activity per mg protein, $R^2 = 0.948$, $p<0.001$ (Figure 5.15). Our coefficient of variation for paired biopsies was: within assay: 3.0%, between assays: 9.4%

A breakdown of activity by site, substrate type and helicobacter status is shown in Table 5.2. ADH activity was higher in the gastric antrum ($p<0.001$). There was a significant difference in ADH activity according to HP status in the gastric antrum but not the gastric body (these differences persist after correction for age, $p<0.005$ for antrum and body).

There was a significant but weak correlation with age and ADH activity in the gastric body $r=-0.30$, $p<0.05$, and gastric antrum $r=-0.23$, $p<0.05$ and this association persists after correcting for HP status $p=0.05$.

There was no significant correlation between the length of time biopsies were frozen (median 123 days, range 15-392 days) and their subsequent activity ($r<0.05$). This remained insignificant after correction for age and HP status ($p=0.74$).

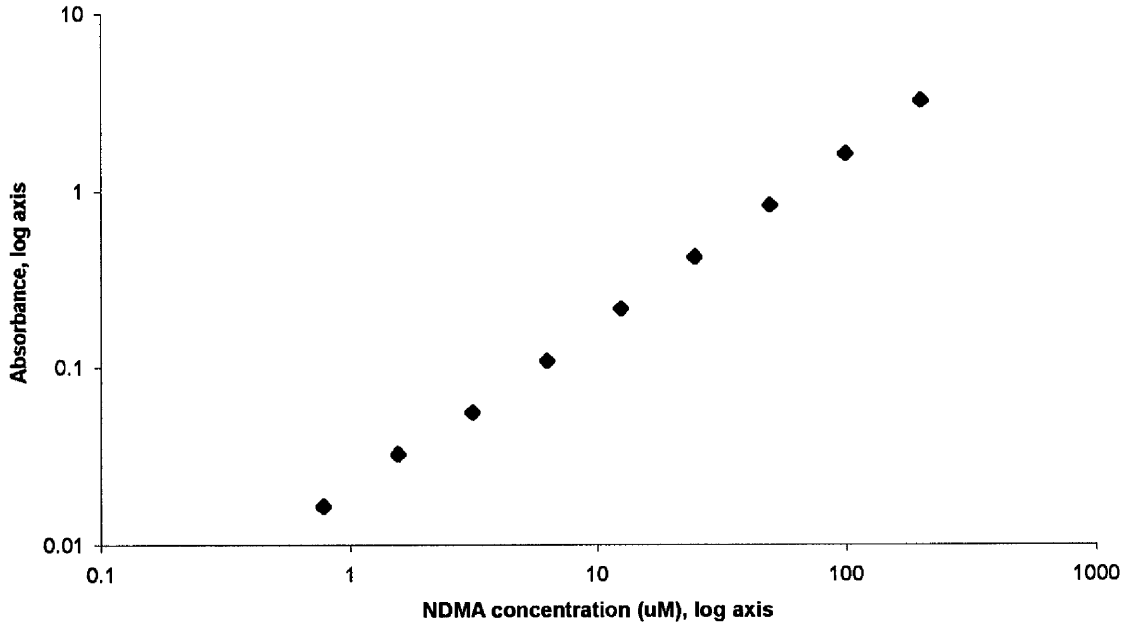

Figure 5.4 Absorbance at 450nm of serial dilutions of N, N-dimethyl-4-nitrosoaniline

This graph demonstrates the linear relationship between absorbance at 450nm and concentration of 267ul of NDMA. Absorbance = 0.01612 · conc (uM). R^2=0.997, $p<0.001$. From the Beer-Lambert law we can determine that the gradient of the line is equal to the product of path length and molar absorptivity coefficient.

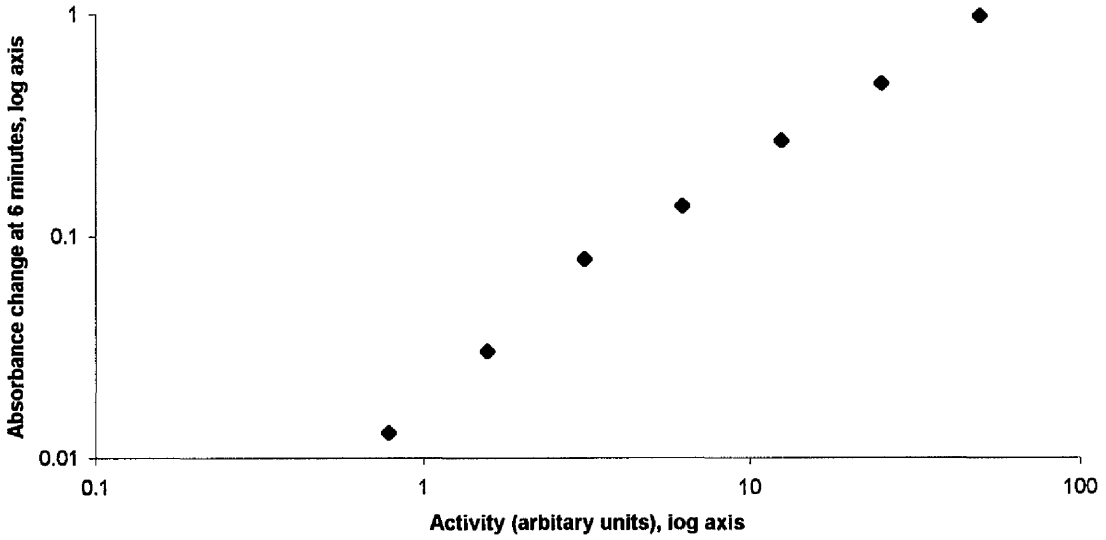

Figure 5.5 Absorbance change of serial dilutions of biopsy material

This graph demonstrates the linear relationship between absorbance change after 6 minutes at 450nm and biopsy activity (R^2=0.998, $p<0.001$). The activity of the undiluted biopsy incubation fluid was arbitrarily defined as 50 units; each subsequent dilution halved the concentration of biopsy material in the reaction well.

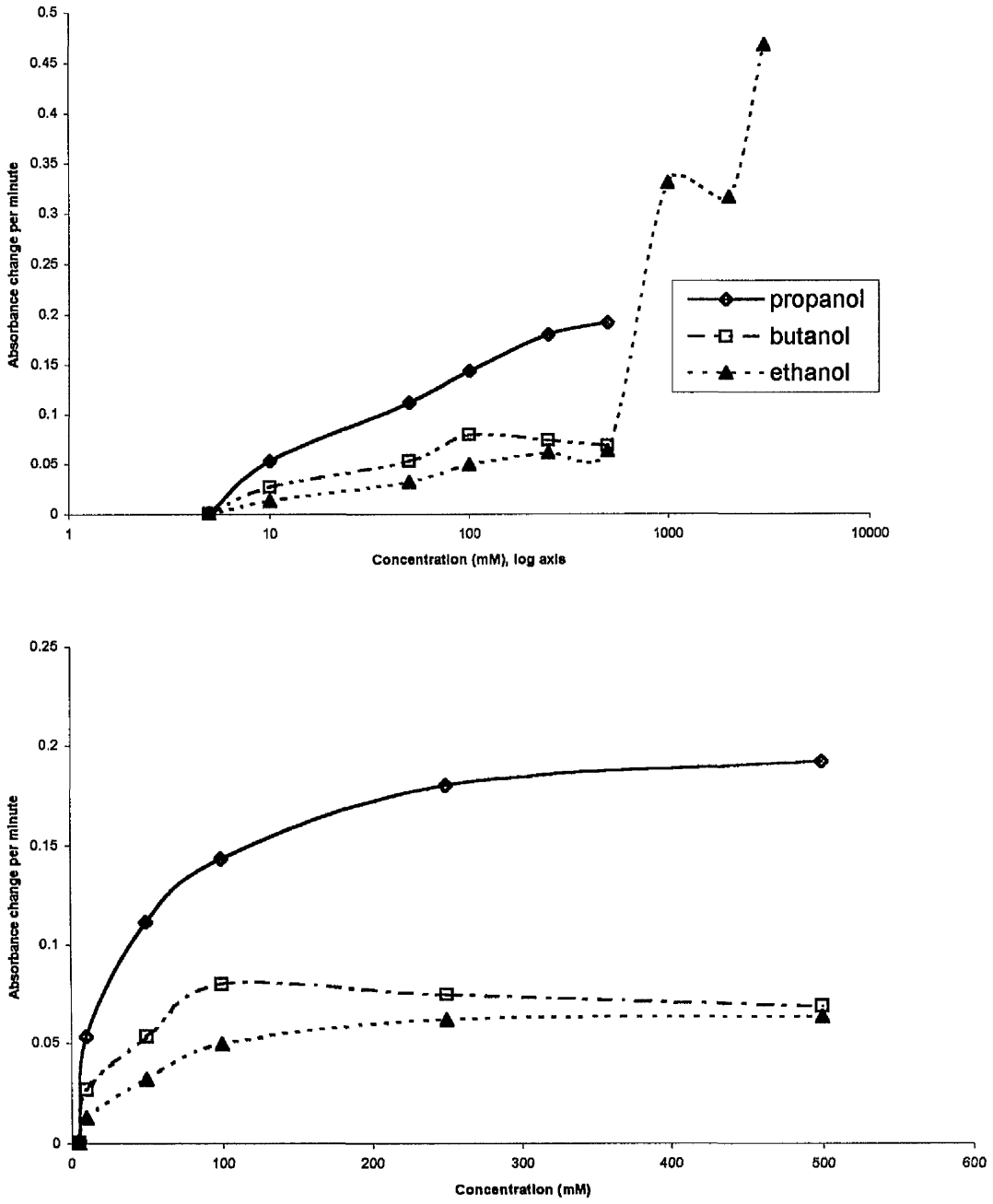

Figure 5.6 The effect of primary alcohol concentration on enzyme activity

These graphs illustrate the effect of varying both the concentration of the primary alcohol substrate, and the type of primary alcohol. The enzyme activity was varied over a wider concentration for ethanol (upper graph) than for the other substrates. The lower graph shows the enzyme activity in detail over the concentration range 5 to 500mM.

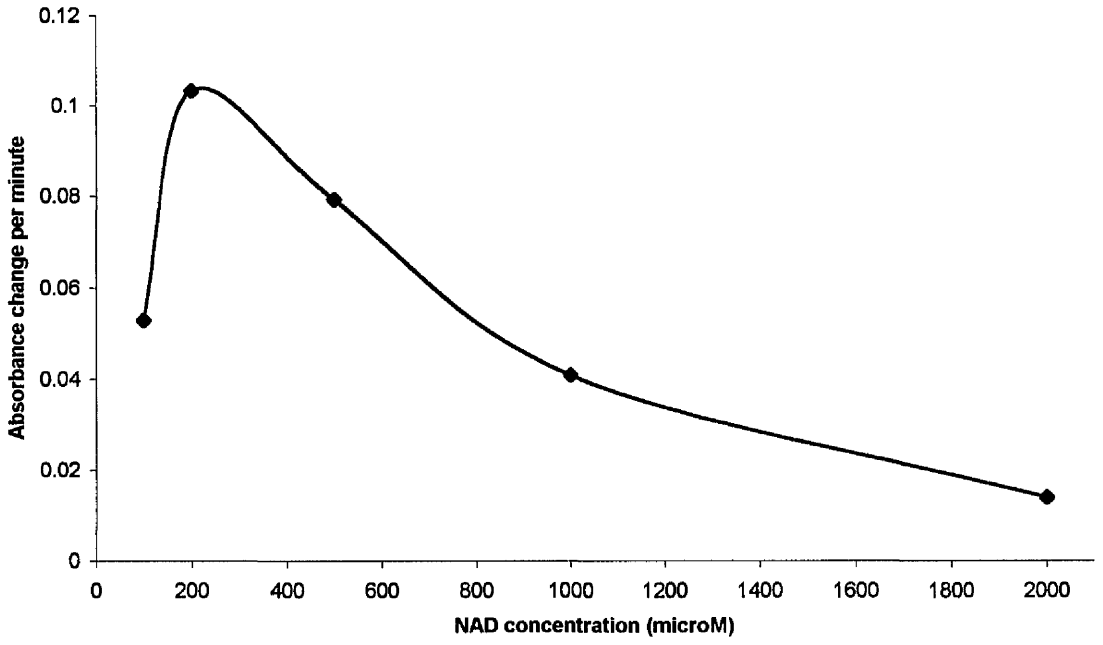

Figure 5.7 The effect of nicotinamide adenine dinucleotide (NAD) concentration on enzyme activity

This graph illustrates the effect of NAD concentration on pooled biopsy specimens, using ethanol as substrate. The optimum NAD concentration is 200 mM.

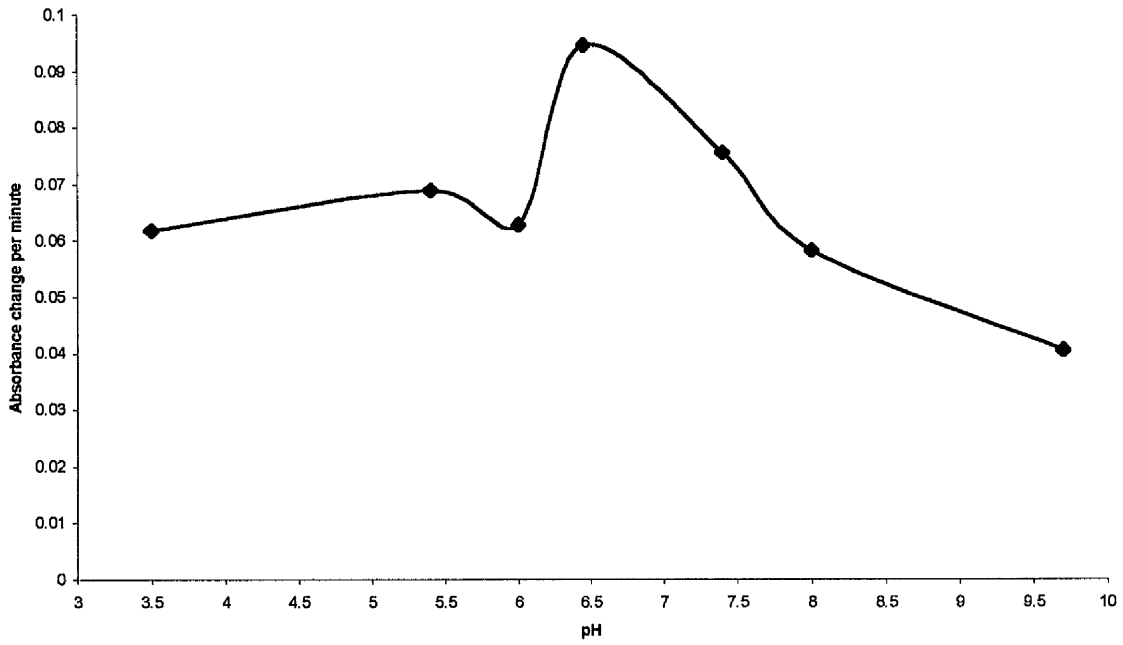

Figure 5.8 The effect of pH on enzyme activity

This graph illustrates the effect of pH on pooled biopsy specimens, using ethanol as substrate. The optimum pH is 6.45.

