EFFECTS OF FEEDING ON THE DISPOSITION OF LORAZEPAM AND DIAZEPAM

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By

Cameron Benjamin Neil Szakacs, B.Sc.

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SUMMARY OF DISSERTATION

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CAMERON BENJAMIN NEIL SZAKACS

Department of Pharmacology
University of Saskatchewan

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Examining Committee:

Dr. Bruce Davis	Dean/Associate Dean/Dean's Designate, Chair College of Graduate Studies and Research
Dr. J.S. Richardson	Chair of Advisory Committee, Department of Pharmacology
Dr. R.J. Herman	Supervisor, Department of Pharmacology
Dr. J.M. Tuchek	Department of Pharmacology
Dr. H.A. Semple	College of Pharmacy and Nutrition

External Examiner:

Dr. Dan Sitar
Department of Pharmacology and Therapeutics
University of Manitoba
Winnipeg, Manitoba

Effects of feeding on the disposition of lorazepam and diazepam

The effects of feeding on drug disposition were investigated using two common benzodiazepines, lorazepam and diazepam, as *in vivo* probes for drugs which are metabolized by direct glucuronide conjugation, and oxidative metabolism, respectively.

The effects of feeding on the disposition of lorazepam was examined in IDDM patients. A tightly-controlled ten-part study in four patients showed lorazepam clearance was significantly lower in euglycemic conditions where low amounts of peripheral glucose were matched with low insulin rates, when compared with studies with amounts of intragastric glucose with low (hyperglycemia) and high (euglycemia) amounts of insulin. This suggests that co-substrate (UDPGA) availability may be a rate limiting step in hepatic glucuronidation in man. Renal clearance of lorazepam glucuronide exhibited a similar pattern, suggesting that renal secretion is depressed in a low-energy state. No dose-dependent insulin response, or effect of insulin species was observed for any studied lorazepam parameter. A study in ten IDDM patients indicated that insulin species influences lorazepam glucuronidation. However, this could not be substantiated in a small crossover study with three IDDM patients, or in the ten-part study. High levels of insulin significantly depressed urine production in IDDM patients, presumably through direct action on the kidney. Glucagon had no effect on lorazepam disposition.

An animal model of EHC of lorazepam was developed in the dog. Interruption of EHC resulted in lower plasma lorazepam concentrations increased lorazepam clearance rates, and decreased volume of distribution. Lorazepam and lorazepam glucuronide concentrations were 10-100 fold greater in bile than in plasma, suggesting a pronounced biliary secretion.

The effects of feeding on diazepam disposition showed that postprandial diazepam peaks are not due to EHC. Feeding caused a decrease in diazepam free

fraction, suggesting that the peaks are the result of changes in plasma protein binding. Perturbation of plasma protein binding with heparin and fat emulsion were in agreement with this conclusion. Concentrations of unbound diazepam increased following meals, suggesting that decreased hepatic metabolism, as well as redistribution from tissues may also play a role in the mechanism of postprandial diazepam peaks.

BIOGRAPHICAL

1963	Born in Kipling, Saskatchewan
1986	B.Sc.(Chemistry), University of Saskatchewan

HONOURS

College of Medicine Arthur Smythe Memorial Scholarship, 1994
Medical Research Foundation of Saskatchewan Ph.D. Scholarship, 1993
College of Medicine Graduate Teaching Fellowship, 1993
Canadian Society for Clinical Investigation Trainee Award, 1991
College of Graduate Studies Ph.D. Scholarship, 1991, 1992
College of Graduate Studies Masters Scholarship, 1989, 1990
Government of Saskatchewan General Proficiency Scholarship, 1981

PUBLICATIONS

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ABSTRACT

The effects of feeding on drug disposition were investigated using two common benzodiazepines, lorazepam and diazepam, as *in vivo* probes for drugs which are metabolized by direct glucuronide conjugation, and oxidative metabolism, respectively.

The effects of feeding on lorazepam disposition were examined in insulin-dependent diabetes mellitus (IDDM) patients. A tightly-controlled ten-part study in four patients showed lorazepam clearance was significantly lower in euglycemic conditions with low levels of peripheral glucose matched with low insulin infusion rates than in studies with high levels of intragastric glucose with low (hyperglycemia) and high (euglycemia) insulin levels. This suggests that co-substrate availability may be a rate-limiting step in hepatic glucuronidation in man. Renal clearance of lorazepam glucuronide exhibited a similar pattern, suggesting that renal secretion is depressed in a low-energy state. No dose-dependent insulin response, or effect of insulin species was observed for any studied lorazepam parameter. High levels of insulin significantly depressed urine production in IDDM patients. Glucagon had no effect on lorazepam disposition. A study in ten IDDM patients indicated that insulin species influences lorazepam glucuronidation; however, this could not be substantiated in a small crossover study with three IDDM patients, or in the ten-part study.

An animal model of enterohepatic circulation (EHC) of lorazepam was developed in the dog. Interruption of EHC resulted in lower plasma lorazepam concentrations, increased lorazepam clearance rates, and decreased volume of distribution. Lorazepam and lorazepam glucuronide concentrations were 10-100 fold greater in bile than in plasma, suggesting a pronounced biliary secretion. These data suggest that the dog model may be useful in studying EHC.

The effects of feeding on diazepam disposition showed that post-prandial diazepam peaks are not due to EHC. Feeding caused a decrease in diazepam free fraction, suggesting

that the peaks are the result of changes in plasma protein binding. Perturbation of plasma protein binding with heparin and fat emulsion were in agreement with this conclusion. Concentrations of unbound diazepam increased following meals, suggesting that decreased hepatic metabolism, and possibly redistribution from tissues may also play a role in the mechanism of postprandial diazepam peaks.

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DEDICATION

This thesis is dedicated to my family.

For Donna, Danielle, and Benjamin.

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LIST OF ABBREVIATIONS

%	percent
℃	degrees Celsius
λ	terminal disposition rate constant
A	amount of drug in the body
ANOVA	analysis of variance
ATP	adenosine triphosphate
AUC	area under the concentration-time curve
AUCG	area under the concentration-time curve of lorazepam glucuronide
AUMC	area under the first moment curve
A_{urine}	amount of drug excreted in the urine
BADF	bile acid dependent bile flow
BAIF	bile acid independent bile flow
cAMP	cyclic 3′,5′-adenosine monophosphate
C_H	hepatic clearance
Ci	curie
CIU	clinical investigation unit
CL	clearance
CL_B	biliary clearance
CL_{int}	intrinsic clearance
Cl_{or}	apparent oral clearance
CL_{org}	clearance for an individual organ
Cl_R	renal clearance
CL_{tot}	total body clearance
CNS	central nervous system
C_p	concentration of drug in plasma

CYP450	cytochrome P450
E	extraction ratio
EHC	enterohepatic circulation
F	bioavailability
FCL	free clearance
FFAs	free fatty acids
f_u	fraction of unbound drug in the plasma
f_{uT}	fraction of unbound drug in tissues
FVd_{ss}	free steady-state volume of distribution
g	gram
GABA	γ-aminobutyric acid
GLUT	glucose transporter
HbA1c	hemoglobin A1c
HPLC	high performance liquid chromatography
hr	hour
i.v.	intravenous
IDDM	insulin dependent diabetes mellitus
IGF-1	insulin-like growth factor-1
kg	kilogram
K_m	Michaelis constant
L	litre
LPL	lipoprotein lipase
m	milli
min	minute
mol	mole
MRT	mean residence time
NAD^+	nicotinamide adenine dinucleotide (oxidized form)

NADH	nicotinamide adenine dinucleotide (reduced form)
NADPH	nicotinamide adenine dinucleotide phosphate (reduced form)
neo/chol	neomycin cholestyramine treatment
ng	nanogram
NIDDM	non-insulin-dependent diabetes mellitus
NSAIDs	nonsteroidal antiinflammatory drugs
Q	blood flow
Q_H	hepatic blood flow
R or/iv	ratio of oral to i.v. metabolites
RUH	Royal University Hospital
$T_{1/2}$	elimination half-life
UDPGA	uridine diphosphate glucuronic acid
UDPGT	uridine diphosphate glucuronyltransferase
V_{area}	volume of distribution (derived from terminal rate constant)
Vd	volume of distribution
Vd_{ss}	steady-state volume of distribution
V_{max}	maximal enzymatic rate
V_P	volume of plasma compartment
V_T	apparent volume of the tissue compartment
WCVM	Western College of Veterinary Medicine
WHO	world health organization
μ	micro

1. INTRODUCTION

1.1. Effects of feeding on drug disposition

The human diet is an extremely complex variable in drug disposition. It varies not only in dietary nutrients, but in also in preparation, volume, and in times of consumption (meal pattern) which may be superimposed on circadian rhythms (Vesell, 1984). In addition to direct dietary effects, feeding-induced physiological changes can also affect how the body handles a drug. For instance, feeding is well known to cause alterations in hepatic blood flow which may affect the distribution and elimination of several drugs. Indeed, some of the effects of diet on drug disposition may not be due to the intake of food itself; as demonstrated by Pavlov's classical experiments, a cephalic phase is clearly important in the body's response to feeding. The duration of a food effect can be highly variable, owing to the type of influence which is exerted. Changes in hepatic blood flow during the absorption of orally administered drug can dramatically change the total amount of drug which reaches the systemic circulation, which may result in significant and clinical effects. On the other hand, changes in the distribution of drugs due to food-induced effects on plasma protein binding might be anticipated to cause only transient changes, the clinical implications of which are dubious (Sellers, 1979). While drug disposition is comprised of distribution and elimination, feeding-induced changes should not be considered mutually exclusive; changes in plasma protein binding may have effects on either component. The effects of feeding on drug disposition can often be readily explained and predicted, however, by a thorough understanding of the pharmacokinetic concepts which control drug distribution and elimination.

1.1.1. Basic pharmacokinetic principles

1.1.1.1. Drug distribution

Drug distribution is the reversible transfer of a drug between one location in the body and another. Because it is commonly accepted that the pharmacological activity and the intensity of action of a drug are directly dependent on its concentration at the receptor site (Colburn, 1987; Rowland and Tozer, 1989; Du Souich *et al.*, 1993), where a drug distributes within the body, as well as its rate of distribution, are of paramount pharmacological importance.

In theory, distribution requires the measurement of the concentration of the drugs within tissues. While this is commonly achieved in animal studies, practical considerations preclude these measurements from being made routinely in man. Instead, drug disposition studies in man rely on the measurement of drug concentrations in readily accessible physiological fluids, usually blood or urine, as a function of time, and the results extrapolated to provide an indirect measure of drug distribution within tissues. The apparent volume of distribution (Vd) describes the relationship between the amount of drug in the body (A) and the concentration of drug within the plasma (C_p):

$$Vd = A / C_p \qquad (1.1)$$

The apparent volume of distribution of a drug is in fact a proportionality constant which possesses no obvious relationship with a drug's real volume of distribution. Because measurement of Vd depends upon serial measurements within the blood, predominant binding to plasma proteins will tend to underestimate a drug's real volume of distribution, just as drugs that are predominantly bound to extravascular tissues will have apparent volumes of distribution which are greater than their true distribution volume. To consider the effects of tissue and plasma protein binding, equation (1.1) can be rewritten as:

$$Vd = V_P + V_T \bullet f_u/f_{uT} \quad (1.2)$$

where V_P is the volume of the plasma, V_T is the apparent tissue volume, f_u is the fraction of unbound drug in the plasma, and f_{uT} is the fraction unbound in tissues (Gibaldi and Perrier, 1982). It can then be seen that a drug's apparent volume of distribution increases with an increase in the unbound fraction in the plasma or a decrease in the unbound fraction to tissue components, and vice versa.

1.1.1.2. Binding of drugs to plasma proteins and tissues

Binding to plasma and tissue proteins is a phenomenon which greatly affects the disposition of many exogenous and endogenous molecules (Goldstein, 1949; Kurz and Fitchtl, 1983; Wallace and Verbeeck, 1987; Rowland and Tozer, 1989; Pacifici and Viani, 1992; Hervé *et al.* 1994). These binding interactions are the result of hydrophobic bonding due initially to electrostatic attraction, and reinforced by hydrogen bonds and Van der Waal's forces (Wilkinson, 1983). Drug-protein complexes are stable and reversible. Although binding to plasma proteins and to tissues can both affect a drug's disposition, far more attention has been paid to plasma protein binding interactions, largely due to the fact that it is much more difficult to determine binding of drugs to tissues than to plasma, and practically impossible to measure protein binding in tissues directly without destroying the integrity of the tissue (Kurz and Fitchl, 1983; Rowland and Tozer, 1989).

Human plasma contains more than 60 proteins and glycoproteins (Tozer, 1981). Two proteins, albumin and α_1-acid glycoprotein, account for the majority of drug-protein binding interactions in plasma (Svensson *et al.*, 1986). Albumin commonly binds acidic drugs, while α_1-acid glycoprotein primarily binds basic drugs. Albumin is the most abundant protein in plasma, constituting approximately 60% of total plasma protein (Kragh-Hansen, 1981; Wilkinson, 1983).

The number of binding sites on plasma proteins are limited. Therefore, all drugs show dose-dependent, or saturable binding. The important concern is whether or not nonlinear binding occurs in the therapeutic or observed concentration range. It can be shown (Tozer, 1981) that the unbound fraction of drug in plasma (f_u) is dependent upon the affinity constant (K) and concentration of the drug-plasma protein complex ([DP]), as well as the total concentration of binding sites on the plasma protein (n•[Pt]), by the equation:

$$f_u = 1 / (1 + K (n\bullet[Pt] - [DP])) \quad (1.3)$$

where n is the number of equivalent binding sites per macromolecule and [Pt] is the molar concentration of protein. Accordingly, the fraction of unbound drug will be virtually constant as long as the concentration of sites occupied by the drug ([DP]) is much less than the total concentration of binding sites on the plasma protein (n•[Pt]). Therefore, the drug concentration at which saturable binding occurs depends upon the concentration of the protein. Under normal physiological conditions, the concentration of albumin is approximately 650μM and acidic drugs of average molecular weight (e.g. 250) show nonlinear binding at concentrations of 50 mg/L or higher. On the other hand, the systemic concentration of α_1-acid glycoprotein is only 15μM, so basic drugs that primarily bind this protein exhibit saturable binding at concentrations above 1mg/L (Tozer, 1981).

Plasma protein concentrations are altered by several different physiological and pathological states (Wilkinson, 1983; Svensson *et al.*, 1986; Pacifici and Viani, 1992). A decrease in albumin concentrations occurs with pregnancy, stress, bed rest and age (neonatal and elderly) as well as in burn patients, chronic renal failure, malnutrition and hepatic disease. The concentration of α_1-acid glycoprotein is decreased with pregnancy and oral contraceptive use, but is increased in patients with burns, Crohn's disease and myocardial infarctions.

Albumin binds to several endogenous compounds such as fatty acids, steroids, bilirubin, as well as a large number of cations and anions, and drugs, especially those with acidic and neutral characteristics. The wide spectrum of chemical moieties that bind to albumin suggest that binding is largely nonselective. On the basis of competitive binding studies, human serum albumin appears to have six binding regions (Kragh-Hansen, 1981; Hervé *et al.*, 1994). Three of the sites are important for drug binding. Site I is commonly referred to as the warfarin and azapropazone binding site. Site II exhibits more selective binding than site I, and is often referred to as the indol and benzodiazepine binding site because of the high degree of binding of such compounds at this domain. Site III is specific for cardenolides and biliary acids (Hervé *et al.*, 1994).

1.1.1.3. Drug elimination

While apparent volume of distribution relates to how a drug distributes in the body, elimination of a drug is described by its clearance. Developed by renal physiologists in the 1930s as a measure of kidney function, in its most general definition, clearance is a proportionality constant (CL) which describes the relationship between a substance's rate of transfer, in amount per unit time, and its concentration in an appropriate reference fluid (Wilkinson, 1987). In general pharmacokinetic considerations, the rate of transfer is the rate of elimination of drug from an organ. Since drug concentrations are most often measured in plasma (C_p), clearance values can be calculated by the following:

$$CL = \text{rate of elimination}/C_p \qquad (1.4)$$

Total clearance of a drug is the sum of clearances through all elimination pathways involved, and is clinically valuable in predicting steady-state concentrations, since rate

of drug delivery is equal to rate of elimination under such circumstances. Clearances may also be calculated for individual organs (CL_{org}) by the following equation:

$$CL_{org} = Q \bullet E \tag{1.5}$$

where Q, the rate of drug delivery to the organ, is the organ's rate of perfusion, and E is the extraction ratio. The practical problems associated with measuring the extraction ratio, which depends upon accurate measurements of arterial and venous substrate concentrations, usually preclude the application of this equation, especially *in vivo*. In addition, organ clearance, as defined by equation 1.5, provides no insight into the physiological processes which determine how the organ eliminates the drug.

Of the organs associated with drug clearance, the two which are the most thoroughly studied are the kidney and the liver. Drug elimination by the kidney is usually associated with drug excretion, although the kidney also possesses significant metabolic capacity (Anders, 1980; Benet, 1996). Renal clearance (CL_R) of a drug is determined by its rate of excretion (amount excreted in the urine during a certain time interval) divided by the concentration in the plasma at the midpoint of the collection interval. A more common approach to calculating renal clearance is by measuring the total amount of drug excreted in the urine during a collection interval (A_{urine}), and dividing by the area under the plasma concentration-time curve (AUC) during that time period.

$$CL_R = A_{urine} / AUC \tag{1.6}$$

The liver is an important organ responsible for the elimination of many drugs. In terms of drug metabolism, the liver possesses a much greater metabolic capacity than any other organ (Wilkinson, 1987; Rowland and Tozer, 1989). Hepatic clearance includes both hepatic metabolism and biliary excretion. Several physiologically based models of hepatic elimination have been developed to predict the effects of changes in the biological variables of elimination on clearance and blood concentration-time profiles, including the well-stirred or venous equilibration model, the parallel-tube

model, the distributed sinusoidal perfusion model, and the dispersion model. All the models provide similar predictions with respect to the overall effects of changes in the physiological determinants in hepatic elimination; however, they differ at a finer level of resolution (Wilkinson, 1987). In the well-stirred model, the liver is considered to be a single well-stirred compartment, with instantaneous and complete mixing of hepatic and portal blood (Rowland *et al.*, 1973; Wilkinson and Shand, 1975). Although this is an obvious oversimplification of true hepatic physiology, an advantage of this model lies in the fact that it can be described with simple mathematics, facilitating predictions of the expected effects of perturbations in the biological determinants which affect hepatic elimination.

In the well-stirred model, only unbound drug is available to be taken up into the hepatocyte. The hepatic clearance (CL_H) is given by:

$$CL_H = Q_H \bullet CL_{int} \bullet f_u / (Q_H + CL_{int} \bullet f_u) \quad (1.7)$$

where Q_H is total hepatic blood flow, CL_{int} is the hepatic intrinsic clearance of unbound drug, and f_u is the free fraction of drug in the blood. The hepatic extraction ratio (E_H) is then:

$$E_H = CL_{int} \bullet f_u / (Q_H + CL_{int} \bullet f_u) \quad (1.8)$$

This model allows a quick estimation of expected changes in clearance after alterations in blood flow, plasma protein binding, and intrinsic clearance which is a measure of the enzyme activity which metabolizes the drug.

Drugs are classified as having low (less than 0.3), intermediate (0.3-0.7), or high (greater than 0.7) extraction ratios. The value of the "well-stirred" model is enhanced by the fact that most drugs appear to have extraction ratios at the extremes (Rowland and Tozer, 1989). For drugs with high extraction ratios, $CL_{int} \bullet f_u >> Q_H$, and equation 1.7 can be reduced to:

$$CL_H = Q_H \quad (1.9)$$

Therefore, highly cleared drugs are dependent only on blood flow, but are independent of both intrinsic clearance and plasma protein binding. Such drugs are said to be perfusion-limited, or to exhibit non-restrictive elimination. For a low extraction drug, the opposite is true, such that $CL_{int} \bullet f_u << Q_H$, and equation 1.7 simplifies to:

$$CL_H = CL_{int} \bullet f_u \qquad (1.10)$$

For drugs that are poorly cleared by the liver (restrictive elimination), changes in blood flow would not affect hepatic clearance, but clearance would be susceptible to alterations in changes in both enzymatic activity and in the drug's free fraction. Similar considerations with respect to hepatic extraction ratio (equation 1.8) show that drugs that possess low extraction ratios are dependent on intrinsic clearance, free fraction and blood flow, while highly extracted drugs are independent of these three parameters. This becomes important when studying the bioavailability (F) of drugs. Drugs that are given orally are absorbed into the portal circulation, and must pass through the liver before reaching the systemic circulation. The fraction of the given dose which reaches the systemic circulation unchanged is a measure of the drug's bioavailability, and can be calculated from the hepatic extraction ratio:

$$F = 1 - E_H \qquad (1.11)$$

Another pharmacokinetic parameter associated with drug elimination is a drug's elimination half-life ($T_{1/2}$). The elimination half-life of a drug is the time it takes for the plasma concentration, or more correctly the amount of drug in the body, to be decreased by 50%. Usually, the elimination half-life is determined by the terminal elimination phase of a drug's concentration-time profile. Several early pharmacokinetic studies relied extensively on elimination half-life to characterize the elimination process (Wilkinson, 1987). In reality, however, elimination half-life is a secondary parameter dependent upon two primary parameters, volume of distribution and clearance, by the equation:

$$T_{1/2} = 0.693 \bullet Vd / CL \qquad (1.12)$$

Changes in half-life may be caused by changes in either clearance or volume of distribution. Moreover, changes can occur in both primary parameters which offset each other such that no effect on half-life is observed. Accordingly, half-life has little value as a measure of intrinsic drug elimination.

1.1.2. Effects of feeding on drug distribution

The distribution of a drug refers to both the extent, and the rate at which a drug distributes in the body. Feeding-induced changes in drug distribution are usually associated with the extent of distribution, and are due largely to changes in plasma protein binding. However, meal-induced changes in blood flow can also affect drug distribution by altering the rate at which drugs are distributed throughout the body.

Most of the fatty acid contained in plasma is present as a component of complex lipids (e.g. phospholipids, triglycerides) which in turn are carried in plasma as constituents of lipoproteins. A small fraction of fatty acid is present in plasma in an unesterified form, commonly called non-esterified free fatty acids (NEFAs), or simply free fatty acids (FFAs). In actuality, FFAs are not "free" in the sense of being unbound, since more than 99% of plasma FFAs are bound to albumin, which facilitates the movement of these poorly soluble endogenous compounds through the systemic circulation (Spector and Fletcher, 1978). The concentration of FFAs within the blood is largely dependent upon nutritional state, with the normal extent of physiological variation ranging between 0.2-2 mM (Ruderman *et al.*, 1995). During periods of fasting, plasma FFAs increase as a result of increased activity of hormone-sensitive adipose lipase. Several hormones which activate adenylyl cyclase, including catecholamines and glucagon, are capable of stimulating this enzyme. Plasma FFAs decrease following a meal as a result of decreased adipose lipase activity, which can

also be inhibited by insulin by decreasing intracellular cAMP, or by increasing the activity of a phosphoprotein phosphatase (Ruderman *et al.*, 1995).

Numerous drugs and endogenous ligands bind to sites on human serum albumin, two of which are major binding sites for many drugs (Table 1.1).

Table 1.1 Specific binding of drugs to human serum albumin[1]

Site I (warfarin binding site)	Site II (indol and benzodiazepine binding site)
azapropazone	benzodiazepines
dansylamide	clofibric acid
dicoumarol	cloxacillin
glyburide	dansylsarcosine
furosemide	ethacrynic acid
phenylbutazone	flucloxacillin
phenytoin	flurbiprofen
sulfadimethoxine	glyburide
sulfinpyrazone	ibuprofen
tolbutamide	iopanoic acid
valproic acid	ketoprofen
warfarin	naproxen

[1] Data adapted from Hervé *et al.*, 1994; Roland and Tozer, 1989; Tillement *et al.*, 1984.

Binding of FFAs to plasma albumin can affect the binding of drugs through competitive or allosteric mechanisms, with the nature of the binding dependent upon the FFA involved. Short to medium chain FFAs can compete directly with drugs for binding at the indol and benzodiazepine binding site (site II), while longer chain FFAs produce effects on drug binding to human serum albumin by allosteric mechanisms by binding to a separate binding site (Menke *et al.*, 1989). *In vivo* studies have shown that ratios

of FFA/albumin above 2 indicate strong displacement of many drugs (Hathcock, 1985).

Food-intake has been reported to increase the plasma protein binding of diazepam, and these changes have been correlated to decreases in plasma FFAs (Naranjo *et al.*, 1980a; Naranjo *et al.*, 1980b). Lower levels of plasma FFAs result in decreased competition between drugs and FFAs for albumin binding sites, leading to quantitatively greater amounts of drug bound to albumin within the plasma. These changes in plasma protein binding have been used to explain the large post-prandial peaks observed with diazepam (Naranjo *et al.*, 1980a). Similar effects would be expected for drugs which bind to site II of albumin. However, the effects of feeding on drug-plasma protein binding can be complex. For instance, the FFA-albumin interaction may be specific according to the FFAs involved. Oleic acid decreases diazepam binding at all molar concentrations of FFA:albumin, however palmitic acid enhances diazepam binding at ratios less than 1, presumably through allosteric co-operativity (Wong and Sellers, 1979). Therefore, the net effect of displacement *in vivo* may depend not only on the quantitative changes in the total level, but also in the ratio of the specific fatty acids to each other. In addition, the effects of FFAs on binding of ligands to site I can be different than those observed for site II. Small amounts of FFAs have been demonstrated to increase the binding affinity of albumin to warfarin, resulting in a reduction of drug transfer of warfarin into the brain and salivary glands (Urien *et al.*, 1989). Moreover, high fat meals can cause an increase in plasma FFAs, due to the actions of lipoprotein lipase on absorbed intestinal chylomicrons (Heimberg *et al.*, 1974; Karpe *et al.*, 1992).

From a kinetic standpoint, displacement of drug from plasma protein binding sites will cause an increase in a drug's volume of distribution (equation 1.2). From a pharmacodynamic point of view, a more important consideration is the effect of drug displacement on free drug concentrations. Since the concentration of free drug in

plasma is believed to reflect the active concentration at receptor sites, changes in plasma protein binding may effect a drug's pharmacological activity. Indeed, based on theoretical simulations, an increase in free fraction will cause an increase in pharmacological effect, although the duration of effect may increase or decrease depending upon the dose and the minimum effective free plasma drug concentration (Levy, 1976). Whether such displacement interactions are clinically significant is debatable, since increased concentrations of displaced drug would be expected to be rapidly buffered throughout the rest of the distribution space, causing a decrease in total drug levels, with only transient changes in free drug concentration and no change in steady-state levels of unbound drug in the plasma (Sellers, 1979; Aarons, 1981; Du Souich, 1993). Given that the drug effect depends upon the magnitude and time-course of the change in free drug, as well as the responsiveness of the drug receptors, the clinical consequences of changes in plasma protein binding are usually small. However, the decrease in total concentration has to be taken into account when measuring drug levels in therapeutic drug monitoring. Regardless of the above considerations, a change in pharmacological response would be anticipated with a change in plasma protein binding for a drug whose distribution is largely confined to the vascular compartment (e.g. warfarin).

Although the majority of feeding-induced changes in drug distribution are associated with changes in plasma protein binding, it should be anticipated that alterations in plasma FFAs levels may affect tissue binding as well. Indeed, more than 60% of total body albumin is located extravascularly (Routledge *et al.*, 1980; Wilkinson, 1983). However, problems in directly assessing tissue binding have limited investigations in this area. In addition, perturbations in binding through displacing agents or changes in protein concentration can be easily applied to studies of plasma protein binding, but such tools are not as readily available for tissue binding studies (Svensson *et al.*, 1986). Therefore, conclusions regarding changes in tissue

binding are often made indirectly, and remain largely speculative and theoretical. By virtue of the large tissue mass, a decrease in tissue binding could lead to a marked decrease in apparent distribution volume. If changes in plasma protein binding also occur, or if the resultant changes in drug concentration exceed the binding capacity of plasma proteins, this would lead to transient increases in the concentrations of free drug within the systemic circulation, and possibly lead to an enhanced pharmacological effect. On the other hand, a displacement from tissue receptor effector sites could lead to a decrease in drug effect. A change in tissue binding would have no consequence on clearance of a drug (equation 1.7), unless of course, protein binding is involved in the uptake or elimination of the drug.

1.1.3. Effects of feeding on drug elimination

The effects of feeding on drug elimination can be divided into chronic and acute categories. Chronic feeding effects are those which occur in response to long-term dietary influences. Because these changes usually involve alterations in enzyme content, the effects are long-lasting. Changes which occur following individual meals are considered acute effects, and are usually transient in nature.

Changes observed in drug elimination with feeding may be a result of changes in blood flow, plasma protein binding, or intrinsic clearance, since all three factors are potentially important in drug clearance (equation 1.7). It is therefore important that all three parameters be considered when determining the mechanism of a food-related effect in drug elimination.

Several studies have shown that feeding causes changes in hepatic blood flow, and that this can affect drug clearance. This is particularly important for highly cleared drugs, in which clearance is flow-dependent. With such drugs, an increase in hepatic flow would cause a transient increase in drug clearance. However, of more importance

is the effect of food on the oral availability of high clearance drugs, since many orally prescribed medications are taken with meals. High protein meals cause an increase in splanchnic blood flow (Melander *et al.*, 1988). Based on assumptions of the well-stirred model, hepatic extraction is inversely related to blood flow (equation 1.8). Therefore, an increase in flow will result in a reduced extraction ratio, which can result in an increase in oral availability. One of the best studied examples of this effect has been done with propranolol. Food intake causes a 50% increase in the AUC of propranolol (Melander *et al.*, 1977). Considering that propranolol is completely absorbed after an oral dose, it follows that the increase in systemic concentrations results from a decrease in hepatic extraction. A common explanation for this effect is that with an increased flow, more drug can escape presystemic metabolism (Walle *et al.*, 1981; Melander *et al.*, 1988). However, increased blood flow cannot account for all the effects, since an increased availability was also noted with carbohydrate-rich meals, which have little effect on hepatic blood flow (McLean *et al.*, 1981), whereas postural-induced changes in hepatic blood flow had no effect on the oral availability of propranolol (Woodruff *et al.*, 1987). Other investigations have implicated amino acids (Semple and Xia, 1995) and glucagon (Semple and Xia, 1994) in reducing hepatic metabolism and/or tissue uptake. Neurophysiological influences have also been shown to be important in the food-effect of propranolol. Power *et al.* (1995) found a greater propranolol AUC following oral administered drug when subjects were strongly teased with an appetizing meal, without actually ingesting any food. Feeding can also decrease the availability of orally administered drugs. This has been clearly documented for hydralazine (Shepherd *et al.*, 1984; Semple *et al.*, 1991).

Feeding-induced changes in plasma protein binding would be expected to influence the total hepatic clearance of drugs which are poorly cleared by the liver (equation 1.10), but have little effect on highly-cleared drugs (equation 1.9). However, since volume of distribution is also dependent upon binding to plasma

proteins (equation 1.2), whether or not a change is observed in elimination half-life will depend upon the relative effects on both parameters. For diazepam, which undergoes restrictive elimination, an increase in plasma protein binding following meals causes post-prandial peaking of diazepam concentrations, However, changes in binding account for only 75% of the increased concentrations of free diazepam, suggesting that meals also affect hepatic metabolism (Naranjo *et al.*, 1980b).

Changes in drug metabolism with feeding can also be due to changes in intrinsic clearance. Drug metabolism may be influenced through changes in hepatic enzyme content. Reducing the amount of dietary protein in animals studies causes a decrease in microsomal P450, as well as reduced rates of drug metabolism through the cytochrome P450 (CYP450) enzyme system (Campbell and Hayes, 1976; Hietanen *et al.*, 1980). Tryptophan and related indoles increase liver protein synthesis; indeed, it is believed that the indoles found in cruciferous vegetables such as cabbage, broccoli, cauliflower and brussels sprouts are responsible for their effects on enhancing drug metabolism (Yang and Yoo, 1988). Dietary components can also have an effect on enzyme activity. It has been suggested that some of the propranolol food-effect may be due to competitive inhibition of the primary conjugation of propranolol (Liedholm and Melander, 1986). Microsomes treated with unsaturated fatty acids exhibit depressed CYP450 and uridine diphosphate glucuronyltransferase (UDPGT) activity (DiAugustine and Fouts, 1969; Lang 1976), possibly by modifying membrane structure. A low-protein diet has been shown to cause an increase in UDPGT activity (Hietanen *et al.*, 1980). Several studies have implicated co-substrate availability as a rate-determining step in the glucuronidative metabolism of drugs. Glucuronidation is the principal phase II reaction of many endogenous and exogenous substrates in all vertebrate species examined (Dutton, 1980), and involves the transfer of glucuronic acid from uridine diphosphate glucuronic acid (UDPGA) to a variety of nucleophilic acceptor groups of endogenous compounds and xenobiotics (e.g. hydroxyl carboxyl,

thiol, amine, and hydroxylamine groups). The enzymes which catalyze the reaction are known collectively as uridine diphosphate-glucuronyl tranferases (UDPGTs). Factors which influence the rate and extent of glucuronidation are therefore the availability of UDPGA and the activity of the UDPGT. A direct precursor of UDPGA synthesis is glucose. Glucose is phosphorylated to glucose-1-phosphate which is combined with uridine triphosphate (UTP) to form UDP-glucose and inorganic pyrophosphate (PPi). The UDP-glucose is then converted to UDPGA by NAD^+-dependent UDP-glucose dehydrogenase. Addition of glucose can restore fasting-induced decreases in glucuronidation rates in perfused livers or isolated hepatocytes (Aw and Jones, 1984; Eacho *et al.*, 1981a; Reinke *et al.*, 1981; Conway *et al.*, 1985), although this has not been observed in all studies (Bànhegyi *et al.*, 1988; Price and Jollow, 1989). Glucose infusion in a perfused rat liver causes an increase in *p*-nitrophenol conjugation as a result of increasing substrate availability for glucuronidation reactions (Reinke *et al.*, 1981). Feeding-related co-substrate availability has also been implicated in drugs that are metabolized by oxidative metabolism. Food restriction (65% of controls) in rats causes an increase in the monooxygenation of *p*-nitroanisole in a perfused liver preparation (Qu *et al.*, 1994). The reason for this is believed to be a stimulation of fatty acid oxidation, elevating the $NADH/NAD^+$ ratio which leads to increased NADPH, a necessary co-factor in oxidative metabolism.

Several studies in humans suggest that the amounts of protein, carbohydrates and fat within the diet are important in the clearance of drugs which are eliminated through oxidative metabolism. The clearance of antipyrine and theophylline in healthy volunteers is significantly greater in subjects fed a high-protein diet, compared to a high-carbohydrate diet (Kappas *et al.*, 1976). Studies by Anderson *et al.* (1979) confirmed these results, and showed that the effects of a high-fat diet are similar to a diet high in carbohydrates. Substituting the type of fat (saturated versus unsaturated) had no effect on the clearance of these oxidatively-metabolized drugs.

Feeding can also affect the metabolism of drugs which are biotransformed by phase II metabolism. Oxazepam glucuronidation was decreased in healthy obese patients on a very low calorie diet; however, this had no effect on the metabolism of antipyrine (phase I) (Sonne *et al.*, 1989). The free clearance of lorazepam was significantly increased with feeding of a high-carbohydrate, low-fat diet, although no difference was observed in the presence of an interrupted enterohepatic circulation (Herman *et al.*, 1994). In the same study, feeding caused no change in the clearance of unbound lorazepam in Gilbert's patients in a control situation, but produced a significant increase when enterocycling was inhibited.

An alternative approach to studying the effects of feeding on drug disposition can be deduced by studying the opposite end of the feeding spectrum, namely the effects of fasting and starvation. Fasting for 72 hours significantly reduces the glucuronidation of bilirubin in rats, due to decreased co-substrate levels (UDP-glucuronic acid), with no change in UDP-glucuronyltransferase activity (Felsher *et al.*, 1979). Reinke *et al.* (1981) showed that glucuronidation of *p*-nitrophenol was significantly lower in fasted rats compared to fed, and fasted-refed rats. Similarly, fasting has been reported to potentiate the hepatoxicity of acetaminophen in the rat (Price and Jollow, 1982) via reduced glucuronidation and sulfation metabolism. Studies on the effects of starvation on mixed function oxidase also show a decrease in metabolism in the fasted state (Thurman *et al.*, 1981).

Feeding can also have effects on non-hepatic routes of elimination. Drugs bound to plasma proteins cannot be filtered at the renal glomerulus. Therefore, feeding-induced changes in plasma protein binding would be expected to affect the renal elimination of drugs or their metabolites which may be highly plasma protein bound and cleared by renal filtration. In addition, changes in urinary pH can affect the reabsorption of drugs from the nephron. Foods which tend to alkalinize the urine are vegetables, citric fruits, almonds, chestnuts and a high intake of dairy products,

whereas bread, bacon corn, lentils, meat, fish and eggs tend to acidify urine (Roberts and Turner, 1988). High intake of sodium within the diet can result in an increased elimination of lithium (Powell and Lamy, 1977).

Biliary clearance of drugs can also be affected by dietary constituents. High-fibre diets can cause a loss of bile acids which can affect the elimination biliary elimination of drugs, as well as the disposition of drugs that participate in an active enterohepatic circulation (Roberts and Turner, 1988)

1.2. ENTEROHEPATIC CIRCULATION

1.2.1 General

Enterohepatic circulation (EHC) is a process whereby a substance secreted into the bile, passes into the lumen of the gastrointestinal tract, where it is subsequently reabsorbed and passes via the portal circulation back to the liver, and is either taken back up into the hepatocytes to complete the cycle, or escapes into the systemic circulation (Figure 1.1).

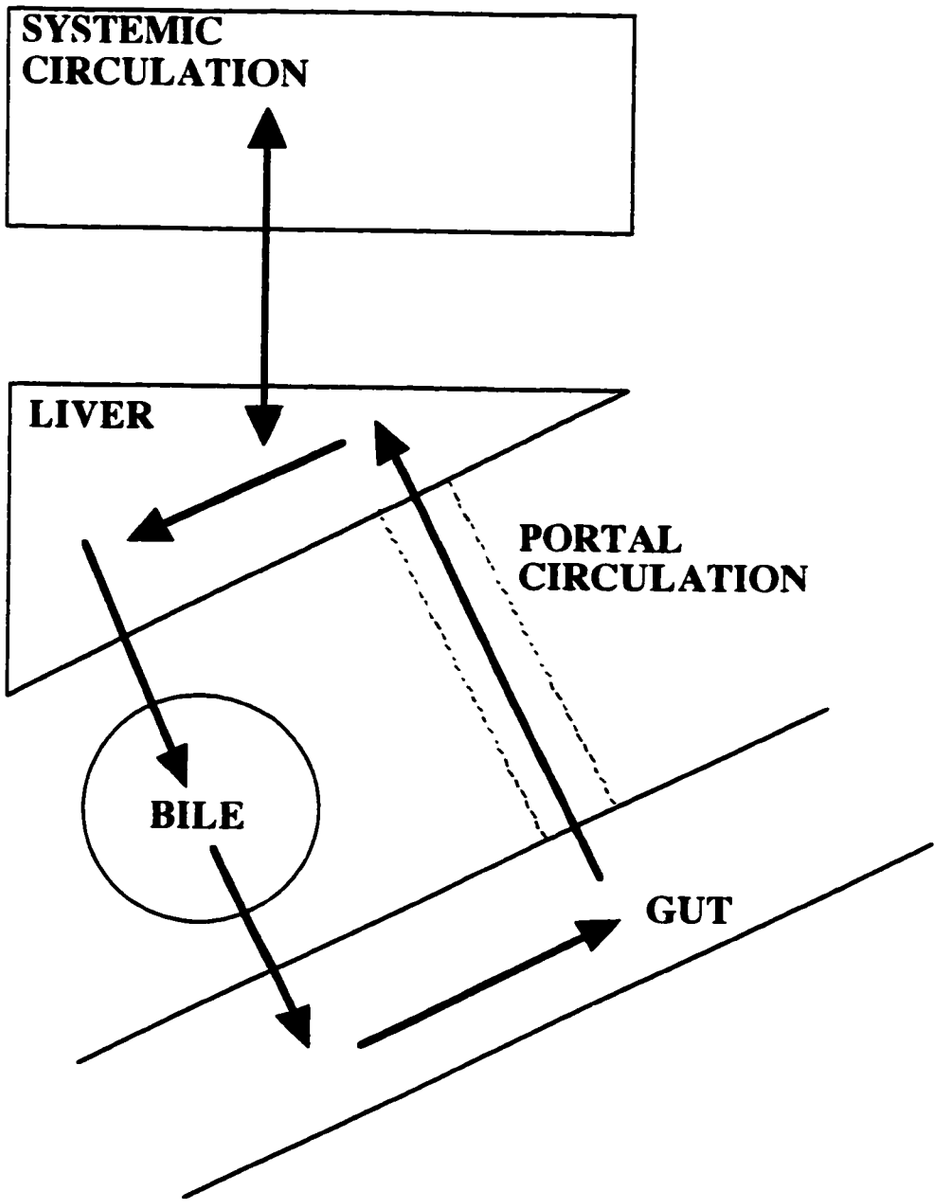


Figure 1.1. Diagrammatic representation of enterohepatic circulation

1.2.2. Physiological regulation of enterohepatic circulation

Enterohepatic circulation is important for the secretion and recovery of bile salts, which are needed to facilitate the digestion and absorption of dietary fat. Other endogenous compounds such as steroids and folate also participate in an EHC (Carey and Cahalane, 1988). The driving force for enterohepatic circulation is the secretion of bile salts (Klaassen and Watkins, 1984). EHC is propelled by the continuous active secretion of bile into the biliary tree, the intermittent contraction of the gallbladder, the peristaltic activity of the gut, active transport by ileocytes, and portal venous flow (Carey and Cahalane, 1988). Bile is formed by hepatic parenchymal cells (hepatocytes) and secreted into bile canaliculi. Within the canaliculi, bile is modified by reabsorption and/or secretion of water and electrolytes. The osmotic gradient created by the high concentration of bile acid in canaliculi (which usually takes Na^+ to maintain electroneutrality) is responsible for water movement during bile formation (Anwer, 1991).

Canalicular bile formation can be divided into bile acid dependent bile flow (BADF) and bile acid independent bile flow (BAIF). BAIF comprises 40% of spontaneous basal bile secretion (Klaassen and Watkins, 1984), which averages 0.5-0.8 mL/min in humans (Rowland and Tozer, 1989). The actual rate of bile flow fluctuates greatly, and is generally reduced at night and stimulated with feeding (Klaassen and Watkins, 1984). Whereas active secretion of bile acids is the mechanism responsible for BADF, BAIF appears to be related to the excretion of a number of different organic and inorganic (e.g. Na^+, Cl^-, HCO_3^-) solutes. BAIF is responsive to a number of exogenous and endogenous agents, including theophylline, barbiturates, insulin, and glucagon (Jones, 1976; Snow and Jones, 1978; Klaassen and Watkins, 1984; Anwer, 1991; Branum *et al.*, 1991, Watkins and Sanders, 1991).

Approximately one-half of the bile produced by the liver enters the gallbladder, with the rest passing directly into the duodenum. The gallbladder concentrates bile by

active secretion of inorganic ions (mainly Na^+, Cl^-, HCO_3^-), with passive movement of water (Carey and Cahalane, 1988). The gallbladder contracts in response to cephalic (vagal) and hormonal (cholecystokinin, motilin) influences during feeding, emptying as much as 80% of its contents within 15-45 minutes. It is important to note that a gallbladder is not necessary for EHC (a rat has no gallbladder, but exhibits a very active EHC). However, the gallbladder is important from a pharmacokinetic perspective, as it introduces a time- and feeding-dependency aspect to the disposition of an enterocycling drug.

Non-ionized bile acids which remain in solution are absorbed passively from all of the gastrointestinal tract. Ionized bile acids are reabsorbed via active sodium-dependent transport in the jejunum and lower ileum. (Watkins, 1991). Reabsorbed bile acids are transported back to the liver via the portal circulation, where they are extensively taken up into hepatocytes on a single pass through the liver by an efficient carrier-mediated system (Klaassen and Watkins, 1984).

1.2.3. Enterohepatic circulation of drugs

Before a drug can be considered to participate in EHC, it must be first taken up into hepatocytes and secreted into bile. Compounds undergoing biliary excretion can be grouped into three main classes based on their bile to plasma concentration ratio (Brauer, 1959; Klaassen and Watkins 1984). Class A substances have a ratio of approximately 1.0, class B substances have ratios between 10 and 1000, and class C compounds have ratios less than 1.0. Drugs for which biliary excretion is important are considered to be class B compounds. In order to achieve such high concentration ratios, class B compounds must be excreted into the bile by energy-dependent, saturable active transport mechanisms. Five separate transport systems are involved in

excreting compounds into bile, each with a unique substrate profile. These are: (1) bile acids; (2) organic anions, such as bilirubin, bromsulfophthalein and glucuronide conjugates; (3) organic cations, of which the prototype is procainamideethobromide (PAEB); (4) neutral compounds, for drugs such as ouabain; (5) metals, such as iron and copper (Klaassen and Watkins, 1984).

Numerous drugs are actively secreted into the bile and undergo EHC (Gregus and Klaassen, 1986). Drugs which are metabolized to glucuronide conjugates are likely candidates to participate in EHC for several reasons. To be excreted into the bile in appreciable amounts, a compound must possess a strong polar group (Dobrinska, 1989; Fleck and Bräunlich, 1991). Glucuronide conjugates are strong acids with pKa values in the range of 3 to 4, and are completely ionized at physiological pH. Secondly, compounds excreted into bile must exceed a molecular weight threshold. The value of this limit shows considerable interspecies variability, and appears to be approximately 325 in the rat, 475 in the rabbit, and between 500-600 in man (Dobrinska, 1989; Fleck and Bräunlich, 1991). Conjugation with glucuronic acid adds 176 daltons to the molecular weight of a drug. To complete the enterocycle, compounds excreted into the bile must be subsequently reabsorbed. Conversion to a polar metabolite decreases the rate of diffusion through the lipophilic membranes of the gut wall; however, glucuronides can be hydrolyzed back to the parent drug through the actions of β-glucuronidases, enzymes produced by anaerobic bacteria of the gastrointestinal flora and present in secretions and sloughed epithelial cells (Watkins, 1991).

Whether or not EHC is important in the disposition of a drug depends upon how much of it is cleared through biliary excretion, as well as how much of the drug makes it back into the systemic circulation via the cycling loop. Experimental evidence from animal studies suggests that a large number of therapeutically important drugs participate in an extensive EHC. However, because of a large interspecies variation in

biliary excretion, these results may not be readily extrapolated to man (Klaassen *et al.*, 1981). Compounds for which EHC has been demonstrated in man include digitoxin (Caldwell *et al.*, 1971), doxycyline (Veng Pederson and Miller, 1980), ranitidine (Miller, 1984), nadolol (Du Souich *et al.*, 1983), pirenzepine (Lee *et al.*, 1986), lorazepam (Herman *et al.*, 1989; Chaudhary *et al.*, 1993, Herman *et al.*, 1994), and several NSAIDs, such as indomethacin (Terhaag and Hermann, 1986; Cole *et al.*, 1992), sulindac (Dobrinska *et al.*, 1983; Dujovne *et al.*, 1983), and piroxicam and tenoxicam (Benveniste *et al.*, 1990).

The ability of the liver to excrete drugs in the bile is expressed as the biliary clearance (CL_B), which is determined by the equation:

$$CL_B = \frac{\text{(bile flow} \bullet \text{concentration in bile)}}{\text{concentration in plasma}} \qquad (1.13)$$

The rate at which a drug is cleared through the bile depends upon the rate of uptake into hepatocytes, the rate of excretion into the bile, and bile flow. The question as to which step is the rate-determining step is debatable. Bile acid depletion induced by cholestyramine significantly decreases the biliary excretion of digitoxin (Greenberger and Thomas, 1973), and bilirubin (Goresky *et al.*, 1974), Concomitant administration of bile acids increases the AUC and oral bioavailability of indomethacin, presumably, by enhancing the frequency of the drug's cycling fraction (Cole *et al.*, 1992). These results suggest that, for some drugs, bile flow may be rate-limiting. For compounds excreted as glucuronide conjugates, the rate-determining step appears to be the rate of excretion into the bile, which in turn is dependent upon the rate of drug glucuronidation, since conversion to a glucuronide is a necessary step for biliary excretion of several xenobiotics. Inhibition of glucuronidation with galactosamine or treatment with diethyl ether (both deplete UDP-glucuronic acid) decreases the biliary excretion of several compounds which are biotransformed to glucuronides, such as bilirubin, diethylstilbestrol, iopanoic and valproic acid (Gregus *et al.*, 1983).

Likewise, treatment with phenobarbital or 3-methylcholanthrene enhances the biliary excretion of such drugs, presumably through induction of hepatic UDP-glucuronyltransferase enzymes (Klaassen and Watkins, 1984).

1.2.3.1. Determination of enterohepatic circulation of drugs

Two common approaches are used in the study of enterohepatic circulation in animals (Gregus and Klaassen, 1986). In the single animal model, bile is readministered into the same animal, with continuous measurement of blood concentration, as well as biliary, fecal, and urinary excretion. The advantage of this model is that it requires a single animal, and EHC is only briefly interrupted. In the second approach, animal pairs are used. Bile excreted by a dosed donor animal is infused into the intestinal lumen of a second animal, from which blood, urine and feces may be analyzed. The advantage of the two animal model is that the second animal is considered to be initially drug-free, and the postabsorptive fate of the drug can be studied. Disadvantages include the requirement of more than one animal, as well as possible differences in the pharmacokinetics between the donor and recipient animal. Diversion of bile to a second animal without supplemental bile salts may also cause a loss of bile acids in the donor animal, leading to a depression in biliary secretion.

There are a number of indirect indicators that a drug may undergo EHC in man. A secondary peak in the plasma concentration-time profile following a meal suggests that a drug may be excreted and concentrated in the gallbladder, with subsequent release and reabsorption after feeding. A biphasic urinary recovery profile implies a similar mechanism. However, a more conclusive method for detecting EHC of a drug in man is to interrupt the cycle in the intestine. The use of nonabsorbable ion-exchange resins, such as cholestyramine, provides a useful non-invasive *in vivo* technique to study EHC in man. Daily cholestyramine administration eight hours after ^{3}H-digitoxin ingestion

reduced the half-life of total serum radioactivity from 11.5 to 6.6 days, and also reduced the effects of the drug on the electorcardiogram (Caldwell *et al.*, 1971). Treatment with cholestyramine led to a 1.5- to 2-fold increase in the clearance of intravenously administered phenprocoumon (Meinertz *et al.*, 1977), doubled the elimination rate of oral tenoxicam and piroxicam (Benveniste *et al.*, 1990), and has been used to increase the excretion rate of chlordecone in patients poisoned with this insecticide (Cohn *et al.*, 1978). Cholestyramine is particularly suited to interrupt the EHC of drugs that are secreted as glucuronides, which are negatively charged at physiological pH. Activated charcoal has also been used to interrupt the EHC of oestriol (Heimer and Englund, 1986) and nadolol (Du Souich *et al.*, 1983). However, caution should be used in interpreting the results from studies using this adsorbent, since activated charcoal avidly binds many drugs, and in effect can act as a sink, trapping drug that passes into the gut from the systemic circulation via passive diffusion. Another method for interrupting the EHC of drugs that cycle as a glucuronide conjugate is through the use of nonabsorbable oral antibiotics to inhibit the anaerobic intestinal flora which produce β-glucuronidase enzymes necessary for hydrolysis of the glucuronide bond. Indeed, oral antibiotics can decrease the plasma concentrations and decrease the clinical efficacy of oral contraceptives, owing to a decrease in EHC (Brewster *et al.*, 1977; Back *et al.*, 1980; Bacon and Shenfield, 1980). These methods can be combined to provide a synergistic effect on the cycling of some drugs eliminated as glucuronides. Treatment with neomycin and cholestyramine produced a 24% increase in the free clearance of lorazepam in humans through interruption of the EHC of that drug (Herman *et al.*, 1989).

More direct methods can be used to investigate enterohepatic circulation in man. These include the use of patients with biliary T-tubes (Colburn *et al.*, 1985; Terhaag and Hermann, 1986: Lee *et al.*, 1986). Such studies possess the obvious advantage of direct measurement of the drug in the bile, but suffer from the concerns that the data

may in part be a reflection of the patient's underlying medical status, and may not mirror the situation in subjects with normal hepatobiliary function. In addition, loss of bile acids through biliary T-tubes can cause in a decrease in bile acid secretion, resulting in an altered biliary secretion and EHC. Biliary excretion of drugs can also be studied using double and triple lumen nasogastric tubes (Dujovne *et al.*, 1982; Lind *et al.*, 1987) to obtain duodenal aspirates, which can be subsequently analyzed for drug excreted via the bile.

1.2.3.2. Pharmacokinetic and clinical implications of enterohepatic circulation of drugs.

The majority of work done on the effects of EHC on pharmacokinetics is based largely on theoretical and computer simulation models (Pang and Gillette, 1978; Chen and Gross, 1979; Shepard *et al.*, 1985a; 1985b; 1989; Shepard and Reuning, 1987; Kurita 1990; Peris-Ribera *et al.*, 1992). By re-introducing "previously eliminated" drug into the circulation, one of the most obvious effects of EHC is to cause an increase in the AUC of a drug. This has important implications on two pharmacokinetic parameters, namely the bioavailability (F), and clearance (CL) of a drug.

In a simulation of the effects of EHC on bioavailability, Shepard and Reuning (1987) demonstrated that systemic availability (F) is determined by the fraction of the dose initially absorbed (f_a*), the fraction of drug excreted into the gut which is reabsorbed with each cycle (f_a), the hepatic extraction ratio (E), and the fraction of extracted drug that is excreted into the bile (f_g) and concentrated in the gallbladder, by the equation :

$$F = f_a^* (1 - E) / (1 - f_a f_g E) \qquad (1.14)$$

The denominator of the equation can be viewed as a factor which quantifies the effect of EHC on bioavailability, since without it, equation 1.14 becomes a standard equation for

systemic availability in the absence of EHC. Given that in the presence of EHC ($1 - f_a f_g E$) is less than unity, oral availability will always be enhanced by enterocycling. The extent of the increase depends upon the hepatic extraction ratio (E), as well as magnitude of the cycling fraction ($f_a f_g$). Since in the presence of first-past hepatic extraction, clearance appears to be greater for an orally administered drug, the extent of EHC is similarly greater following oral dosing. However, EHC cannot increase systemic availability to values greater than unity. The maximum systemic availability exists when $E = 0$, and will depend, therefore, only upon the fraction absorbed. If $E \neq 0$, and EHC does occur, oral availability cannot be greater than intravenously administered drug, even though a greater percentage of the drug cycles following oral administration.

The total clearance of a drug is determined by dividing the dose by the AUC of the plasma-concentration time profile. For drugs which are excreted in the bile and completely reabsorbed, the cycling loop acts as a distributional compartment from which drug can redistribute back to the systemic circulation. This will increase the AUC of the drug, and as such, will reduce the "effective" clearance of a drug (Herman *et al.*, 1989, Peris-Ribera *et al.*, 1992). Although EHC cannot directly influence intrinsic clearance, this parameter may be subsequently underestimated if enterocycling is not taken into account. By slowing the rate at which the drug is eliminated from the body, EHC will also lengthen effective half-life, which will also increase the time need to reach steady-state conditions (Shepard *et al.*, 1985a). Frequency of gallbladder contractions has no effect on the AUC of a drug which undergoes EHC (Shepard and Reuning, 1987). However, area under the first moment curve (AUMC) is affected by time-dependent factors (Shepard *et al.* 1989), suggesting that the variable nature of gallbladder contraction affects both mean residence time (MRT), (a nonparametric correlate of elimination half-life) and volume of distribution at steady-state, both of which are dependent upon AUMC.

In addition to the theoretical effects on drug pharmacokinetics, EHC can affect the accurate characterization of a drug's disposition by introducing sporadic secondary peaks, usually late in a drug's concentration-time profile, during which time blood sampling tends to be much less frequent, and less likely to be noticed. Collection of a sample at the top of such a peak would lead to gross overestimation of plasma concentrations during that time interval, and an underestimation of clearance and overestimation of the elimination half-life (Shepard *et al.*, 1985b)

EHC can also influence a drug's pharmacodynamic activity. EHC-induced post-prandial peaks could cause an increase in drug activity due to an increase in the plasma concentrations; however, case reports of this nature are not readily found. Decreased effective clearance rates leads to increased systemic concentrations, which can be beneficial, as in the case with oral contraceptives (Brewster *et al.* 1977; Bacon and Shenfield, 1980) or detrimental, as demonstrated by chlordecone poisoning (Cohn *et al.*, 1978). EHC can also have some unique pharmacodynamic effects, by virtue of its physiology. In animal studies and in man, the high incidence of intestinal ulceration associated with NSAIDs is believed to be at least partly due to extensive enterohepatic circulation (Brune and Beck, 1991; Yamada *et al.*, 1993). EHC may also play a permissive role in the origin of certain carcinomas. It is an epidemiological fact that people who eat a high-meat, high-fat "western" diet have a much greater incidence of colon and intestinal cancers (Reddy and Wynder, 1973; Reddy *et al.*, 1992). A western diet is also associated with high fecal β-glucuronidase activities (Reddy *et al.*, 1974; Ling and Hanninen, 1992). It has been hypothesized that the increased incidence of colon and intestinal cancers may be the direct result of enterohepatic cycling which concentrates and releases carcinogenic compounds such as polycyclic aromatic hydrocarbons (PAHs) from non-toxic glucuronide conjugates within the lower bowels (Hill, 1988).

1.3. BENZODIAZEPINES

1.3.1. General

Benzodiazepines represent one of the most widely used class of drugs in the world. It has been estimated that in the United States, a total of 1.6% of all adults between 18 and 79 years use antianxiety drugs daily for a period of one year or longer (Mellinger *et al.*, 1984). In 1979 and 1984, benzodiazepines accounted for 9% and 7%, respectively, of all prescriptions dispensed in Great Britain (Guentert, 1984). First identified as a class in the 1930s, benzodiazepines were introduced into clinical therapeutics in 1960 with the release of chlordiazepoxide (Librium®). Previous to that time, general practitioners had extensively used barbiturates for the management of anxiety and sleep disorders. After the introduction of benzodiazepines, the use of barbiturates declined dramatically. Between 1964 and 1974, the number of prescriptions for barbiturates fell by 50% (Beaumont, 1990), during which time benzodiazepines attained remarkable popularity. In 1963 diazepam (Valium®) was introduced, and within 9 years, became the most frequently prescribed drug in the United States, selling 50 million prescriptions annually (Guentert, 1984). Compared to barbiturates, benzodiazepines are much safer in overdose, and have fewer and less severe side effects, and fewer drug interactions. They are believed to be less liable to induce dependence with subsequent withdrawal states (Beaumont, 1990).

Since the introduction of chlordiazepoxide, more than 3000 benzodiazepines have been synthesized, more than 120 have been tested for biological activity, and approximately 35 are used clinically throughout the world (Hobbs *et al.*, 1996). The large number of benzodiazepines in clinical use not only reflects the therapeutic advantages of this class of compounds, but also reflects their diverse clinical applications. Benzodiazepines are generally classified as anxiolytic and hypnotic agents. They are useful in controlling anxiety during minor surgery, endoscopy and

other investigative procedures, as well as in the management of acute alcoholic and hallucinogenic withdrawal (Guentert, 1984). As hypnotic agents, benzodiazepines significantly decrease the latency of sleep onset, increase the duration of stage 2 non-rapid eye movement (NREM) sleep (which represents approximately 75% of total sleep) and decrease the duration of rapid eye movement (REM) and slow-wave sleep (Trevor and Way, 1992). Benzodiazepines are also used for the treatment of epilepsy and for controlling skeletal muscle spasms of various etiologies. Benzodiazepines are used extensively as a preanesthetic medication, and are used intravenously for induction and maintenance of anesthesia (Kennedy & Longnecker, 1996).

The general structure of the benzodiazepines is shown in Figure 1.2. The term benzodiazepine refers to the portion of the structure composed of a benzene ring (A) joined to a seven-member diazepine ring (B). Almost all of the clinically used benzodiazepines contain a 1,4-diazepine ring, as well as a 5-aryl substituent (ring C). Position 7 of ring A contains a strongly electronegative group, such as a halogen, which enhances the drug's pharmacological potency. Position 2 of ring B often possesses a keto group, however, the chemical nature of positions 1 to 3 can vary widely, and can include a fused imidazolo or triazolo ring between positions 1 and 2. Introduction of hydroxyl groups at position 3 of ring B shortens the benzodiazepine's biological half-life.