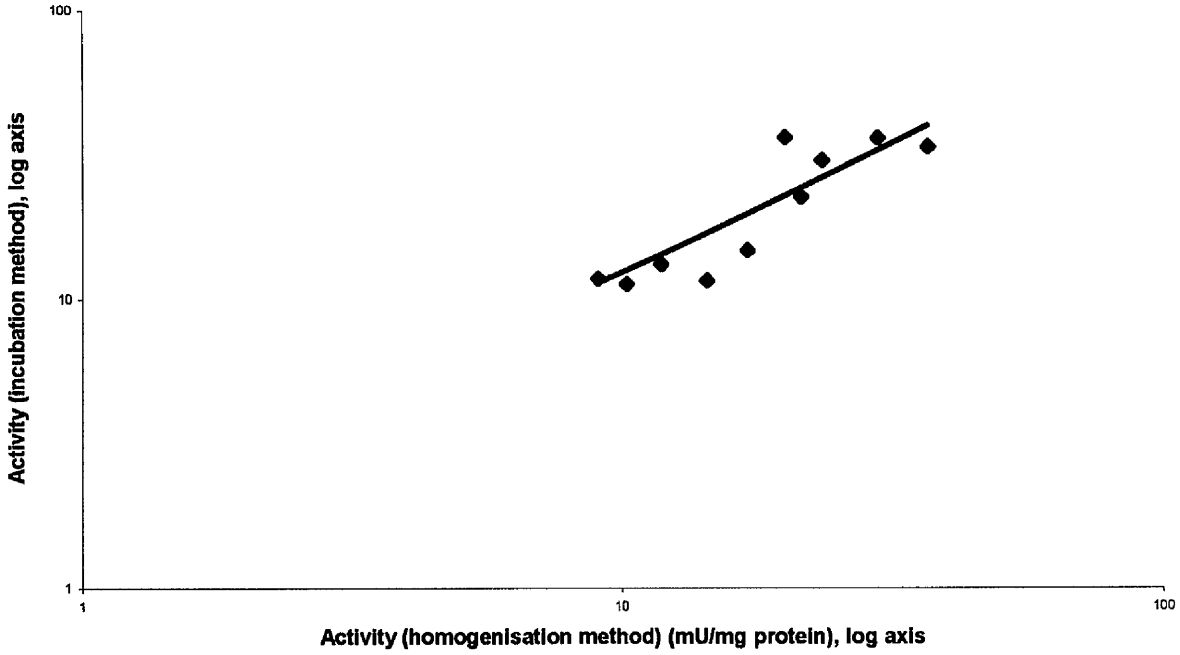

Figure 5.9 Plot of enzyme activity obtained by incubation and homogenization methods

This graph illustrates the linear relationship between the mean activity of paired antral biopsies prepared by homogenisation, and the mean activity of paired antral biopsies, from the same subject, prepared by incubation. Activity (incubated)= 1.0305·activity (homogenized) + 2.1315; (r=0.89, $p<0.001$, R^2=0.79)

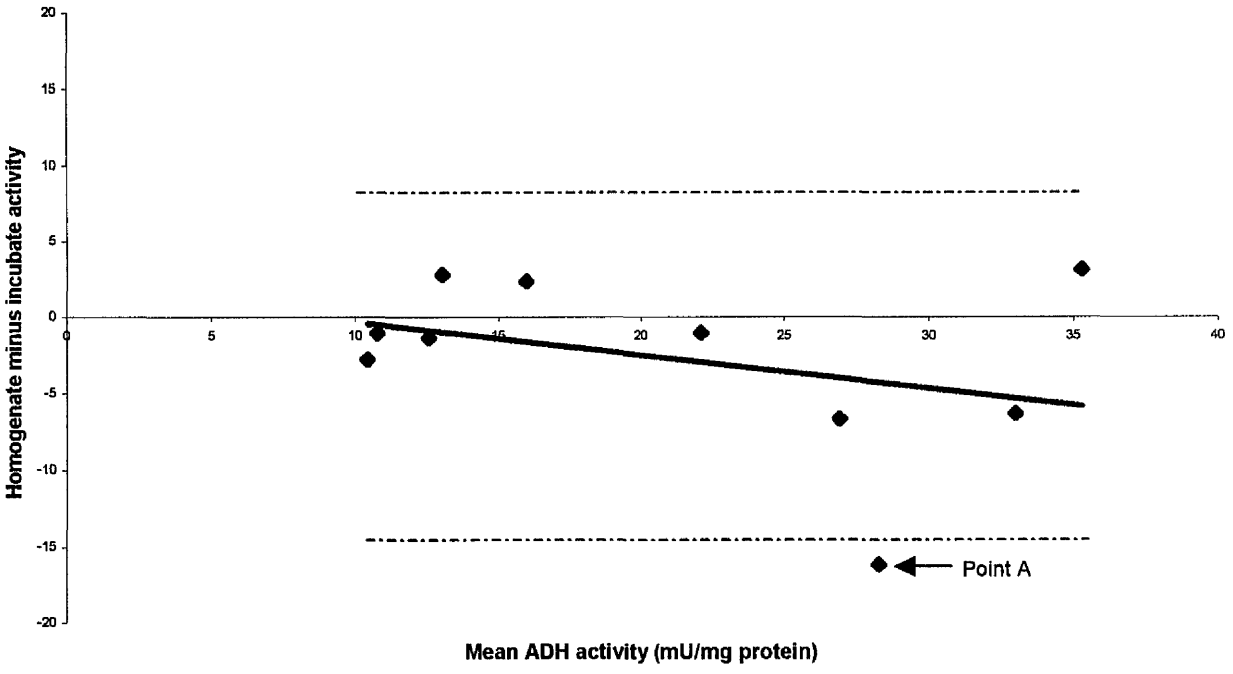

Figure 5.10 Bland and Altman plot comparing incubation and homogenization methods

This graph shows a Bland and Altman plot (plot of difference between methods versus mean activity of both methods). The solid line illustrates the regression line. The equation of this line is: difference in activity=1.78- 0.216·mean activity This non- significant regression line may suggest a minor trend towards over-estimation of the enzyme activity when using the incubation method in samples of high activity (r=-0.35, p=0.32, R^2=0.123). The mean of the difference between the methods (95% CI) is –2.73 (-14.18- 8.73). The dotted lines illustrate the 95% confidence limits. Point A has a disproportionately great influence on these parameters. Excluding this point would give the following parameters: difference in activity=0.49- 0.086·mean activity; r=-0.23, p=0.55, R^2=0.052; mean difference (95% CI) = -1.23 (-8.37- 5.91).

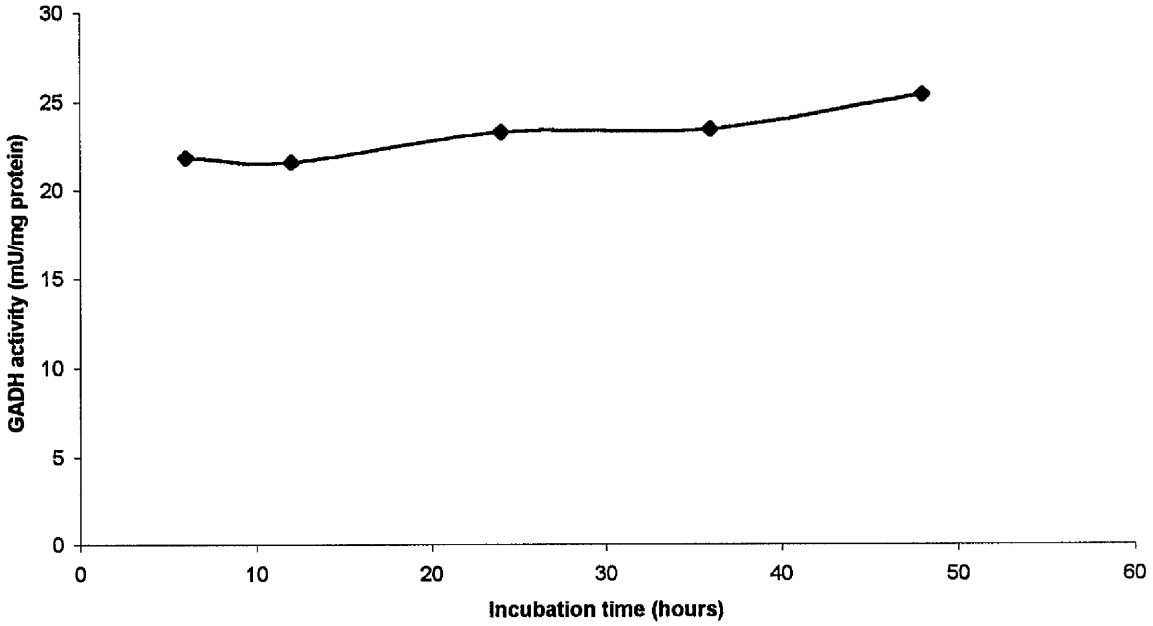

Figure 5.11 The effect of incubation time on enzyme activity

This graphs illustrates the minimal effect of incubation time in the range 6- 48 hours on the GADH activity detected in the incubation fluid. The activity at 48 hours (25.40mU/mg protein) is 16% greater than that at 6 hours (21.85mU/mg protein)

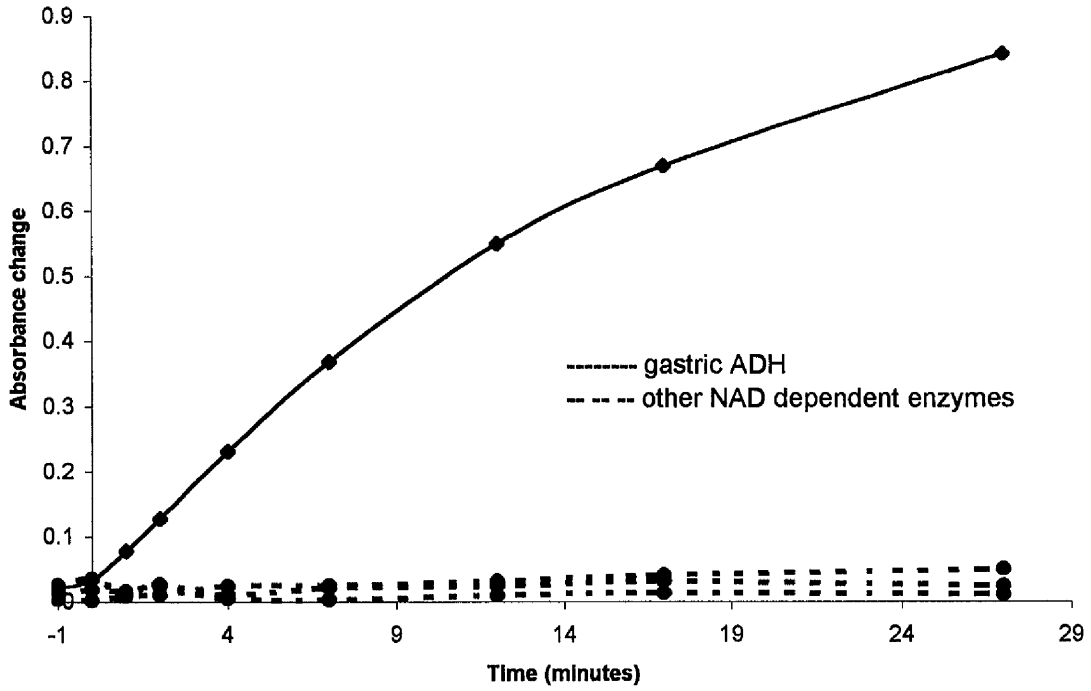

Figure 5.12 Activity of gastric alcohol dehydrogenase compared to other NAD dependent oxido-reductase enzymes

This graph illustrates that the contribution of other oxido-reductase enzymes to the activity detected by this assay is negligible.

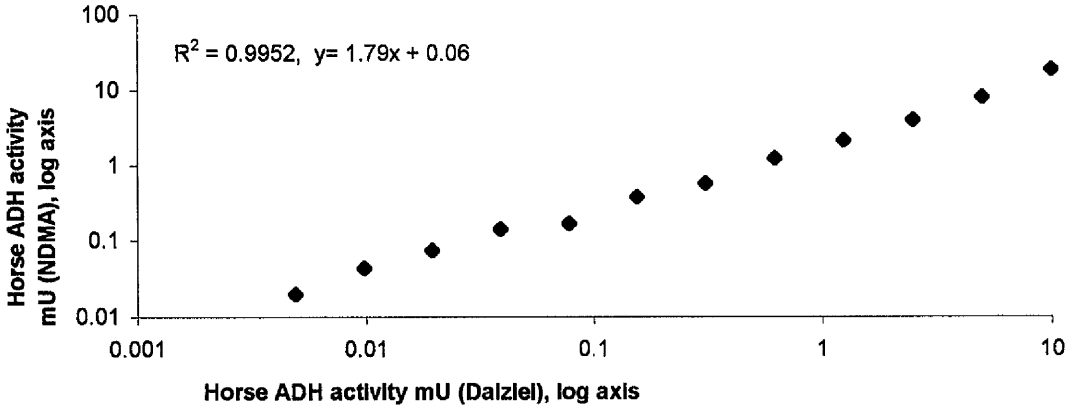

Figure 5.13 Horse liver ADH activity by Dalziel's method and NDMA method

This graph illustrates the linear relationship between ADH activity assessed by UV spectrophotometry (Dalziel, 1957), and by the NDMA method.