Benzodiazepines are weakly basic drugs, which are often classified not by chemical structure, but according to their elimination half-life. Greenblatt *et al.* (1981) described three categories, dividing benzodiazepines into long-acting, intermediate- and short-acting, and ultra-short acting. Long-acting agents have half-lives exceeding 24 hours and often have long half-life active metabolites. These drugs accumulate extensively during repeated administration. Examples of long-acting agents include diazepam and clorazepate. Intermediate- and short-acting benzodiazepines include temazepam, lorazepam and oxazepam. These drugs have half-lives ranging from 5-24

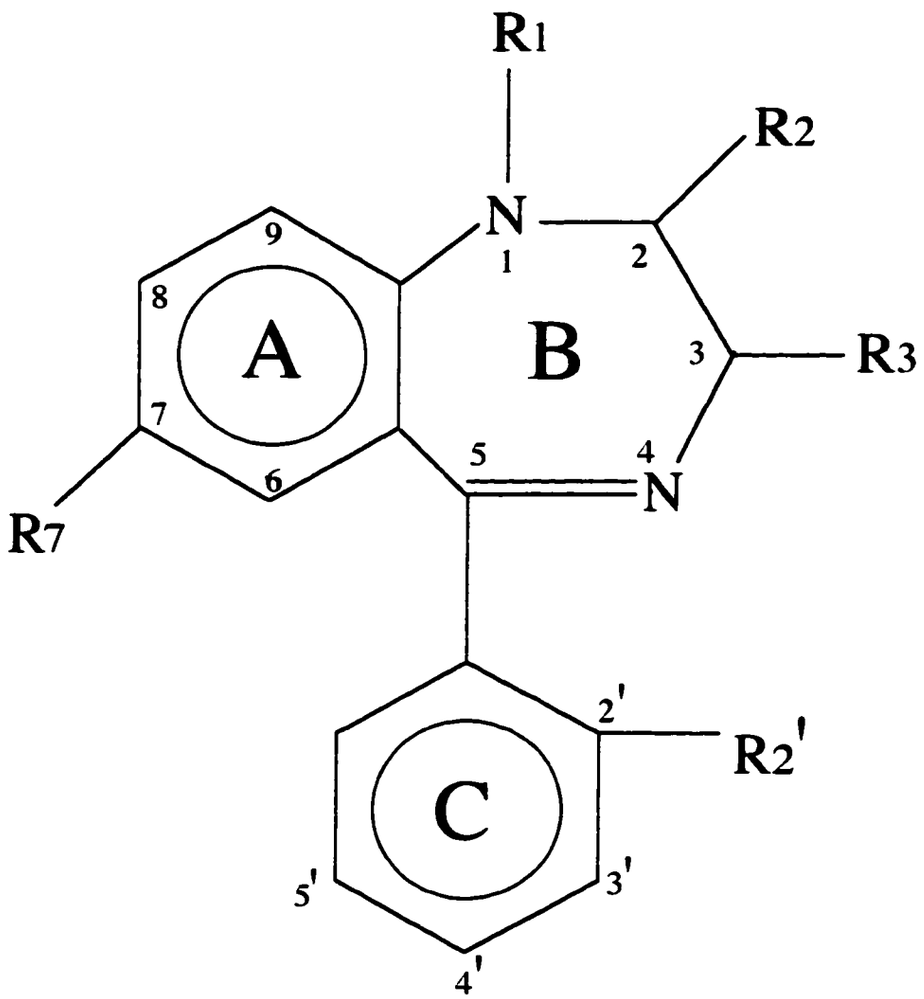


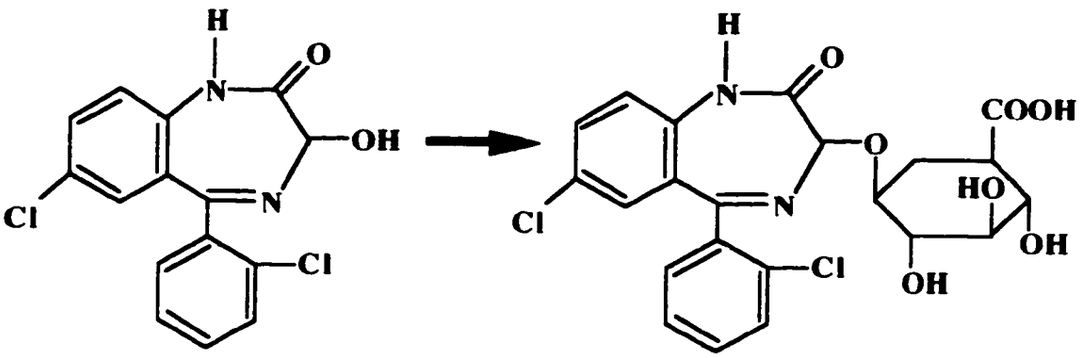
Figure 1.2 **General chemical structure of the benzodiazepines. R's denotes chemical substituents.**

hours, and active metabolites are uncommon. Ultra-short acting benzodiazepines have half-lives under 5 hours, and include triazolam and midazolam.

Early studies on the mechanism of action of the benzodiazepines at a molecular level revealed that benzodiazepines bind to a specific high affinity site on glycoproteins within the neuronal cell membrane (Möhler and Okada, 1977; Squires and Braestrup, 1977). Since that time, further studies have shown that these sites are integral components of the $GABA_A$ receptor, through which γ-aminobutyric acid (GABA), the principal inhibitory neurotransmitter of the CNS, mediates most of its effects on neuronal excitability. The $GABA_A$ receptor is an integral part of the membrane-bound chloride channel which is impermeable to ions in the absence of GABA. $GABA_A$ receptor activation results in a chloride influx into the cell and the increase of negative charge hyperpolarizes the cell. Benzodiazepines bind directly to the $GABA_A$ receptor/chloride channel complex and allosterically modulate its activity. In the presence of benzodiazepines, the affinity of the $GABA_A$ receptor for GABA is enhanced. Similarly, GABA allosterically alters benzodiazepine binding (Hobbs *et al.*, 1996). Benzodiazepine agonists facilitate GABAergic transmission by increasing the frequency of openings of the chloride channels (Twyman *et al.*, 1989). The discovery of inverse agonists which act at the benzodiazepine receptor has demonstrated that the channel gating function of GABA can also be allosterically modulated in a negative, or inhibitory, manner.

1.3.2. Lorazepam

Lorazepam (7-Chloro-5-(2-chlorophenyl)-1,3-dihydro-3-hydroxy-2H-1,4-benzodiazepin-2-one) is a 3-hydroxybenzodiazepine; a group extensively metabolized in the liver by glucuronidation of the 3-OH group, yielding pharmacologically inactive conjugates. The pathway of lorazepam metabolism is shown in Figure 1.3.



LORAZEPAM LORAZEPAM GLUCURONIDE

Figure 1.3. Glucuronidation of lorazepam

1.3.2.1. Absorption

Lorazepam is readily absorbed from the gastrointestinal tract in humans. Peak concentrations appear in the plasma 1.5-2 hours after oral dosing (Greenblatt *et al.*, 1976; 1979a). A lag time of approximately 30 minutes associated with absorption of lorazepam tablets probably represents a delay in drug going into solution, since Herman *et al.* (1989) found lag times of only a few minutes when administering an oral solution of lorazepam. Absorption of lorazepam appears to follow first-order processes, with absorption half-lives in the range of 10-15 minutes, and absorption being essentially complete within 1-2 hours. Absolute systemic availability of lorazepam is extensive, often reported as 100% of the administered dose, with no difference between intravenous, intramuscular, sublingual and oral routes (Greenblatt *et al.*, 1982; Herman *et al.*, 1989).

1.3.2.2. Disposition

The disposition of lorazepam is characterized by multicompartmental kinetics; intravenous data are often fitted to both two- and three-compartment models (Greenblatt *et al.*, 1979a; Herman *et al.*, 1989). The apparent volume of distribution of lorazepam in humans is approximately 1.0-1.3 L/kg (Greenblatt *et al.*, 1982). Binding of lorazepam to plasma proteins has been estimated to be 90% (Greenblatt, 1981; Herman *et al.*, 1989). Accordingly, the volume of distribution of free drug is approximately 14.4 L/kg (Herman *et al.*, 1989), suggesting extensive tissue distribution.

Lorazepam is extensively metabolized to a 3-OH glucuronide conjugate in man (Figure 1.3). After intravenous lorazepam, Greenblatt *et al.* (1979a) was able to recover 77% of the administered dose as lorazepam glucuronide recovered in urine within 72 hours, with a projected cumulative excretion of approximately 82%. Less than 1% of the dose was excreted unchanged in the urine, and only 6% was excreted as

oxidized metabolites (Kyriakopoulos *et al.*, 1978; Verbeeck *et al.*, 1976; Greenblatt *et al.*, 1979a). As such, lorazepam is often used as a marker of hepatic glucuronidative capacity in a number of pathological and physiological conditions (Kraus *et al.*, 1978; Greenblatt *et al.*, 1979b; Crom *et al.*, 1987, 1991; Kearns *et al.*, 1990).

Total clearance rates of lorazepam are estimated to be 0.7-1.2 mL/min/kg (Greenblatt, 1981), corresponding to free drug clearance rates of approximately 9-11 mL/min/kg (Kraus *et al.*, 1978; Herman *et al.*, 1989). A study by Kraus *et al.* (1978) found that liver disease and age had no effect on lorazepam clearance, while Greenblatt *et al.* (1979b) reported that the total clearance of lorazepam in 15 elderly subjects was significantly (22%) reduced compared to 15 healthy young subjects. Lorazepam clearance is reduced in patients with spinal cord injuries (Segal *et al.*, 1991), but increased in patients with cystic fibrosis (Kearns *et al.*, 1990), as well as in patients suffering from burn trauma (Martyn and Greenblatt, 1988). Whether or not these physiological and pathological conditions truly affect lorazepam clearance is not certain, since these studies report effects on total clearance values. Without considering possible effects on plasma protein binding, and without measuring free clearance values, changes in hepatic clearance based on total clearance rates can only be speculative. Crom *et al.* (1991) found no difference in unbound lorazepam clearance between children and adults. Herman *et al.* (1994) reported lower clearance rates of free lorazepam in patients with Gilbert's syndrome, the extent of which depended upon their feeding state, and interruption of enterohepatic circulation.

Considerable differences exist in the elimination half-lives reported for lorazepam. While Greenblatt and co-workers (1976; 1979a) found values of 13-16 hours, half-lives of 22 hours were reported by Kraus *et al.* (1978). Using simultaneous oral/iv administration, Herman *et al.* (1989) reported that the half-life of lorazepam determined for the orally administered dose was significantly shorter than that determined by the intravenous route. The authors pointed out that in a review of

lorazepam kinetics (Greenblatt, 1981), half-lives of oral lorazepam were generally shorter than those obtained from intravenous data, suggesting a difference in intravenous and oral lorazepam pharmacokinetics, possibly due to differences in clearance by the two routes.

Lorazepam glucuronide rapidly appears in the systemic circulation following intravenous lorazepam administration, and reaches maximal values in normal subjects within 6-12 hours (Greenblatt *et al.*, 1976; Herman *et al.*, 1989). Lorazepam glucuronide disappears in parallel with its parent drug, strongly suggesting that the elimination rate of lorazepam glucuronide is determined by its formation rate (Greenblatt *et al.*, 1979a). The plasma protein binding of lorazepam glucuronide has been estimated to be roughly 65% (Verbeeck *et al.*, 1976). This metabolite is recovered quantitatively in the urine of normal subjects. However, high concentrations of lorazepam glucuronide accumulate in patients with chronic renal failure (Verbeeck *et al.*, 1976).

Several studies suggest that lorazepam participates in a significant EHC in man. Lorazepam has been shown to enterocycle in ponies; following intravenous administration, 24% of the dose was recovered in the bile as the glucuronide (Greenblatt and Engelking, 1988). A closely related benzodiazepine, oxazepam, has been shown to enterocycle in dogs (Alván *et al.*, 1977). Following oral administration of ^{14}C-labeled lorazepam, Greenblatt *et al.* (1976) found 7% of the radioactivity in the faeces (Greenblatt *et al.*, 1976), suggesting either that lorazepam is not completely absorbed or that lorazepam or its metabolites undergo EHC in man. Delayed fecal excretion up to the fifth day following administration of radiolabeled lorazepam further hinted to an EHC in humans (Elliot, 1976). Recently, Herman *et al.* (1989) showed that lorazepam undergoes significant enterohepatic circulation in human beings. Using neomycin (an antibiotic effective against both gram-positive and gram-negative bacteria) and cholestyramine (a biliary sequestrant) to block the lorazepam enterocycling resulted

in a 25-35% increase in the oral and intravenous lorazepam clearance rates and decreases in total volume of distribution and elimination half-life. These findings were subsequently confirmed by multiple-dose studies, where treatment with neomycin and cholestyramine increased lorazepam clearances 5-45%, consistent with interruption of an enterohepatic circulation of the drug (Chaudhary *et al.*, 1993). Moreover, lorazepam EHC was subject to a circadian variation and produced different pharmacokinetic effects dependent upon whether or not the studies were done during a day-time or night-time dosing interval. In the presence of neomycin and cholestyramine, night-time clearances were significantly greater compared to the day; however, recoveries of urinary glucuronide were greater during the day compared to the night. This suggests that with fasting during the night, lorazepam glucuronide is trapped within an inactive enterohepatic circulation which is reactivated with feeding during the day.

Enterohepatic cycling makes the pharmacokinetics of a drug considerably more complex. In addition to adding a distributional compartment of the enteric circuit, it also reduces a drug's effective clearance. Lorazepam is often used as an *in vivo* probe to estimate hepatic glucuronidation (Crom *et al.*, 1987; Relling et al, 1987). However, without considering the effects of EHC, these measurements may not accurately reflect hepatic glucuronidative capacity (Herman *et al.*, 1989).

1.3.3. Diazepam

Diazepam (7-chloro-1,3-dihydro-1-methyl-5-phenyl -2H-1,4-benzodiazepin-2-one) is the prototype of a group of benzodiazepines (e.g. chlordiazepoxide, flurazepam) which form active N-dealkylated metabolites. Subsequent slow hydroxylation at position 3 results in metabolites which persist longer in the body than the parent drug. Diazepam is primarily metabolized to desmethyldiazepam, which is further

biotransformed to oxazepam. A secondary and less important route of diazepam metabolism is hydroxylation to temazepam. Since all three metabolites are pharmacologically active, the pharmacological effects of diazepam may persist for some time after diazepam's concentrations have been effectively reduced through metabolism. The major metabolic pathways of diazepam are shown in Figure 1.4.

1.3.3.1. Absorption

Diazepam is rapidly absorbed following oral administration with peak plasma concentrations occurring in 30-90 minutes (Kaplan *et al.*, 1973; Hillestad *et al.*, 1974; Klotz *et al.*, 1975; Greenblatt *et al.*, 1978). Assuming a first-order process, absorption half-lives are in the range of 15-25 minutes. Diazepam is a weak base (pKa = 3.3), and is absorbed most effectively at the high pH found in the duodenum. Absorption of diazepam is virtually complete, with the a bioavailability (F) reported as unity (Kaplan *et al.*, 1973). This is consistent with diazepam's low extraction ratio in man (Klotz *et al.*, 1976b).

1.3.3.2. Disposition

The kinetics of diazepam exhibit multicompartmental characteristics. Klotz *et al.* (1976a) fitted intravenous data to a two-compartmental model, whereas Greenblatt *et al.* (1980) used either two- or three-compartments, and Kaplan *et al.* (1973) used a three-compartmental model to fit their data. Three-compartment models appear to be more appropriate for highly lipid soluble compounds, reflecting a rapid initial distribution into the brain and other highly perfused organs, an intermediate redistribution phase into less well perfused tissues, and a slow elimination phase (Hobbs *et al.*, 1996). Both animal and human studies have demonstrated that diazepam

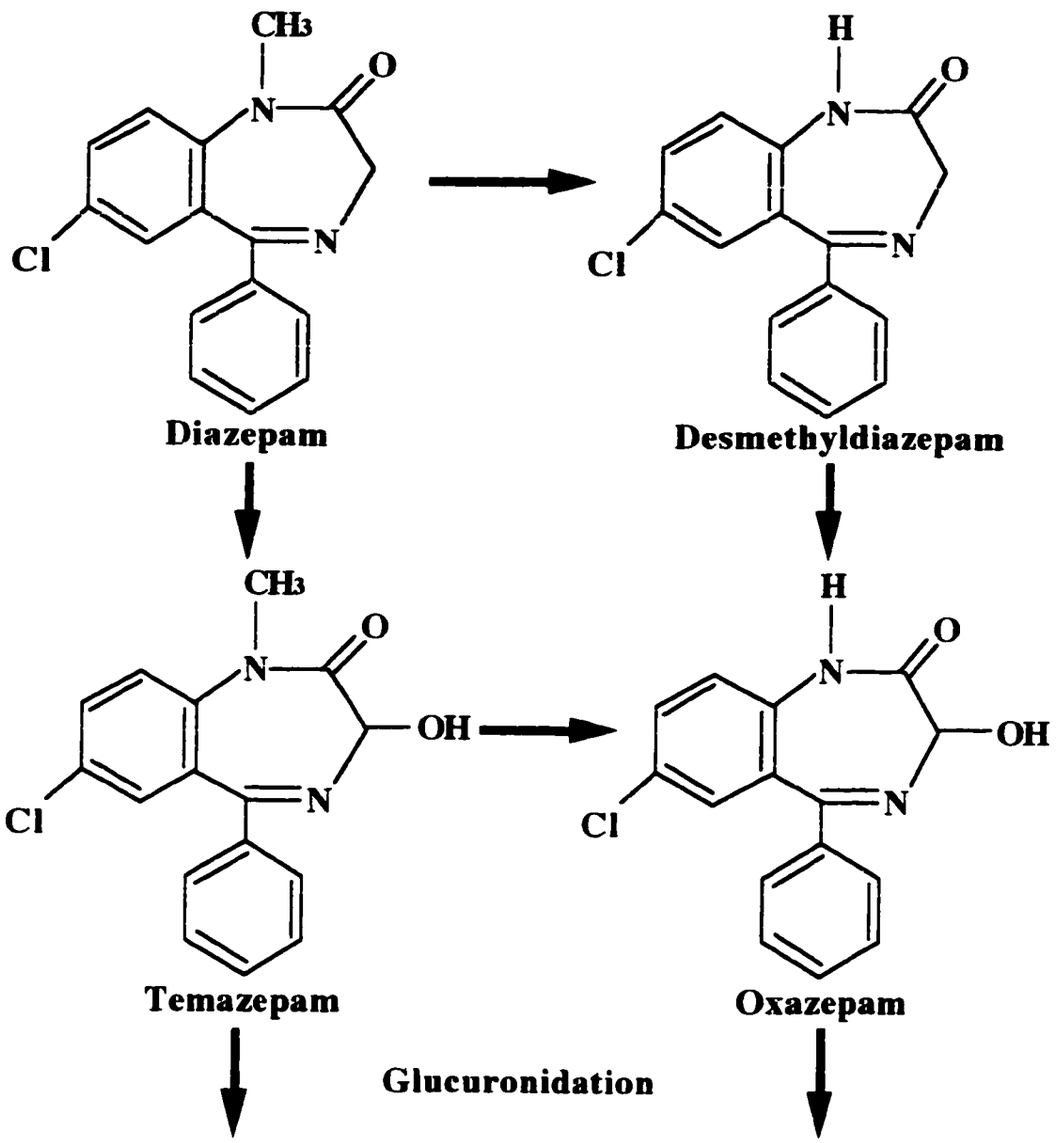


Figure 1.4. **Metabolic pathways of diazepam biotransformation.**

has no difficulty crossing the blood-brain barrier and, following distribution, concentrations in the cerebrospinal fluid (CSF) are equivalent to free drug concentrations found in the plasma (Colburn and Jack, 1987).

Several studies have shown a pronounced inter-individual variation in the disposition of diazepam. For example, a 30-fold variation in plasma concentration was recorded in patients given the same oral dose (Mandelli *et al.*, 1978).

The apparent volume of distribution of diazepam has been estimated to be approximately 1.0 L/kg in normal males, regardless of whether the estimates were calculated as Vd_{ss} (Klotz *et al.*, 1975) or V_{β} (Greenblatt *et al.*, 1980). Both these groups of investigators found that the volume of distribution of diazepam increases significantly with age. Gender also appears to be an important factor, as females have larger distribution volumes than males (Greenblatt *et al.*, 1980; MacLeod *et al.*, 1979). Binding to plasma proteins, principally albumin, is extensive, with diazepam free fractions reported to be approximately 1-2% (Abel *et al.*, 1979; Greenblatt *et al.*, 1980). There is some disagreement within the literature on the effect of age on the plasma protein binding of diazepam. Several studies (Abel *et al.*, 1979, Greenblatt *et al.*, 1980, Davis *et al.*, 1985) have reported that binding of diazepam to plasma proteins was decreased in with age, while others (Klotz *et al.*, 1975; Johnson *et al.*, 1979) found no correlation. Greenblatt *et al.* (1980) could only detect a difference between young and elderly females; no significant difference was found between young and elderly male subjects. Although Greenblatt *et al.* (1980) could not detect a difference in plasma protein binding based on gender, Abel *et al.* (1979) found that 40% of female subjects in their study had larger free fractions than males. In addition to intersubject variation, plasma protein binding of diazepam shows considerable intrasubject variation. Naranjo and co-workers (1980a) reported a diurnal variation in diazepam free fraction, associated with the intake of meals. Further studies indicated that the

variation was a result of meal-induced changes in plasma free fatty acids, which can compete with diazepam for binding sites on plasma albumin (Naranjo *et al.*, 1980b)

Correcting for the effects of plasma protein binding, the volume of distribution of unbound diazepam is approximately 90L/kg (Greenblatt *et al.*, 1980), suggesting extensive tissue distribution. This is not surprising considering the drug's lipophilic nature. Abernethy *et al.* (1983) found a 3-fold increase in diazepam's volume of distribution in obese subjects compared to normal-weight patients. Binding to tissue proteins may also be an important factor, since 60% of the body's albumin is extravascular (Routledge *et al.*, 1980; Wilkinson, 1983). Indeed, in experiments studying the tissue distribution of benzodiazepines in rats, Scavone *et al.* (1987) calculated that the largest fraction of total body stores was in skeletal muscle (approximately 40% for diazepam), followed by fat and liver. In studies using rabbits, diazepam binding is characterized by binding ratios in plasma and tissues of the same order of magnitude (Fichtl and Hurz, 1981).

Diazepam is extensively metabolized in man by the pathways shown in Figure 1.4. Less than 5% of an intravenous dose is excreted unchanged in the urine (Kaplan *et al.*, 1973). The major metabolic reaction of diazepam is demethylation, with the mean fraction of the dose converted to desmethyldiazepam being 0.62 (Bertilsson *et al.*, 1989). Working with human liver microsomes, Inaba *et al.* (1988) reported that the intrinsic clearance for the generation of temazepam was higher than that for the formation of desmethyldiazepam. Bertilsson *et al.* (1993) suggest that this discrepancy can be explained by *in vitro* work in their laboratory which indicates that two distinct enzymes are involved in the 3-hydroxylation of diazepam in human hepatic microsomes, one enzyme system which possesses a low V_{max} at therapeutic concentrations, and a separate enzyme system which is characterized by a high K_m and V_{max} at high substrate concentrations.

Estimates of clearance of diazepam indicate a pronounced inter-individual variability. Being a drug that is extensively metabolized by the liver, it is not surprising that diazepam clearance values are lower in patients with liver disease (Klotz *et al.*, 1975; Klotz *et al.*, 1977b). Aging is believed to reduce the clearance of drugs which undergo oxidative metabolism, although studies on the effects of age on diazepam clearance show some discrepancy. Greenblatt *et al.* (1980) reported total body clearances of 0.39 (range 0.28-0.77) mL/min/kg in 11 young males, a significant difference ($p<0.01$) compared to the clearance values of 0.24 (range 0.11-0.34) mL/min/kg calculated for 11 elderly male subjects. Interestingly, total body clearance of diazepam was higher in 11 young and 11 elderly females compared to males, although there was no difference in total diazepam clearance between the young (0.51, range 0.35-0.93 mL/min/kg) and elderly females (0.48, range 0.27-0.81 mL/min/kg). This is in contrast to the findings of MacLeod *et al.* (1979), who concluded that age did not appear to influence clearance and also that average total body clearance values were less in females. As well, Klotz *et al.* (1975), found no effect of age *per se* on diazepam clearance. All three of the aforementioned studies based their conclusions on total clearance values. Being a low clearance drug in humans, it follows that the clearance of diazepam is dependent upon free fraction (Wilkinson and Shand, 1975). Greenblatt *et al.* (1980) calculated free diazepam clearances, reporting rates of 29.9 (range 18.3-56.9), 43.6 (range, 22.4-76.7), 14.9 (range 7.0-25.4), and 28.0 (range 13.9-45.1) mL/min/kg in young males and females, and elderly males and females, respectively. However , estimates were based on single plasma protein measurements drawn in the nonfasting state 3 days after the dose of diazepam.

Given the large interindividual and intraindividual variability in plasma protein binding of diazepam, it is reasonable to assume that the variability of diazepam clearance is in part due to changes in diazepam free fraction. However, Giles *et al.* (1981) reported that in young healthy subjects, plasma protein binding is not principally

related to interindividual variation in mean diazepam disposition, but rather ascribed 93% of the variation in total clearance to variations in the intrinsic clearance of the drug.

Although previous work has focused on factors such as age and gender, recent findings in the field of pharmacogenetics suggest that much of the variability in diazepam clearance may be controlled by genetic factors. Studies have shown that a polymorphism in the enzymes responsible for oxidative hydroxylation of S-mephenytoin, namely CYP2C19 (Goldstein *et al.*, 1994), co-segregates with the rate of diazepam metabolism. The total clearance of diazepam and desmethyldiazepam in individuals phenotyped as poor metabolizers of S-mephenytoin was approximately half that found in extensive metabolizers (Bertilsson *et al.*, 1989). The difference could not be explained by alteration in the route of metabolism, as formation of the desmethyl metabolite was identical between the two groups, suggesting that both diazepam and desmethyldiazepam are metabolized by CYP2C19. Polymorphism of S-mephenytoin also shows interethnic differences; less than 5% of the Caucasian population are classified as poor metabolizers, compared to almost 15% in native Chinese (Bertilsson *et al.*, 1992), 13% in Koreans (Sohn *et al.*, 1992), while the incidence in Japanese subjects is 18-23% (Jurima *et al.*, 1985; Nakamura *et al.*, 1985). In a study in Korean subjects, poor metabolizers of S-mephenytoin had lower diazepam clearances and longer mean half-lives than extensive metabolizers (Sohn *et al.*, 1992). Interestingly, no differences in clearance or half-life of diazepam were found in a study of native Chinese poor and extensive metabolizers of S-mephenytoin (Zhang *et al.*, 1990), with both groups exhibiting values similar to Caucasian poor metabolizers. This supports findings from a study which found that Orientals had a lower clearance of diazepam than Caucasians (Kumana *et al.*, 1987). A study in twins (Alda *et al.*, 1987) failed to detect any hereditary influence on the disposition of diazepam; however, diazepam concentrations were determined only for 24 hours after dosing.

Given the substantial amount of variation in both volume of distribution and clearance, the elimination half-life of diazepam varies widely depending on the population studied. Greenblatt *et al.* (1980) reported elimination half-lives of 36.0 hours (range 20.4-66.7 hours) in a group of young males, while elderly males had a mean value of 98.5 hours (range 50.2-173.3 hours). The dramatic increase was attributed to significant decreases in total and unbound clearances, as well as to an increase in volume of distribution of total drug. Others have not shown the clearance changes. In these cases, increases in half-life are reported to be due to an increase in volume of distribution.

Due to the low clearance of diazepam and its demethylated product, desmethyldiazepam, extensive accumulation of both compounds is observed after multiple doses. This is clinically important since diazepam is most often used therapeutically on a subchronic or chronic schedule. In addition, high levels of desmethyldiazepam have been shown to decrease the clearance of diazepam (Klotz and Reimann, 1981).

1.3.3.2.1. Post-prandial diazepam peaks

Peaks occur in the concentration-time profile of diazepam that coincide closely in time with the ingestion of meals (Linnoila *et al.*, 1975; Korttila *et al.*, 1976; Korttila and Kangas, 1977; Naranjo *et al.*, 1980a; 1980b). Enterohepatic circulation of diazepam has been considered by several investigators to explain these deviations from log-linear decline in plasma concentrations (Van de Kleijn *et al.*, 1971; Baird and Hailey, 1972, 1973; Sellman *et al.*, 1975). However, the molecular weight threshold of compounds excreted in bile is approximately 500 daltons (Dobrinska, 1989; Eustace *et al.*, 1975), which is considerably greater than diazepam's molecular weight of 285. Indeed, in studies using biliary T-tubes in patients with normal liver function (Eustace

et al., 1975; Klotz *et al.*, 1975) and in patients with liver disease (Hepner *et al.*, 1977), biliary diazepam and desmethyldiazepam concentrations were insufficient to have an effect on drug disposition, although these measurements were made after acute rather than chronic dosing where accumulation of drug and or metabolite are more likely to occur. Thus, diazepam does not appear to participate directly in an enterohepatic circulation. However, one of its metabolites, oxazepam, is known to undergo significant enterohepatic circulation in mice (Van der Kleijn *et al.*, 1971), dogs (Alván *et al.*, 1977) and guinea-pigs (Bertagni *et al.*, 1978). Evidence of enterohepatic circulation of oxazepam was found in patients with renal failure (Odar-Cederlöf *et al.*, 1977). However, this may not be readily extrapolated to a normal physiological condition, since uremia caused a 60-fold increase in systemic oxazepam glucuronide levels. To suggest that oxazepam contributes to an enterohepatic circulation of diazepam poses the presence of a metabolism-reformation pathway. A study with 2 postcholecystectomy patients with biliary T-tubes, found only minimal amounts of total oxazepam recovered in the bile within 48 hours after oxazepam dosing (Shull *et al.*, 1976) while another study reported only low levels of labile benzodiazepine conjugates in the bile of 4 patients with biliary T-tubes given diazepam, despite fluctuations in systemic diazepam levels (Eustace et al, 1975).

More recent studies suggest that post-prandial diazepam peaking is the result of changes in binding to plasma proteins, and as such, a redistribution of diazepam from peripheral tissues to the vascular compartment. Naranjo *et al.* (1980a) observed circadian fluctuations in diazepam plasma protein binding, with higher diazepam free fractions values between 11 p.m. and 8 a.m., and lower diazepam free fractions after 9 a.m.. The diurnal changes in diazepam plasma protein binding were temporally related to food intake. Further work by this group correlated the decrease in diazepam free fraction with a concomitant decrease in total free fatty acids following meals (Naranjo *et al.*, 1980b). Free fatty acids decrease diazepam binding to albumin *in vitro* (Tsutsumi

et al., 1975; Sellers *et al.*; 1980) and *in vivo* (Colburn and Gibaldi, 1978). In contrast to the aforementioned studies, several investigators have failed to observed meal-induced changes in diazepam binding to plasma proteins (Klotz *et al.*, 1977a; Korttila and Kanges, 1977), suggesting that diazepam post-prandial peaks are not caused by changes in plasma protein binding.

1.4. DIABETES MELLITUS

1.4.1. General

Diabetes mellitus is an heterogeneous disorder, characterized by chronic hyperglycemia and disturbances of carbohydrate, fat and protein metabolism associated with an absolute, or relative deficiency in insulin secretion and/or insulin action (Bennett, 1994). The term diabetes mellitus is derived from the Greek word *diabetes*, meaning siphon, referring to the copious amounts of urine produced, and the Latinized Greek word *mellítus*, which means "honeyed" or "sweet" (Lawrence, 1994).
The World Health Organization (WHO) classification of diabetes mellitus includes a number of different clinical classes and statistical risk classes. In its classic form, two common clinical types of diabetes mellitus are recognized: insulin-dependent diabetes mellitus (IDDM), also known as Type I diabetes mellitus; and non-insulin-dependent diabetes mellitus, also known as Type II diabetes mellitus. Earlier nomenclature designated IDDM as juvenile-onset, and NIDDM as adult-onset diabetes; however, since these designations relate nothing of the underlying cause of the disease, and because either disorder may present at any age, their use is no longer recommended (Bennett, 1994). These types of diabetes differ both in clinical presentation and presumed etiology.

IDDM is characterized by an insulin deficiency secondary to an almost complete autoimmune destruction of pancreatic β-cells (insulin-producing), with maintenance of the α- (glucagon-secreting) and δ -cells (somatostatin-secreting) of the islets of Langerhans (Eisenbarth *et al.*, 1994). Patients present with classical symptoms of diabetes, such as polydipsia, polyuria, excessive hunger, fatigue, weight loss and/or ketoacidosis. The incidence of IDDM is influenced by genetic factors; approximately 50% of identical twins are concordant for the disease (Eisenbarth *et al.*, 1994). However, 90% of patients who develop IDDM do not have a first-degree relative with diabetes, suggesting that genetics alone is not responsible for the disease. Current theories imply that genetics predispose individuals to developing IDDM, with the autoimmune destruction of the pancreatic β-cells precipitated by environmental factors. A growing body of evidence implicates viral infections as possible precipitating events in the pathogenesis of IDDM (Yoon *et al.*, 1979; King *et al.*, 1983; Pak *et al.*, 1988; Guberski *et al.*, 1991). A steady increase in the incidence rate of IDDM in Europe, but not in North America, gives further support to environmental factors playing a role (LaPorte *et al.*, 1995). The estimated prevalence rate of IDDM in the United States is 1.2/1000 (LaPorte *et al.*, 1995), while the cumulative incidence rate of IDDM, has been estimated to be 10.5 per 1000 by the age of 70 (Krolewski and Warram, 1995). IDDM accounts for approximately 7% of all cases of diabetes mellitus (LaPorte *et al.*, 1995). Treatment of IDDM requires life-long exogenous insulin therapy.

NIDDM may also present with classical diabetic symptoms, but is often asymptomatic. Because of the lack of overt symptoms, complications of the disorder, such as retinopathy, neuropathy or atherosclerosis may be the first clinical indications of the disease. NIDDM is the result of a lack of insulin action (insulin resistance), or a disorder in insulin secretion. Patients with fully developed NIDDM syndrome usually exhibit both (Bennett, 1994). In most individuals with NIDDM, basal insulin levels are normal or elevated, with the degree of elevation correlated with the degree of obesity.

However, insulin secretory responses to oral glucose loads are markedly reduced (Khan, 1995b). A strong genetic link is associated with the pathogenesis of NIDDM. The cumulative incidence rates of NIDDM in the general population is 5.8% by the age of 60, but is 28.0% in the offspring of two parents with NIDDM. (Krolewski and Warram, 1995). Obesity is also a significant risk factor in developing NIDDM. The estimated prevalence rate of NIDDM in the United States is 3.1% (Kenney *et al.*, 1995). However, considering the asymptomatic nature of the disorder, the actual rate could be much higher. The cumulative incidence rate of NIDDM by the age of 70 has been estimated to be 111 per 1000 (Krolewski and Warram, 1995). NIDDM accounts for 90% of all diabetic cases (Kenney *et al.* 1995). Standard treatment of NIDDM includes diet, oral hypoglycemic agents, and if necessary, insulin.

1.4.2. Physiological actions of insulin

Insulin is a 5800 dalton peptide hormone produced by β-cells within the pancreatic islets of Langerhans. Synthesized as a single chain precursor of 110 amino acids called preproinsulin, it is converted to proinsulin (86 amino acids) upon translocation through the rough endoplasmic reticulum, and to insulin in the Golgi complex (Davis and Granner, 1996). Insulin is comprised of two peptide chains held together by two disulfide bonds; the A chain of human insulin contains 21 amino acids, and the B chain contains 30 amino acids.

Insulin is the primary hormone responsible for signaling the storage and utilization of glucose and other nutrients and inhibition of glucose production (Khan, 1995a). Insulin acts as an anabolic hormone by stimulating transport systems and enzymes for the intracellular utilization and storage of glucose, amino acids and fatty acids, while inhibiting catabolic processes such as the breakdown of glycogen, fat, and protein. The cellular actions of insulin are mediated by binding to the extracellular

domain of insulin receptors, and to a lesser degree, to insulin-like growth factor 1 (IGF-1) receptors. These receptors are found in classic tissues involved with fuel metabolism, such as muscle, liver, and fat, but also in other tissues, including the brain, circulating blood cells and gonadal cells (Davis and Granner, 1996). Insulin and IGF-1 receptors belong to a family of receptors known as ligand-activated protein tyrosine kinases. Insulin binding causes rapid autophosphorylation of tyrosine residues on the intracellular domain of the receptor, which results in an increased tyrosine kinase activity of the receptor towards other intracellular substrates, including insulin-receptor substrate-1 (IRS-1). This initiates a complex cascade of biochemical interactions, leading to several physiological, biochemical and molecular events. Increased glucose uptake is stimulated by the translocation of intracellular glucose transporters (GLUT-4 and GLUT-1) to the cellular membrane (Davis and Granner, 1996). This is an immediate action of insulin, and is reversible; upon the removal of insulin, the transporters return to the intracellular pool. A more long-term effect of insulin is the regulation of genes. Insulin is a powerful regulator of gene transcription, and is known to regulate nearly 100 genes, including mitogen-activated protein (MAP) kinases (O'Brien and Granner, 1991).

The plasma half-life of insulin injected into the systemic circulation is 5 to 6 minutes in diabetic and non-diabetic populations (Kahn, 1995a). Insulin is degraded primarily in the liver, kidney and muscle. Approximately one-half of the insulin released into the portal circulation is taken up in a single pass through the liver, and never reaches the systemic circulation (Khan, 1995a).

Until recently, all patients with IDDM were treated with animal insulin preparations. However, advances in the field of biotechnology have been able to produce synthetic forms of human insulin identical to the endogenous hormone. It is generally accepted that human insulin and animal insulin are identical in their physiological actions, (Sjöbom *et al.*, 1990; Ferrer *et al.*, 1992). A growing body of

evidence, however, suggests otherwise. Small changes in the structure of the insulin molecule has been shown to have dramatic effects on its activity *in vitro* (Tager *et al.*; 1979; King and Kahn, 1981). Human insulin differs from porcine insulin by one (B-30) amino acid, and from bovine insulin by three (B-30, A-8, A-10) amino acid residues. *In vitro* binding studies with monoclonal anti-insulin receptor antibodies have shown that human insulin and porcine insulin interact differently with the insulin receptor (Sesti *et al.*, 1991). Several studies have shown a difference between human and porcine and/or bovine insulin in the physiological effects in man (Lingelfelser *et al.*, 1992; Dahlan *et al.*, 1993; Kern *et al.*, 1994), although other studies have failed to find any differences in either the effects (Sjöbom *et al.*, 1990; Ferrer *et al.*, 1992) or the kinetics (Thorsteinsson *et al.*, 1987) of the different insulin species.

1.4.3. Effect of diabetes mellitus on drug disposition

1.4.3.1. Distribution

Factors which can alter the extent of drug distribution in a disease state include changes in binding to tissues and plasma proteins, as well as alterations in weight and body surface, and the lean/fat body mass ratio (Klotz, 1976). Also, the rate of distribution may be affected by changes in blood flow and cardiac output. In general, little attention has been paid to studying the effects of diabetes on the distribution of drugs (O'Connor and Feely, 1987). Moreover, there appears to be considerable inconsistencies among various studies that have investigated this subject (Gwilt *et al.*, 1991), due in part to the different forms of the disease. For example, Samela *et al.* (1980) reported an increase in the volume of distribution of antipyrine in patients with NIDDM compared to controls, while Zysset and Weitholtz (1988) found a decrease in antipyrine's volume of distribution in NIDDM patients, and no change in IIDDM

subjects. The volume of distribution of acetaminophen is reported to be increased in IDDM patients, but not different from controls in patients with NIDDM (Adithan *et al.*, 1988).

Changes in plasma protein binding may be anticipated in diabetic patients. These may result from of non-enzymatic glycosylation of plasma proteins, or through binding interactions due to FFAs, which may be elevated as a result of disturbances in fuel metabolism. Mereish *et al.* (1982) showed that binding of salicylate decreased with glycosylation of albumin. A study by Ruiz-Cabello and Erill (1984) showed a decrease in plasma protein binding of diazepam and sulfisoxazole in serum incubated with 20 mM glucose, and an increase in the free fraction of both drugs in the serum of diabetic patients. No details were given as to whether the patients had IDDM or NIDDM. The authors suggested that post-translational modification of albumin due to glycosylation caused interference with binding of sulfisoxazole to site I, without affecting site II. Decreased diazepam binding was believed to be due increased FFAs. In a separate study, IDDM patients showed no change in plasma protein binding of phenytoin, but a significant increase in the binding of valproic acid, which was positively correlated with FFAs (Gatti *et al.*, 1987). Studies of plasma protein binding of warfarin (bound to albumin) and lidocaine (bound to α_1-acid glycoprotein) found a decrease in binding of both drugs in IDDM subjects, and an increased binding of warfarin and no change in binding of lidocaine in patients with NIDDM (O'Byrne *et al.*, 1993). The IDDM group also had lower plasma concentrations of albumin, possibly through diabetes-induced nephropathy. Together, these studies suggest that in patients with IDDM, binding of drugs to site I and II is generally decreased, although the underlying mechanism may be different. In patients with NIDDM, effects on plasma protein binding appear to be less consistent.

One of the main implications of a change in plasma protein binding is a disruption of the relationship between total plasma drug concentration and clinical

effect. A decrease in binding to plasma proteins would increase the volume of distribution of the drug, resulting in lower total concentrations, with no change in free drug concentrations.

1.4.3.2. Elimination

A considerable body of work has been done investigating the effect of diabetes on the elimination of xenobiotics. The most important measure of drug elimination is clearance. Inasmuch as the total clearance of poorly cleared drugs is dependent upon free fraction and intrinsic clearance (equation 1.7), diabetic-induced changes in either of these parameters would be expected to affect their elimination.

Much of the work on the effects of diabetes on drug metabolism has been done with animals. Studies in the rat show that drugs which are oxidatively metabolized by the CYP450-mediated system are modified by diabetes in a substrate-specific and sex-specific manner. Alloxan, streptozotozin, or 6-aminonicotinamide-induced diabetes in male rats decreases the activities of aminopyrine-N-demethylase, aryl hydrocarbon (benzo[a]pyrene) hydroxylase, and hexobarbital oxidation, but increases aniline hydroxylase activity (Stohs *et al.*, 1979; Reinke *et al.*, 1978; Faas and Carter, 1980; Chawalit *et al.*, 1982; Skett and Joels, 1985; Eacho and Weiner, 1980a). In female rats, induction of diabetes increases the metabolism of aminopyrine, hexobarbital, aniline and benzo[a]pyrene. Insulin treatment was able to reverse the changes in diabetic rats, but had no effect on metabolism in normal rats. Streptozotocin-induced diabetes decreases the 3-hydroxylation and N-demethylation of diazepam, the N-deethylation of lidocaine and the N-oxidation and N-demethylation of imiprimine in male rats, but had no effect on imipramine 2-hydroxylase (Skett and Joels, 1985). There was no effect in female rats on any of the enzymes studied. Interestingly, Skett *et al.* (1984) found that streptozotocin-induced diabetes and castration had similar

effects on drug metabolism in the male rat, and that streptozotocin had little effect in the castrated male rat, suggesting a possible link between androgens and insulin in the control of drug metabolism.

Changes in CYP450 levels in diabetic rats have been attributed to an increase in the content of a specific cytochrome P450 isozyme, namely, CYP2E1 (Dong *et al.*, 1988; Favreau and Schenkman, 1988). Although CYP2E1 is found in untreated animals, it is predominant in female rats (Waxman *et al.*, 1989). CYP2E1 is one of the most highly conserved P450 genes; of the genes cloned from mammals in the gene families CYP2 and CYP3, only CYP2E1 shows enough structural similarities between rat and human species to be given the same gene number (Ingelman-Sundberg and Johansson, 1995). Expression of CYP2E1 has been detected in leukocytes from uncontrolled diabetics (Song *et al.*, 1990), and is induced by ethanol (Gonzales, 1992) and starvation (Johansson *et al.*, 1991). Whether changes in CYP2E1 content is responsible for altered drug metabolism in diabetic animals remains to be seen. Recent evidence suggests the gene is correlated to glucose homeostasis, with acetone as its natural endogenous substrate (Ingelman-Sundberg and Johansson, 1995).

Diabetes can also affect drug-conjugating enzymes, and like oxidative metabolism, the effects are substrate- sex-, and species-dependent. Diabetes inhibits the glucuronidation of 1-naphthol (Morrison and Hawksworth; 1984; Grant and Duthie; 1987; Watkins *et al.*, 1988), phenolphthalein (Grant and Duthie; 1987) and testosterone (Chawalit *et al.*, 1982; Watkins and Mangels; 1987; Watkins *et al.*, 1988), increases the conjugation of diethylstilbestrol (Watkins *et al.*, 1988), and can either increase (Price and Jollow, 1982) or have no effect (Morrison and Hawksworth; 1984) on the glucuronidation of acetaminophen. Glucuronidation of 4-nitrophenol (Rouer *et al.*, 1981; Eacho *et al.*, 1981a; 1981b; Morrison and Hawksworth 1984; Morrison *et al.*, 1986; Vega *et al.*, 1986) is either enhanced or inhibited in male diabetic animals, depending on the species, but has no effect on females. Insulin was able to reverse the

diabetes-induced effect on glucuronidation in some (Eacho *et al.*, 1981b; Morrison and Hawksworth, 1984; Vega *et al.*, 1986; Vega *et al.*, 1992) but not all (Vega *et al.*, 1992) studies.

In addition to its effects on drug metabolizing enzymes, diabetes has also been shown to effect the hepatobiliary clearance of drugs. Insulin deficiency for one month in streptozotocin-induced male diabetic rats had no effect on the total clearance of acetaminophen, but significantly reduced its volume of distribution, and reduced the biliary excretion of glucuronide and sulfate conjugates by 75 and 50%, respectively (Watkins and Sherman, 1992). No change was noted in either biliary flow or UDPGA levels. Conversely, the volume of distribution and biliary clearance of digoxin was increased significantly in the diabetic rats. Diabetes had no effect on bilirubin pharmacokinetic parameters. Administration of insulin reversed most, but not all, of the changes in acetaminophen and digoxin kinetics.

The large substrate-, species-, and sex-differences in the effects of diabetes in animals make extrapolation to the human population difficult. Furthermore, there is evidence suggesting that the effect of experimentally-induced diabetes depends upon the diabetogenic agent used (Chawalit *et al.*, 1982, Vega *et al.*, 1992).

Compared to work with animals, studies on the effects of diabetes on drug metabolism in humans are sparse and conflicting (Gwilt *et al.*, 1991). This is further complicated by the multiple forms of the disease, as well as the complications of chronic diabetes including changes in the liver. Dainith *et al.* (1976) reported that patients with NIDDM treated with insulin had an increased metabolism and a decreased elimination half-life of antipyrine, while there was no alteration of antipyrine elimination in diabetics patients who were controlled by only diet or with oral hypoglycemics. A different study using age- and weight-matched controls found that antipyrine metabolism was significantly decreased in NIDDM patients, but was increased (not significantly) in IDDM (Zysset and Weitholtz, 1988). Dajani *et al.*

(1974) demonstrated a decrease in the rate of metabolism of phenacetin in diabetics, which was reversed with insulin treatment. However, no mention was made of the type of diabetes that was being studied. Finally, in a study in which oxazepam was administered twice to 6 diabetic patients during uncontrolled and controlled diabetes, the average AUC and elimination half-life of oxazepam was 34 and 39% higher during the uncontrolled state; however, these differences were not significant (Scott *et al.*, 1988).

2. PRESENT INVESTIGATION

2.1 General

Feeding can exert profound influences on drug disposition (Vesell, 1984; Anderson, 1988). These pharmacokinetic interactions can include changes in a drug's distribution, as well as in its elimination, both of which can potentially lead to changes in pharmacodynamic effects. The purpose of the present investigation is to study the influence of feeding on the pharmacokinetics of two benzodiazepines, lorazepam and diazepam, which are to be used as indirect tools to assess feeding-effects on drug disposition.

Lorazepam is a model of benzodiazepines which are conjugated directly to glucuronide metabolites. On the other hand, diazepam can be considered to be the prototype of the benzodiazepines which are metabolized extensively through hepatic oxidation (phase I metabolism). Moreover, diazepam is prototypical of a drug having extensive distribution into tissues. This classification not only designates how the body handles these drugs, but also their potential therapeutic uses (Greenblatt *et al.*, 1981). Feeding effects on lorazepam disposition would be anticipated to demonstrate changes mediated by alterations in drug metabolism, whereas changes in diazepam disposition caused by food intake would be expected to caused by changes in drug distribution.

Work by others has suggested that the rate of hepatic glucuronidation may be limited by the availability of UDP-glucuronic acid (Otani *et al.*, 1976; Moldéus *et al.*, 1978; Conway *et al.*, 1987; Felsher *et al.*, 1979; Hjelle *et al.*, 1985; Price and Jollow, 1984; Reinke *et al.*, 1981, Singh and Schwarz, 1981; Shipley and Weiner, 1987).

Studies in our laboratory show that the disposition of lorazepam in normal controls and in patients with Gilbert's syndrome is altered following the ingestion of a a high-carbohydrate, low-fat meal (Herman *et al.*, 1994). However, the effects of feeding may be complicated by the hormonal and neural responses induced by food intake. Because patients with IDDM lack endogenous insulin, this population provides a unique opportunity to study drug disposition in euglycemic and hyperglycemic conditions in the absence of the insulin response. This group also permits the possibility of studying the effects of high and low levels of insulin on hepatic glucuronidation, since insulin has been implicated in possessing a role in drug metabolism (Eacho *et al.*, 1981b; Morrison and Hawksworth, 1984; Vega *et al.*, 1986; Vega *et al.*, 1992). Insulin is also a potent stimulus for bile flow (Klaassen and Watkins, 1984). Studies with patients with IDDM also enable the opportunity of examining the potential differences in the physiological actions of human and animal insulins, which have been recently suggested in the literature. Patients with IDDM also have abnormal glucagon physiology. Since it has been shown that glucagon can stimulate glucuronidation (Ricci *et al.*, 1989), and is also a potent choleretic agent in man (Branum *et al.*, 1991), the effects of exogenous glucagon on the disposition of lorazepam were also examined in this group.

Because the pharmacokinetics of lorazepam in man are complicated by enterohepatic circulation, studies of the effects of feeding on lorazepam disposition must also consider enterocycling. To help to understand the effects of EHC on lorazepam disposition, a second objective of this investigation was to develop an animal model of EHC in the dog. The animals most frequently used for studies on biliary excretion of xenobiotics are the rat and the dog (Siegers, 1991). The rat, however, has no gallbladder, and also has a bile flow that exceeds that of man by a factor of 20; biliary flow between man and dog are comparable. A related benzodiazepine, oxazepam, undergoes enterohepatic circulation in the dog (Alván *et al.*, 1977). Since

lorazepam undergoes enterohepatic cycling in humans (Herman *et al.*, 1989), and ponies (Greenblatt and Engelking, 1988) it was decided that this would be used as a probe drug in the development of a model of enterohepatic circulation in the dog.

Food intake causes significant post-prandial peaks in the concentration-time profile of diazepam. Several investigators have suggested these peaks are due to enterohepatic circulation (Van de Kleijn *et al.*, 1971; Baird and Hailey, 1972, 1973; Sellman *et al.*, 1975). Others have disputed these claims, and report that post-prandial diazepam peaks are the result of changes in plasma protein binding (Naranjo *et al.*, 1980a; 1980b). However, changes in binding have not been observed by all investigators (Klotz *et al.*, 1977a; Kortilla and Kangas, 1977). Because of the magnitude of these peaks during multiple-dosing, which is the usual way in which drugs are taken, this investigation sought to elucidate the true mechanism of post-prandial diazepam peaks, and to examine if these peaks were associated with an alteration in free concentration of diazepam, which could lead to possible pharmacodynamic consequences. Previously, it has been shown that treatment with neomycin and cholestyramine is effective in interrupting the enterohepatic circulation of lorazepam (Herman *et al.*, 1989). If post-prandial diazepam peaks are the result of enterohepatic circulation, interrupting the cycling should produce significant changes in the disposition of this drug. If, on the other hand, these peaks are due to altered binding due to nutritional effects on plasma free fatty acids, perturbation of plasma protein binding through the use of heparin and intravenous fat emulsion should provide information on the interaction between diazepam, free fatty acids, and plasma proteins.

2.2 Hypotheses

1. Feeding affects the disposition of lorazepam by altering its metabolism.
2. The disposition of lorazepam is altered by insulin in IDDM patients in a dose-dependent manner.
3. Animal and human insulin have differential effects on the disposition of lorazepam in man.
4. The disposition of lorazepam is altered by glucagon in patients with IDDM.
5. Lorazepam undergoes EHC in the dog, and the dog would make a useful animal model for examining EHC in man.
6. The effects of feeding on diazepam disposition are due to changes in drug distribution. Post-prandial peaks are the result of EHC, and these can be eliminated by interrupting the cycling pathway with neomycin and cholestyramine treatment.

2.3. Objectives

1. To determine the effects of glucose on the pharmacokinetics of lorazepam in man, using patients with IDDM as a test population.
2. To determine the effects of insulin, and insulin species on hepatic glucuronidation.
3. To evaluate the dog as an animal model of EHC of lorazepam.
4. To determine the mechanism of post-prandial diazepam peaks.

3. EFFECT OF FEEDING AND INSULIN ON THE DISPOSITION OF LORAZEPAM IN PATIENTS WITH INSULIN-DEPENDENT DIABETES MELLITUS

3.1. GENERAL INTRODUCTION

Ingestion of a high carbohydrate meal has been shown to affect the rates of elimination of drugs which are metabolized directly to glucuronide conjugates (Sonne *et al.*, 1989; Herman *et al.*, 1994). Moreover, *in vitro* studies suggest that substrate availability (UDPGA) is a rate-limiting step in the glucuronidation of xenobiotics (Felsher *et al.*, 1979; Reinke *et al.*, 1981). Assessing the effect of glucose on hepatic glucuronidation *in vivo* can be difficult, since exogenously administered glucose triggers a number of physiological responses, such as insulin secretion, which may have direct effects on how the drug is handled within the body. This problem can be partially overcome by studying the disposition of drugs in patients with IDDM, who, by virtue of their lack of endogenous insulin secretion, cannot counterregulate exogenous glucose administration. Such studies also allow for the investigation of the effects of insulin on hepatic drug metabolism.

The effects of feeding and insulin on the disposition of lorazepam in insulin dependent diabetic patients was studied using three separate experimental protocols. Accordingly, each study will be dealt with separately.

3.2. DISPOSITION OF LORAZEPAM IN INSULIN-DEPENDENT DIABETES MELLITUS WITH AND WITHOUT INTERRUPTION OF ENTEROHEPATIC CIRCULATION[1]

3.2.1. INTRODUCTION

Diabetes mellitus is a chronic disease characterized by an absolute or relative lack of insulin and inappropriate hyperglycemia. In addition to the metabolic abnormalities which accompany diabetes, several studies have shown that the glucuronidation of drugs and various other substances may be altered in the diabetic state in animals (Eacho *et al.*, 1981b; Price and Jollow, 1982; Morrison and Hawksworth, 1984; Morrison *et al.*, 1986; Vega *et al.*, 1986). Unfortunately, the data on the effects of diabetes on drug metabolism in humans are more sparse, and are often conflicting (Daintith *et al.*, 1976; Adithan *et al.*, 1988; 1989; Zysset and Weitholtz, 1988).

In humans, lorazepam is extensively metabolized through glucuronidation by the liver, and as such, is often used as an *in vivo* marker of this metabolic pathway. Recently, Herman *et al.* (1989) demonstrated that lorazepam undergoes significant EHC in man, and that this cycle can be blocked by treatment with neomycin and cholestyramine (neo/chol). Therefore, lorazepam clearance in the presence of neo/chol is a better estimator of hepatic glucuronidative capacity, and the difference between cycling-intact and cycling-interrupted clearance provides a measure of the extent of EHC. The objective of this study was to examine the effects of diabetes on the hepatic glucuronidation and EHC of lorazepam in humans.

[1] A version of this chapter has been published. R.J. Herman, A. Chaudhary, C.B. Szakacs, D. Woo, R. Lane, and M.A. Boctor. Eur. J. Clin. Pharmacol. 48: 253-258. (1995).

3.2.2. MATERIALS AND METHODS

3.2.2.1. Subjects

Ten non-smoking males between the age of 18-37 years diagnosed with insulin-dependent diabetes mellitus were recruited for the study by advertisements posted throughout the Royal University Hospital (RUH) and the University of Saskatchewan, by advertisement in local newspapers, and by referral through participating endocrinologists. Patients were diagnosed as having insulin dependent diabetes mellitus (IDDM) on the basis of fasting and post-prandial blood sugars above the World Health Organization (WHO) criteria for normal subjects. All diabetic subjects were non-smoking. Participants were required to pass a complete medical evaluation and were healthy (apart from their diabetes), with no evidence of long-term complications of their disease, such as hypertension, proteinuria, retinal changes or peripheral neuropathy. Patients were instructed to abstain from alcohol and other medications one week before and throughout the study interval. All subjects were required to sign a consent form which was approved by the University Advisory Committee on Ethics in Human Experimentation.

3.2.2.2. Experimental methods

Subjects presented themselves to the Clinical Investigation Unit (CIU) of the hospital at approximately 7:30 a.m. after an overnight fast. An intravenous infusion of 0.9% saline was established in each arm; one to withdraw blood samples (through a 4-way stopcock) and the other to deliver the lorazepam. After blank blood and urine samples were collected, 2 mg of lorazepam (Wyeth Laboratories, Toronto, Ont.) were administered by rapid intravenous injection. Time zero was designated as the start of the time of injection. Blood samples were collected after 1, 3, 5, 10, 15, 30, and 45 minutes and at 1, 1.5, 2, 3, 4, 5, 6, 8, 10, 12, 16, 24, 30, 36, and 48 hours. All urine

was collected during the 48 hour study interval. Subjects remained fasted until after the 2.5 hour sample, at which time their blood sugar was measured. They were then given their regular morning insulin and a standard diabetic breakfast. A second meal was given at noon, followed by a mid-afternoon snack, and an evening meal. All meals were high-carbohydrate, low-fat meals. Following the 12 hour blood sample, the intravenous catheter was removed, and the patients were allowed to leave the CIU, returning periodically thereafter for further blood and urine collections. A second study was carried out two weeks later, identical to the first, except that patients were started on neomycin 1 g every 6 hours one day prior to the study, and given cholestyramine 4 g every 4 hours beginning with the 2.5 hour blood sample. Neomycin and cholestyramine were continued together throughout the remainder of the study interval.

3.2.2.3. Analytical methods

3.2.2.3.1. HPLC assay of lorazepam

Lorazepam concentrations in plasma and urine were measured by solvent extraction and HPLC according to previously published methods (Herman *et al.*, 1989). Measured volumes of biological samples (1.0 mL, plasma; 0.1 mL, urine) were placed in glass culture tubes and alkalinized with 1.0 mL of saturated sodium borate (Fisher Scientific Co., Fair Lawn, NJ) solution. An internal standard of 100 ng of chromatographically pure desmethyldiazepam (a gift from Hoffman LaRoche, Mississauga, Ont.) was added, and the mixture was twice extracted with freshly distilled diethyl ether (Omnisolv grade, BDH Inc., Toronto, Ont.). The ether was evaporated to dryness under a gentle stream of N_2 gas, and the precipitate reconstituted with 100 µL of mobile phase prior to HPLC injection. The HPLC system consisted of an SP8700 solvent delivery system (Spectra-Physics, San Jose, CA) which pumped an isocratic solvent mixture (70:30) of 0.1% sodium phosphate buffer (sodium phosphate

monobasic; Fisher Scientific Co.), pH 3.0, and acetonitrile (Omnisolv grade BDH Inc.) at 1.8 mL/min across a 15 x 0.39 cm, 5μm particle size NovaPak® C-18 column (Waters Associates, Milford, MA). A graded three minute wash of 2% tetrahydrofuran (BDH Inc.) in acetonitrile was applied at the end of each run to remove late-eluting peaks. Samples were injected using a WISP model 710B autosampler (Waters Associates). Detection was by ultraviolet absorbance (Model 441 ultraviolet detector, Waters Associates) set at 230 nm, the absorbancy maximum for lorazepam. This was connected to an SP4290 integrating recorder (Spectra Physics). Peak heights determined by the integrating recorder were used to calculate peak height ratios of lorazepam to desmethyldiazepam. Lorazepam concentrations in biological samples were interpolated from a calibration curve obtained from similarly prepared blank plasma spiked with lorazepam. Each calibration curve consisted of six points in the concentration range of 5-100 ng/mL, and a new calibration curve was prepared with each set of unknown samples. Calibration curves were linear from 5-200 ng/mL, with a limit of detection of 3 ng/mL. The coefficients of variation for within-assay measurements were 7.6% at 5 ng/mL, and 1.5 % at 60 ng/mL, while the corresponding inter-assay coefficient of variation for the same concentrations were 8.3% and 2.0%, respectively. At least two samples with known lorazepam concentrations were added to each set of extractions to act as quality controls, and the entire run was repeated if the results of these measurements fell outside the cumulative 95% confidence intervals for the expected concentration levels. Figure 3.1 is a representative chromatogram showing lorazepam and desmethyldiazepam peaks (chromatogram shown is an actual patient sample, and represents a lorazepam concentration of 55.5 ng/mL). Figure 3.2 shows a standard calibration curve.

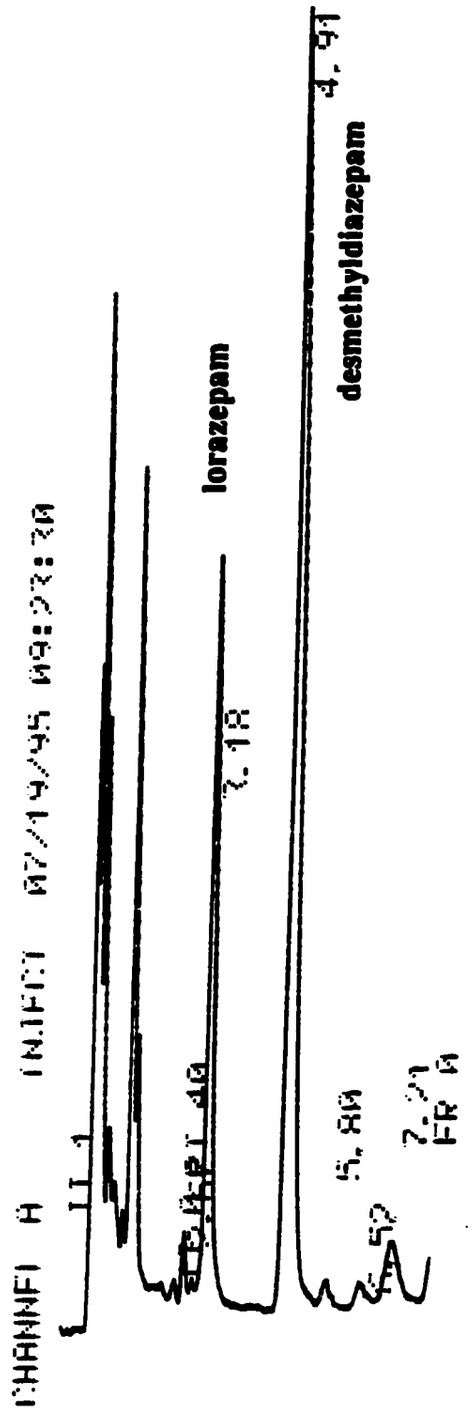


Figure 3.1. Representative HPLC chromatogram showing the separation of lorazepam and desmethyldiazepam (internal standard).

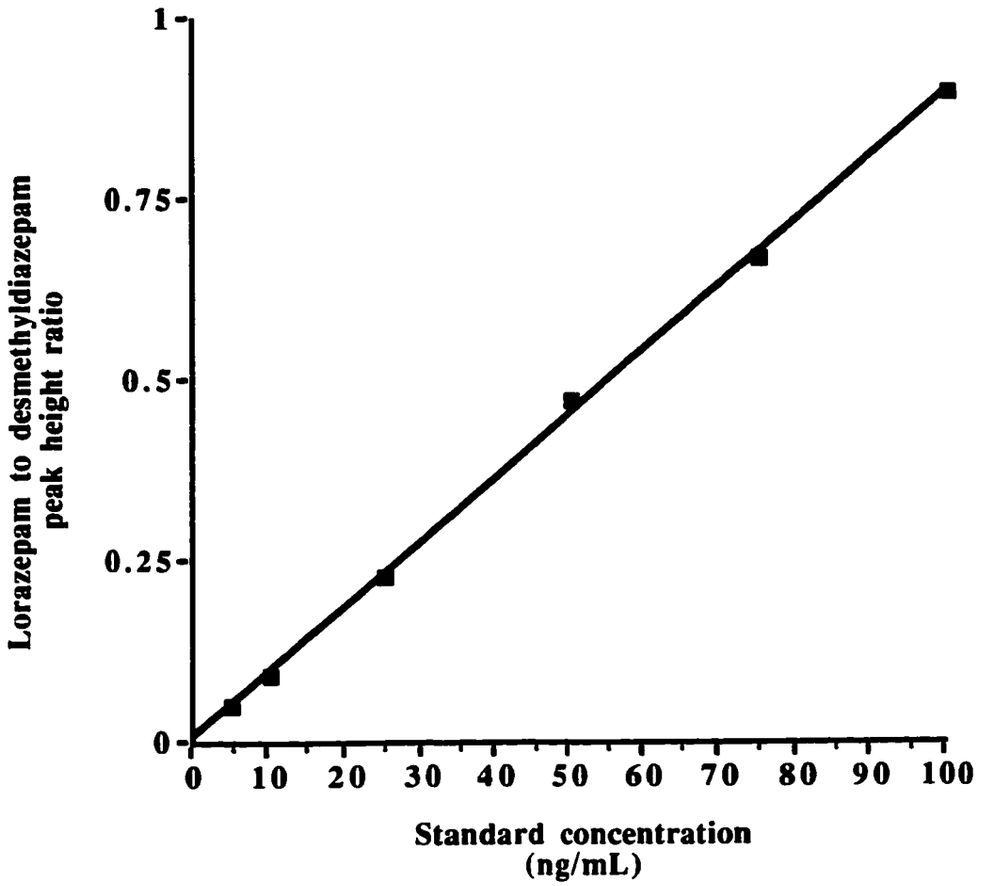


Figure 3.2. Representative standard curve for determination of lorazepam concentration in plasma using high performance liquid chromatography.