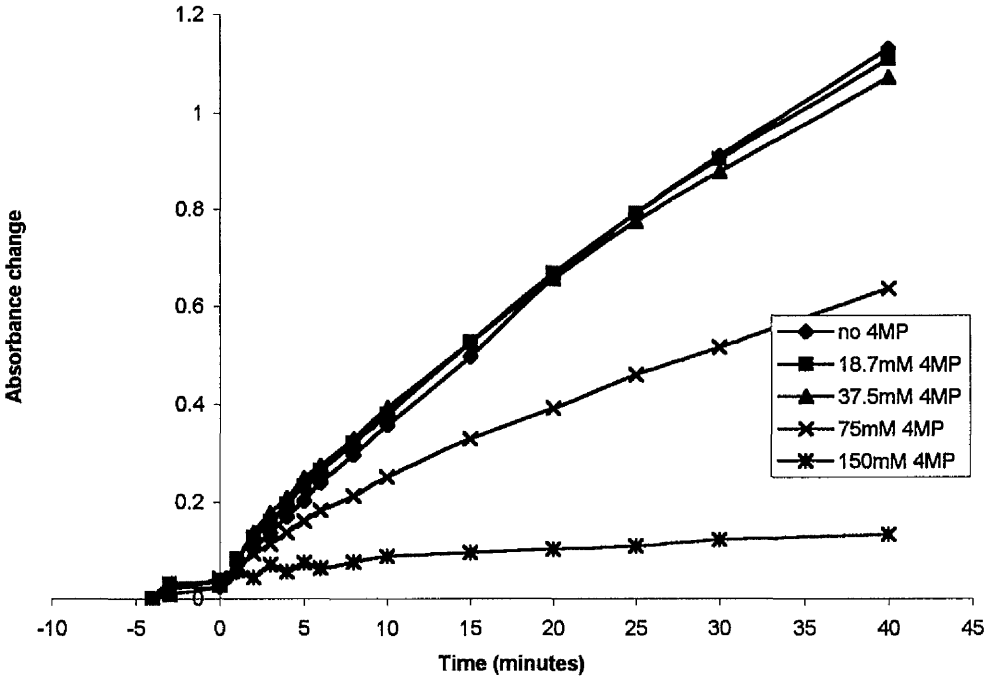

Figure 5.14 Activity of a suspension of *Helicobacter pylori*, with differing concentrations of 4 methyl pyrazole

This graph illustrates the effect of varying 4 methyl pyrazole (4MP) concentration on *Helicobatcer pylori* alcohol dehydrogenase (ADH) activity. Significant inhibition of activity is apparent at 75mM 4MP, and almost complete inhibition at 150mM. The mammalian enzymes (human gastric ADH and horse liver ADH) were completely inhibited at 18.7mM 4MP.

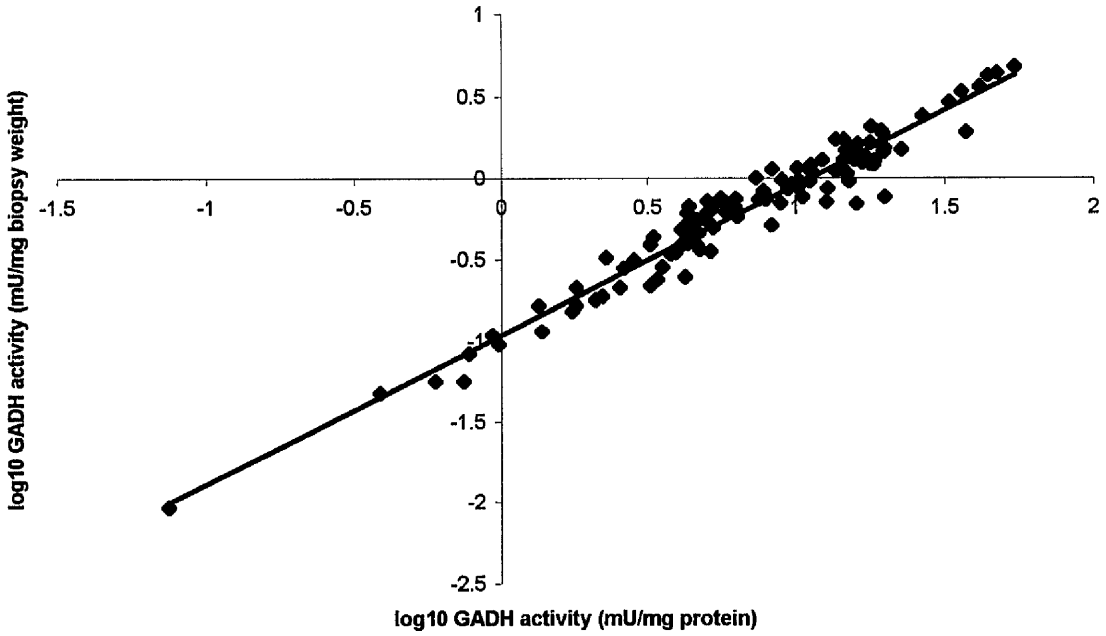

Figure 5.15 Relationship between gastric alcohol dehydrogenase activity in biopsy samples when expressed by weight or protein content

This graph illustrates the linear relationship (R^2=0.948, p<0.001) between enzyme activity expressed as biopsy weight (mU/mg) and protein content (mU/mg protein) in 110 biopsy specimens.

Table 5.2 The effect mucosal type (antral or body) and the effect of *Helicobacter pylori* infection, on gastric alcohol dehydrogenase activity

	Antrum		Body	
Overall	10.9 (3.9- 17.8)		5.2 (3.7- 10.2)	
HP+ve	8.9 (2.0-15.4)	} p<0.005	6.3 (3.5-12.6)	} p=NS
HP-ve	18.3 (8.8- 25.0)		5.1 (4.3- 7.1)	

Gastric alcohol dehydrogenase (ADH) median activity (inter- quartile range) is quoted in mU (umol·min^{-1}·mg protein $^{-1}$·10^{3}). HP= *Helicobacter pylori*. NS= not statistically significant. Gastric antral ADH activity is significantly greater than that of gastric body, p<0.001.

5.6 Discussion

NDMA is a yellow- green coloured aldehyde- like dye that becomes colourless when reduced to its hyroxylamine form by ADH in the presence of NADH. Dunn and Bernhard, 1971 described its reaction with liver ADH, and Skursky et al., 1979 subsequently reported a method for detecting ADH in serum using NDMA.

Many of the traditional gastric ADH assays rely on specimens obtained at surgical resection (Yin et al., 1997), (Buhler and Wartburg, 1982), (Roine et al., 1992a), (Hernandez-Munoz et al., 1990), (Roine et al., 1990), (Haber et al., 1996) or by using non- standard biopsy forceps (Brown et al., 1995), or combined biopsies (Thuluvath et al., 1994), giving an average specimen weight greater than the one in this study of 7.8mg. Almost all assays employ a homogenisation stage. The aim of this study was to develop a method that would allow a large number of specimens to be processed more rapidly, and to minimise any experimental error due to differences in the completeness of homogenisation between samples. The incubation technique, coupled with the increased sensitivity of the NDMA assay, allowed reliable detection of ADH in the incubation supernatant.

The results obtained from incubation were in close agreement with those obtained by homogenisation. There was only a modest increase in enzyme

activity with an increasing duration of incubation. For convenience, an incubation beyond 24 hours was felt to be of only marginal benefit.

Gastric alcohol dehydrogenase can utilise many primary alcohols as substrate, and hence the assay was performed with ethanol, butanol, and propanol. Although higher activities are seen with the primary alcohols other than ethanol, it is the activity with ethanol as substrate that is likely to be of interest to most researchers. Likewise, although the enzyme activity rises as ethanol concentration increases, the concentrations that are easily achievable in social drinking are likely to be of most relevance. The two plateau phases in activity versus concentration are likely to represent concentrations at which one isozyme of ADH is fully saturated, but where the concentration has not yet reached the K_m (Michaelis constant) value of other isozymes. The continued rise in concentration above 3M ethanol may represent Class III (χ) ADH, which is not saturatable by ethanol.

The variation in gastric ADH activity due to gender, HP status, age and ethnic group has been discussed in Chapter 2. These differences have been suggested by some to contribute to increased susceptibility to the effects of alcohol. Differing ADH activities in different sites in the GI tract, particularly in relation to the local activity of acetaldehyde dehydrogenase may have a role in local tissue damage and carcinogenesis, because acetaldehyde has been shown to be a potential carcinogen (Seitz et al., 1990), (Hori et al., 1997).

In this Caucasian population studied, a small but statistically significant age effect was demonstrated. The lower activity of antral ADH in HP positive

subjects has been noted by others (discussed in Chapter 2), and is probably a consequence of a predominantly antral gastritis.

The activity observed with this method for ADH derived from Baker's yeast was markedly below the manufacturer's published activity (using a standard spectrophotometric technique). However, the estimated activity of mammalian ADH was similar using the NDMA and the spectrophotometric techniques. This may be because the non- mammalian enzyme has less affinity for NDMA. The prokaryotic ADH enzymes required higher concentrations of 4MP for inhibition, suggesting that they also have a lower affinity for 4MP.

In conclusion, this study has demonstrated a method for assaying ADH in gastric endoscopic biopsies which is sensitive, rapid and robust, giving results for differences in ADH activity by helicobacter status and age which are similar to those found when using more traditional methods.

Chapter 6: The effect of *Helicobacter pylori* eradication on blood alcohol concentration, gastric emptying, and the systemic delivery of alcohol

6.1 Introduction

It has been noted for some time that when alcohol (ethanol) is administered orally there is incomplete delivery of the alcohol dose to the systemic circulation (Julkunen et al., 1985), (Di Padova et al., 1987a). This discrepancy is due to pre-systemic metabolism (also called first pass metabolism) of alcohol, which must occur in either the gastrointestinal tract or the liver (via the portal circulation).

Gastric mucosa contains alcohol dehydrogenase that is capable of metabolising ingested alcohol to acetaldehyde (Pestalozzi et al., 1983), (Buhler and Wartburg, 1982), (Yin et al., 1997). Gastric metabolism of alcohol may therefore account for a proportion of the first pass effect. Whether this proportion is clinically significant compared with hepatic first pass metabolism is a matter of great controversy (Gentry et al., 1994a), (Levitt, 1994a), (Gentry et al., 1994b). *Helicobacter pylori* (HP) infection is associated with lower gastric alcohol dehydrogenase (GADH) activity (discussed in detail in Chapter 2).

Eradication of HP has been shown to increase the activity of GADH (Gupta et al., 1994), (Simanowski et al., 1998), (Kechagias et al., 2001). To date, only one study has addressed the effect of therapy for HP on alcohol metabolism (Simanowski et al., 1998), suggesting an increase in first pass metabolism following HP suppression. However, it failed to take account of gastric

emptying, or address the unusual pharmacokinetic properties of alcohol. Hepatic alcohol metabolism does not obey first- order kinetic principles, and the contribution of the liver to pre-systemic metabolism will therefore vary with the rate of alcohol delivery to the portal circulation. This will vary with the rate of gastric emptying (Kechagias et al., 1998), (Amir et al., 1996). Hence, by acting as a reservoir, the stomach can both increase exposure to GADH (Amir et al., 1996), and increase hepatic first pass metabolism (Holford, 1987). Therefore, HP eradication could influence pre-systemic alcohol metabolism both by affecting gastric emptying, as well as by increasing GADH activity.

The aims of this study are: 1) To determine if HP eradication alters the amount of alcohol reaching the systemic circulation following oral administration, and 2) By assessing GADH activity and the rate of gastric emptying, this study also aims to determine the relative contribution of each of these factors to any observed changes.

6.2 Patients and methods

6.2.1 Patients

18 male patients, mean age 42 (range 23- 67), who were attending for upper GI endoscopy as a routine part of investigation for dyspepsia, dysphagia, or weight loss, were recruited. The criteria for inclusion were: HP positive, male sex, Caucasian ethnic origin, age less than 70, and non-malignant upper GI pathology that warranted HP eradication therapy on clinical grounds (duodenal or gastric

ulceration; gastritis; duodenitis; scarring from previous ulceration). Subjects were excluded if they had a history of: alcoholism, (current or previous); liver disease; malignancy (either in the past, or at index endoscopy), previous gastric surgery; other severe illness (diabetes, epilepsy, ischaemic heart disease, cerebrovascular disease, severe respiratory disease). They were also excluded if they had taken any of the following medications in the preceding 2 weeks: H_2 receptor antagonists, proton pump inhibitors, other anti-ulcer therapies (except for simple antacids), aspirin, anticonvulsants, and other medication known to significantly affect hepatic metabolism or GADH activity. All subjects gave informed written consent and the local ethics committee approved this study. This study conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

6.2.2 Initial endoscopy

In addition to samples required for diagnostic purposes, a biopsy was taken with standard endoscopic biopsy forceps (Olympus FB3K) for histological examination from the gastric body and antrum. Two biopsies were also taken from both the gastric antrum and body for determination of GADH activity, by the methods detailed in Chapter 5. An endoscopic urease (CLO^{TM}) test was also taken from the gastric antrum. Subjects were considered to be HP positive if the CLO test became positive within 24 hours, and there was evidence of HP on histology of gastric biopsies.

6.2.3 Initial administration of alcohol

Subjects were asked to delay taking eradication therapy until this part of the study had been performed, which took place within one week of the initial endoscopy. Subjects abstained from alcohol for 24 hours, and fasted for 4 hours prior to alcohol administration. An intravenous (IV) cannula was inserted, and blood was taken for a standard biochemical screen (which was performed by the hospital laboratory staff) including liver function tests, and baseline blood ethanol measurement. They were given a meal consisting of 100mls of Ensure oral dietary supplement (Abbott Laboratories Ltd., Queenborough, Kent UK) (1 Calorie/ml), and 2 slices of toast with 10g of butter. Thirty minutes after their meal, subjects were given 0.3 g/kg of ethanol, in the form of vodka, given with a sufficient volume of pure orange juice (approximately 130- 200ml) (0.4 Calories/ ml), such that the concentration of ethanol in the beverage was 12% weight/ volume. In addition, the alcoholic mixture contained 1g of dissolved paracetamol (acetaminophen). Blood was taken from the IV cannula for estimation of blood alcohol and serum paracetamol concentrations at the following times: 0, 10, 15, 20, 25, 30, 35, 40, 50, 60, 80, 100, 120, 150, 180 minutes. Both alcohol and paracetamol were analysed by enzymatic methods (alcohol- Sigma ADH, paracetamol- Cambridge Life Sciences), using a Cobas MIRA analyser (Roche Diagnostics, Basel, Switzerlan). The alcohol and paracetamol measurements were performed by Mr W Borland (Department of Biochemistry, Glasgow Royal Infirmary).