3.2.2.3.2. Measurement of lorazepam glucuronide

The concentration of lorazepam glucuronide in biological samples was estimated as lorazepam equivalents obtained following overnight hydrolysis with β-glucuronidase/sulphatase (from *Helix pomatia*; Sigma Chemicals Co., St. Louis, MO) at 37 °C.

3.2.2.3.3. Plasma protein binding measurements

The binding of lorazepam to plasma proteins was determined by equilibrium dialysis using a Spectrum equilibrium dialyzer (Spectrum Medical Industries, Inc., Los Angeles, CA) with Spectra/Por molecular porous membrane tubing (molecular weight cutoff 12000-14000) (Spectrum Medical Industries). Unbound fraction was calculated by a method which corrected for volume shifting (Tozer *et al.*, 1983; Tozer, 1984). Two 100 μL aliquots were taken from 1.2 mL of plasma spiked with 5×10^{-2} μCi (approximately 300 ng) of ^{14}C-lorazepam (specific activity 52 mCi/mmol, Amersham Corp., Arlington Heights, IL). The remaining 1 mL was dialyzed against an equal volume of 0.067M phosphate buffer, pH 7.4, for 4 hours at 37°C. Duplicate 100 μL aliquots of post-dialysis buffer and pre-dialysis plasma were subsequently measured for radioactivity by liquid scintillation counting. Ten mL of scintillation cocktail (Ready Micro, Beckman Instruments Inc., Mississauga Ont.) were added to each sample, and the radioactivity measured with a LS5801 Beckman scintillation counter using automated quench compensation (Beckman Instruments Inc.). The unbound fraction (f_u) was calculated as:

$$f_u = D_{b'} / (D_p - D_{b'}) \qquad (3.1)$$

where $D_{b'}$ is the radioactivity of drug in the post-dialysis buffer, and D_p is the corresponding pre-dialysis plasma radioactivity.

3.2.2.4. Pharmacokinetic methods

3.2.2.4.1. Non-linear regression analysis

Log-linear concentration-time profiles were constructed from the plasma lorazepam concentrations. The resulting curves were subsequently fit to a 2-compartment and 3-compartment model according to the following equations:

$$C_t = Ae^{-\alpha t} + Be^{-\beta t} \quad (3.2)$$

$$C_t = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\gamma t} \quad (3.3)$$

Initial estimates of exponents A, B, and C, and rate constants α, β, and γ, were determined by the method of residuals (Gibaldi and Perrier, 1982a). Using these initial estimates, the data were fitted by iterative, weighted, non-linear least-squares regression (SAAM 23, Resource facility for kinetic analysis; run on a VAX8650 digital computer, Digital Equipment Corp., Maynard MA) to determine a "weighted best fit" of the data, and to provide final estimates of the exponents and rate constants. Final determination of the number of compartments to which the model was fitted was resolved statistically by the F-test (Boxenbaum *et al.*, 1974) and the Akaike information criterion (Yamaoka *et al.*, 1978).

3.2.2.4.2. Compartmental methods

Compartmental methods were used to determine terminal disposition rate constants, necessary to calculate the area under the curve from the last measured concentration (Ct_{last}) to infinity AUC ($Ct_{last-\infty}$). This area was calculated by dividing the last measured concentration by the terminal disposition rate constant, λ (β or γ), derived from the non-linear least-squares regression analysis:

$$AUC\ (Ct_{last-\infty}) = Ct_{last} / \lambda \quad (3.4)$$

Area under the curve to the last measured concentration AUC ($0\text{-}Ct_{last}$) was determined by the trapezoidal rule to peak concentrations, and the log trapezoidal rule thereafter

(Rowland and Tozer, 1989). The total area under the curve from time zero to infinity AUC (0-∞) was calculated by simply adding these two areas:

$$AUC\ (0\text{-}\infty) = AUC\ (0\text{-}Ct_{last}) + AUC\ (Ct_{last}\text{-}_{\infty}) \quad (3.5)$$

The terminal disposition rate constant was also used to estimate the elimination half-life ($T_{1/2}$):

$$T_{1/2} = \ln 2 / \lambda \quad (3.6)$$

3.2.2.4.3. Noncompartmental methods

Noncompartmental methods do not require the assumption of a specific compartmental model for either a drug or its metabolite. Analysis is based on the estimation of the area under the curve of drug concentration versus time (AUC). The methods are based on statistical moment theory, where the time course of drug concentration in plasma is regarded as a statistical distribution (Gibaldi and Perrier, 1982b). Statistical moments are calculated based on the product of concentration and time (C x t). Thus, the zero moment of the drug concentration is the area under the product of concentration and time (time raised to the zeroth power) versus time curve, or simply the AUC. The first moment of the concentration is the area under the product of concentration and time (raised to the first power) versus time curve, or the AUMC. Although higher moments may be calculated, pharmacokinetic analysis is usually limited to zero and first moments, as higher moments are more prone to unacceptable levels of computational error (Gibaldi and Perrier, 1982b).

Once the area under the curve was estimated, total body clearance (CL) was calculated by the equation:

$$CL = Dose / AUC\ (0\text{-}\infty) \quad (3.7)$$

The apparent volume of distribution at steady-state (Vd_{ss}), was calculated as:

$$Vd_{ss} = (Dose \bullet AUMC) / AUC^2 \quad (3.8)$$

Renal clearance (CL_R) of lorazepam glucuronide was calculated as:

$$CL_R = LzGl_{urine} / AUCG \quad (3.9)$$

where $LzGl_{urine}$ is the total amount of lorazepam glucuronide excreted in the urine during the study interval, and AUCG is the area under the curve of the glucuronide during the same time period.

Clearance and apparent volume of distribution were calculated for unbound drug (FCL and FVd_{ss}) by dividing the corresponding parameters by the drug's unbound fraction in plasma, f_u:

$$FCL = CL / f_u \quad (3.10)$$

$$FVd_{ss} = Vd_{ss} / f_u \quad (3.11)$$

3.2.2.5. Statistical methods

Data were analyzed by analysis of variance (ANOVA) using repeated measures by two different statistical packages: BMDP (University of California Press; run on a VAX8650 digital computer, Digital Equipment Corp.); and SUPERANOVA (Abacus Concepts Inc., Berkeley, CA; run on a Macintosh PowerPC 6100, Apple Computer Inc., Cupertino, CA), with $p \leq 0.05$ for acceptance of significance. Significant differences found with BMDP were tested by least significant difference (LSD). Significant differences between means in data analyzed by SUPERANOVA were tested using least square means and planned contrasts. Paired t-tests were performed using the STATSVIEW (Abacus Concepts Inc.) statistical package.

3.2.3. RESULTS

The characteristics of the diabetic subjects are shown in Table 3.1. The mean age of the 10 subjects was 26.2 ± 5.7 years, and the mean duration of diabetes since the time of diagnosis was 12.6 ± 5.8 years (range 1-19 years). Two of the patients had fasting bilirubin levels that were slightly above (both 23 μmol/L) the normal range for our laboratory (0-22 μmol/L). These normalized following ingestion of a standard breakfast meal, indicating that they also had Gilbert's syndrome. All subjects had relatively good insulin/glycemic control, as indicated by the measurement of HbA1c and glycated protein (fructosamine). HbA1c is a stable fructose derivative of hemoglobin formed from non-enzymatic reactions with glucose and other sugars, which along with glycated protein, provides a measure of long-term glycemic control.

Table 3.1. Characteristics of diabetic subjects in lorazepam disposition studies with and without interruption of enterohepatic circulation.

Subject	Age (years)	Weight (kg)	D.D. (years)	T.B.I (μmol/L)	HbA1c (%)	G.Protein (μmol/L)
JW	28	80.6	13	8	5.7	348
LK	28	88.4	1.0	19	6.8	362
DO	22	85.6	15	23	6.6	317
BS	37	54.6	5.5	10	9.4	518
RG	22	80.0	19	23	7.5	405
RH	20	66.1	8	10	7.8	405
GB	18	73.8	15	10	6.5	415
DH	29	82.3	15	13	7.2	354
BJ	28	84.0	18	14	7.7	371
DZ	30	80.4	16	12	7.0	364
Mean	26.2	77.6	12.6	14.2	7.2	385
S.D.	5.7	10.2	5.8	5.5	1.0	55

D.D. duration of diabetes since time of diagnosis
T.B.I. total bilirubin (normal range; 0-22 μmol/L)
HbA1c hemoglobin A1c (normal range; 3.5-5.9%)
G. Protein glycated protein (normal range; ≤ 285 μmol/L)

Table 3.2. Insulin prescriptions of diabetics in lorazepam disposition study with and without interruption of enterohepatic circulation.

Patient	Insulin prescription
JW	HUM N 24, R 12 (am); HUM N 16, R 8 (pm)
LK	HUM N 26, R 8 (once daily)
DO	HUM N 32, R 7 (am); HUM N 26, R 5 (pm)
BS	HUM N 30 (once daily)
RG	HUM N 23, R 15 (am); HUM N 20, R 8 (pm)
RH	NPH 30, TO 12 (am); NPH 16, TO 10 (pm)
GB	NPH 24, TO 14 (am); NPH 10 TO 10 (pm)
DH	NPH 28, TO 14 (once daily)
BJ	NPH 53, TO 14 (am); TO 8 (pm)
DZ	NPH 22, TO 5 (am); NPH 2, TO 2 (pm)

HUM N	human intermediate acting insulin (Humulin N or Novolin NPH), 100 units/mL.
HUM R	human short-acting insulin (Humulin R or Novolin Toronto), 100 units/mL.
NPH	NPH insulin (Isophane Insulin), 100 units/mL.
TO	Toronto insulin (Regular Crystalline Zinc Insulin), 100 units/mL

The insulin prescription of each of the patients is shown in Table 3.2. At the outset of the study, five patients were on long-term, stable treatment with human insulin, and five patients were on combined beef/pork insulin. Three of the ten patients took their insulin once a day, while the other seven were on a twice-daily split-mixed regimen. The mean daily insulin prescription of the ten study patients was 56 units (range 30-75 units).

Short-term glycemic control during control and neomycin/cholestyramine treatments was monitored by blood glucose measurements taken in the morning and afternoon of the first day of each study. The results are shown in Table 3.3. Statistical analysis was done by analysis of variance (ANOVA) with repeated measures with 1 between factor (insulin species) and 2 within factors (study treatment, time of day). There were no differences in

blood glucose measurements in human vs. beef/pork treated patients (10.3 ± 5.0 vs. 7.8 ±4.4 mmol/L, p=0.23). However, blood glucose levels were significantly greater during control studies than in studies with neomycin/cholestyramine (11.2 ± 4.7 vs. 6.9 ± 3.9 mmol/L, p=0.001). Also, there was a significant interaction between insulin and the type of study (p=0.04), with patients treated with human insulin showing a greater decrease in blood sugar with neomycin/cholestyramine treatment (13.5 ± 5.6 vs. 7.1 ± 3.2 mmol/L) than patients treated with beef/pork insulin (8.9 ± 4.1 vs. 6.7 ± 4.6 mmol/L).

Table 3.3. Blood glucose measurements (mmol/L) in diabetic subjects during the morning and afternoon of control and neomycin/cholestyramine treatment studies.

Subject	Control		Neomycin/cholestyramine	
	a.m.	p.m.	a.m.	p.m.
Patients on human insulin				
JW	11.1	16.7	11.0	9.0
LK	11.2	9.0	9.0	4.0
DO	11.0	22.0	9.0	9.0
BS	9.0	17.0	2.0	2.0
RG	11.0	17.0	9.0	7.0
Patients on beef/pork insulin				
RH	16.7	11.1	17.0	11.0
GB	10.2	11.0	7.0	4.0
DH	2.0	9.0	2.0	9.0
BJ	5.3	10.2	6.4	4.4
DZ	5.2	8.8	2.6	3.6

The mean pharmacokinetic parameters of lorazepam in the ten diabetic patients are shown in Table 3.4, along with comparative data from fourteen, healthy, age-, gender-, and weight-matched (age, 22.9 ± 3.3 years; weight, 76.4 ± 13.6 kg) non-diabetic controls (Herman *et al.*, 1989; Chaudhary *et al.*, 1993).

Table 3.4. Mean pharmacokinetic parameters of lorazepam in diabetic and normal subjects.

Parameter	Diabetics (n=10)	Normals[1] (n=14)
FCL	7.73 ± 3.69	6.96 ± 2.58
FVd_{ss}	13.9 ± 4.79	12.7 ± 3.30
$T_{1/2}$	24.4 ± 9.44	23.7 ± 6.84
f_u	0.09 ± 0.01	0.10 ± 0.02

Data are mean ± S.D.

[1] normal data adapted from Herman *et al.*, 1989; and Chaudhary *et al.*, 1993.

FCL systemic clearance of free lorazepam (mL/min/kg).

FVd_{ss} apparent volume of distribution at steady-state of free lorazepam (L/kg).

$T_{1/2}$ elimination half-life of lorazepam (hr).

f_u lorazepam free fraction in plasma.

The clearance of unbound lorazepam (7.73 ± 3.69 mL/min/kg), the apparent volume of distribution of free lorazepam at steady-state (13.9 ± 4.79 L/kg), the drug's terminal elimination half-life (24.4 ± 9.44 hours), and free fraction (0.09 ± 0.01) were not significantly different in the ten diabetic patients and the non-diabetic subjects.

The percentage of lorazepam recovered as lorazepam glucuronide in the urine of the diabetic subjects during the 48 hour study interval was 61.5 ± 13.3 %, similar to that in normal subjects (72.9 ± 11.7 %) when corrected for the difference in collection time (60 hours in normal controls).

Table 3.5. Mean pharmacokinetic parameters of lorazepam and lorazepam glucuronide in 10 diabetic subjects during control and neomycin/ cholestyramine studies.

Parameter	Control	Neomycin/cholestyramine
FCL	7.73 ± 3.69	11.7 ± 5.98[1]
FVd_{ss}	13.9 ± 4.79	11.6 ± 2.70[1]
$T_{1/2}$	24.4 ± 9.44	13.7 ± 6.26[1]
f_u	0.09 ± 0.01	0.10 ± 0.01
AUCG	821 ± 340	658 ± 171
AUCG/AUC	1.69 ± 0.87	1.67 ± 0.87

Data are mean ± S.D.

[1] significantly different from control ($p \leq 0.05$), determined by ANOVA with repeated measures.

FCL systemic clearance of free lorazepam (mL/min/kg).

FVd_{ss} apparent volume of distribution at steady-state of free lorazepam (L/kg).

$T_{1/2}$ elimination half-life of lorazepam, (hr).

f_u lorazepam free fraction in plasma.

AUCG area under the curve of lorazepam glucuronide in plasma during 48 hours, (ng/mL-hr).

AUCG/AUC ratio of area under the curve of lorazepam glucuronide to area under the curve of lorazepam in plasma.

The effect of treatment with neomycin and cholestyramine (neo/chol) on the mean pharmacokinetic parameters of lorazepam in the ten diabetic patients is shown in Table 3.5. Neo/chol treatment caused a 63 ± 80% increase in the clearance of free lorazepam ($p \leq 0.05$), a 14 ± 17% reduction in the apparent volume of distribution of the free drug ($p \leq 0.05$), and a 38 ± 30% decrease in lorazepam's elimination half-life ($p \leq 0.05$). There was no change in lorazepam binding to plasma proteins with neo/chol treatment, compared to control studies. The magnitude and direction of these changes were similar to those observed with neo/chol treatment in non-diabetic subjects (Herman *et al.*, 1989). The area under the plasma concentration-time curve of lorazepam glucuronide was the same between the two study conditions, as was the ratio of area under the curve of lorazepam glucuronide to lorazepam in plasma (AUCG/AUC).

The urinary recoveries of lorazepam and lorazepam glucuronide in the diabetic patients during the 48 hour collection intervals are shown in Table 3.6. The amount of unchanged lorazepam was less than 1% of the administered dose in both arms of the study. Similarly, no difference was observed between control and neomycin/cholestyramine treatment in the recovery of lorazepam glucuronide, or in the renal clearance of the glucuronide.

Table 3.6. Lorazepam and lorazepam glucuronide determined in urine in control and neomycin/cholestyramine-treated diabetic patients (n=10).

Parameter	Control	Neomycin/cholestyramine
UA_{Lz}	0.9 ± 0.9	0.7 ± 0.6
UA_{LzGl}	61.5 ± 13.3	66.0 ± 19.4
CL_R	0.42 ± 0.24	0.50 ± 0.23

Data are mean ± S.D.
No significant differences were detected between control and neomycin/cholestyramine treated studies, as determined by ANOVA with repeated measures.
UA_{Lz} % of dose recovered as unchanged lorazepam in urine during 48 hours.
UA_{LzGl} % of dose recovered as lorazepam glucuronide in urine during 48 hours.
CL_R renal clearance of lorazepam glucuronide (mL/min/kg).

Although no differences were observed between diabetic patients and non-diabetic controls in the response to neo/chol treatment, differences were observed between diabetic patients treated with beef/pork and patients treated with human insulin (Table 3.7). Diabetic patients treated with human insulin showed no response to neo/chol treatment, while neo/chol caused a large increase in lorazepam clearance in

Table 3.7. Clearance of unbound lorazepam (mL/min/kg) during control and neomycin/cholestyramine treatment in 10 individual diabetic subjects.

Subject	Control	Neomycin/ cholestyramine	% change
Patients on human insulin			
JW	4.89	5.36	9.61
LK	5.70	5.79	1.58
DO	8.17	7.98	-2.33
BS	13.6	12.5	-8.02
RG	7.58	7.70	1.58
Mean ± S.D.	7.98 ± 3.41	7.87 ± 2.83	0.47 ± 6.44
Patients on beef/pork insulin			
RH	4.92	10.9	122
GB	4.01	7.98	99.0
DH	4.63	15.5	236
BJ	9.80	21.6	120
DZ	14.1	21.2	50.8
Mean ± S.D.	7.48 ± 4.34	15.4 ± 6.05[1,2]	125 ± 67.9[1]

Statistical analysis performed by ANOVA with repeated measures with 1 between factor (insulin species) and 1 within factor (treatment)
[1] significantly different from patients on human insulin ($p<0.05$)
[2] significantly different from control ($p<0.05$)

patients treated with beef/pork insulin. In addition, lorazepam clearance in the presence of neo/chol treatment (a reflection of hepatic glucuronidative capacity) was significantly greater in beef/pork-treated diabetics compared to either patients receiving human insulin or non-diabetic controls. There was no difference in clearance of free lorazepam during neo/chol treatment between diabetics treated with human insulin and non-diabetic controls.

Typical concentration-time profiles of lorazepam during control and neomycin/cholestyramine treated studies for a patient treated with human insulin (JW) or beef/pork insulin (DZ) are shown in Figures 3.3 and 3.4.

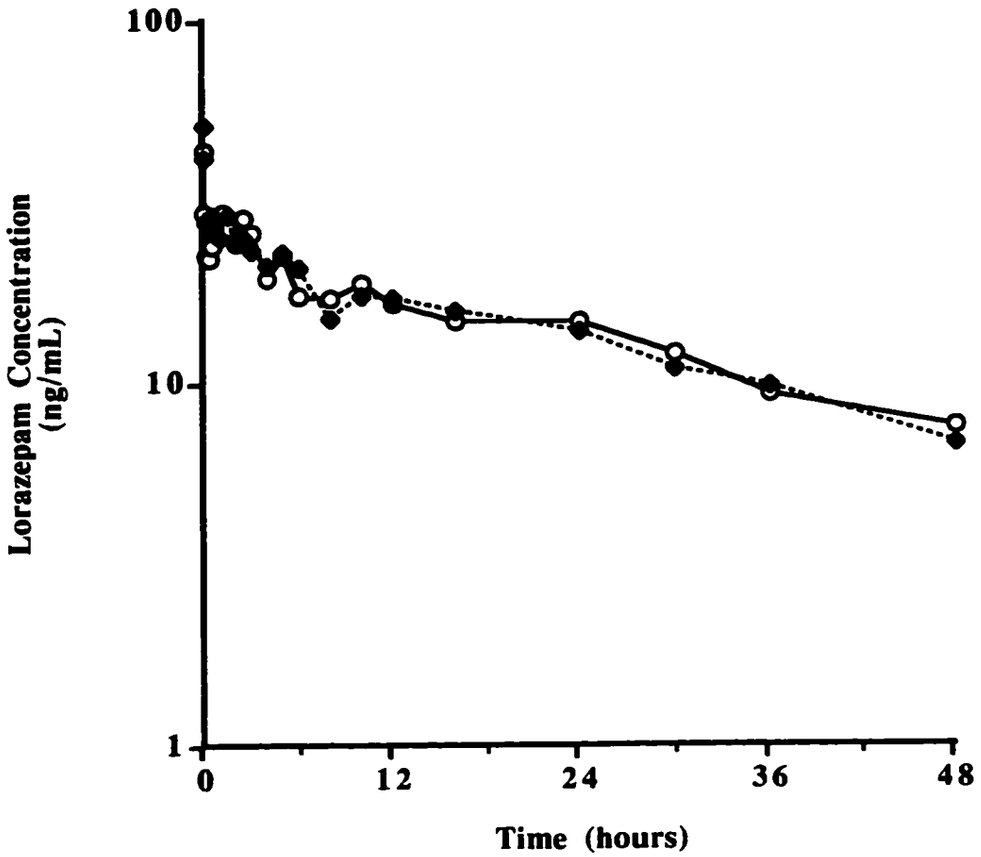


Figure 3.3. Concentration-time profile of lorazepam in an insulin-dependent diabetic subject (JW) treated with human insulin. Open circles/solid line indicate control study. Solid diamonds/dashed line indicate neomycin/cholestyramine treatment

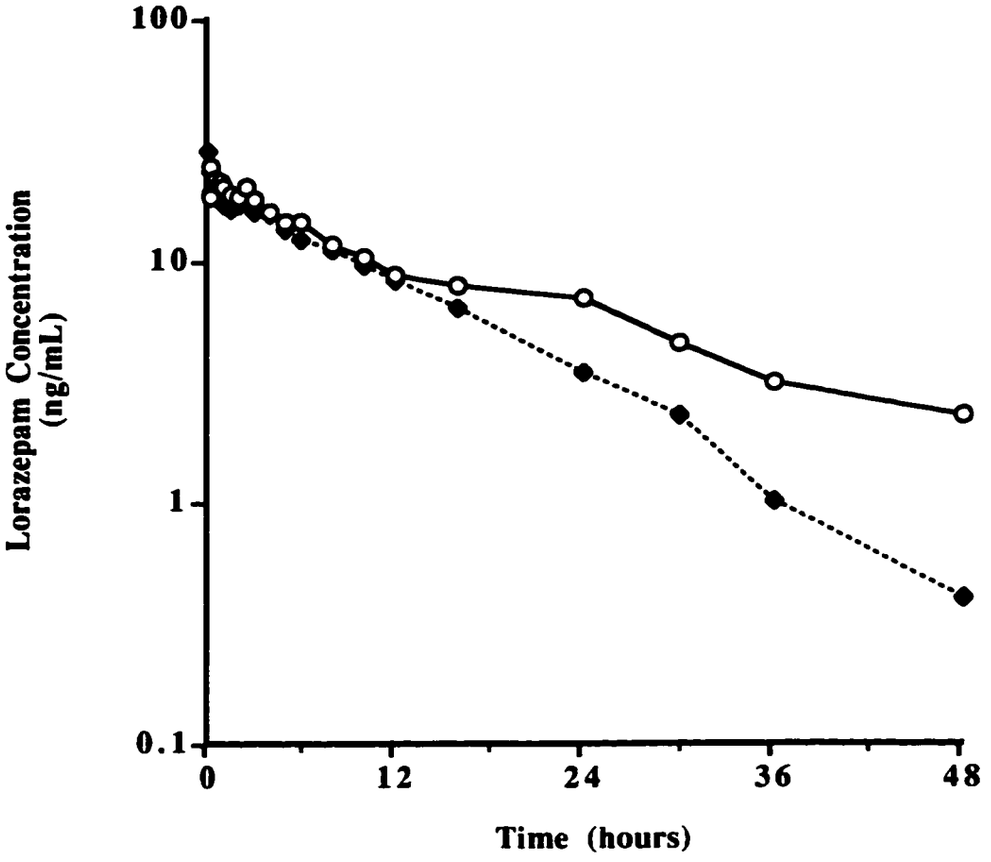


Figure 3.4. Concentration-time profile of lorazepam in an insulin-dependent diabetic subject (DZ) treated with beef/pork insulin. Open circles/solid line indicate control study. Solid diamonds/dashed line indicate neomycin/cholestyramine treatment.

There were no differences in apparent volume of distribution of lorazepam during neomycin/cholestyramine treatment between any of the study groups. However, the elimination half-life of lorazepam was significantly (40-55%) shorter in neo/chol-treated diabetics treated with beef/pork insulin compared to human insulin treated diabetics and normal controls ($p<0.05$ for both).

The ratio of the AUC of the metabolite to parent drug (AUCG/AUC) was identical between patients treated with human insulin (1.63 ± 0.82) and beef/pork insulin (1.74 ± 1.00), and in normal controls (1.54 ± 0.51) when measured in the absence of neo/chol (Figure 3.5). In studies with neo/chol treatment, the ratio of AUCG/AUC decreased in the human insulin treated diabetics (1.11 ± 0.19), an effect similar to that observed in non-diabetic controls (1.39 ± 0.55). However, the ratio in beef/pork insulin-treated patients increased with neo/chol treatment (2.23 ± 0.94), such that it became significantly greater in patients treated with beef/pork insulin than in patients treated with human insulin and in non-diabetic controls ($p \leq 0.05$, for both). The renal clearance of lorazepam glucuronide did not differ between any of the subgroups, or between the cycling-intact or cycling-interrupted state.

Finally, there was a significant positive correlation ($r \geq 0.9$, $p \leq 0.05$) between the clearance of free lorazepam and Hb1Ac in patients treated with human insulin, but not in diabetics treated with beef/pork insulin (Figure 3.6). A similar but weaker correlation was noted with glycated protein ($r \geq 0.8$, $p \leq 0.07$) in human insulin treated diabetics, but no such relationships were observed for patients treated with beef/pork insulin.

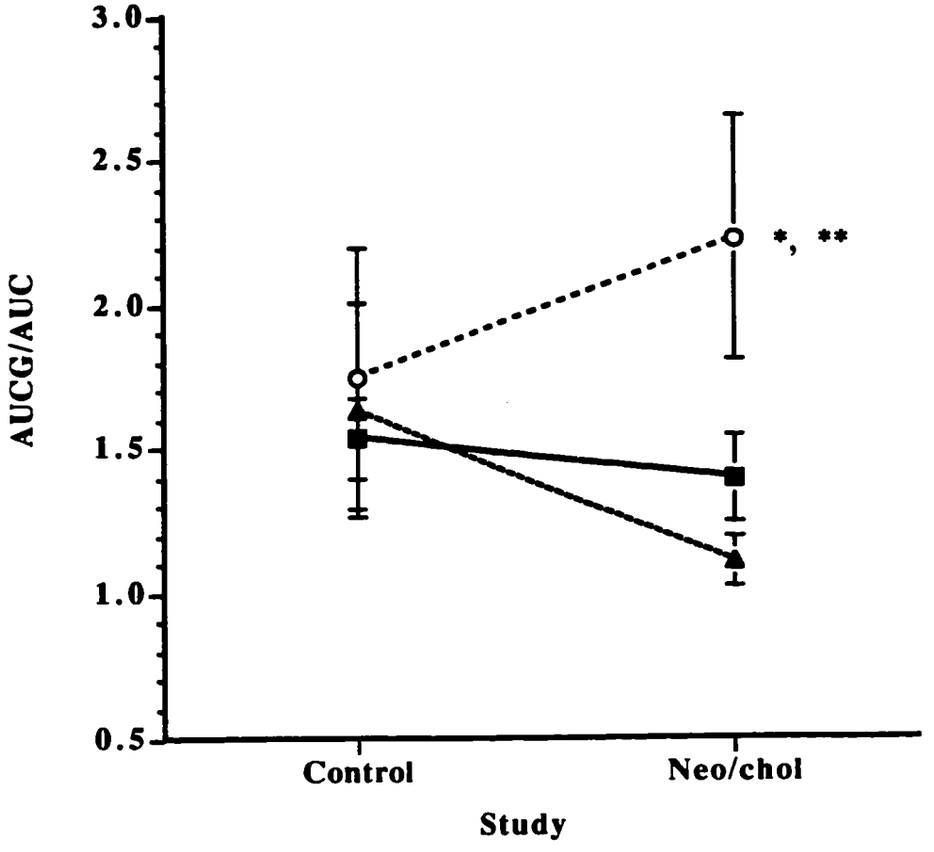


Figure 3.5. Ratio of the area under the curve of lorazepam glucuronide to lorazepam (AUCG/AUC) in response neomycin/cholestyramine treatment in insulin dependent diabetics treated with human (closed triangles) and beef/pork (open circles) insulin and in non-diabetic controls (closed squares).