6.2.4 Eradication therapy

The eradication therapy consisted of seven days of lanzoprazole 30mg twice daily, amoxycillin 500mg twice daily, and metronidazole 400mg three times daily (all taken orally).

6.2.5 Subsequent endoscopy and alcohol administration

Repeat upper GI endoscopy, with the same biopsy protocol as described for initial endoscopy, was performed on average 9 weeks (range 6-17 weeks) after commencing eradication therapy to confirm HP eradication. The alcohol administration procedure was repeated within two week of the second endoscopy. It was performed at the same time of day as the initial study to minimise diurnal variation in alcohol metabolism (Palmer et al., 1991) or gastric emptying.

The aim of this study is to assess the effect of HP eradication, rather than the efficacy of an eradication regime at causing changes in alcohol metabolism. The analysis of the results was therefore based on successful eradication rather than intention to treat.

6.2.6 Estimation of total absorbed alcohol dose

As a consequence of its non- linear pharmacokinetics, the area under the curve (AUC) for blood alcohol concentration (BAC) is not directly related to the dose of alcohol absorbed (Holford, 1987). Increasing the rate of absorption of alcohol will increase the AUC, despite the dose remaining constant. In addition, an increase in alcohol dose will cause a disproportionately greater increase in AUC (Wilkinson, 1980) (discussed in detail in Chapter 3).

The overall alcohol elimination rate can be described in man using the Michaelis- Menten equation (Holford, 1987) (see Appendix A.2, Equation 3). The equation parameters V_{max} (maximum velocity) and K_m (Michaelis constant) were estimated from the portion of the BAC versus time curve after absorption and distribution were complete, using the integrated form of the equation, as described by Lundquist (Lundquist and Wolthers, 1958) (see Appendix A.3, Equation 6). By using these parameters, the total amount of alcohol reaching the systemic circulation can be estimated at any given time by using the formula described by Gentry et al., 1992 and Lin et al., 1976 (Appendix A.4, Equation 7), where the dose absorbed will be proportional to the AUC of elimination rate determined by the Michaelis- Menten formula over time for each value of blood alcohol concentration.

For reasons discussed in detail later, volume of distribution was not directly measured in this study. The volume of distribution was estimated from published data (Wagner et al., 1989), (Lands, 1998), (Holford, 1987), and this was used to provide an estimate of bioavailability.

Despite concerns about its validity in relation to total absorbed dose, the AUC of BAC versus time is perhaps still the most useful expression of the duration and extent of exposure of the body to alcohol, which may be a major factor in determining systemic toxicity, and hence the AUC of BAC versus time was also calculated.

6.2.7 Gastric emptying

Paracetamol is absorbed in the jejunum but not the stomach, and measurement of serum paracetamol concentration can therefore provide useful information about the rate of gastric emptying (Van Wyk et al., 1990), (Clements et al., 1978). Changes in gastric emptying were assessed by comparing both the magnitude of the peak paracetamol concentration, and the time to peak paracetamol concentration. Subjects remained seated throughout the alcohol administration study to minimise changes in gastric emptying associated with posture.

6.3 Statistics

Student's paired t- test was used to compare subject parameters before and after HP eradication. The area under the curve was calculated using the trapezoidal method. Correlations were performed using Pearson's product moment correlation for normally distributed data, and Spearman's rank correlation for non- parametric data.

Calculations were performed using Excel version 9.0 (Microsoft Corp., Seattle, Washington, USA); SPSS version 9.0 (SPSS Inc., Chicago, Illinois, USA); Minitab version 11.2 (Minitab Inc, Philadelphia, Pennsylvania, USA). The estimation of the parameters of the Michaelis- Menten equation were performed using non- linear least squares regression curve fitting software (Curve Expert version 1.34, D Hyams, Starkville, Mississippi, USA). All tests were 2- tailed and significance was taken as $p<0.05$.

6.4 Results

The initial endoscopic findings of the 18 subjects are shown in Table 6.1. Three subjects withdrew from the study prior to the second endoscopy. Eradication therapy was successful in 10 out of the remaining 15 subjects. In those with successful HP eradication, GADH activity rose significantly in the gastric antrum, pre- eradication 4.79mU/mg protein, post eradication 17.16mU/mg protein, $p<0.03$, but not the gastric body, pre- eradication 4.70mU/mg protein, post eradication 6.07mU/mg protein, $p=0.89$ (Table 6.2). There was no change following successful eradication therapy in AUC for BAC versus time, or in the total absorbed dose of alcohol/ volume of distribution, or in the estimated bioavailability of alcohol (Table 6.2). There was also no change in the magnitude, or time to peak, of the serum paracetamol concentration following successful eradication therapy, suggesting the eradication of HP did not influence the rate of gastric emptying after the test meal (Table 6.2). In the five

subjects in whom eradication therapy was unsuccessful (Table 6.3), there was no significant change in any of these parameters.

There was no significant change in weight following eradication therapy: successful eradication group (median); pre-eradication 81.4kg, post eradication 83.0kg, p=0.39: unsuccessful eradication group; pre-eradication 69.5kg; post eradication 71.4kg, p=0.44.

The mean values for blood alcohol concentration versus time before and after eradication therapy are shown in Figures 6.1 and 6.2, for the successful eradication and unsuccessful eradication groups, respectively. Corresponding values for serum paracetamol are shown in Figures 6.3 and 6.4. The cumulative dose of alcohol eliminated per litre of volume of distribution versus time is shown in Figures 6.5 and 6.6, for the two groups.

There was no correlation in the baseline data between the estimated bioavailability and ADH activity in the gastric antrum (r=-0.23, p=0.39), gastric body (r=-0.27, p=0.33), or the mean of the body and antrum (r=-0.27, p=0.33). Bioavailability correlated with peak serum paracetamol concentration (r=-0.54, p=0.02), but not the time of the paracetamol peak (r=-0.26, p=0.30). The group in whom HP was successfully eradicated showed no correlation between the change in estimated bioavailability and change in gastric ADH activity, or change in paracetamol measurements (data not shown). The change in AUC for BAC correlated strongly with the change in alcohol dose absorbed/ Vd (r=0.83, $p<0.01$).

Table 6.1 Initial endoscopic findings in eighteen male subjects

Endoscopic Diagnosis	Number of Subjects
Gastritis	5
Duodenitis	6
Duodenal scarring	4
Duodenal ulcer	5
Gastric ulcer	1
Gastric erosions	2
Oesophagitis	2

Note that more than one endoscopic diagnosis is recorded for some subjects.

Table 6.2 Changes in gastric alcohol dehydrogenase activity and alcohol metabolism in the group in whom eradication therapy was successful

	GADH activity (antrum)	GADH activity (body)	Alcohol absorbed/Vd	Alcohol AUC	Estimated % bioavailability	Time of paracetamol peak	Peak paracetamol concentration
Pre-eradication therapy	4.79 (3.4- 11.5)	4.70 (3.3- 16.8)	9.46 (7.9- 9.9)	606 (398- 734)	64 (53-66)	35 (29- 52)	94 (68- 109)
Post-eradication therapy	17.16 (7.1- 25.0)	6.07 (3.2- 8.6)	10.45 (8.2- 12.1)	689 (533- 783)	70 (55- 81)	32.5 (20- 65)	86 (63- 111)
Increase after therapy (95% CI)	13 (1.1- 20.1)	0.53 (-7.7- 7.5)	1.05 (-0.67- 3.35)	83 (-74- 240)	7.1 (-4.5- 23)	-2 (-30- 32)	-5 (-22- 14)
p value	0.03*	0.89	0.17	0.36	0.17	0.96	0.62

All values are medians. Ranges are interquartile ranges except where 95% CI is indicated. AUC= area under the curve of blood alcohol concentration versus time (mmol·min). GADH= gastric alcohol dehydrogenase (mU/mg protein). Vd= volume of distribution. Alcohol absorbed/ Vd expressed in mmol/l. Paracetamol concentration is expressed in umol/l, time to peak paracetamol expressed in minutes. Asterisk * indicates significant p value.

Table 6.3 Changes in gastric alcohol dehydrogenase activity and alcohol metabolism in the group in whom eradication therapy was unsuccessful

	GADH activity (antrum)	GADH activity (body)	Alcohol absorbed/Vd	Alcohol AUC	Estimated % bioavailability	Time of paracetamol peak	Peak paracetamol concentration
Pre-eradication therapy	14.77 (4.6- 16.4)	9.41 (3.7- 12.6)	9.07 (7.4- 10.2)	556 (404- 770)	61 (49- 68)	53 (29- 60)	70 (59- 105)
Post-eradication therapy	15.09 (3.5- 28.0)	10.14 (6.9- 12.0)	7.76 (6.8- 10.2)	506 (397- 739)	52 (46- 69)	43 (35- 50)	77 (70- 99)
Increase after therapy (95% CI)	0.44 (-7.9- 16.4)	1.85 (-4.2- 6.6)	-0.25 (-1.7- 0.8)	-47 (-150- 100)	-1.7 (-11.4- 5.2)	-4 (-28- 20)	5 (-23- 27)
p value	0.39	0.57	0.36	0.61	0.36	0.67	0.84

All values are medians. Ranges are interquartile ranges except where 95% CI is indicated. AUC= area under the curve of blood alcohol concentration versus time (mmol.min). GADH= gastric alcohol dehydrogenase (mU/mg protein). Vd= volume of distribution. Alcohol absorbed/ Vd expressed in mmol/l. Paracetamol concentration is expressed in umol/l, time to peak paracetamol expressed in minutes.

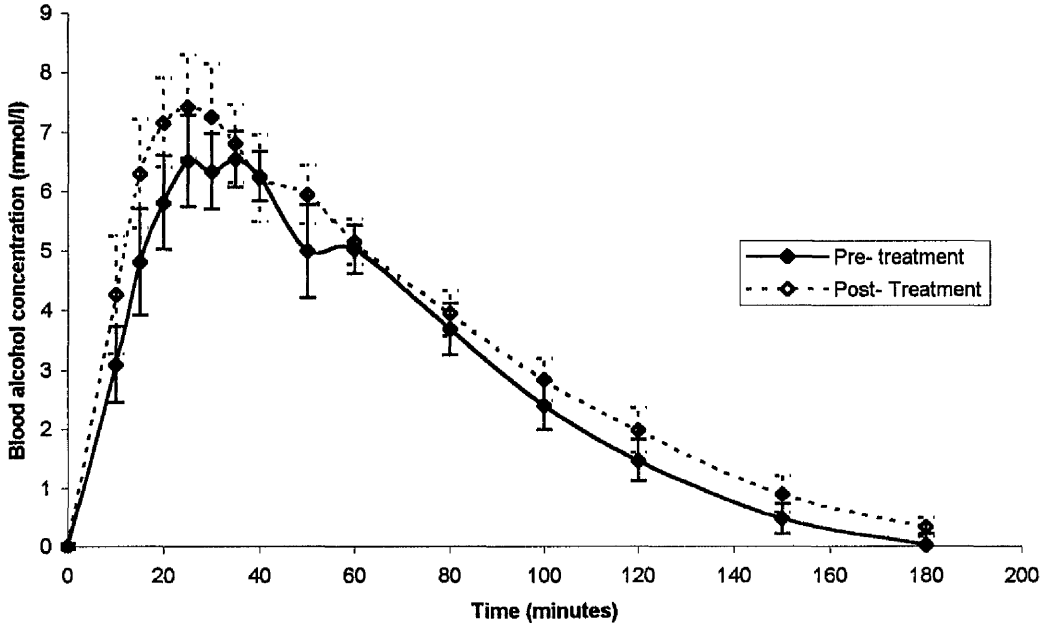

Figure 6.1 Blood alcohol vs. time- Successful eradication group

Mean blood alcohol concentration versus time curves for the successful eradication group. Error bars show the standard error of the mean. There is no significant difference in area under the curve pre and post eradication (606 versus 689 mmol·min, respectively, p=0.36).

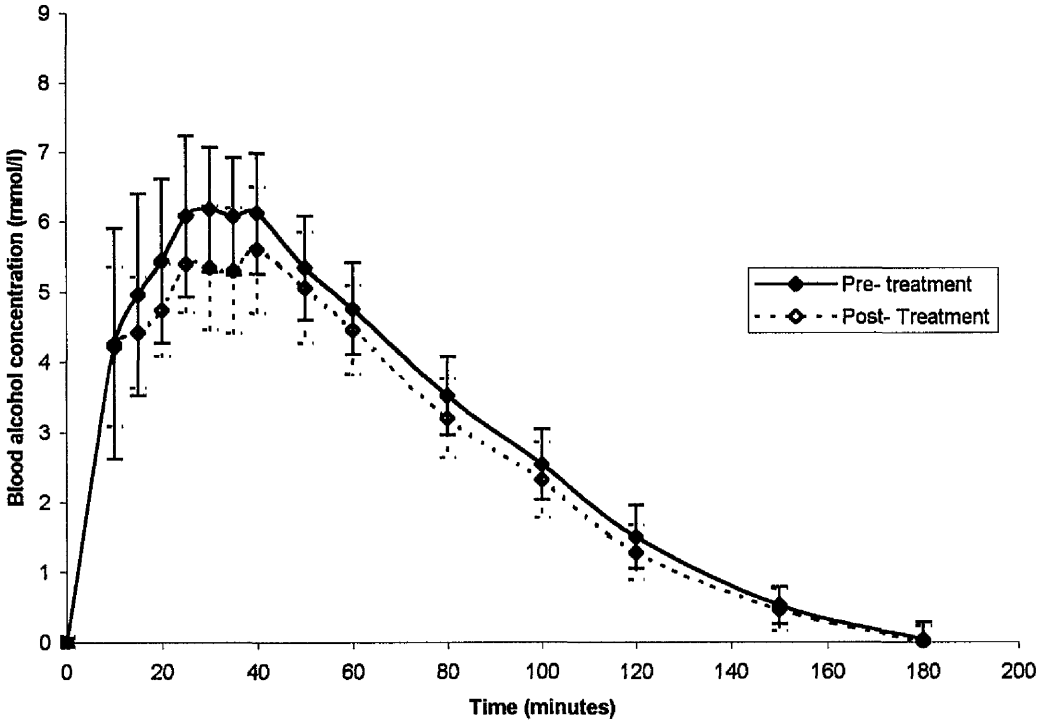

Figure 6.2 Blood alcohol vs. time- Unsuccessful eradication group

Mean blood alcohol concentration versus time curves for the unsuccessful eradication group. Error bars show the standard error of the mean. There is no significant difference in area under the curve pre and post eradication (556 versus 506 mmol·min, respectively, p=0.61).