* indicates significantly different ($p \leq 0.05$) from human insulin

** indicates significantly different ($p \leq 0.05$) from non-diabetic controls

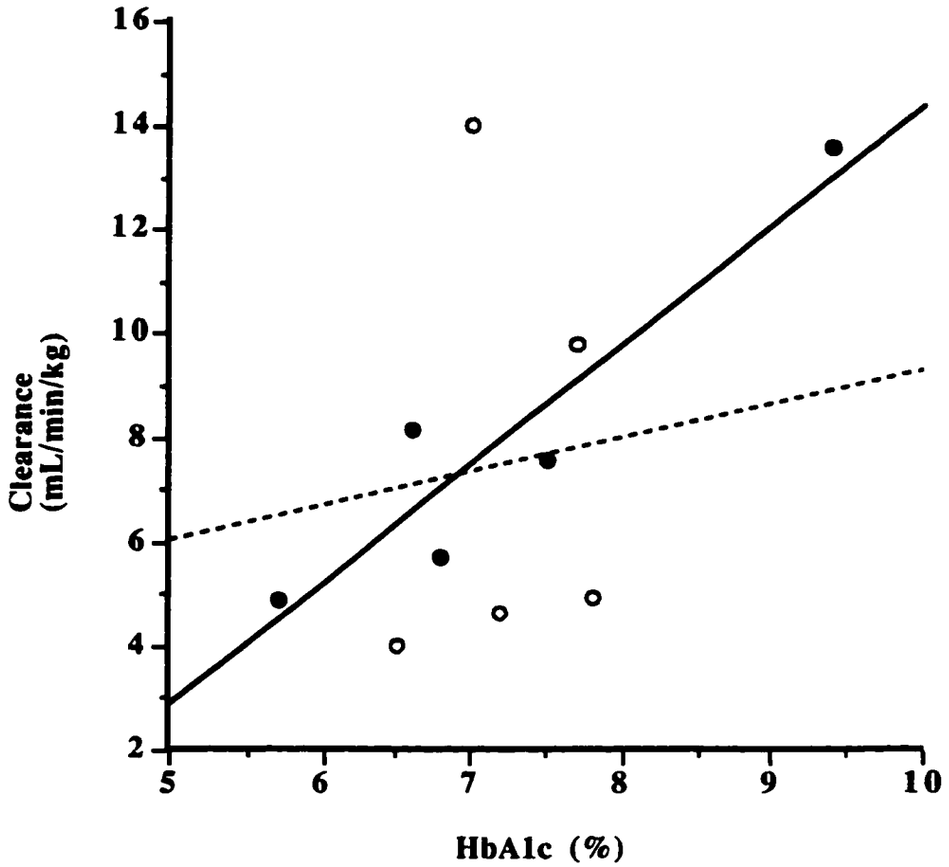


Figure 3.6. Relationship between clearance of unbound lorazepam and HbA1c in diabetic patients treated with human insulin (solid circles/solid line) and beef/pork insulin (open circles/ dashed line).

3.2.4. DISCUSSION

The results from this study indicate that there is no difference in the pharmacokinetics of lorazepam in patients with IDDM and non-diabetic controls. The clearance of unbound lorazepam, the steady-state volume of distribution of free drug, and the binding of lorazepam to plasma proteins in diabetic patients were similar to values found in normal control subjects. Moreover, there were no differences in any of these parameters between diabetic patients treated with either human insulin or with beef/pork insulin.

Within the literature, there are considerable discrepancies with respect to the effect of diabetes on drug disposition. Indeed, a recent review of drug kinetics in diabetes (Gwilt *et al.*, 1991) noted that many inconsistencies are found in the reported effects of diabetes on drug distribution and clearance, often due to inappropriate controls, the possible presence of undetected or unreported complications of diabetes, as well as the failure to distinguish between IDDM and NIDDM. Although studies in patients with NIDDM often report no change in either drug distribution (Daintith *et al.*, 1976; Murali *et al.*, 1983; Adithan *et al.* 1988; Zysset and Weitholtz, 1988) or drug clearance (Murali *et al.*, 1983; Zysset and Weitholtz, 1988; Adithan *et al.*, 1989), several studies of drug disposition in patients with IDDM patients have detected differences in one or both parameters when compared to non-diabetic controls (Murali *et al.*, 1983; Zysset and Weitholtz, 1988; Adithan *et al.*, 1988; 1989). However, a common problem with these studies is that the majority are performed with orally administered drug, and values of clearance and volume of distribution are almost always reported for total (bound and unbound) drug. Thus, the reported effects of diabetes on drug distribution and clearance may not be an accurate reflection of true drug disposition changes.

An unexpected finding of this investigation was that the free clearances of lorazepam, and the change in lorazepam clearance in response to neo/chol treatment,

were significantly greater in IDDM patients treated with beef/pork insulin compared to human insulin-treated diabetics and to non-diabetic controls. Insofar as neo/chol treatment interrupts the EHC of lorazepam, and the clearance of lorazepam in the presence of an interrupted EHC can be considered to be an accurate reflection of hepatic glucuronidation, this suggests that beef/pork and human insulin may possess differential influences on the metabolism of lorazepam by the liver. Since a negative correlation was noted between glycemic control and free lorazepam clearance for human-insulin treated diabetic patients, but no correlation was observed with beef/pork treated patients, this suggests that human insulin may inhibit (directly or indirectly) the hepatic glucuronidation of lorazepam in humans. This is further supported by the finding that the clearance of free lorazepam in non-diabetic individuals is decreased by a continuous feeding of a high-carbohydrate diet, which in turn would be expected to increase circulating insulin concentrations (Herman *et al.*, 1994). Furthermore, the ratio of AUCG/AUC is significantly greater in the IDDM patients treated with beef/pork insulin, compared to the human-insulin treated diabetics and the non-diabetic controls. Since no change was observed in the renal clearance of lorazepam glucuronide in any of the subgroups or between any of the studies, this ratio can be considered to be an indirect measure of metabolic clearance (Rowland and Tozer, 1989), indicating an increase in lorazepam glucuronide formation in diabetic patients treated with beef/pork insulin.

Work by others provides support for the observation that differences in lorazepam disposition may be due to the differential effects of insulin species. Several investigators have suggested that there may be differences in some of the effects of beef/pork and human insulins in certain human tissues. *In vitro* binding studies with monoclonal anti-insulin receptor antibodies have shown that human insulin and porcine insulin interact differently with the human insulin receptor (Sesti *et al.*, 1991). In addition, several studies have shown a difference between human and porcine and/or

bovine insulin in the physiological effects in man (Kern *et al.*, 1990; Lingelfelser *et al.*, 1992; Dahlan *et al.*, 1994).

Several other factors, such as insulin dose, glycemic control, and diet, may have also contributed to the observed differences in lorazepam disposition. Although there was no difference in the morning and afternoon blood sugar measurements between patients treated with beef/pork and human insulins, the measurements showed considerable variability throughout the two studies. Moreover, while each patient was studied for a total of 48 hours, only the first 12 hours were performed within the confines of CIU of the hospital, such that there was no control of either patient behaviour or dietary intake during the final 36 hours. Finally, these observations were made in separate groups, so that differences within the two patient groups may have also been a factor.

3.3. DISPOSITION OF LORAZEPAM WITH AND WITHOUT ENTEROHEPATIC CIRCULATION IN PATIENTS ON HUMAN AND BEEF/PORK INSULIN: A CROSSOVER STUDY

3.3.1. INTRODUCTION

Previous studies on the disposition of lorazepam in patients with IDDM detected a difference in the effects of interruption of EHC of lorazepam in patients treated with human insulin when compared to patients treated with beef/pork insulin (section 3.2). Since these observations were made in two different patient groups, the purpose of this

study was to examine whether these differences could be found within the same patients treated with both types of insulin in a crossover study design.

3.3.2. MATERIALS AND METHODS

All materials and methods used in this study were identical to those described in section 3.2.1, with the following exceptions:

3.3.2.1. Subjects

Three non-smoking males, between the age of 20-30 years, diagnosed with insulin-dependent diabetes mellitus, were recruited for the study.

3.3.2.2. Experimental methods

The protocol of the study was identical to the previous study, wherein a single 2 mg dose of lorazepam was administered intravenously on two separate occasions, with simultaneous neomycin and cholestyramine treatment given during one study interval, to block the enterohepatic circulation of lorazepam. Patients were initially studied on the insulin type (human or beef/pork) with which they had been previously stabilized (study 1). Subjects were then switched over to the other insulin type, and following a minimum four week adjustment period, the disposition of lorazepam with and without neomycin and cholestyramine was restudied in each patient (study 2). Patients were then switched back to their original insulin prescription, and following another four week adjustment period, were restudied a third and final time (study 3) (a total of six individual studies per patient).

3.3.3. RESULTS

The characteristics of the three patients who participated in this study are shown in Table 3.8. The mean age of the subjects was 25 ± 5.0 years (range 20-30 years), and their mean weight was 77.8 ± 9.0 kg (range 71.2-88.0 kg). The average duration of diabetes since time of diagnosis was 12.7 ± 4.9 years (range 7-16 years). None of the patients had fasting bilirubin levels above the normal range of our laboratory. Two of the subjects (DH and KK) presented with relatively good glycemic control, as indicated by HbA1c and glycated protein measurements. Both measures were moderately elevated in the third diabetic patient (CS).

Table 3.8. Characteristics of three diabetic subjects in a crossover study of the effects of human and beef/pork insulins on the response to neomycin/cholestyramine treatment.

Subject	Age (years)	Weight (kg)	D.D. (years)	T.B.I. (μmol/L)	HbA1c (%)	G.Protein (μmol/L)
DH	30	88.0	16	17	8.5	348
CS	20	71.2	7	7	11.5	473
KK	25	74.2	15	12	8.4	355
Mean	25	77.8	12.7	12	9.5	392
S.D.	5	9.0	4.9	5	1.8	70

D.D. duration of diabetes since time of diagnosis.
T.B.I. total bilirubin (normal range; 0-22 μmol/L).
HbA1c hemoglobin A1c (normal range; 3.5-5.9%).
G. Protein glycated protein (normal range; ≤ 285 μmol/L).

Of the three subjects, two (CS and KK) were initially treated with human insulin, while the other (DH) was treated with beef/pork insulin. The two patients treated with human insulin were on split-mixed insulin regimens, while the beef/pork treated patient received once daily insulin injections. Two patients (DH and CS) were

treated with a combination of short- and intermediate-acting insulin, and the other patient (KK) used a combination of short- and long-acting insulin. Two patients (DH and CS) completed all three phases of the study (a total of six separate studies). The third subject (KK) completed only the first two phases. Table 3.9 shows the insulin prescription for the three individuals during all phases of the study.

Table 3.9. Insulin prescription of three diabetic patients during the crossover study.

Subject	Study	Insulin	Insulin Prescription
DH	1	beef/pork	NPH 30, TO 20 (once daily)
	2	human	HUM N 40, R 15 (once daily)
	3	beef/pork	NPH 34, TO 20 (once daily)
CS	1	human	HUM N 27, R 10 (am); HUM N 10, R 8 (pm)
	2	beef/pork	NPH 29, TO 10 (am); NPH 9, TO 10 (pm)
	3	human	HUM N 28, R 11 (am); HUM N 9, R 10 (pm)
KK	1	human	HUM U 16, TO 6 (am); NOV U 16, TO 10 (pm)
	2	beef/pork	ULT 19, TO 7 (am); ULT 13, TO 10 (pm)

HUM N	human intermediate-acting insulin (Humulin N or Novolin NPH), 100 units/mL.
HUM R	human short-acting insulin (Humulin R or Novolin Toronto), 100 units/mL.
HUM U	human long-acting insulin (Humulin U or Novolin ULT), 100 units/mL.
NPH	NPH insulin (Isophane Insulin), 100 units/mL.
TO	Toronto insulin (Regular Crystalline Zinc Insulin), 100 units/mL.
ULT	ultralente insulin (Extended Zinc Insulin), 100 units/mL.

Blood glucose measurements were taken during the morning and afternoon of each kinetic study. The results are shown in Table 3.10. There was no difference between blood glucose determinations in the three patients when comparing studies done during human versus beef/pork insulin treatment (11.13 ± 0.89 vs. 10.59 ± 3.54 mmol/L, $p=0.83$), however more variability was observed in the measurements during

the beef/pork insulin treatment. Neomycin/cholestyramine treatment produced lower blood glucose measurements compared to control studies (9.76 ± 1.96 vs. 11.97 ± 2.6 mmol/L, p=0.08), although the difference was not significant.

Table 3.10. Blood glucose measurements (mmol/L) in the morning/afternoon in the crossover study with 3 IDDM patients treated with human and beef/pork insulin in control and neo/chol studies.

	Human		Beef/pork		Study 3[1]	
Subject	control	neo/chol	control	neo/chol	control	neo/chol
DH	12.4/9.8	10.5/8.8	10.6/13.0	6.4/14.4	6.3/9.7	13.1/12.4
CS	11.5/11.5	10.1/11.4	12.9/20.2	6.9/13.7	8.1/14.0	8.6/10.9
KK	10.8/13.8	9.5/13.5	4.0/13.1	4.1/7.8	N/A	N/A

[1] DH treated with beef/pork insulin; CS treated with human insulin, KK did not participate

The plasma concentration-time profile of lorazepam during control studies in one individual (CS) who completed all three phases of the study is shown in Figure 3.7. For purposes of clarity, profiles during studies with neo/chol treatment are not included in this figure. The concentration-time profiles showed pronounced variability between the different studies. More importantly, there was a considerable difference in the two studies in which the patient was treated with the same (human) insulin. Similar result were noted within the other individuals, as well as during neo/chol treated studies. Because of the lack of reproducibility of results, the study was stopped prematurely. As such, only two of the patients (DH and CS) completing all six studies, while only four studies were performed on the third (KK) subject.

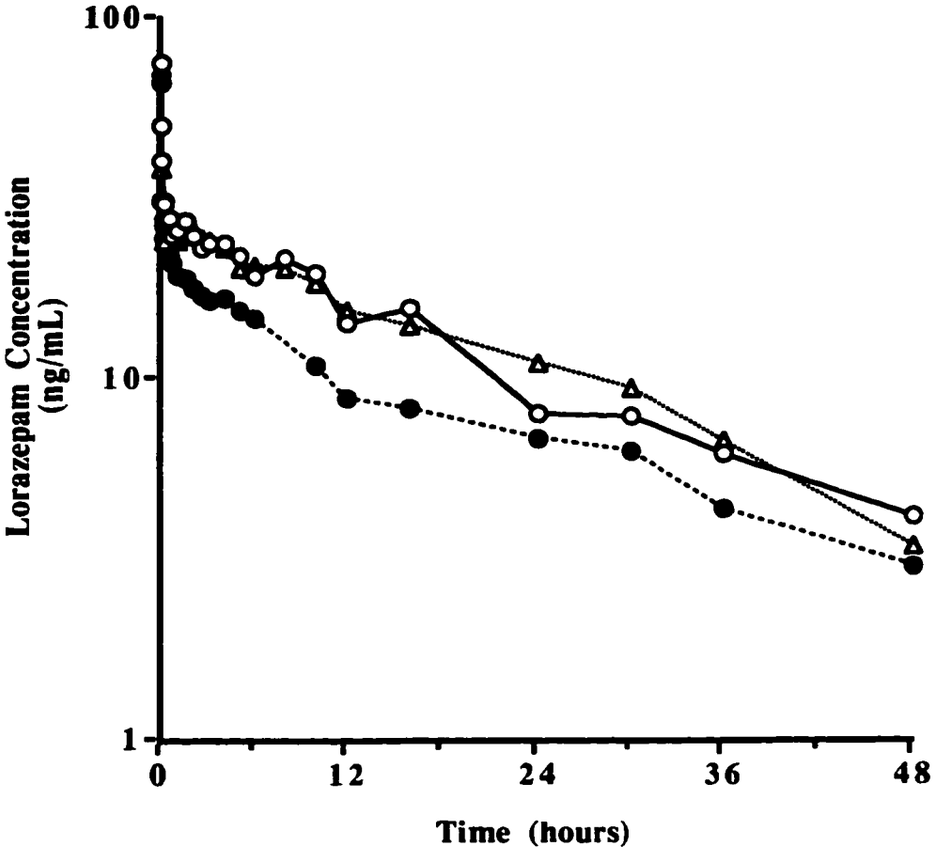


Figure 3.7. Plasma concentration-time profiles of lorazepam in a single IDDM patient (CS) studied under control conditions on 3 separate occasions (twice on human insulin; once on beef/pork insulin)
Circles indicate studies performed during human insulin treatment.
Triangles indicate studies performed during beef/pork insulin treatment.

Table 3.11. Mean pharmacokinetic parameters of lorazepam in 3 diabetic subjects under control conditions and during treatment with neomycin/cholestyramine.

Parameter	control	neomycin/cholestyramine
AUC	444 ± 91	416 ± 82
FCL	11.45 ± 1.32	13.01 ± 1.52
FVd_{ss}	14.06 ± 1.49	13.76 ± 2.66
$T_{1/2}$	14.51 ± 2.28	11.94 ± 2.70
f_u	0.076 ± 0.007	0.076 ± 0.012

Data are mean ± S.D.
No significant differences were observed between any parameters comparing control to neomycin/cholestyramine treatment, as determined by analysis of variance with repeated measures.

AUC	area under the concentration-time profile of lorazepam in plasma (ng/mL-hr).
FCL	systemic clearance of free lorazepam (mL/min/kg).
FVd_{ss}	apparent volume of distribution at steady-state of free lorazepam (L/kg).
$T_{1/2}$	elimination half-life of lorazepam (hr).
f_u	lorazepam free fraction in plasma.

The mean pharmacokinetics of lorazepam in the three subjects with and without neomycin/cholestyramine treatment is shown in Table 3.11. As only two of the three patients completed all three parts of the crossover study, the reported values (unless otherwise stated) represents data from only the first two studies (a single crossover, or 2 studies, per individual, for a total of 6 studies).

The clearance of free lorazepam under control conditions was 11.45 ± 1.32 mL/min/kg. This is considerably higher than values found in previous studies of non-diabetic individuals (7.73 ± 3.69 mL/min/kg) or in the earlier study with diabetics (6.96 ± 2.58 mL/min/kg). Similarly, lorazepam's terminal disposition half-life was less in these three diabetic subjects; 14.51 ± 2.28 hours vs. 24.4 ± 9.44 (normals) and 23.7 ± 6.84 (previous diabetic study). Lorazepam's unbound fraction was lower (0.076 ± 0.007) compared to normals (0.09 ± 0.01) and previously studied diabetics (0.10 ±

0.01). The volume of distribution of free lorazepam was similar between all studies.

The clearance of unbound lorazepam increased with neomycin/cholestyramine treatment in all cases by a mean of 14% (range 1.8-38.5); however, this difference was not significant (p=0.13). Neo/chol treatment had no effect on the volume of distribution of free lorazepam (p=0.39). The mean half-life of lorazepam decreased by 18% with neo/chol treatment (p=0.17), with 5 of the 6 studies showing a decrease in half-life (range 12.9-40.7%), and 1 study showing an 19.1% increase in half-life following administration of neo/chol. There was no difference in the unbound fraction of lorazepam between the two studies (p=0.93).

Parameters determined for lorazepam glucuronide are shown in Table 3.12. Urinary recovery of lorazepam glucuronide was similar between control and neomycin/cholestyramine treatments. However, both were greater than previous recovery values for lorazepam glucuronide in normal volunteers and in previous studies of diabetic patients. The area under the curve of lorazepam glucuronide in plasma was significantly greater (p=0.03) during neomycin/cholestryamine treatment compared to control, as was the ratio of metabolite to parent drug (AUCG/AUC) (p=0.007). Both of these were unexpected given the effect of neo/chol binding lorazepam glucuronide in the gut, and were in contrast to previous findings in normal and diabetic subjects, where no difference was observed between these two measures. Renal clearance of lorazepam glucuronide was slightly greater during the control studies, but this difference was not significant (p=0.12).

Table 3.12. Pharmacokinetic parameters of lorazepam glucuronide in control and neomycin/cholestyramine-treated studies (n=3).

Parameter	Control	Neomycin/cholestyramine
$UA_{L\text{-}Gl}$	86.4 ± 14.0	84.6 ± 9.5
AUCG	708 ± 232	962 ± 243[1]
CL_R	0.61 ± 0.22	0.44 ± 0.14
AUCG/AUC	1.59 ± 0.75	2.26 ± 0.68[1]

Data are mean ± S.D.

[1] significantly different from control at $p \leq 0.05$, as determined by analysis of variance with repeated measures.

$UA_{L\text{-}Gl}$ % of the dose recovered as lorazepam glucuronide in urine during 48 hours.

AUCG area under the curve of lorazepam glucuronide in plasma during 48 hours (ng/mL-hr).

CL_R renal clearance of lorazepam glucuronide (mL/min/kg).

AUCG/AUC ratio of area under the curve of lorazepam glucuronide to area under the curve of lorazepam in plasma.

The individual clearances of free lorazepam in each subject on the different insulins and their response to neomycin/cholestyramine treatment are shown in Table 3.13. No consistent differences were observed in the response to neomycin/cholestyramine treatment when the diabetic subjects were treated with the different insulins. Treatment with neomycin/cholestyramine caused the free clearance of lorazepam to increase by a mean of 19.5% (range 4.4-38.5%) when the patients were treated with human insulin, and a mean increase of 8.2% (range 1.8-12.4%) when the patients were treated with beef/pork insulin. In the third study in which patients were switched back to their original insulin and the response to neo/chol treatment on lorazepam kinetics was restudied, both patients who participated showed very different responses when compared to their initial studies (Table 3.13). The patient studied twice while treated with beef/pork insulin (DH) had a 32.9% increase in clearance of free lorazepam with neo/chol treatment during the second study, compared to an initial response of only 4.4%. Conversely, neo/chol treatment in the patient

studied twice while treated with human insulin (CS) increased the free clearance of lorazepam by 1.9% during the final crossover study, compared to an initial increase of 38.5%.

Table 3.13. Clearance of free lorazepam (mL/min/kg) and response to neomycin/cholestyramine treatment in three insulin dependent diabetic patients on human and beef/pork insulin in a crossover study.

	Human			Beef/pork			Study 3[1]		
Subject	CNTL	N/C	% change	CNTL	N/C	% change	CNTL	N/C	% change
DH	12.39	12.94	4.4	13.10	13.34	1.8	12.38	16.45	32.9
CS	10.80	14.96	38.5	9.31	10.28	10.4	12.94	13.19	1.9
KK	11.30	13.06	15.6	11.78	13.45	12.4	-	-	-
Mean	11.50	13.65	19.5	11.39	12.35	8.2	N/A	N/A	N/A
S.D.	0.81	1.13	17.4	1.92	1.79	5.6			

[1] DH treated with beef/pork insulin; CS treated with human insulin, KK did not participate.

CNTL control studies
N/C neomycin/cholestyramine treatment
N/A not applicable (patients DH and CS were studied on different insulins)

No differences were observed in lorazepam's free volume of distribution, elimination half-life, or unbound fraction, or in any of the parameters calculated for lorazepam glucuronide on human vs. beef/pork insulin.

3.3.4. DISCUSSION

Previous studies (section 3.2) suggested that there may be a difference in the effects of human and beef/pork insulin on lorazepam disposition. Because these effects were observed in different individuals, part of the variability of the results could be due to interindividual differences in lorazepam disposition. Accordingly, the present study attempted to reproduce the observed differences in insulin effects without the effects of interindividual variability, employing a crossover design to study the effects of human and beef/pork insulin within individuals. Unfortunately, results of this study were highly variable, and were not reproducible. In particular, markedly different lorazepam kinetics were observed in two patients who were studied twice on the same insulin preparation. For these reasons, the study was stopped prematurely.

Because of the noted lack of reproducibility, little strength can be given to any conclusions which might be drawn from this study. In addition, most of the measured parameters differed from values found in previous studies from our laboratory. Accordingly, differences due to either neo/chol treatment, or due to the type of insulin with which the patients were being treated, would not be expected to be detected. Since these studies were performed repeatedly within the same individuals, this investigation suggests that variables other than interindividual differences, such as insulin dose and/or dietary intake, may be influential in lorazepam disposition in patients with IDDM. No attempt was made to control either of these parameters in this study. Therefore, results from this study imply that the investigation of lorazepam disposition in patients with IDDM must be performed under strict experimental conditions, and that possible confounding variables, such as insulin dose and feeding, should be tightly controlled.

3.4. EFFECTS OF GLUCOSE, INSULIN, GLUCAGON, AND INTERRUPTION OF ENTEROHEPATIC CIRCULATION ON THE DISPOSITION OF LORAZEPAM IN PATIENTS WITH IDDM

3.4.1. INTRODUCTION

Previous studies of lorazepam disposition in patients with IDDM suggested that differences existed in patients treated with human insulin and with beef/pork insulin (section 3.2). In particular, patients treated with beef/pork insulin showed an increased clearance of free drug during interruption of EHC with neo/chol treatment, as well as a greater response to neo/chol treatment. This suggested that human insulin may be an inhibitor of hepatic glucuronidation, whereas beef/pork insulin was not. However, other factors, such as insulin dose and diet, may have played a part in these differences, since no attempt was made to control these factors. In addition, these observations were made in two separate groups, with only 5 individuals in each group. An attempt to account for interindividual variation using a crossover design study with 3 IDDM patients failed to reproduce these initial findings (section 3.3). However, in that study, lorazepam pharmacokinetic parameters were not reproducible, and highly variable within subjects. The purpose of this study was to study the independent effects of glucose and insulin on the disposition of lorazepam in IDDM patients, using a insulin/glucose clamping technique, which is well-known and accepted for studying glucose and insulin actions. Because patients with IDDM also have abnormalities in glucagon regulation, the effects of this hormone on lorazepam kinetics were also examined.

3.4.2. MATERIALS AND METHODS

All materials and methods used in this study were identical to those described in section 3.2.1, with the following exceptions:

3.4.2.1. Subjects

Six healthy, non-smoking males, between the age of 19-40 years, and diagnosed with insulin dependent diabetes mellitus, were recruited for this study.

3.4.2.2. Experimental methods

The disposition of lorazepam was examined ten times in each individual under a number of different conditions. For each study, the subjects presented themselves to the CIU at approximately 8:00 p.m. after an evening meal, but without taking their long-acting evening insulin. They had started neomycin, 1 g every 6 hours, the same morning, and were started on cholestyramine, 4 g every 6 hours, immediately on arrival at the CIU. Neomycin and cholestyramine were continued throughout the entire study interval. An intravenous infusion of 0.9% saline was established in one arm, with an inline four-way stopcock for withdrawing blood samples.

The protocol of the remainder of the experiment depended upon the conditions under which the subjects were studied. For a low insulin /low blood sugar study, a second intravenous infusion was placed in the opposite arm, through which 5% dextrose in water was infused at a constant rate of 100 mL/hour (approximately 480 kcal/day) by an infusion pump (Lifecare 5000 infusion system, Abbott Laboratories, North Chicago IL). An infusion of insulin diluted in 0.9% saline (approximately 1 unit/20 mL), was piggy-backed on top of the dextrose infusion and delivered by a second infusion pump at a rate of approximately 1-2 units per hour. Blood glucose measurements (Medisense Companion 2 Blood Glucose Sensor, Medisense Inc., Waltham MA) were made frequently thereafter (at least once every hour throughout the

day and the night) with the insulin infusion adjusted to maintain blood glucose between 4 and 5 mmoL/L. At 7:30 a.m., the patient was instructed to empty his bladder, and blank blood samples were collected. At 8:00 a.m., 2 mg of lorazepam (Wyeth Laboratories) were administered by rapid intravenous infusion into the arm opposite the blood sampling arm, and blood samples were collected at 1, 3, 5, 10, 15, 30, and 45 minutes, and 1, 1.5, 2, 3, 4, 6, 8, 10, 13, 16, 20 and 24 hours. Additional blood samples were collected at 0, 5, and 10 hours for plasma protein binding determinations. All urine was collected throughout the 24 hour study period. Subjects were instructed to remain semirecumbant until noon, after which time they were allowed to move freely. During the study period, patients were allowed to drink water, but remained fasted throughout.

The protocol for a low insulin/high blood glucose study was identical to the low insulin/low blood glucose study, except that the 5% dextrose in water intravenous infusion was replaced with an enteral infusion of a concentrated glucose solution (Polycose, Ross Laboratories, Columbus OH) delivered through a nasogastric tube directly into the stomach by an enteral infusion pump (Flexiflo III enteral pump, Ross Laboratories) at a rate of 50-100 mL/hour (approximately 2000-4000 kcal/day). Insulin infusion rates were patterned after the rates required during the basal low insulin/low blood sugar study, and glucose infusion rates were subsequently adjusted to maintain blood glucose levels between 15-20 mmoL/L. The high insulin level/low blood glucose studies were identical to the low insulin/high blood glucose procedures; however, insulin infusion rates were increased to approximately 4-8 units/hour in order to maintain blood glucose levels in the 4-5 mmoL/L. For practical reasons, a high insulin level/high blood glucose level protocol was not done. Each of the three aforementioned studies was carried out on each subject using both human insulin (Humulin R-(U-100), Eli Lilly, Scarborough, Ontario) and beef/pork insulin (Illetin II Pork and Beef Regular-100 unit, Eli Lilly). In addition to these six studies, each patient completed another

three studies (basal euglycemic, hyperglycemic, and hyperinsulinemic euglycemic) while being treated with beef/pork insulin, in which they received glucagon (Eli Lilly) infused into a hand vein by a pulsatile infusion pump (Autosyringe Travenol model AS6H infusion pump, Travenol Laboratories, Inc., Hooksett NH). Glucagon (1 mg/mL) was diluted 1:1 in sterile water and infused at a rate of 12μL (6μg) every 30 minutes, for a total of 288 μg per 24 hour study. This infusion rate was based on similar glucagon infusion rates used by other investigators in normal (Branum *et al.*, 1991; Attvall *et al.*, 1992) and IDDM subjects (Ørskov *et al.*, 1991). In addition, it has been shown that the physiological effects of glucagon are better maintained when given as intermittent pulses rather than continuous infusion (Attvall *et al.*, 1992).

A tenth study examined the elimination of lorazepam in these patients without neo/chol under a low insulin level/low blood glucose level protocol, with glucagon infusions.

The insulin species for the first study was chosen according to the type of insulin on which the patient initially presented. To establish basal insulin requirements for each individual, and for safety considerations, the initial study on each type of insulin was a low insulin /low blood glucose experiment. Basal insulin requirements were determined as those required to maintain blood sugars between 4-5 mmol/L during intravenous infusion of 5% dextrose in water. The rate of the dextrose infusion (100 mL/hr) was chosen to facilitate the study of the effects of low glucose levels on lorazepam disposition, without causing ketoacidosis. Subsequent glucose (Polycose) and insulin rates were determined based on this initial basal study. The remaining studies on each insulin species were randomized, with a minimum two week period between each. Efforts were made to complete all studies on that type of insulin before the patient was switched to the other insulin type. A summary of the experimental protocol is shown in Table 3.14.

Table 3.14. Protocol for ten-part lorazepam disposition studies in insulin-dependent diabetes mellitus patients.

Insulin type	Insulin infusion rate	Glucose infusion rate	Blood glucose level	Glucagon infusion	Neo/chol
human	low	low	low	no	yes
human	low	high	high	no	yes
human	high	high	low	no	yes
beef/pork	low	low	low	no	yes
beef/pork	low	high	high	no	yes
beef/pork	high	high	low	no	yes
beef/pork	low	low	low	yes	yes
beef/pork	low	high	high	yes	yes
beef/pork	high	high	low	yes	yes
beef/pork	low	low	low	yes	no

3.4.3. RESULTS

Six diabetic patients participated in the study. Of the six, four individuals completed all ten parts of the study. One participant left the study after completing three of the ten separate phases, due to employment relocation requirements. A second subject dropped out of the study for undetermined personal reasons after completing only one part. The results presented hereafter pertain only to the four individuals who completed the entire study protocol.

The characteristics of the four diabetic patients are shown in Table 3.15. The mean age of the subjects 30.5 ± 9.4 years (range 19-40 years), with a mean weight of 76.5 ±13.8 kg (range 59-89 kg). The average length of time since the original diagnosis of diabetes in these individuals was 13.5 ± 7.5 years (range 7-23 years). All patients had normal total bilirubin levels following an overnight fast, indicating that none had Gilbert's syndrome, a disease which can influence lorazepam disposition (Herman *et al.*, 1994).

Table 3.15. Baseline characteristics of 4 insulin-dependent diabetes mellitus patients in lorazepam kinetic studies examining the effects of blood glucose, insulin, glucagon and enterohepatic circulation.