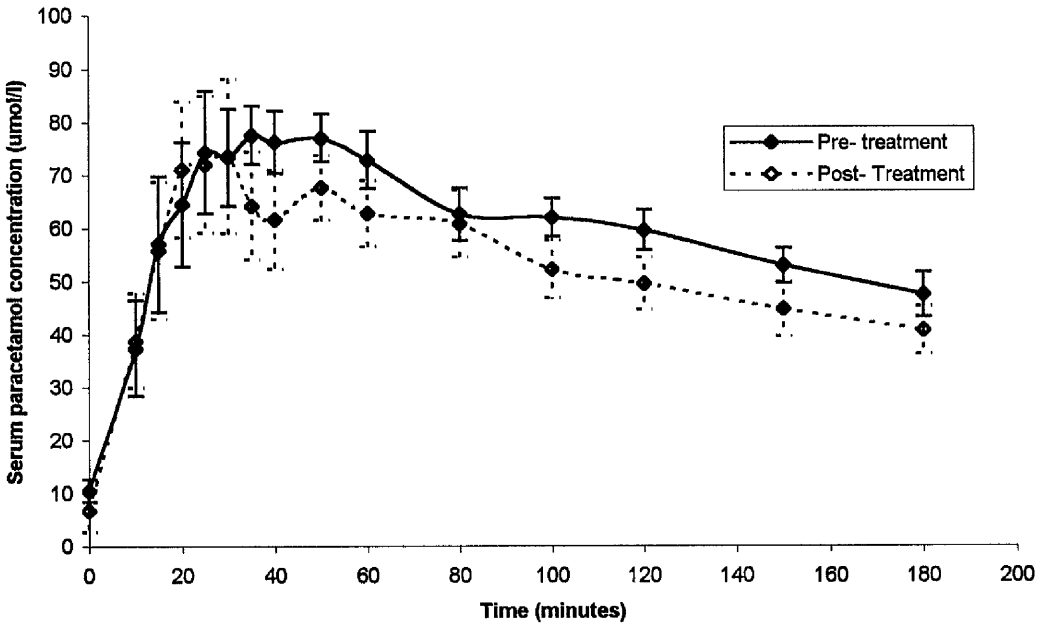

Figure 6.3 Serum paracetamol vs. time- Successful eradication group

Mean serum paracetamol concentration versus time curves for the successful eradication group. Error bars show the standard error of the mean. There is no significant difference pre- and post- eradication for the time of paracetamol peak (35 versus 32.5 min, respectively, p=0.96), or for the peak paracetamol concentration (94 versus 86 umol/l, respectively, p=0.62).

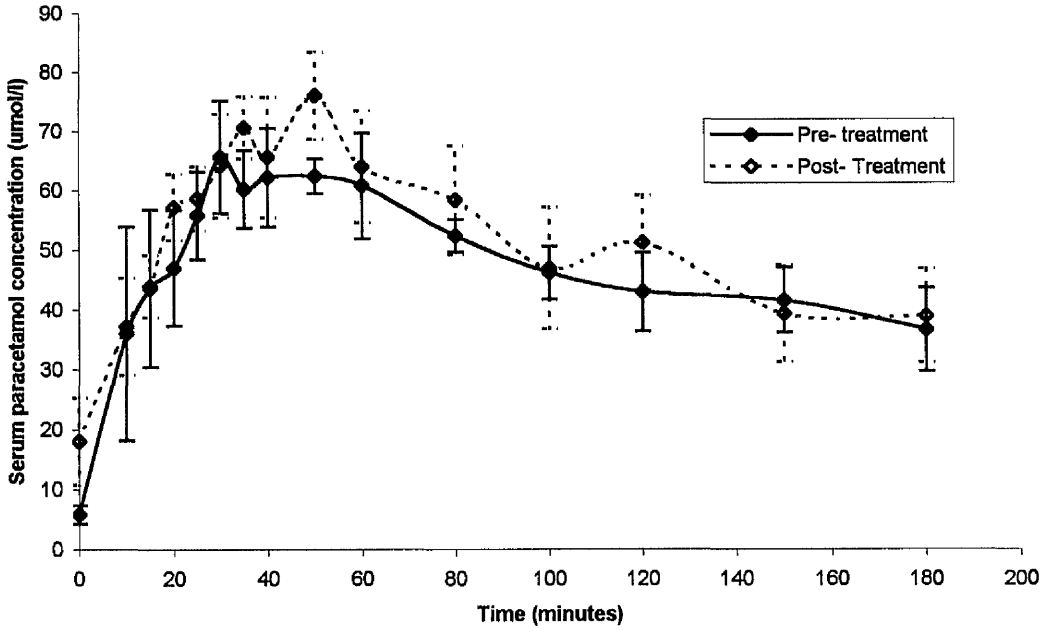

Figure 6.4 Serum paracetamol vs. time- Unsuccessful eradication group

Mean serum paracetamol concentration versus time curves for the unsuccessful eradication group. Error bars show the standard error of the mean. There is no significant difference pre- and post- eradication for the time of paracetamol peak (53 versus 43 min, respectively, p=0.67), or for the peak paracetamol concentration (70 versus 77 umol/l, respectively, p=0.84)

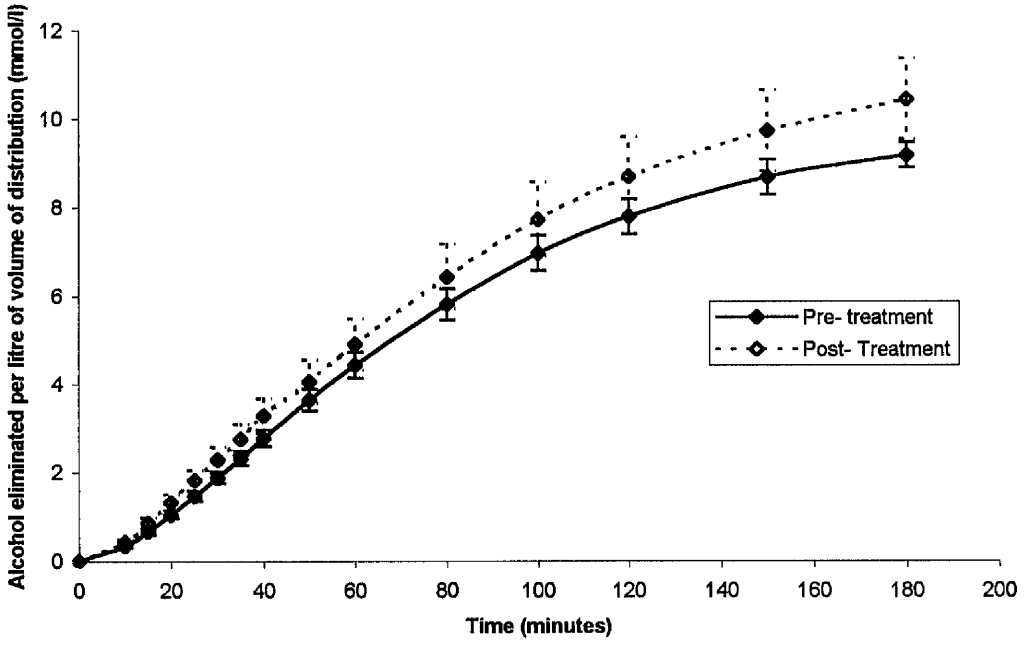

Figure 6.5 Cumulative dose of alcohol eliminated/ volume of distribution versus time- Successful eradication group

Mean cumulative amount of alcohol eliminated per litre of volume of distribution, for the successful eradication group. Error bars show the standard error of the mean. There is no significant difference pre- and post- eradication (9.46 versus 10.45 mmol/l, respectively, p=0.17).

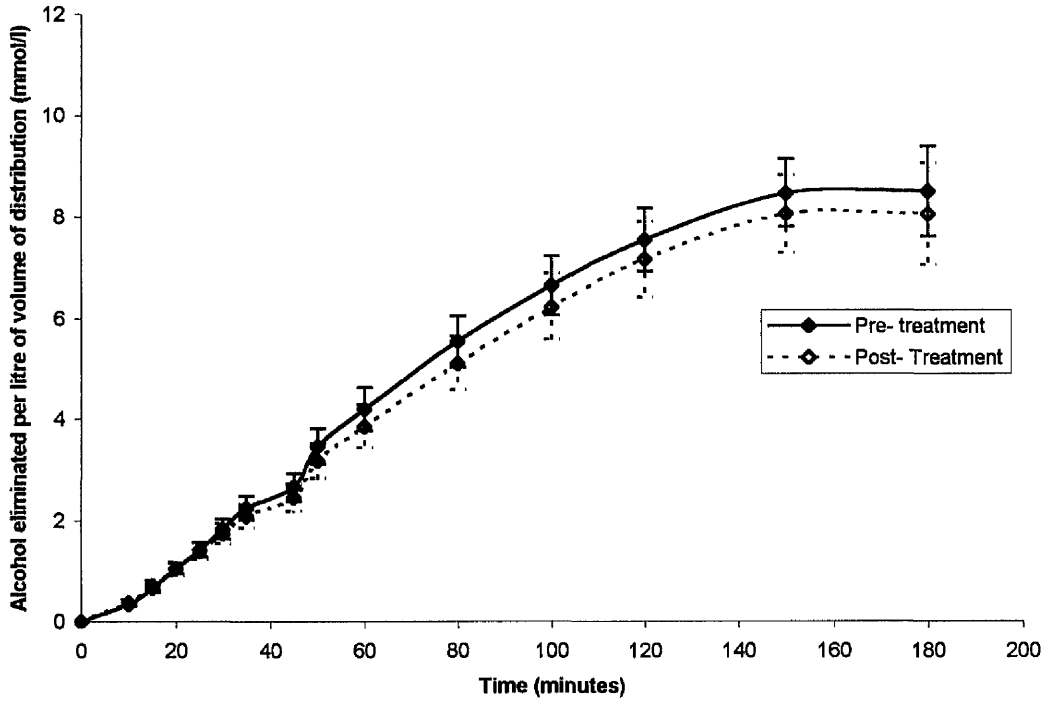

Figure 6.6 Cumulative dose of alcohol eliminated/ volume of distribution versus time- Unsuccessful eradication group

Mean cumulative amount of alcohol eliminated per litre of volume of distribution, for the unsuccessful eradication group. Error bars show the standard error of the mean. There is no significant difference pre- and post- eradication (9.07 versus 7.76 mmol/l, respectively, p=0.36).

6.5 Discussion

This study has demonstrated that a rise in GADH activity does not change the pre- systemic metabolism of alcohol. The relative importance of GADH in pre-systemic alcohol metabolism, and therefore its role in determining susceptibility to alcohol related disease, has been hotly debated over recent years (Gentry et al., 1994a), (Levitt, 1994a), (Gentry et al., 1994b). Factors affecting GADH activity were discussed in detail in Chapter 2.

Recruitment was deliberately restricted to non- elderly male subjects of Caucasian ethnic origin. They were not taking any medication likely to interfere with GADH, and a period of several weeks was scheduled between eradication therapy and repeat alcohol administration. The test meal (Lin et al., 1976) and alcohol concentration (Roine et al., 1991) were chosen to reasonably delay gastric emptying, as this is associated with increased pre- systemic metabolism (Oneta et al., 1998). The study was designed in this way to maximize the likelihood of the subjects having high potential GADH activity post eradication and hence a significant gastric pre- systemic metabolism, if this indeed exists, and to minimise variation in hepatic alcohol metabolism and gastric emptying. The median baseline bioavailability of approximately 60– 65% implies that the study design has been successful in facilitating significant pre- systemic metabolism.

The discovery that HP eradication increases GADH activity (Gupta et al., 1994), (Simanowski et al., 1998), (Kechagias et al., 2001) has allowed researchers an opportunity to assess the importance of GADH by increasing its activity.

Previously, researchers studying gastric first pass metabolism only had the opportunity to lower GADH activity with medication (discussed in Chapter 2), or alter gastric motility (Amir et al., 1996), (Dziekan et al., 1997), (Kechagias et al., 1998), (Oneta et al., 1998). To date there has been only one study, which was of a similar size to this study, addressing the effect of HP eradication on alcohol first pass metabolism (Simanowski et al., 1998). Its findings were in contrast to the present study, as it suggested that treatment for HP was associated with an increase in alcohol first pass metabolism (i.e. a fall in alcohol bioavailability). However, that study can be criticised on a number of points. Firstly, no attempt was made to estimate gastric emptying time. Secondly, an unusual eradication regime was given (amoxycillin and ranitidine for 2 weeks), which the authors admit is likely to suppress but not eliminate HP, and administration of alcohol took place only 5 days after this treatment had stopped. They also failed to take account of the non- linear pharmacokinetics of alcohol. In the present study, the lack of positive results in the subjects who received eradication therapy but remained HP positive suggests that there were no significant effects due to our eradication medication.

Frezza et al., 1990 suggested that lower GADH activity in women may account for their increased susceptibility to alcohol secondary to higher bioavailability. They found a significant correlation between GADH activity and the extent of first pass metabolism. No such correlation was identified in the present study, or in other studies (Oneta et al., 1998), (Simanowski et al., 1998). However, the Frezza study also made the common mistake of assuming that the dose of absorbed alcohol could be calculated by methods which are appropriate only to

first- order drugs (Sweeney, 1990). The AUC may provide a useful marker for the extent of exposure of the body to alcohol, but it should not be regarded as proportional to the total dose of alcohol absorbed. Later studies by this group (Baraona et al., 2001), (Amir et al., 1996), (Roine et al., 1991), (Gentry et al., 1999), (Lim et al., 1993), (Di Padova et al., 1992) and other researchers (Lin et al., 1976), (Levitt et al., 1997) have employed the integration methods that was also used in the present study.

The overall bioavailability of alcohol will vary with the rate of gastric emptying (discussed in detail in Chapter 2). Therefore, the stomach may increase first pass metabolism purely because it delays the transit of alcohol into the small intestine, even without consideration of the effects of GADH. In addition, delayed gastric emptying will increase the exposure time of luminal alcohol to GADH. In this study, as in others, (Oneta et al., 1998), it was therefore considered crucial to have some indication of the rate of gastric emptying. A significant correlation between alcohol bioavailability and gastric emptying time was demonstrated. Other studies have reported either a direct correlation (Oneta et al., 1998), (Pedrosa et al., 1996), or a strong association between bioavailability and gastric emptying (Amir et al., 1996), (Kechagias et al., 1998), (Dziekan et al., 1997), (Levitt et al., 1997). Unmeasured differences in gastric emptying rates may account for the different result observed by Simanowski et al., 1998.