Subject	Age (years)	Weight (kg)	D.D. (years)	T.B.I. (μmol/L)	HblAc (%)	G.P. (μmol/L)	D.I.R. (units)
SP	19	89	7	10	8.9	376	76
BS	40	59	8	8	11.3	479	30
RA	36	86	23	11	6.5	282	45
KR	27	72	16	9	6.3	237	77
Mean	30.5	76.5	13.5	9.5	8.3	344	57
S.D.	9.4	13.8	7.5	1.3	2.4	107	23

D.D. duration of diabetes since time of diagnosis.
T.B.I. total bilirubin (normal range; 0-22 μmol/L)
HbA1c hemoglobin A1c (normal range; 3.5-5.9%).
G. P. glycated protein (normal range; $\leq$ 285 μmol/L).
D.I.R. daily insulin requirements.

Three of the four patients presented with good glycemic control, indicated by HbA1c and glycated protein measurements close to the normal range; the fourth exhibited higher values. The daily insulin requirements varied from 30-76 units, with an average of 57 $\pm$ 23 units.

Table 3.16 shows the mean blood glucose levels as well as the absolute amount of insulin and glucose infused during each 24 hour study. Mean blood glucose levels during euglycemic clamping very close to the preset value of 5 mmoL/L in all treatment groups. Blood glucose levels during hyperglycemic conditions were near the upper end of the predetermined range. Insulin requirements tended to be slightly higher during the low insulin/euglycemic studies compared to the low insulin/ hyperglycemic studies; however, the differences were not significant (p=0.65). Similarly, the amount of insulin infused during hyperinsulinemic euglycemic clamping was higher, although not significantly

Table 3.16. Mean blood glucose levels, total insulin, and total glucose infused during lorazepam kinetic studies examining the effects of blood glucose, insulin, glucagon and enterohepatic circulation in four insulin-dependent diabetic subjects.

Treatment	Parameter	Conditions		
		low/low	low/high	high/low
HU	blood glucose	4.9±0.7	19.6±1.9	5.2±0.8
	insulin	30.2±11.6	39.4±7.7	211.9±138.5
	total glucose	687±42	3530±840	4711±701
B/P	blood glucose	5.0±0.4	20.1±1.6	5.2±0.9
	insulin	24.9±12.5	38.8±8.4	149.3±33.8
	total glucose	665±13	4210±1392	4296±1169
B/P-G	blood glucose	5.0±0.7	19.3±1.1	5.3±0.6
	insulin	27.0±14.1	36.5±10.3	150.4±64.8
	total glucose	680±19	3541±1461	4151±1248
B/P-G/no NC	blood glucose	5.7±0.6		
	insulin	23.5±16.9		
	total glucose	667±20		

All values are means ± S.D.
blood glucose blood glucose levels (mmoL/L).
insulin total amount of infused insulin per 24 hour study (units).
total glucose total amount of infused glucose per 24 hour study (mmoL).
low/low low insulin, euglycemia studies.
low/high low insulin, hyperglycemia studies.
high/low high insulin, euglycemia studies.
HU studies using human insulin.
B/P studies using beef/pork insulin.
B/P-G studies using beef/pork insulin with glucagon infusions.
B/P-G/no NC studies using beef/pork insulin with glucagon infusions and no neomycin/cholestyramine treatment.

(p=0.50), during the treatment with human insulin compared to the other treatment groups, due to a higher amount of administered enteral glucose in these studies.

The plasma concentration-time curves of lorazepam for each of the ten studies in a single diabetic patient (SP) are shown in Figure 3.8. Lorazepam concentrations fell quickly during the first 1-2 hours, then decreased in a smooth log-linear fashion over the remainder of the study interval. The large number of curves makes it difficult to discern individual clamping studies; however, the curves demonstrate the reproducibility of the results in this study, unlike the findings in the previous crossover study (section 3.3).

The effect of the different insulin treatments, glucagon, and blood glucose levels on the area under the plasma concentration-time curve and the total body clearance of 2 mg of intravenously administered lorazepam is summarized in Table 3.17. The model used for the statistical analysis was a repeated measures ANOVA with 2 within factors (treatment and conditions) and no between factors. Each analysis involved 4 individuals, with 9 studies per individual, for a total of 36 studies. (The same model was used for all other variables, unless otherwise stated). There was no effect of treatment on either the AUC or total clearance of lorazepam ($p=0.7$ for both) when comparing studies carried out while patients were treated with either human insulin, beef/pork insulin, or beef/pork insulin with glucagon. However, there was a significant effect ($p=0.01$) of experimental conditions, as the AUC of lorazepam was 15-20% higher during low insulin/euglycemic conditions (501 ± 84 ng/mL-hr) than both the low insulin/hyperglycemic studies (438 ± 38 ng/mL-hr, $p=0.02$) and the high insulin/euglycemic studies (413 ± 51 ng/mL-hr, $p=0.006$). Similarly, the clearance of lorazepam was lower during low insulin/euglycemic conditions (0.94 ± 0.25 mL/min/kg) compared to low insulin/hyperglycemic (1.05 ± 0.21, $p=0.03$) and high insulin/euglycemic (1.11 ± 0.22 mL/min/kg, $p=0.005$) studies (Figure 3.9). There was no interaction between treatment and conditions.

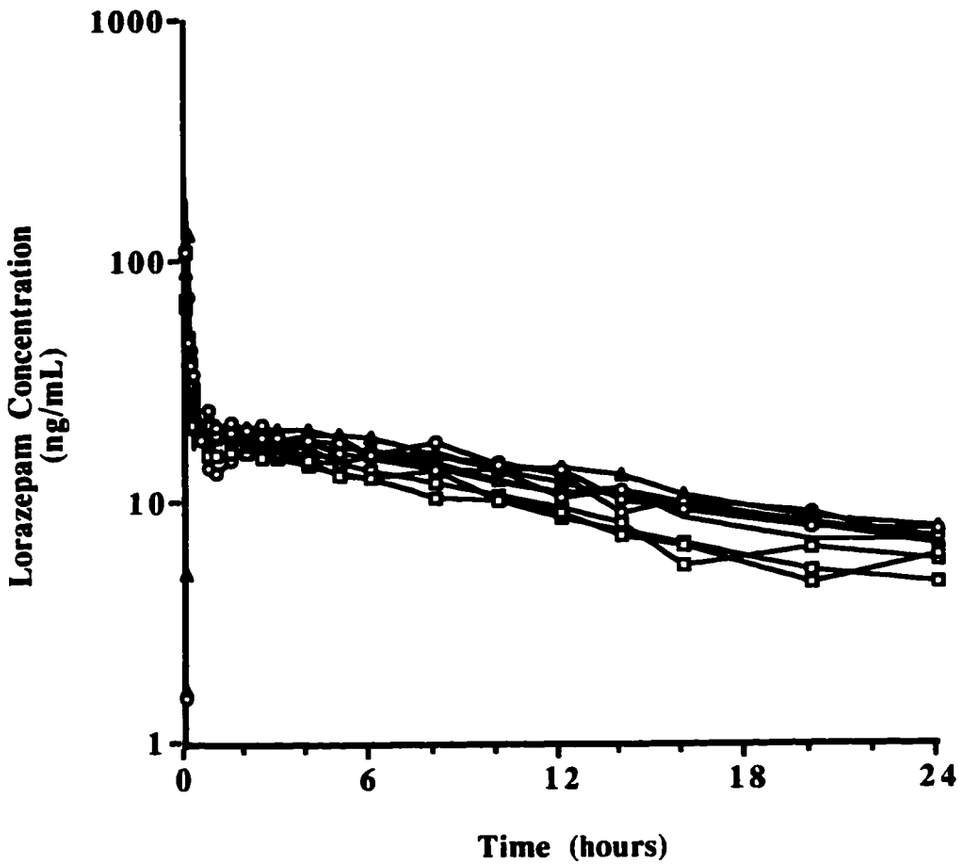


Figure 3.8. Plasma concentration-time profiles of lorazepam during ten separate kinetic studies in a single diabetic patient (SP). Triangles indicate studies done with human insulin. Circles indicate studies done with beef/pork insulin. Squares indicate studies done with beef/pork insulin and glucagon infusions.

Table 3.17. AUC and total systemic clearance of lorazepam during lorazepam kinetic studies examining the effects of blood glucose, insulin, glucagon and enterohepatic circulation in four insulin dependent diabetic subjects.

Treatment	Parameter	Conditions			Mean
		low/low	low/high	high/low	
HU	AUC	521±55	447±36	428±30	466±57
	CL	0.90±0.27	1.04±0.28	1.07±0.26	1.00±0.26
B/P	AUC	504±48	446±20	404±37	452±54
	CL	0.92±0.22	1.04±0.20	1.12±0.23	1.03±0.22
B/P-G	AUC	478±140	420±43	409±83	435±96
	CL	1.01±0.30	1.08±0.19	1.13±0.24	1.07±0.23
Mean	AUC	501±8	438±34[1]	413±51[1]	
	CL	0.94±0.25	1.05±0.21[1]	1.11±0.22[1]	

All values are means ± S.D.

[1] significantly different from low/low conditions, as determined by ANOVA using repeated measures.

AUC area under the plasma concentration time curve (ng/mL-hr).
CL total systemic clearance of lorazepam in (mL/min/kg).
low/low low insulin, euglycemia studies.
low/high low insulin, hyperglycemia studies.
high/low high insulin, euglycemia studies.
HU studies using human insulin.
B/P studies using beef/pork insulin.
B/P-G studies using beef/pork insulin with glucagon infusions.

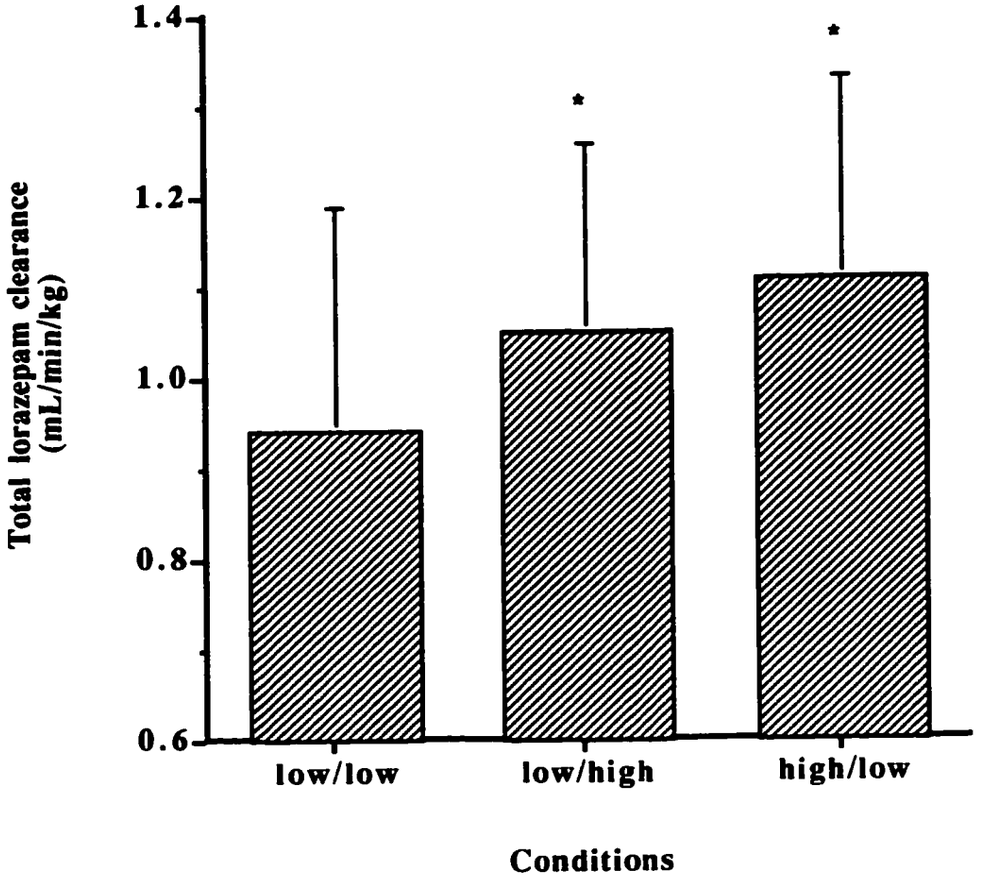


Figure 3.9. The effect of insulin and blood glucose level on total lorazepam clearance (mL/min/kg) in 4 insulin dependent diabetic patients.

low/low indicates low insulin levels, euglycemia
low/high indicates low insulin levels, hyperglycemia
high/low indicates high insulin levels, euglycemia
*** indicates significantly different from low/low ($p<0.05$)**
Error bars represent standard deviation

Table 3.18. Clearance of unbound lorazepam during lorazepam kinetic studies examining the effects of blood glucose, insulin, glucagon and enterohepatic circulation in four insulin dependent diabetic subjects.

Treatment	FCL			Mean
	low/low	low/high	high/low	
HU	13.3±4.5	15.8±4.4	14.5±3.4	14.5±3.9
B/P	13.2±3.8	15.1±2.9	15.6±3.5	14.6±3.3
B/P-G	13.5±3.5	14.9±3.5	14.8±3.6	14.4±3.3
Mean	13.3±3.6	15.3±3.3	14.9±3.2	

All values are means ± S.D.
No significant differences were observed between any treatment or conditions within the studies, as determined by analysis of variance with repeated measures.

FCL	clearance of free lorazepam (mL/min/kg).
low/low	low insulin, euglycemia studies.
low/high	low insulin, hyperglycemia studies.
high/low	high insulin, euglycemia studies.
HU	studies using human insulin.
B/P	studies using beef/pork insulin.
B/P-G	studies using beef/pork insulin with glucagon infusions.

The mean systemic clearance of free lorazepam was not significantly different between the human insulin, beef/pork insulin, and beef/pork insulin with glucagon infusion treatment groups (Table 3.18). Similar to results obtained for total systemic clearance, there was no effect of treatment among the various studies ($p=0.95$). Statistical analysis of free clearance values showed that the difference between the various conditions was not significant ($p=0.30$), though clearance of free lorazepam tended to be lower during low insulin, euglycemic clamp studies when compared to both the low insulin, hyperglycemic and the high insulin, euglycemic clamp studies.

Table 3.19. Percent of unbound lorazepam in plasma during lorazepam kinetic studies examining the effects of blood glucose, insulin, glucagon and enterohepatic circulation in four insulin dependent diabetic subjects.

Treatment	% unbound			Mean
	low/low	low/high	high/low	
HU	6.80±0.74	6.62±0.42	7.39±0.56	6.93±0.63
B/P	7.05±1.12	6.89±0.12	7.26±0.43	7.07±0.65
B/P-G	7.50±1.12	7.43±1.26	7.74±1.37	7.56±1.14
Mean	7.12±0.96	6.98±0.78	7.46±0.83	

All values are means ± S.D.
No significant differences were observed between any treatment or conditions within the studies, as determined by analysis of variance with repeated measures.
% unbound percentage of unbound lorazepam in plasma.
low/low low insulin, euglycemia studies.
low/high low insulin, hyperglycemia studies.
high/low high insulin, euglycemia studies.
HU studies using human insulin.
B/P studies using beef/pork insulin.
B/P-G studies using beef/pork insulin with glucagon infusions.

No differences in the unbound fraction of lorazepam were detected in the four IDDM patients in any of the various treatment or conditions groups.

Table 3.20. Volume of distribution of lorazepam at steady-state during lorazepam kinetic studies examining the effects of blood glucose, insulin, glucagon and enterohepatic circulation in four insulin dependent diabetic subjects.

Treatment	Vd_{ss}			Mean
	low/low	low/high	high/low	
HU	0.916±0.175	0.927±0.225	0.983±0.284	0.942±0.212
B/P	0.859±0.154	0.941±0.197	0.909±0.212	0.903±0.175
B/P-G	0.844±0.177	0.813±0.142	0.928±0.163	0.862±0.154
Mean	0.873±0.156	0.894±0.183	0.940±0.206	

All values are means ± S.D.
No significant differences were observed between any treatment or conditions within the studies, as determined by analysis of variance with repeated measures.
Vd_{ss} volume of distribution at steady-state of lorazepam (L/kg).
low/low low insulin, euglycemia studies.
low/high low insulin, hyperglycemia studies.
high/low high insulin, euglycemia studies.
HU studies using human insulin.
B/P studies using beef/pork insulin.
B/P-G studies using beef/pork insulin with glucagon infusions.

Table 3.20 shows the steady-state volume of distribution of lorazepam during the different studies. Statistically, there was no effect of treatment ($p=0.07$) or conditions ($p=0.08$) on the volume of distribution at steady-state of lorazepam.

Table 3.21. Elimination half-life of lorazepam during lorazepam kinetic studies examining the effects of blood glucose, insulin, glucagon and enterohepatic circulation in four insulin dependent diabetic subjects.

Treatment	$T_{1/2}$			Mean
	low/low	low/high	high/low	
HU	10.4±1.9	8.7±0.3	8.5±0.6	9.2±1.0
B/P	10.1±0.6	9.0±0.3	8.6±1.4	9.2±1.4
B/P-G	9.9±1.1	8.2±0.2	9.6±2.1	9.2±1.4
Mean	10.1±1.1	8.6±0.4	8.9±1.4	

All values are means ± S.D.
No significant differences were observed between any treatment or conditions within the studies, as determined by analysis of variance with repeated measures.

$T_{1/2}$	elimination half-life of lorazepam (hr).
low/low	low insulin, euglycemia studies.
low/high	low insulin, hyperglycemia studies.
high/low	high insulin, euglycemia studies.
HU	studies using human insulin.
B/P	studies using beef/pork insulin.
B/P-G	studies using beef/pork insulin with glucagon infusions.

Table 3.21 shows the effects of insulin species and glucagon, as well as the effects of glucose on the elimination half-life of lorazepam in the four IDDM patients. There was no effect of treatment (p=0.98) on lorazepam's half-life. The elimination half-life was 10-15% longer during low insulin/euglycemic studies; however these differences did not reach significance (p=0.067). Changes in half-life would be expected considering that differences were detected in total clearance of lorazepam (Table 3.17); however, these were probably not detected due to the observed changes in volume of distribution (Table 3.20).

Table 3.22. Renal clearance of lorazepam glucuronide during lorazepam kinetic studies examining the effects of blood glucose, insulin, glucagon and enterohepatic circulation in four insulin dependent diabetic subjects.

Treatment	CL_R			Mean
	low/low	low/high	high/low	
HU	0.45±0.10	0.61±0.26	0.62±0.22	0.51±0.14
B/P	0.39±0.03	0.58±0.14	0.57±0.15	0.54±.016
B/P-G	0.41±0.07	0.57±.014	0.63±0.20	0.56±.020
Mean	0.42±0.07	0.59±0.17[1]	0.61±0.18[1]	

All values are means ± S.D.
[1] significantly different from low/low conditions ($p<0.05$), as determined by analysis of variance with repeated measures.
CL_R renal clearance of lorazepam glucuronide, in mL/min/kg.
low/low low insulin, euglycemia studies.
low/high low insulin, hyperglycemia studies.
high/low high insulin, euglycemia studies.
HU studies using human insulin.
B/P studies using beef/pork insulin.
B/P-G studies using beef/pork insulin with glucagon infusions.

Renal clearance of lorazepam glucuronide was similar whether patients were treated with human insulin, beef/pork insulin, or with beef/pork insulin with glucagon infusions (Table 3.22). However, repeated measures ANOVA showed a significant effect of experimental conditions ($p=0.02$). The renal clearance of lorazepam glucuronide during low insulin/euglycemic studies (0.42±0.07 mL/min/kg) was significantly lower than values achieved during both low insulin/hyperglycemic studies (0.59±0.17 mL/min/kg, $p=0.02$), and hyperinsulinemic/euglycemic studies (0.61±0.18 mL/min/kg, $p=0.01$) (Figure 3.10). There was no interaction between treatment and experimental conditions ($p=0.97$).

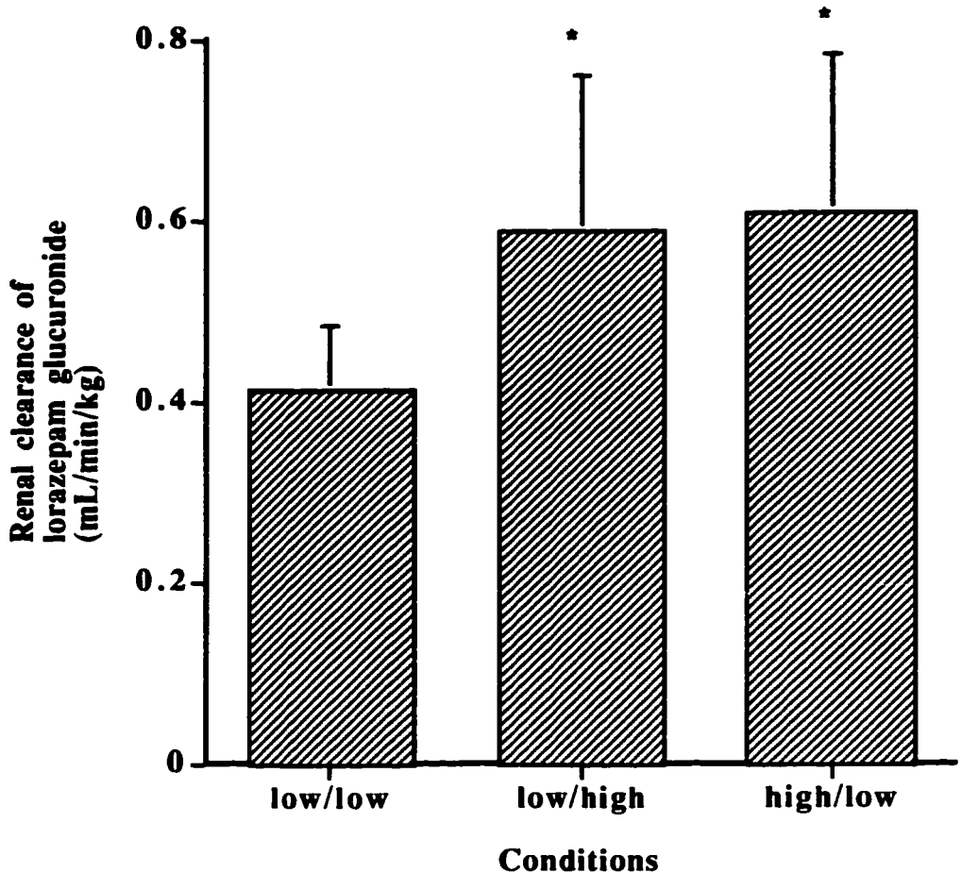


Figure 3.10.The effect of insulin and blood glucose level on renal lorazepam glucuronide clearance (mL/min/kg) in 4 insulin dependent diabetic patients.

low/low indicates low insulin levels, euglycemia
low/high indicates low insulin levels, hyperglycemia
high/low indicates high insulin levels, euglycemia
*** indicates significantly different from low/low ($p<0.05$)**
Error bars represent standard deviation

Table 3.23. Ratio of area under the plasma concentration curve of lorazepam glucuronide to area under the plasma concentration curve of lorazepam (AUCG/AUC) during lorazepam kinetic studies examining the effects of blood glucose, insulin, glucagon and enterohepatic circulation in four insulin dependent diabetic subjects.

Treatment	AUCG/AUC			Mean
	low/low	low/high	high/low	
HU	1.85±0.57	1.74±0.48	1.65±0.84	1.75±0.59
B/P	2.03±0.50	1.75±0.65	1.77±0.66	1.85±0.56
B/P-G	2.14±0.31	1.80±0.50	1.67±0.42	1.87±0.43
Mean	2.01±0.44	1.76±0.50	1.70±0.60	

All values are means ± S.D.
No significant differences were observed between any treatment or conditions within the studies, as determined by analysis of variance with repeated measures ($p<0.05$).

AUCG/AUC	ratio of the area under the plasma concentration curve of lorazepam glucuronide to the area under the plasma concentration curve of lorazepam
low/low	low insulin, euglycemia studies.
low/high	low insulin, hyperglycemia studies.
high/low	high insulin, euglycemia studies.
HU	studies using human insulin.
B/P	studies using beef/pork insulin.
B/P-G	studies using beef/pork insulin with glucagon infusions.

No effect of the different insulin treatments, or glucagon infusions was observed on the ratio of the area under the concentration-time curve of lorazepam glucuronide to the area under the plasma concentration-time curve of lorazepam (AUCG/AUC) in the four diabetic patients ($p=0.75$) (Table 3.23). The ratio was higher during low insulin, euglycemic clamp conditions (2.01 ± 0.44) compared to studies done during low insulin, hyperglycemic (1.76 ± 0.50) and high insulin, euglycemic (1.70 ± 0.60) conditions; however the effect of the different conditions on AUCG/AUC did not reach significance ($p=0.086$).

Table 3.24. Volume of urine excreted during lorazepam kinetic studies examining the effects of blood glucose, insulin, glucagon and enterohepatic circulation in four insulin dependent diabetic subjects.

Treatment	Urine			Mean
	low/low	low/high	high/low	
HU	3631±652	3898±1483	2004±546	3177±1249
B/P	3591±891	3308±1527	1868±978	2922±1317
B/P-G	3724±360	3220±1072	1780±526	2908±1079
Mean	3648±609[1]	3475±1284[1]	1884±653	

All values are means ± S.D.

[1] significantly different from high/low conditions ($p<0.05$), as determined by analysis of variance with repeated measures.

Urine — total volume of urine excreted during 24 hours (mL).
low/low — low insulin, euglycemia studies.
low/high — low insulin, hyperglycemia studies.
high/low — high insulin, euglycemia studies.
HU — studies using human insulin.
B/P — studies using beef/pork insulin.
B/P-G — studies using beef/pork insulin with glucagon infusions.

During each of the lorazepam kinetic studies, all urine was collected over the entire twenty-four hour period. Table 3.24 summarizes the results of the volume of urine excreted by the four diabetic patients during the various studies, and Table 3.25 shows the corresponding urinary excretion of glucose. Although the type of insulin treatment, as well as infusions of glucagon, had no effect on the amount of urine produced ($p=0.80$), urinary excretion was significantly influenced by the amount of infused insulin ($p=0.001$) (Figure 3.11). There was no difference ($p=0.55$) in urinary excretion between low insulin/euglycemic clamp studies (3648 ± 609 mL) and low insulin/hyperglycemic conditions (3475 ± 1284 mL). The volume of urine excreted during high insulin/euglycemic studies (1884 ± 653 mL) was significantly lower than

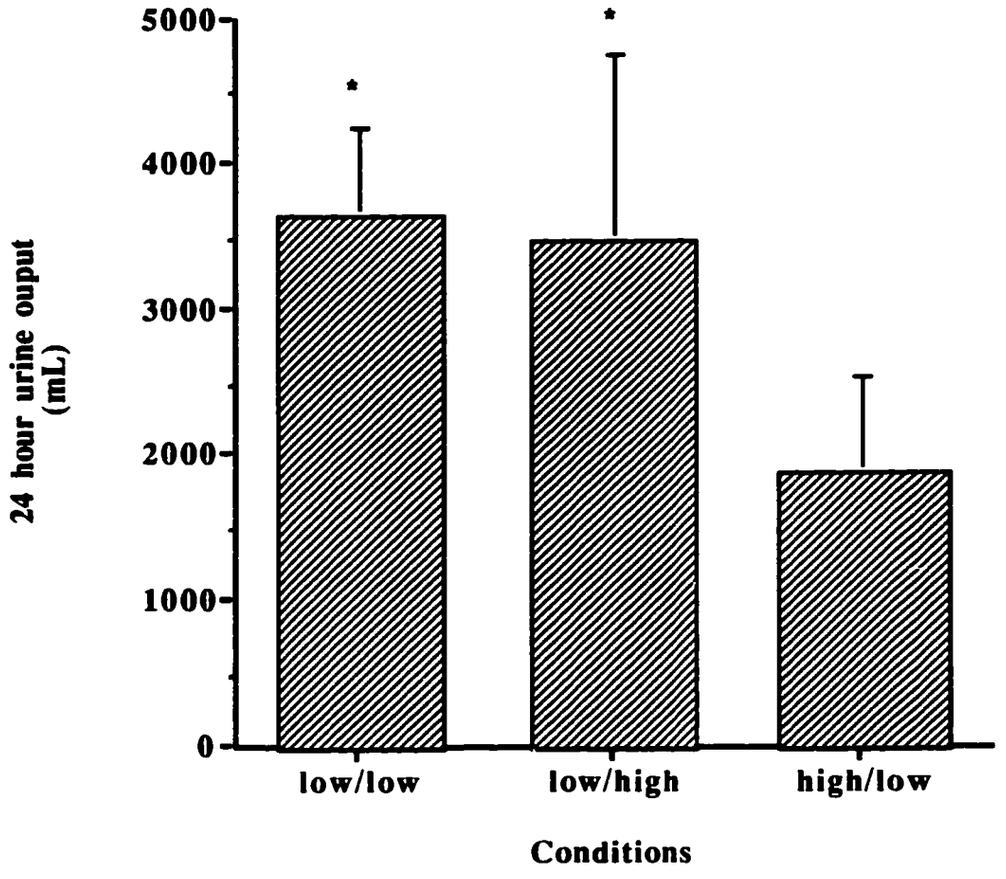


Figure 3.11.The effect of insulin and blood glucose level on 24 hour urinary output (mL) in 4 insulin dependent diabetic patients.

low/low indicates low insulin levels, euglycemia
low/high indicates low insulin levels,hyperglycemia
high/low indicates high insulin levels, euglycemia
*** indicates significantly different ($p<0.05$) from high/low**
Error bars represent standard deviation

Table 3.25. Amount of glucose excreted in urine during lorazepam kinetic studies examining the effects of blood glucose, insulin, glucagon and enterohepatic circulation in four insulin dependent diabetic subjects.

Treatment	Urinary glucose			Mean
	low/low	low/high	high/low	
HU	1.5±1.0	1636±741	0.6±0.5	546±893
B/P	1.3±0.5	1678±915	1.5±1.7	560±954
B/P-G	0.8±0.2	1383±532	0.8±0.5	462±735
Mean	1.2±0.7[1]	1566±688	1.0±1.1[1]	

All values are means ± S.D.

[1] significantly different from low/high conditions ($p<0.05$), as determined by analysis of variance with repeated measures.

Urinary glucose — total amount of glucose excreted in urine excreted during 24 hours (mmoL).
low/low — low insulin, euglycemia studies.
low/high — low insulin, hyperglycemia studies.
high/low — high insulin, euglycemia studies.
HU — studies using human insulin.
B/P — studies using beef/pork insulin.
B/P-G — studies using beef/pork insulin with glucagon infusions.

either the low insulin/euglycemic ($p=0.007$), or low insulin/hyperglycemic ($p=0.001$) studies. The effect of the conditions on urine output was consistent among the different treatment groups, as there was no interaction between treatment and conditions ($p=0.92$).

Excretion of urinary glucose was higher ($p = 0.0005$) during studies performed during hyperglycemic conditions than euglycemic studies. Interestingly, this did not correlate with in an increase in urinary output (Table 3.24). Indeed, urine output was similar in the low insulin/euglycemic and high insulin/hyperglycemic studies. No effect of insulin treatments on urinary glucose excretion were noted; however, this was expected, given the large variability in urinary glucose between the different conditions.

Table 3.26. The effect of interruption of enterohepatic circulation on the pharmacokinetics of lorazepam in four insulin dependent diabetic subjects

Parameter	Cycling-interrupted	Cycle-intact	p-value[1]
AUC	478 ± 140	520 ± 71	0.40
CL_{tot}	1.01±0.30	0.94 ± 0.32	0.68
FCL	13.5 ± 3.5	12.6 ± 3.5	0.71
% unbound	7.50 ± 1.12	7.13 ± 1.52	0.51
Vd_{ss}	0.844 ± 0.177	0.900 ± 0.159	0.18
AUCG/AUC	2.14 ± 0.07	1.64 ± 0.75	0.22
CL_R	0.41 ± 0.07	0.55 ± 0.23	0.21
Urine	3724 ± 360	3465 ± 1183	0.65

All values are means ± S.D.