In this study, as in many other studies concerned with alcohol first pass metabolism, a choice was made not to administer alcohol intravenously (Dziekan et al., 1997), (Kechagias et al., 1998), (Mallat et al., 1994), (Lin et al., 1976),

(Roine et al., 1991), (Roine et al., 1990), (Roine et al., 1992a), (Palmer et al., 1991), (Kawashima et al., 1996). The advantage of IV administration is that it allows calculation of volume of distribution, hence allowing calculation of total dose absorbed, and in situations where there is repeated testing, it may correct for differences in metabolic rate or volume of distribution. However, because this study is comparing the dose absorbed on two occasions, provided the volume of distribution remains constant, then it does not need to calculated in order to draw a comparison. An estimated volume of distribution was used to provide an estimate of bioavailability, but the accuracy of this estimate does not affect the change in bioavailability- this is why the p value for the change in absorbed dose/ Vd is the same as that for the change in bioavailability (Tables 6.2 and 6.3).

The other potential advantage of IV administration, namely the correction of day to day variability, is difficult to justify with regard to alcohol. In studies where alcohol has been administered intravenously prior to an oral dose (Amir et al., 1996), (Oneta et al., 1998), (Pedrosa et al., 1996), (Pozzato et al., 1994), (Gentry et al., 1999), (Battiston et al., 1997), (Casini et al., 1994), (Lim et al., 1993), (Di Padova et al., 1992), (Caballeria et al., 1989b), (Simanowski et al., 1998), the IV study has not been performed on the same day as oral administration. Whilst this minimises any systematic effect on hepatic ADH of a prior alcohol load (e.g. depletion of cofactors, altered redox potential), by it will obviously not correct for day to day variation in alcohol metabolism. One study (Ammon et al., 1996) gave IV and oral alcohol simultaneously, using a heavy isotope technique to separately analyse blood alcohol derived from IV and oral routes. This approach

was flawed, however, as the additional alcohol from the IV route will increase hepatic ADH saturation and therefore decrease hepatic FPM.

Studies in which IV administration of alcohol was repeated on the same subjects have not shown any systematic change in pharmacokinetic parameters (Amir et al., 1996), (Oneta et al., 1998), (Pedrosa et al., 1996), (Gentry et al., 1999), (Di Padova et al., 1992), except for some studies in which Cimetidine was given (Pozzato et al., 1994), (Battiston et al., 1997). However, despite a change in AUC in these studies, volume of distribution remained the same. It was therefore felt that a systematic effect on the volume of distribution was very unlikely, and that IV alcohol administration was unnecessary.

The aim of this study was to test the hypothesis that GADH significantly contributes to alcohol bioavailability. Despite a rise in GADH activity, the results showed no overall change in bioavailability. As this was a small study (although comparable in size to most other work in this field), the possibility of type II error should be considered. However, from interpretation of the 95% confidence intervals, these results would be consistent with at most a 7% relative decrease (4.5% absolute decrease) in the dose of alcohol absorbed following eradication therapy, and hence this study is unlikely to be failing to detect an increase in first pass metabolism of any magnitude.

6.6 Conclusion

Under these test conditions, a rise in gastric alcohol dehydrogenase activity following *Helicobacter pylori* eradication is not associated with a change in total dose of absorbed alcohol, and was not associated with a change in gastric emptying. This provides further evidence to suggest that gastric alcohol dehydrogenase is unlikely to play any clinically significant role in the pre-systemic metabolism of alcohol. The eradication of *Helicobacter pylori* is unlikely to confer any advantages in terms of decreased systemic exposure to alcohol.

SECTION 3 STUDY OF THE CHANGES IN HEPATIC BIOCHEMICAL TESTS AND MARKERS OF FIBROSIS FOLLOWING ALCOHOL WITHDRAWAL

Chapter 7: Effect of alcohol withdrawal on liver transaminase levels and markers of liver fibrosis

7.1 Introduction

In clinical practice it has been observed that some alcoholic patients deteriorate both clinically and biochemically over a few days following withdrawal from alcohol. Case reports and studies of alcoholic hepatitis (Sabesin et al., 1978), (Helman et al., 1971) also suggest biochemical liver function tests may worsen after alcohol withdrawal. However, there have only been a few studies addressing hepatic biochemical changes during alcohol withdrawal in subjects across the full spectrum of alcoholic liver disease. These studies were mostly retrospective, and their results were conflicting (Hemmingsten et al., 1980), (Nielsen, 1965), (Marshall et al., 1983), (Salum, 1972)

Abrupt alcohol withdrawal might cause liver damage by several mechanisms, such as worsening of hepatic hypoxia, or an increase in cytochrome- dependent xenobiotic metabolism, which were discussed in detail in Chapter 1.

Studies of fibrotic markers have shown an increase in collagen metabolites following alcohol withdrawal. However, the conclusions of these studies are unclear because of difficulties in interpreting the results with the type of assay used (Risteli and Risteli, 1995).

The aim of this study was to determine the pattern of change of standard biochemical tests in alcohol withdrawal, and to use non-invasive markers of liver disease to look for further evidence of hepatic damage.

7.2 Methods

7.2.1 Patients

Twenty- two male patients (median age 44, range 29-65), all of whom had consumed >150g of alcohol daily for >1 year (median 10 years) were recruited from inpatient admissions to the alcohol rehabilitation unit at Ruchill Hospital, Glasgow. Admissions were asked consecutively to participate in the study, provided they were not excluded by the criteria below. Patients were admitted for elective alcohol withdrawal, rather than management of liver disease. A patient population with clinically compensated liver disease was chosen for this study. Acute illness often precipitates alcohol withdrawal in chronic alcoholics, and could potentially confound the results. Subjects with evidence of a medical condition precipitating alcohol withdrawal or who had hepatic decompensation (jaundice, ascites, encephalopathy, or gastrointestinal haemorrhage) at baseline were therefore excluded. Patients with evidence of liver disease of another aetiology (positive hepatitis A, B, or C serology, positive anti-mitochondrial, anti-smooth muscle or anti-nuclear antibody, or a history of gallstones) were excluded from the study. These subjects were managed as inpatients for 1 week following alcohol withdrawal, and they were asked to return as outpatients

during the following week. They were withdrawn from the study if they resumed drinking. All subjects gave informed written consent and the local ethics committee approved this study. This study conforms to the ethical guidelines of the 1975 Declaration of Helsinki.

7.2.2 Clinical measurements

Patients were examined daily for signs of liver disease and possible complications such as infection, gastro-intestinal bleeding, ascites, oedema or pancreatitis. An estimate of body composition was made on admission by measurement of body mass index (BMI), triceps skin fold thickness and mid upper arm circumference. The percentage body fat was calculated from these measurements (Lean et al., 1996). In addition, daily measurement of alcohol withdrawal symptoms were performed by ward nursing staff, using the Clinical Institute Withdrawal Assessment Scale for Alcohol (revised):- CIWA-Ar (Sullivan et al., 1989). Alcohol intake was assessed on the basis of clinical history obtained by medical and nursing staff experienced in alcohol dependence.

7.2.3 Laboratory measurements

7.2.3.1 Standard hospital laboratory tests

The first blood samples were obtained on hospital admission within 72 hours of last alcohol consumed (median 22 hours). These samples were processed as part of routine hospital assays by the hospital laboratory personnel for the following: alanine transaminase (ALT), albumin, alkaline phosphatase (ALP), amylase, aspartate transaminase (AST), bilirubin (BR), blood ethanol, coagulation profile, creatine kinase (CK), full blood count, gamma glutamyl transferase (GGT), urea and electrolytes. These were repeated daily for seven days. The patients were usually discharged at day 7, and returned on day 14 to repeat these tests. The arbitrary criteria used in this study to define worsening of liver function tests were: a 50% rise from the baseline value, with the elevated result outwith the normal laboratory reference range. This was chosen prior to commencement of the study in an attempt to exclude values that rose simply as a result of minor biological and analytical variation. Continuing abstinence was assessed by blood alcohol measurement, random breath alcohol measurement, and self- reporting.

7.2.3.2 Markers of hepatic fibrogenesis

Plasma samples from day 1, 7 and 14 were frozen at –20°C within 4 hours of being obtained, and later analysed in one batch, to minimise analytical variation. Two different commercial radio-immunoassay kits were used for amino-terminal pro-collagen III peptide (PIIINP). They were manufactured by Orion Diagnostica (Espoo, Finland), and Cis Bio International (Paris, France). Tissue inhibitor of metalloproteinase-1 (TIMP-1) was assayed using a commercial colorimetric ELISA assay from Amersham Life Science (Buckinghamshire, England). All

plasma samples were analysed in duplicate. Our intra-assay coefficients of variation for these assays were: TIMP1:- 2.9%, PIIINP (Orion):- 4.8%, PIIINP (Cis):- 4.8% at levels measured in the present study. Plasma from 11 healthy age-matched males who had no history of excess alcohol consumption or physical illness was used to provide a normal range for PIIINP and TIMP1.

The Orion assay, which uses an antibody to human PIIINP, is specific for the intact pro-collagen peptide in human serum (Risteli et al., 1988), and is regarded as a marker for collagen synthesis (Schuppan et al., 1995). Two hundred microlitres of the plasma sample were mixed with a known amount of ^{125}I labelled PIIINP and then with PIIINP rabbit antiserum. The rabbit antiserum-PIIINP complex was separated by addition of bovine anti- rabbit antibody bound to plastic beads that were retained after aspirating the remainder of the fluid after centrifugation. The residual radioactivity is therefore inversely proportional to the amount of PIIINP present in the plasma sample. Dilutions of a standard PIIINP solution provided by the manufacturer were used to create a curve of radioactivity versus PIIINP concentration to allow determination of plasma PIIINP concentration from corresponding radioactivity counts.

The Cis assay uses an antibody to bovine PIIINP and it is sensitive for both intact PIIINP and smaller PIIINP fragments in human serum (Rohde et al., 1979). It is regarded as a marker for both collagen synthesis and degradation (Schuppan et al., 1995). The assay tubes supplied by the manufacturer are pre-coated with PIIINP antibody. Twenty microlitres of plasma were added to each tube. After a period of incubation and decantation of the sample fluid, ^{125}I

labelled anti-PIIINP antibody was added. After further incubation and decantation, the activity in each tube was measured. This is proportional to the amount of ^{125}I labelled antibody- PIIINP- solid phase antibody complex present, and hence the radioactivity varies directly with the amount of PIIINP in each plasma sample. Measurement of the activity of dilutions of the manufacturer's standard was used to construct a curve from which plasma PIIINP concentration could be determined.

For both radioimmunoassays, activity was measured on a Beckman- Coulter LS gamma counter (Beckman Coulter Ltd., High Wycombe, Buckinghamshire, UK), with integrated software that performed calculations for the standard curve.

The TIMP1 assay is a colorimetric ELISA assay that uses a 96- well microtitre plate method. The wells of the plate supplied by the manufacturer are coated with anti human TIMP1 antibody. The plasma samples were diluted with assay buffer to a dilution of 1:10 for control subjects, and a dilution of 1:20 (or higher if required) for the alcoholic subjects. One hundred microlitres of the diluted plasma sample or dilutions of the manufacturer's standard were added to each well. After incubation and aspiration, a solution of TIMP1 antibody conjugated with horseradish peroxidase (HRP) was added. After further incubation and aspiration, 3, 3',5, 5' -tetramethylbenzidine was added. This is oxidised by HRP, undergoing colour change. The amount of peroxidase enzyme (present as a HRP labelled antibody- TIMP1- solid phase antibody complex), and hence the extent of colour change, is therefore proportional to the amount of TIMP1 in the plasma sample. After further incubation, the HRP reaction was terminated by sulphuric

acid, resulting in a further colour change. The absorbance at 450nm was measured using a Dynatech MR5000 automated microtitre plate reader (Dynatech Labs Ltd., Billinghurst, West Sussex, UK). An absorbance versus concentration curve was obtained from dilutions of the manufacturer's standard, and integrated software was used to estimate the sample TIMP1 concentrations from the standard curve.

7.3 Statistics

In the comparison of baseline data, unpaired normal data were analysed using Student's t-test, after square root of logarithmic transformation, where appropriate. Non-parametric data were analysed using the Mann-Whitney U Test. Estimation of the 95% confidence interval for prevalence, and calculation of the coefficient of variation by one- way analysis of variance, were performed by the methods described by Bland (Bland, 1995). For the analysis of repeated measures data, within-subjects analysis of variance was used (Tabachnick and Fidell, 1996). All tests were two- tailed, and statistical significance was taken as $p<0.05$. Statistical analyses were performed using SPSS version 9.0 (SPSS Inc, Chicago, Illinois, USA) and Minitab version 11.2 (Minitab Inc, Philadelphia, Pennsylvania, USA).

7.4 Results

Data were analysed on an intention to include basis. Twenty- one (95%) of the subjects remained in the study for the first week to complete the initial intensive assessment phase, and 13 subjects (59%) remained on Day 14.

7.4.1 Biochemical liver function tests

Gamma GT, bilirubin, ALP, and CK all tended to improve or remain the same in the seven days following alcohol withdrawal (data not shown). Aspartate amino-transferase and ALT also tended to improve in the majority of subjects (Figure 7.1), and there was no significant change in transaminase levels when the group was considered as a whole, but a number of subjects did show a marked worsening in liver transaminase levels (Figure 7.2).

The change in transaminases was of similar proportion for AST and ALT. Using the pre-defined criteria described above we separated the subjects into 2 groups, shown graphically as an improving transaminase group (Figure 7.1) and as a worsening transaminase group (Figure 7.2). Seven out of 22 subjects enrolled showed a worsening in transaminases (32%, 95% confidence interval 12%-51%). Despite a rise in ALT, subject A in Figure 7.1 was included in the improving transaminase group as he did not meet the pre- defined criteria for the other group.

There was no statistically significant difference between the groups with respect to the baseline biochemical, haematological, or clinical parameters (Table 7.1). The normal or mildly elevated baseline biochemical results for both groups are consistent with our intention to recruit subjects with clinically compensated liver disease.

The group with worsening transaminases received significantly greater amounts of chlordiazepoxide (median 140mg per day) during the first week of admission compared to the remaining subjects (median 36mg per day), p=0.003. The CIWA scores were not different between the two groups (median 16, worsening transaminase group, vs. 12, improving transaminase group, p=0.3).

Only one subject received paracetamol containing medication (subject B in Figure 7.2). He took a total of 1g of paracetamol on days 3, 4, 6 and 8, and 2g on day 7. He had the most marked transaminase rise of any subject in this study.

The rate of complications during admission was similar in the two groups, all of which were respiratory infections (one of seven in the worse transaminase group versus three of fifteen in the improving transaminase group, p>0.9).

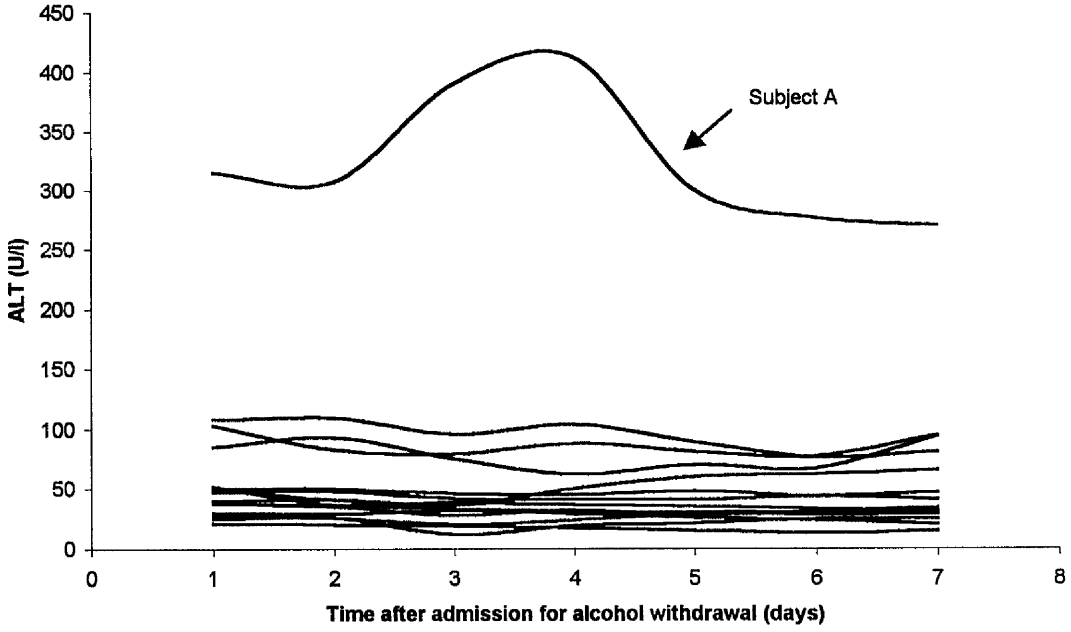

Figure 7.1 Change in serum alanine amino-transferase following alcohol withdrawal- improving transaminase group

This graph shows the serum ALT levels measured for seven days after alcohol withdrawal in the fifteen subjects with improving hepatic transaminases (ALT reference range 5- 40 U/l). Subject A showed a transaminase rise but did not meet pre- defined criteria for inclusion in the worsening transaminase group (see text).

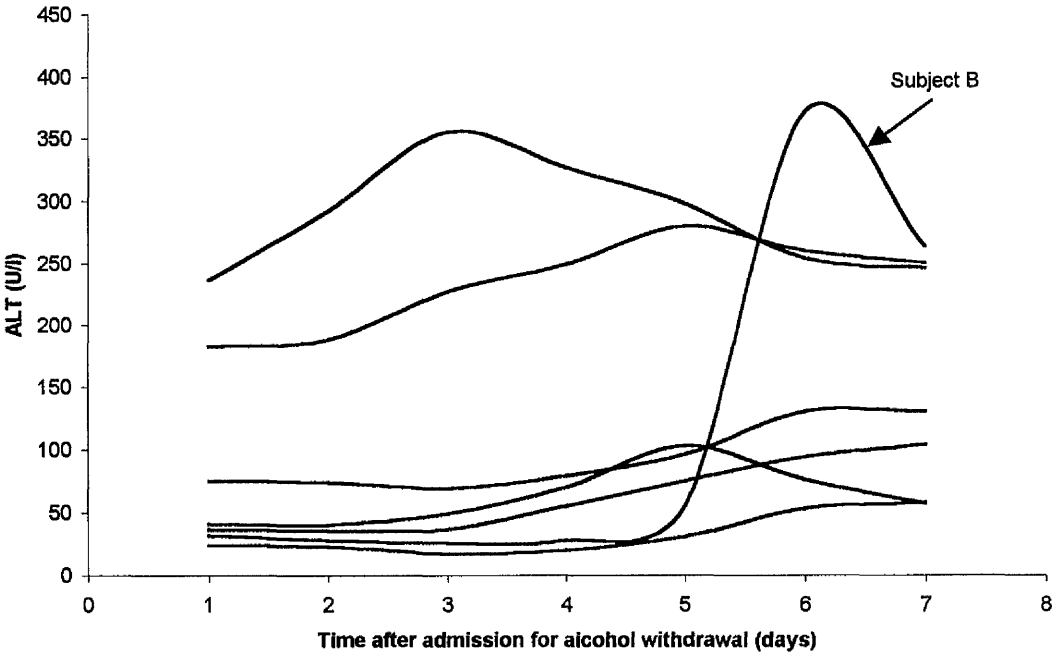

Figure 7.2 Change in serum alanine amino-transferase following alcohol withdrawal- worsening transaminase group

This graph shows the serum ALT levels measured for seven days after alcohol withdrawal in the seven subjects with worsening hepatic transaminases (ALT reference range 5- 40 U/l). Subject B was the only subject to have taken paracetamol (see text).

Table 7.1 Comparison of day one clinical and laboratory parameters between the group of subjects with worsening transaminases and the others

Baseline clinical, biochemical or haematological parameter	Group with worsening transaminases	Group without worsening transaminases	p value
Age (years)	42 (9.0)	45 (11.5)	0.56
Alcohol consumed daily (g)	358 (129)	362 (126)	0.95
Years of alcohol abuse	11 (1.75- 17)*	10 (4- 20)*	0.47
Body fat (percentage)	22 (6.3)	18 (5.9)	0.16
Cigarettes smoked per day**	20 (0- 40)*	20 (9- 35)*	0.97
Duration of abstinence prior to admission (hours)	6.5 (1.6- 31.5)*	24 (13- 45)*	0.12
Blood ethanol (mg/dl) **	0 (0- 147.2)*	0 (0- 36.5)*	0.96
Albumin (g/l)	42 (4.0)	44 (4.4)	0.47
AST (U/l)	60 (32- 120)*	50 (31.0- 134.0)*	0.99
ALT (U/l)	75 (32- 183)*	43 (28- 96)*	0.49
Bilirubin (μmol/l)	13 (6- 19)*	8 (5- 15)*	0.37
Gamma-GT (U/l)	178 (66- 309)*	69 (53- 233)*	0.51
Alkaline Phosphatase	192 (53)	216 (51)	0.39
Haemoglobin (g/l)	146 (9.5)	154 (9.8)	0.10
Amylase	160 (55)	213 (132)	0.84
Creatine kinase (U/l)	81 (71- 137)*	49 (38- 97)*	0.21
White blood cell count ($x10^9$/litre)	6.8 (5.9- 9.5)*	6.6 (5.5- 8.1)*	0.58
Prothrombin Time (seconds)	12 (0.6)	12.4 (0.7)	0.37
PIIINP (U/ml, Cis method)	0.36 (0.30- 0.74)*	0.46 (0.31- 0.51)*	0.64
PIIINP (ug/l Orion method)	2.82 (1.11- 5.67)*	2.44 (1.76- 3.52)*	0.74
TIMP1 (ng/ml)	1108 (422)	1268 (418)	0.90

Data shown are means with standard deviation in brackets, except: *where data are non- parametric, and medians are shown with inter-quartile range in brackets. Two -sample (unpaired) Student's t-test performed throughout (with transformation, if appropriate), except: ** Mann- Whitney U-test.

7.4.2 Markers of hepatic fibrogenesis

The TIMP1 levels were markedly higher in the alcoholic group than in the control group (mean 1177 vs. 227ng/ml, $p<0.001$). The PIIINP concentrations from the alcoholic patients were significantly lower than in control subjects in the Cis assay (median 0.43 vs. 0.53 U/ml, $p=0.03$), but not in the Orion assay (median 2.57 vs. 3.23 ug/l, $p=0.96$), implying that the difference between these groups is predominantly because of the PIIINP fragments (Figure 7.3).

There was an increase in the PIIINP levels following alcohol withdrawal in both assays (Orion $p<0.02$, Cis $p<0.01$). The percentage median increase was: Orion 44% and 75%, Cis 10% and 42% at days 7 & 14, respectively. As the major rise was seen with the Orion assay, this would be consistent with a rise mainly caused by intact PIIINP. The TIMP1 levels did not change following alcohol withdrawal ($p=0.22$), (Figure 7.3).

There was no significant difference between the group with improving transaminases and the others with respect to day 1 TIMP1 or PIIINP measured by either assay (Cis median 0.36U/ml vs. 0.46U/ml, $p=0.64$; Orion median 2.82ug/l vs. 2.44ug/l, $p=0.74$; TIMP1 mean 1108ng/ml vs. 1268ng/ml, $p=0.9$, in worsening and improving transaminase groups, respectively).

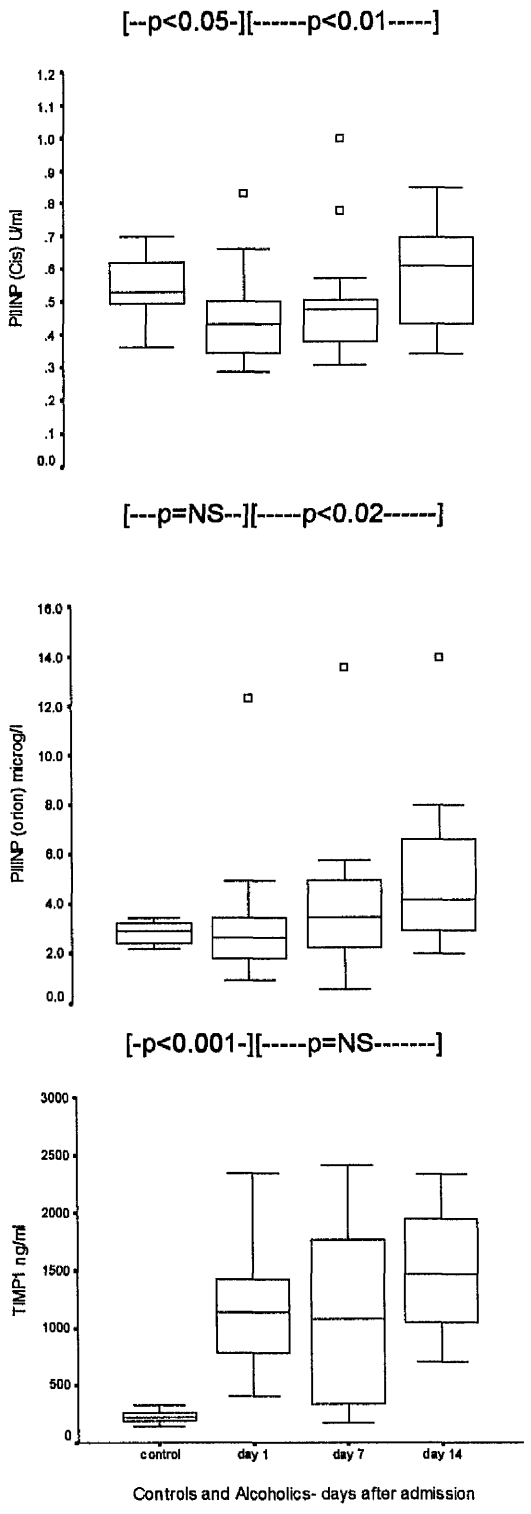

Figure 7.3 Markers of hepatic fibrogenesis following alcohol withdrawal
Boxplot showing plasma concentrations of PIIINP (Cis), PIIINP (Orion) and TIMP1 in controls and alcoholics undergoing alcohol withdrawal. The large boxes represent the inter-quartile range. The horizontal bar represents the median. □ represents outlying values. Statistical significance is indicated for the difference between alcohol withdrawal subjects and control subjects, and for the individual difference in alcoholics following withdrawal. NS= non-significant.

7.5 Discussion

The pathophysiology of hepatic damage in alcoholism is not fully understood. However, there is evidence for mechanisms by which hepatic damage may occur in alcohol withdrawal, which were discussed in Chapter 1.

The final outcome of long-term liver damage in alcoholic liver disease is cirrhosis. A large proportion of the collagen deposited, especially in the early stages of cirrhosis, is type III (Popper and Udenfriend, 1970). The precursor molecule of type III collagen, type III procollagen, is secreted with peptide extensions called propeptides (PIIIP) attached to the amino (PIIINP) and carboxy (PIIICP) terminals. Intact PIIINP is released when procollagen molecules aggregate to form the type III collagen fibre (Prockop et al., 1979). However, some PIIINP is not released from collagen after the collagen fibre has been formed. Type III collagen fibres with PIIINP still attached have been demonstrated in primate liver (Sato et al., 1986a). If collagen to which PIIINP remains attached is subsequently degraded, then smaller fragments of PIIINP may be released. Thus, a PIIINP assay that is sensitive to both intact PIIINP and PIIINP fragments reflects both synthesis and degradation (the Cis assay) (Hahn, 1984; Schuppan et al., 1995). Assays measuring intact PIIINP only (the Orion assay) reflect mainly collagen synthesis (Risteli et al., 1988; Schuppan et al., 1995). Plasma PIIINP levels correlate well with hepatic prolyl hydroxylase levels (Torres-Salinas et al., 1986). Direct mathematical comparison of the two assays was not possible, but by comparing the pattern of changes in these two

assays, this study has attempted to determine whether a change in PIIINP is a result of changes in intact PIIINP or PIIINP fragments. There was no assay available to measure PIIINP fragments alone.

The PIIINP can be metabolised by hepatic sinusoidal cells (Smedsrod, 1988), and serum levels may therefore be affected by changes in clearance resulting from a decrease in hepatic blood flow during alcohol withdrawal.

Failure to degrade excess collagen is important in the development of hepatic cirrhosis. The role of TIMP1 in inhibiting collagen degradation was discussed in Chapter 1. There is good evidence that plasma levels of TIMP1 give an accurate reflection of liver collagen metabolism, when other serious systemic diseases can be excluded. Hepatic collagenase activity decreases as hepatic fibrosis progresses (Maruyama et al., 1982). Plasma TIMP1 levels correlate with fibrosis scores on liver biopsy (Murawaki et al., 1993; Murawaki et al., 1997). Plasma TIMP1 (and PIIINP) concentrations rise as alcoholic liver disease progresses in histological severity (Sato et al., 1986b; Nouchi et al., 1987; Li et al., 1994). Plasma TIMP1 levels are not known to be influenced by hepatic blood flow.

Although there was no overall change in transaminases following alcohol withdrawal when considering all the subjects, there was a subset showing a marked transaminase increase. The subject with the largest transaminase rise was the only subject to consume paracetamol, albeit at modest doses. The potential for paracetamol-induced hepatotoxicity (Lieber, 1993) might therefore be markedly increased, being manifest even if a dose well below the recommended

daily maximum dose was ingested at a critical time following alcohol withdrawal.

A large retrospective Scandinavian study (Salum, 1972) showed an increase in AST and ALT in 8.7% and 7.0% respectively of subjects withdrawing from alcohol. However, liver function tests were only checked on the basis of clinical need, and in most subjects these were not checked on more than two occasions during the first week of withdrawal. They found that an elevation in transaminases was associated with more severe symptoms of delirium tremens, which is supported by the finding in the present study of a greater requirement for benzodiazepines. The worsening transaminase group are likely to have experienced a symptomatically more severe alcohol withdrawal despite similar CIWA scores, because they required more benzodiazepines, which will tend to reduce their CIWA score. Sympathetic over-activity contributes to the symptoms observed in alcohol withdrawal and can further increase hepatic oxygen demand (Hadengue et al., 1988) and hence those with more severe symptoms may also have a greater degree of hepatic ischaemia resulting in altered transaminases.

A retrospective study of liver function tests and liver biopsy findings in alcohol withdrawal suggested that those with worsening liver function tests might have a histologically more severe disease (Marshall et al., 1983). The retrospective nature of that study may have introduced bias. In contrast, in the present study, no difference in biochemical or clinical parameters was found to suggest the subgroup with worsening transaminases had more severe disease. It was felt

unethical to perform liver biopsy in these subjects with clinically compensated disease.

The control subjects in this study appear to have a higher level of PIIINP fragment than alcoholic subjects on day 1. This observed difference may have resulted from a decreased collagen degradation in alcoholics whilst still drinking, which would be consistent with the findings of increased serum TIMP in alcoholics. Lieber's group also noted a higher PIIINP level in controls versus alcoholics with fatty liver when using an assay that was very sensitive for PIIINP fragments, but not when using a different assay which was less sensitive (Sato et al., 1986b; Nouchi et al., 1987; Li et al., 1994). Lieber's group has also shown that alcoholics have an increase in PIIINP following alcohol withdrawal (Nouchi et al., 1987; Li et al., 1994). However, these studies did not employ an assay exclusively for intact PIIINP and were hence unable to conclude if the observed changes were likely to be due to intact peptide or peptide fragments.

This study demonstrates a rise in PIIINP following alcohol withdrawal that is likely to be due to intact PIIINP. Although this may imply increased fibrogenesis, it may reflect decreased hepatic clearance. The marked increase in TIMP1 in alcoholics compared to controls suggests that there may be significant inhibition of collagen degradation even in this group of patients with clinically compensated disease.

7.6 Conclusions

These results suggest that in alcoholics with clinically compensated liver disease, alcohol withdrawal is usually accompanied by an improvement in liver transaminase levels. However, a minority of subjects show a worsening of transaminases. The period following alcohol withdrawal may be a time of marked increased susceptibility to the effects of even small amounts of paracetamol. Although the changes observed in transaminases and PIIINP are small and not associated with major differences in short-term clinical outcome, they may reflect mild liver injury and either a minor increase in fibrosis or a decrease in hepatic clearance. The cumulative effect of repeated episodes of withdrawal may be important, and hence further studies looking at the effect of gradual withdrawal of alcohol on clinical and biochemical parameters would be of value. The most striking observation is the markedly increased plasma TIMP1 levels in these alcoholics compared with controls, supported by the finding of decreased PIIINP fragments, suggesting that decreased collagen degradation may be present prior to the development of clinically overt liver disease.

SECTION 4 CONCLUSIONS

Chapter 8-Conclusions

This thesis has three major conclusions. Firstly, it concludes that a colorimetric assay based on the reduction of N, N-dimethyl-4-nitrosoaniline (NDMA) by alcohol dehydrogenase in the presence of an alcoholic substrate offers significant advantages over conventional ultraviolet spectrophotometric methods for the detection of alcohol dehydrogenase. The NDMA method developed in his thesis is sensitive, specific, robust, and can be readily applied to the detection of gastric alcohol dehydrogenase (GADH) activity in the small amount of tissue in standard endoscopic gastric biopsies. Use of an incubation stage to process the biopsies allows the preparation time associated with a homogenisation stage to be eliminated, without a significant loss in the enzyme activity detected.

Secondly, it has been demonstrated that a rise in gastric alcohol dehydrogenase activity following *Helicobacter pylori* eradication is not associated with a change in total dose of absorbed alcohol, and was not associated with a change in gastric emptying, despite using test conditions designed to maximise any potential first pass metabolism of alcohol as a consequence of GADH activity. This suggests that that this enzyme plays at most a minor role in determining alcohol bioavailability. The eradication of *Helicobacter pylori* is unlikely to confer any advantages in terms of decreased systemic exposure to alcohol.

Thirdly, the study of alcoholics with clinically compensated liver disease suggested that alcohol withdrawal is usually accompanied by an improvement in liver transaminase levels. However, a minority of subjects show a worsening of transaminases, and there was evidence of an increased susceptibility to the

effects of small amounts of paracetamol ingested during the withdrawal period. The changes observed in transaminases and markers of hepatic fibrogenesis were small and not associated with a difference in short-term clinical outcome. However, these changes may reflect mild liver injury and either a minor increase in fibrosis or a decrease in hepatic clearance. The cumulative effect of repeated episodes of withdrawal may be important, and hence further studies looking at the effect of gradual withdrawal of alcohol on clinical and biochemical parameters would be of value. The markedly increased plasma TIMP1 levels in these alcoholics compared with controls, supported by the changes observed in procollagen III peptide, suggesting that decreased collagen degradation may be present prior to the development of clinically overt liver disease.

SECTION 5 APPENDIX AND REFERENCES

Appendix: Equations relating to alcohol pharmacokinetics

A.1 The Widmark Equation

This equation represents the earliest attempt to describe the elimination of alcohol mathematically, and was based on the assumption that the elimination rate of alcohol is constant regardless of the blood alcohol concentration (Mellanby, 1919).

$$A = p \cdot r \cdot (C_T + \beta \cdot t) \qquad (1) \text{ The Widmark equation}$$

Where A= amount of alcohol absorbed, C_T= blood concentration at a given time t, p= body weight, β is Widmark's constant (equivalent to the gradient of the linear part of the BAC versus time curve), and r= fraction of body weight in which alcohol is distributed. Volume of distribution is equivalent to p multiplied by r, and the (hypothetical) dose at time zero (denoted C_0) will equal the amount of alcohol absorbed divided by the volume of distribution. This equation assumes absorption and distribution is complete by time zero. The equation therefore becomes:

$$C_0 = C_T + \beta \cdot t \qquad (2)$$

A.2 The Michaelis- Menten equation

This equation describes the enzyme velocity (V) (which, in turn can be considered as the rate of generation of product, or the rate of consumption of substrate), where S is substrate concentration, V_{max} is maximum enzyme velocity, and K_m is the Michaelis constant, defined as the substrate concentration required to produce a reaction rate which is half of V_{max} (and which is therefore also a measure of enzyme- substrate affinity).

$$V = \frac{V_{max} \cdot S}{K_m + S} \qquad (3)$$ The Michaelis- Menten equation

A.3 Derivation of the integrated form of the Michaelis- Menten equation

The reaction velocity is equal to the rate of change of substrate concentration with time, hence:

$$\frac{dS}{dt} = \frac{V_{max} \cdot S}{K_m + S} \qquad (4)$$

where t is time. By inversion:

$$\frac{dt}{dS} = \frac{K_m + S}{V_{max} \cdot S}$$

$$\Rightarrow t = \int \frac{K_m + S}{V_{max} \cdot S} dS$$

$$\Rightarrow t = \int \left(\frac{K_m}{V_{max} \cdot S} + \frac{S}{V_{max} \cdot S} \right) dS \qquad (5)$$

$$\Rightarrow t = \int \left(\frac{K_m}{V_{max} \cdot S} + \frac{1}{V_{max}} \right) dS$$

$$\Rightarrow t = \ln S \cdot \frac{K_m}{V_{max}} + \frac{S}{V_{max}} + k$$

At time zero (t_0), $S=S_0$, hence:

$$0 = \ln S_0 \cdot \frac{K_m}{V_{max}} + \frac{S_0}{V_{max}} + k$$

$$\Rightarrow k = -\ln S_0 \cdot \frac{K_m}{V_{max}} - \frac{S_0}{V_{max}}$$

Putting this back into equation (5) gives:

$$t = \ln S \cdot \frac{K_m}{V_{max}} + \frac{S}{V_{max}} - \ln S_0 \cdot \frac{K_m}{V_{max}} - \frac{S_0}{V_{max}}$$

$$\Rightarrow V_{max} \cdot t = S - S_0 + K_m \cdot \ln \frac{S}{S_0}$$

Where ln= natural logarithm. As we are interested in the decline of S, rather than an increase, the rate of change gives us a negative velocity, hence the equation becomes:

$$V_{max} \cdot t = S_0 - S + K_m \cdot \ln \frac{S_0}{S}$$

$$\Rightarrow t = \left(S_0 - S + K_m \cdot \ln \frac{S_0}{S} \right) \cdot \frac{1}{V_{max}} \quad (6)$$

With this equation, we can estimate parameters for S_0, K_m and V_{max} using non-linear curve fitting software, by plotting S and t.

A.4 Estimating the total dose of alcohol absorbed by using the Michaelis-Menten equation

The amount of a substance that has been delivered systemically by any given time point must equal the amount present in the body compartment(s) plus the amount eliminated. For a single compartment model, this can be represented as:

$$A_t = Vd \cdot S_t + E_t$$

Where, at time t, A_t=amount of substance delivered systemically, Vd= volume of distribution, S_t= the concentration of the substance (or substrate) in the compartment fluid and E_t= amount eliminated.

As described by Gentry et al., 1992 and Lin et al., 1976, for a drug that obeys Michaelis- Menten elimination kinetics, by integrating the Michaelis- Menten equation for all values of S from t_0 to time t:

$$A = Vd \cdot \left(S_t + \int_0^t \frac{S_t \cdot V_{max}}{K_m + S_t} dt \right)$$

Once absorption is complete, and the substance has been eliminated completely, S_t will equal zero, and the equation therefore becomes:

$$A = Vd \cdot \int_0^t \frac{S_t \cdot V_{max}}{K_m + S_t} dt \qquad (7)$$

A.5 Relationship between total bioavailability and concentration

Total bioavailability is the product of hepatic bioavailability (HB), and the fraction of orally administered dose being absorbed by the gut (f).

$$TB = HB \cdot f$$

Hepatic bioavailability (HB) is the ratio of amount of substance leaving the liver relative to amount entering it.

$$HB = \frac{\text{amount leaving liver}}{\text{amount entering liver}}$$

$$\Rightarrow HB = \frac{\text{amount leaving liver}}{Q \cdot [S]}$$

$$\Rightarrow HB = \frac{Q \cdot [S] - \text{amount metabolised}}{Q \cdot [S]} \qquad (8)$$

$$\Rightarrow HB = \frac{Q \cdot [S] - Cl_T \cdot [S]}{Q \cdot [S]}$$

$$\Rightarrow HB = 1 - \frac{Cl_T}{Q}$$

Where total hepatic clearance is Cl_T, Q is liver portal venous flow, and S is substrate concentration.

In systems approaching hepatic enzyme saturation, and in the absence of significant protein binding, total hepatic clearance (Cl_T) is related to intrinsic hepatic clearance (Cl_{INT}) thus:

$$Cl_T = \frac{Q \cdot Cl_{INT}}{Q + Cl_{INT}}$$

Substituting this into equation (8) gives:

$$HB = 1 - \frac{Cl_T}{Q}$$

$$\Rightarrow HB = 1 - \frac{\frac{Q \cdot Cl_{INT}}{Q + Cl_{INT}}}{Q} \qquad (9)$$

$$\Rightarrow HB = 1 - \frac{Cl_{INT}}{Q + Cl_{INT}}$$

$$\Rightarrow HB = \frac{Q}{Q + Cl_{INT}}$$

Intrinsic hepatic clearance (Cl_{INT}) is a function of hepatic enzymatic activity and for alcohol can thus be described in terms of the Michaelis- Menten equation:

$$\text{clearance} = \frac{\text{amount removed}}{\text{concentration}}$$

Therefore, over a given unit of time,

$$Cl_{INT} = \frac{\frac{V_{max} \cdot S}{K_m + S}}{S}$$

$$\Rightarrow Cl_{INT} = \frac{V_{max}}{K_m + S}$$

Substituting this into (9) gives:

$$HB = \frac{Q}{Q + Cl_{INT}}$$

$$\Rightarrow HB = \frac{Q}{Q + \frac{V_{max}}{K_m + S}}$$

$$\Rightarrow HB = \frac{Q}{\frac{Q \cdot K_m + Q \cdot S + V_{max}}{K_m + S}}$$

$$\Rightarrow HB = \frac{Q(K_m + S)}{Q(K_m + S) + V_{max}}$$

$$\Rightarrow HB = \frac{1}{1 + \frac{V_{max}}{Q(K_m + S)}} \qquad (10)$$

With increasing alcohol dose K_m, V_{max}, and Q are relatively constant (with respect to hepatic bioavailability). Therefore:

$$\lim_{S \to \infty} \left(\frac{1}{1 + \frac{V_{max}}{Q(K_m + S)}} \right) = 1$$

i.e. the hepatic bioavailability will increase towards unity (100%) with increasing substrate concentrations.

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