[1] comparisons made by paired t-tests.

Cycling-interrupted — studies done with beef/pork insulin with glucagon infusions with neomycin/cholestyramine treatment.

Cycling-intact — studies done with beef/pork insulin with glucagon infusions without neomycin/cholestyramine treatment.

AUC — area under the plasma concentration time curve (ng/mL-hr).

CL_{tot} — total systemic clearance of lorazepam (mL/min/kg).

FCL — clearance of free lorazepam (mL/min/kg).

% unbound — percentage of unbound lorazepam in plasma.

Vd_{ss} — volume of distribution at steady-state of lorazepam (L/kg).

AUCG/AUC — ratio of the area under the plasma concentration curve of lorazepam glucuronide to the area under the plasmaconcentration curve of lorazepam.

CL_R — renal clearance of lorazepam glucuronide (mL/min /kg).

Urine — 24-hour urinary output (mL).

The effect of interruption of enterohepatic circulation (EHC) on the pharmacokinetics of lorazepam is summarized in Table 3.26. Of the ten kinetic studies done in each diabetic subject, only one was performed with an intact EHC; the rest all had the EHC of lorazepam interrupted through the concomitant use of neomycin and cholestyramine. The single study in which enterocycling was allowed to remain intact was performed while the diabetic patients were treated with beef/pork insulin and with pulsatile glucagon infusions (the rationale for this was based on the fact that glucagon is

a potent choleretic, and the suggestion that a greater EHC exists in patients treated with beef/pork insulin (section 3.2)). Interruption of enterohepatic circulation of lorazepam with neomycin/cholestyramine treatment did not cause a significant change in any of measured parameters when compared to similar studies performed with an intact EHC. Neomycin/cholestyramine treatment caused a mean decrease of 7.0% (range 3.1-10.4%) in lorazepam AUC and a mean increase of 8.2% (range 7.4-9.8%) in total lorazepam clearance in three of the four individuals when compared to identical studies done with beef/pork insulin with glucagon infusions during low insulin/low blood glucose levels. Neomycin/cholestyramine treatment led to 7.6% increase in lorazepam AUC and a 8.4% decrease in total lorazepam clearance in the fourth subject. Interruption of EHC was associated with an increase in lorazepam's volume of distribution at steady-state by 21% and 7.3% in two of the diabetic subjects, however one patient showed only a 2% increase, while the other showed a 0.5% decrease. Renal clearance of lorazepam glucuronide was greater in all four diabetic patients in studies without neomycin/cholestyramine, with the increase ranging from 4.8-84%.

3.4.4. DISCUSSION

The purpose of this study was to examine the effects of glucose and insulin on the pharmacokinetics of lorazepam in patients with IDDM. Previous work (sections 3.2 & 3.3) had suggested that both variables might have important effects on the disposition of lorazepam within a diabetic population. However, the design of the previous studies did not permit the study of both variables as possible independent factors. Accordingly, it was hoped that the tightly-controlled protocol of this present investigation might be successful in determining the influence that these variables have in affecting lorazepam disposition in patients with IDDM, and in so doing, provide

some insight into the effects of these factors in drug disposition which may be applied to the general population.

The disposition of lorazepam in IDDM patients was clearly affected by exogenous glucose in this study. Total lorazepam clearance was significantly lower during low insulin/euglycemic clamping compared to either the low insulin/hyperglycemic or the high insulin/euglycemic clamping (Table 3.17, Figure 3.9). The variable which appears to be responsible for the difference between these studies was the amount of exogenous glucose administered. Blood glucose levels, *per se*, were not a controlling factor, since lorazepam clearance rates were similar between low insulin/high blood glucose studies (blood glucose ~ 20 mmoL/L) and high insulin/euglycemic studies (blood glucose ~ 5 mmoL/L). Similarly, the rate or species of insulin infusion did not influence lorazepam clearances. During the low insulin/euglycemic clamp studies, low amounts of glucose (677±27 mmoL / 24 hr study) were delivered directly into the peripheral circulation though an arm vein. The amount of glucose administered during the low insulin/hyperglycemia (3762±1189 mmoL / 24 hr study) and the high insulin/hyperglycemic (4386 ± 996 mmoL / 24 hr study) studies was approximately 6-fold greater. The route of administration may also be important, since glucose given directly into the stomach enters the portal circulation where high concentrations pertain and where it is available to be taken up and utilized by the liver. Glucose delivered into the systemic circulation, particularly in the presence of insulin is largely taken up onto skeletal muscle, and would have considerably less hepatic effects. The observation that high levels of glucose infusion enhance lorazepam metabolism suggests that substrate availability may be important in controlling the glucuronidation of this drug.

Renal clearances of lorazepam glucuronide during the low insulin/hyperglycemic and the hyperinsulinemic/euglycemic clamp studies were similar to values reported by Greenblatt *et al.* (1976) for healthy subjects, but approximately

50% higher than rates observed by Herman *et al.*, (1994) in studies with fed healthy normal volunteers. Glucose affected the renal excretion of lorazepam glucuronide similar to the effects on total lorazepam clearance. The renal clearance of lorazepam glucuronide significantly increased with high levels of administered exogenous glucose (Table 3.22, Figure 3.10). These results are identical to those reported by Herman *et al.* (1994), who found an increased renal clearance of lorazepam glucuronide in both normal subjects and Gilbert's patients treated with neo/chol who were continually fed a high-carbohydrate, low-fat diet, when compared to fasted studies. Since the kidney possesses significant levels of glucuronidative enzymes (Dutton, 1980; Anders, 1980), one possible explanation is that renal glucuronide formation increased with exogenous glucose in the same fashion as in the liver. This would imply extrahepatic metabolism of lorazepam in man. In a study in dogs with hepatic and total splanchnic devascularization, Gerkens *et al.* (1981) concluded that the liver is the major site of lorazepam glucuronidation in dogs; however, appreciable extrahepatic glucuronidation was also attributed to the gastrointestinal mucosa. Although there are no such direct studies in man, indirect evidence for extrahepatic metabolism can be found in studies comparing oral and i.v. metabolite ratios (R or/iv). For drugs completely eliminated by the liver, R or/iv is equal to 1; higher ratios indicate extrahepatic metabolism. Based on such observations, Herman *et al.* (1989) reported significant extrahepatic metabolism of lorazepam in normal subjects. However, further work by this group (Herman *et al.*, 1994) concluded that renal saturation of lorazepam glucuronide secretion was likely the underlying cause of observed changes in R or/iv, a situation which makes the use of such ratios invalid for determining extrahepatic metabolism, and that extrahepatic metabolism of lorazepam was unlikely to occur to any level of significance in humans.

A second possibility to explain the changes in renal clearance during these studies is a reduction in renal elimination of the metabolite during the low

insulin/euglycemic studies. It is unlikely that the decrease in renal clearance of lorazepam is caused by a decrease in glomerular filtration rate, since urine volumes were comparable between the low insulin/low blood glucose study and the low insulin/high blood glucose study, which were both significantly higher than the high insulin/low blood glucose study (Table 3.22, Figure 3.10). Since the reduced clearance is not due to reduced filtration, it follows that the reduction must be due to either a decreased tubular secretion or an increased reabsorption. Glucuronides are organic anions which are actively secreted at the renal tubule (Miners and Mackenzie, 1991). Probenicid treatment has been shown to decrease the renal clearance of glucuronide metabolites (Smith *et al.*, 1985). Since active transport is an energy-dependent mechanism fueled by adenosine triphosphate (ATP), it is possible that the low-energy state caused by the low amount of glucose and insulin resulted in a decreased active secretion of the glucuronide. However, whether or not this is responsible for the changes in the renal clearance of lorazepam glucuronide remains speculative.

The amount of administered glucose had no effects on any other measured lorazepam parameter. Glucose had no significant effect on the free clearance of lorazepam (Table 3.18), in contrast to the effects observed on total clearance. This would suggest that the glucose-related changes in total clearance were the result of changes in lorazepam free fraction; however, glucose had no significant effect on lorazepam protein binding (Table 3.19). Although the difference was not statistically significant, clearance of unbound lorazepam was lower during the low insulin/euglycemic studies (Table 3.18). In addition to the small number of study subjects, part of the reason for the failure to reach significance may be due to the variable nature of the free fraction determinations. While no effect of treatment or conditions was detected, the fraction of unbound lorazepam appeared to be higher during the hyperglycemic/euglycemic clamps and in the studies done with glucagon

infusions. Since clearance of unbound lorazepam depends upon total clearance and unbound fraction, the variability introduced through the free fraction determinations may have been enough to obscure any significant difference in free clearance values.

The ratio of the area under the curve of the lorazepam glucuronide to the area under the curve of lorazepam (AUCG/AUC) can be viewed as a surrogate measure of metabolic clearance (Rowland and Tozer, 1989; Herman *et al.*, 1995), provided that glucuronide clearance does not change. Given that lorazepam clearance increases with higher levels of exogenous glucose, one would expect the ratio to also increase. However, studies using high amounts of exogenous glucose had lower AUCG/AUC ratios ($p=0.086$) than the studies that used low amounts of glucose (Table 3.23). Since renal clearances of lorazepam glucuronide were also significantly higher during the high glucose studies (Table 3.22, Figure 3.10), this likely accounts for the lower AUCG/AUC ratios during these studies.

The finding of an increased clearance of lorazepam during administration of high intragastric glucose is supported by work done in other laboratories. It has been suggested that the synthesis of UDPGA is a rate-limiting step in the glucuronidation of several xenobiotics (Otani *et al.*, 1976; Moldéus *et al.*, 1978; Conway *et al.*, 1987; Felsher *et al.*, 1979; Hjelle *et al.*, 1985; Price and Jollow, 1984; Reinke *et al.*, 1981, Singh and Schwarz, 1981; Shipley and Weiner, 1987). A high rate of glucuronidation is dependent upon a sufficient supply of UDPGA (Bock, 1995). The K_m values of UDPGTs for UDPGA are usually reported to be in the range of 0.1-0.4 mM, which is approximately the concentration of UDPGA in the liver. Therefore, changes in cellular metabolism which increase or decrease the availability of UDPGA cause corresponding changes in the rate of glucuronidation (Reinke *et al.*, 1994). Indeed, in an energy depleted state, the supply of co-substrates can limit the rate of conjugation (Hjelle *et al.*, 1985; Dills *et al.*, 1987). Fasting has been shown to cause a decrease in rates of glucuronidation attendant upon a decrease in UDPGA synthesis (Aw and Jones, 1984;

Bànhegyi *et al.*, 1988; Conway *et al.*, 1985; Conway *et al.*, 1987; Felsher *et al.*, 1979; Minnigh and Zemaitis, 1982; Price *et al.*, 1987; Price and Jollow, 1988). Addition of glucose can restore fasting-induced decreases in glucuronidation in perfused livers or isolated hepatocytes (Aw and Jones, 1984; Eacho *et al.*, 1981a; Reinke *et al.*, 1981; Conway *et al.*, 1985), although this has not been observed in all studies (Bànhegyi *et al.*, 1988; Price and Jollow, 1989). Studies with oxazepam, a benzodiazepine structurally related to lorazepam, showed that a low calorie, low carbohydrate diet depressed oxazepam clearance, but had no effect on antipyrine clearance in man (Sonne *et al.*, 1989). Also, the clearance of unbound lorazepam was greater in normal volunteers during a steady intake of a high-carbohydrate, low-fat diet compared to values measured in the same patients during an extended fast (Herman *et al.*, 1994). In that same study, patients with Gilbert's syndrome given neomycin and cholestyramine to interrupt EHC, had greater clearances of unbound lorazepam fed compared to fasting. Decreased UDPGA levels are also associated with a decrease in the biliary excretion of drugs, through decreased formation of glucuronide metabolites (Gregus *et al.*, 1983).

Another explanation, namely, enhanced activity of the enzyme UDPGT, could also be a factor in the increase in the hepatic clearance of lorazepam, although work by others does not support this. Studies with rats show that fasting for 3 days has no effect on absolute UDPGT activity towards either 4-nitrophenol (Marselos and Laitinen, 1975), or bilirubin (Felsher *et al.*, 1979). In acutely fasted rats, the hepatic clearance of acetaminophen was impaired at high substrate levels when compared to fed animals; however, at therapeutic levels of drug, the apparent rate constant of UDPGT for acetaminophen was unchanged, indicating that the effect of fasting was due to a decrease in UDPGA availability, and not due to a decrease in enzyme activity (Price *et al.*, 1987). In studies with Brazilian squirrel monkeys, a 24-hour fast caused UDPGA

and UDP-glucose levels to be reduced by one-half; however, hepatic bilirubin UDPGT activity significantly increased in a compensatory manner (Myers *et al.*, 1990).

Although several pharmacokinetic parameters of lorazepam were dependent upon the amount of glucose that was administered, insulin which was infused did not appear to have any effects on lorazepam disposition. Indeed, the only parameter which appeared to be dependent upon insulin levels was urine production. Urine output during the hyperinsulinemic/euglycemic clamping was approximately one-half the values measured during the low insulin/low blood glucose and the low insulin/high blood glucose studies (Table 3.24, Figure 3.11). This was unexpected given that the polyuria associated with diabetes mellitus is commonly perceived to be due to the decreased reabsorption of water secondary to the increased concentrations of glucose in the collecting duct (Lawrence, 1994). Urine output could not be correlated with urinary glucose excretion, which was low during the high insulin/euglycemic studies and the low insulin/euglycemic studies, but much higher during the high blood glucose studies (Table 3.25). This suggests that the low urine output during the high insulin/euglycemic studies was due to the high levels of insulin which were infused during the studies. This is in agreement with work by other investigators who have found that high levels of insulin causes sodium retention in normal individuals (Schloeder and Stinebaugh, 1970; Skøtt *et al.*, 1989; Galvan *et al.*, 1995). Furthermore, inhibition of basal insulin secretion in normal subjects with somatostatin causes a natriuresis (DeFronzo *et al.*, 1978). It has also been shown that IDDM patients show an impaired sodium excretion following NaCl infusion (Stenvinkel *et al.*, 1991) and have a 10-15% increase in total exchangeable sodium (O'Hare *et al.*, 1985; Feldt-Rasmussen *et al.*, 1987). Although the exact mechanism is unknown, the effect is believed to occur at the proximal tubular reabsorption site (Stenvinkel *et al.*, 1991). This increased exchangeable sodium has been suggested to be a principle causative factor in hypertension in IDDM (O'Hare *et al.*, 1985; Feldt-Rasmussen *et al.*, 1987). It

may also play a role in the cardiovascular complications in NIDDM, since these patients are also hyperinsulinemic. Unfortunately, urinary excretion of electrolytes were not measured in this study, which may have been useful in corroborating these observations.

Glucagon levels in IDDM patients are routinely higher than normal, especially in subjects with poor control. Glucagon is known to be a potent stimulator of bile flow in man (Branum *et al.*, 1991), and has been demonstrated to stimulate bilirubin and *p*-nitrophenol glucuronidation *in vitro* (Ricci *et al.*, 1989). It was hypothesized that if alterations in lorazepam disposition in IDDM patients were related to glucagon, additional glucagon may cause an enhanced effect in a dose-dependent manner. Glucagon infusions were studied in patients treated with beef/pork insulin since previous work (section 3.2) had indicated that IDDM patients treated with beef/pork insulin had a greater rate of hepatic glucuronidation as well as a greater response to interruption of EHC with neo/chol treatment. Thus, it was felt that changes due to glucagon would most likely be detected when patients were treated with this insulin species. However, pulsatile glucagon infusions had no effect on lorazepam pharmacokinetics. This may partially be due to the fact that IDDM patients have been shown to have a reduced hepatic glucagon response compared to normal subjects (Ørskov *et al.*, 1991).

Interruption of EHC is important in studies of lorazepam disposition in that it provides a more accurate reflection of hepatic glucuronidation (Herman *et al.*, 1989). Since it was not one of the objectives of the present investigation to study the effects of EHC *per se*, only one of the ten studies was done without neo/chol treatment. The rationale for the choice of studying EHC on beef/pork insulin with infusions of glucagon was based on findings from our earlier studies that had shown that lorazepam clearances were greater on beef/pork insulin and we expected that, if anything, glucagon would have potentiated these differences. When compared to studies

conducted under similar experimental conditions, neo/chol treatment increased both the total and free clearance of lorazepam by 7%, although the differences were not significant (p=0.68, 0.71, respectively). The magnitude of this effect was considerably lower than what has been observed in other studies (Herman *et al.*, 1989; 1994); however, several reasons may account for the perceived lack of effect of neo/chol treatment during these studies. Firstly, the study without neo/chol was done during a low insulin/euglycemic protocol, when hepatic clearances were found to be quite low. Therefore, any effect of interruption of EHC may be more difficult to detect. Secondly, since this study could only be compared to an identical study with neo/chol treatment, a paired t-test was used to test differences, which with only 4 subjects, requires a substantial difference between the different treatment groups. Thirdly, hepatobiliary secretion is dependent upon active transport from within the hepatocyte into bile canaliculi. As with renal tubular secretion, this mechanism may be inhibited during the low-energy state anticipated during the low insulin/euglycemic studies. Finally, insulin is known to be a potent choleretic agent (Jones *et al.*, 1984). Low levels of insulin might cause a reduction in bile flow, which could reduce lorazepam's cycling fraction.

Diabetes mellitus has been shown to affect drug disposition in both animals and in man. Many studies have demonstrated that hepatic glucuronidation is reduced in experimentally-induced diabetic animals, although the effects are substrate specific, sex-related, and are dependent upon the species being examined. In chemically-induced diabetic animals, insulin is able to reverse most, but not all, of the observed changes, suggesting that insulin has important effects on hepatic glucuronidation (section 1.4.3). We did not examine the effects of acute insulin deficiencies; however, insulin *per se* had no effect on lorazepam elimination. Moreover, results from this study suggest that IDDM does not affect the disposition of lorazepam in humans. The values determined for total clearance of intravenous lorazepam in the four IDDM patients in this study were similar to those reported for normal subjects (Greenblatt,

1981), suggesting that diabetes has no effect on this measure in otherwise healthy IDDM patients. Although several investigators have reported changes in the oxidative metabolism of drugs in patients with IDDM (Daintith *et al.*, 1976; Adithan *et al.*, 1989), reports on the effects of diabetes on the elimination of drugs that are extensively metabolized by glucuronidation are scarce. Dajani *et al.* (1974) reported a reduction in the elimination of acetaminophen in IDDM patients. These results were also noted by others (Adithan *et al.*, 1988). However, the fraction of acetaminophen that is eliminated by glucuronidation in man is approximately 60%, which is considerably less than the 90% fraction commonly reported for lorazepam (Greenblatt *et al.*, 1979a; Miners and Mackenzie, 1991). Thus, it is possible that the decrease in the metabolism of acetaminophen in patients with IDDM may occur through effects in non-glucuronide pathways of elimination. The differential effects of IDDM on the disposition of these two drugs may also be related to the large difference in their therapeutic doses.

Lorazepam free clearance rates in the IDDM patients in this study were in agreement with values reported in a similar study in fed and fasted with a neo/chol interrupted EHC (Herman *et al.*, 1994). In that study, continuous feeding of a high-carbohydrate, low-fat diet to neo/chol-treated normal subjects resulted in a decrease (not significant) in lorazepam free clearance. This is in contrast with the present study, in which free clearance values appeared to increase with high levels of glucose, although the change was not statistically significant (Table 3.18). Feeding caused a significant ($p<0.05$) increase in lorazepam free clearance in Gilbert's patients (Herman *et al.*, 1994). Clearance rates of unbound lorazepam in the present study were approximately double those reported for cycling-interrupted values of intravenous lorazepam in a steady-state kinetic study of lorazepam kinetics in normal subjects (Chaudhary *et al.*, 1993). The reason for the discrepancy between the two studies is not known, but may be due to the differences in steady-state vs. acute lorazepam administration.

The volume of distribution of lorazepam in the diabetic patient in this study were in agreement to those reported by Greenblatt *et al.* (1977) in 5 healthy male subjects. While changes in drug volume of distribution in diabetes have been reported (Adithan *et al.*, 1988,1989; Zysset & Weitholtz, 1988), factors controlling drug distribution, such as binding to plasma proteins and to tissues, often affect drugs differently. Therefore, diabetic induced changes in the distribution of one drug may have little or no effect on another drug's distribution. The fraction of unbound lorazepam within the diabetic subjects was very similar to values found in healthy males by Kraus *et al.* (1978), but was approximately 25% lower than previous determinations made in our laboratory in normal subjects (Herman *et al.*, 1989; Chaudhary *et al.*, 1994; Herman *et al.*, 1994). As with volume of distribution, the characteristics of drug binding to plasma proteins is highly dependent upon the drug involved, depending upon the type of plasma protein involved, as well the particular binding sites. Although the plasma protein binding of a related benzodiazepine, diazepam, has been shown to be altered in diabetic patients (Ruiz-Cabello and Erill, 1984, McNamara *et al.*, 1988), the observed change was an increase in binding, as opposed to the decrease noted in the present study with lorazepam.

The elimination half-lives of lorazepam were approximately 25-40 % lower than the half-lives reported by Greenblatt *et al.* (1977, 1982) in normal subjects; however, this is fully expected since the studies with the diabetic patients were done with an interrupted EHC. Indeed, the differences are in keeping with the predicted effects of EHC on the pharmacokinetics of lorazepam (Herman *et al.*, 1989).

3.5. SUMMARY

The disposition of lorazepam in patients with IDDM was investigated in three separate studies. Although the lack of reproducibility found within the second study (section 3.3) preclude its contribution to overall conclusions, results from the initial

study with the 10 IDDM patients (section 3.2), and from the final study in which lorazepam kinetics were examined repeatedly in 4 diabetic patients (section 3.4), suggest that the disposition of lorazepam is not affected by this disease state. No differences were found in the pharmacokinetics of lorazepam between these patients and non-diabetic controls. However, since these data were obtained from studies with healthy IDDM patients with relatively good diabetic control, these results may not be representative of lorazepam disposition in untreated, or poorly-treated insulin-dependent diabetes, or in patients who are suffering from complications of the disease.

Although all three studies examined the disposition of lorazepam in patients with IDDM, several differences in results were noted among them. A clear difference was observed in the initial study in the free clearance of lorazepam, and in the response to the interruption of EHC with neo/chol, which appeared to be dependent upon the insulin species with which the patient was treated. In particular, patients treated with beef/pork insulin had significantly greater free clearances of lorazepam during neo/chol treatment, as well as a much greater response to neo/chol treatment, when compared to patients treated with human insulin. However, the observed effect of insulin species on lorazepam disposition in the initial study could not be reproduced or substantiated in either the crossover study, or in the final study.

Why beef/pork and human insulins appeared to have differential effects on lorazepam disposition in the first study, but not in the other two is not immediately clear. An intuitive assumption might be that the patient numbers in the final two studies were too small, and the tests were not statistically powerful enough to detect a difference. Indeed, according to power curves (Pearson and Hartley, 1951), the power to detect a difference in insulin effect in the final ten-part study was very low (<0.2). However, with the amount of observed variance, the power of the test would have been less than 0.4 even with 60 degrees of freedom in the denominator of the F-statistic (corresponds to number of patients). This does not imply that there was a high degree

of random variability in the ten-part study. Rather, each patient showed highly reproducible results. If the true degree of difference in lorazepam clearance according to insulin species was that which was observed in the initial study, the power of the 10-part study to detect such a change would have been 0.98. The fact that differences in lorazepam clearance and renal lorazepam glucuronide clearance were detected with the experimental design, despite of only having 4 subjects, suggests that the small number of patients is not responsible for not being able to detect a difference due to insulin species.

A another possibile explanation for the lack of observed effect of insulin treatment is that the time frame in the two studies which did not show effects was too restrictive. Patients may have to be treated with one insulin for an extended period of time before the true effects of the insulin on hepatic metabolism become apparent. Although a possible explanation, this is unlikely, since during the 10-part study, completion of the ten separate kinetic studies took approximately one year to complete.

A second possibility is that no difference exists, in which the question then becomes, why was a difference found between human and beef/pork treated IDDM patients in the initial disposition study. One reason may be that the initial study was not a crossover design. Therefore, there may have been other differences between the two groups that could account for the differences in lorazepam disposition. Furthermore, as clearly demonstrated in the final study, glucose clearly has an effect on the clearance of lorazepam, which suggests that feeding may be an important regulatory factor in lorazepam disposition. During the study where a difference between the insulins was noted, there was no attempt to control the caloric intake of the study subjects, which would be expected to introduce added variability to the results. Indeed, a large amount of variability existed in the morning and afternoon blood sugar measurements, and patients treated with human insulin demonstrated a greater decrease in blood sugar with neo/chol treatment than did subjects who were treated with beef/pork insulin (Table

3.3). While the clamping study showed no effect of blood sugars on lorazepam elimination, it did not address the possibility of the effects of low blood glucose levels. It is possible that depletion of glucose, or the neurochemical events that accompany hypoglycemia, may affect hepatic glucuronidation. Finally, further evidence which points away from the likelihood of the proposed difference between human and beef/pork insulin is that no effect of insulin species was detected in the clamping study, in which the separate effects of glucose, insulin, glucagon, and EHC were tightly regulated. Indeed, under these rigidly-controlled conditions, results were clearly reproducible and experimental variability was greatly reduced, such that the effects of exogenous glucose on lorazepam disposition were readily apparent.

It should be noted that the effects of glucose on lorazepam kinetics, as well as the effects of insulin on urine production were observed in patients with IDDM under nonphysiologic administration of high concentrations of insulin and glucose, and extrapolation to normal subjects or diabetic patients should be done with caution. Nevertheless, these findings do possess pharmacological importance. The effect of glucose on the elimination of lorazepam demonstrates that under certain physiological condition, co-substrate availability may be a rate-limiting step of hepatic glucuronidation *in vivo*. Similar effects observed with the renal elimination of lorazepam glucuronide indicate that glucose may be an important factor in the renal elimination of drugs and their metabolites. Whether this is due to a direct glucose effect, or through an indirect effect on the energy-state on the kidney is not known. This study also provides evidence for the argument that there is no difference between the effects of human and beef/pork insulin in man, at least with respect to their putative effects on hepatic glucuronidation and on renal function. The results of this study also indicate that insulin may possess an important physiological role in renal function. This may be important in understanding the precipitating events which initiate hypertension in diabetic patients, as well as in the general population.

4. ENTEROHEPATIC CIRCULATION OF LORAZEPAM IN DOGS

4.1 Introduction

Lorazepam is metabolized almost exclusively by the liver to its 3-OH glucuronide and excreted in urine. As such, the drug is commonly employed as an *in vivo* probe of hepatic conjugative elimination. However, recently, it has been shown that lorazepam undergoes significant enterohepatic circulation (EHC) in man (Herman *et al.*, 1989). Enterocycling complicates the pharmacokinetics of lorazepam, and must be quantitated or excluded in order to use the drug as a valid estimator of glucuronidation. EHC can be assessed indirectly in man by blocking the cycling fraction with concomitant treatment with neomycin and cholestyramine. However, the effectiveness of this technique is not known. Although more direct studies can be done with biliary T-tubes, these experiments suffer other methodological shortcomings in that the underlying condition necessitating T-tube drainage may affect drug elimination, and diversion of bile itself, may alter the physiological state of biliary secretion. Thus development of a viable animal model would permit a more direct measure of the effects of EHC on the disposition of lorazepam. Like man, lorazepam is extensively metabolized to the glucuronide metabolite in the dog (Ruelius, 1978). In addition, dogs are considered to be good biliary excretors (Fleck and Bräunlich, 1991), and bile flow in dogs and humans is very similar (Klaassen and Watkins, 1984). Oxazepam, a benzodiazepine closely related to lorazepam, has been shown to enterocycle in beagles (Alván *et al.*, 1977).

4.2. MATERIALS AND METHODS

4.2.1. Experimental methods

Four mixed-breed male dogs, weighing between 19.7-26.0 kg (mean = 22.9 kg) were used for the study. All dogs were purchased through the Western College of Veterinary Medicine (WCVM), University of Saskatchewan, and were deemed healthy before inclusion within the study.

4.2.1.1. Surgical model

All surgical preparations for the study were performed by Dr. J. Fowler D.V.M. and Dr. M. Papich, D.V.M., in the departments of Surgery and Physiology, WCVM, University of Saskatchewan.

Following an overnight fast, each dog was brought into a designated operating room. The dog was given an intravenous preanesthetic, and was then intubated, clipped and prepared for sterile surgery. Surgery was performed under general anesthesia using a halothane/O_2 mixture.

During the surgical procedure, two catheters were placed for blood sampling. The first was placed in the jugular vein. After securing the catheter to the animal with sutures, the catheter was looped and brought to the back of the dog's neck, where the wound was dressed wrapped in gauze. A second catheter was placed in the portal vein. After ensuring blood could with withdrawn through the catheter, it was sutured in place. Using a small incision, a choledochostomy tube was placed into the dog's gallbladder to collect and sample bile. The incision was then closed and the tube tested to ensure it was held firmly in place. An additional catheter was placed entering the gut distal to the pylorus in which "oral" drug and collected bile could administered. Finally, an occludable cuff was placed around the common bile duct to provide a means to interrupt biliary flow. The cuff was filled with water, and tested to see that it was

able to effectively constrict the common bile duct. The catheters controlling the occludable cuff, along with the free ends of the hepatic vein catheter, the catheter placed in the gut, and the choledochostomy tube were externalized through the dog's skin and brought up towards the dog's back. Following surgical closure of the animal, the entire abdomen was wrapped in gauze, in which the catheters were secured so that approximately 1-2 inches of each catheter was exposed through the gauze "jacket" at the top of the dog's back.

Following surgery, animals were allowed to heal for at least two weeks before any experiments were conducted. During that time, and between experiments, catheters were kept patent by daily flushing with heparinized saline solution. Occasionally, catheters for blood sampling would become blocked such that fluid could be infused, but not withdrawn. The blockage could often be removed through repeated infusions of saline, however, if this failed, the catheter was replaced.

4.2.1.2. Pharmacokinetic studies

Following an overnight fast, dogs were taken to an experimental study room within the WCVM and fitted with a urinary catheter, after which blank blood, bile and urine samples were obtained. At the designated starting time, each dog was given between 4-10 mg of lorazepam solution (Wyeth Laboratories) infused into the tube leading directly into the gut. At the same time, a bolus intravenous infusion of 50 μCi of ^{14}C lorazepam (Amersham Corp., Arlington Heights, IL) was delivered through the jugular catheter, which was subsequently washed thoroughly with saline. During an initial pilot study, jugular and portal blood samples were collected at 3, 6, 10, 15, 30, and 45 minutes, and 1, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 8.5, 9, 10, 12, 16, 17, 18, 20, 24, 32, 40, 48, 56, 64, 72, 80, 88, 96, 104, 112, 120, 128, 136, 136, and 144 hours. Later studies used similar collection times; however, due to the short half-life of lorazepam in dogs, sample collection was terminated after 24, and in subsequent

studies, after 8 hours. Bile samples were collected hourly (except in the pilot study), and urine was collected throughout the entire study interval.

A number of different experiments were used to study the putative enterocycling of lorazepam in each of the dogs. All studies employed the simultaneous administration of oral non-labeled lorazepam with intravenous radiolabelled drug. In the initial pilot study, the kinetics of lorazepam was studied in a single dog ("Billy Rubin") with an intact enterohepatic circulation throughout the entire experiment. Studies in a second dog ("Gill") employed four different protocols: an initial study in which bile was re-infused via the gut catheter (cycling-intact); a second study and third study in which neomycin (500 mg every 6 hours) and low (1 g every 2 hours) or high (4 g every 2 hours) doses of cholestyramine were used to block lorazepam's enterohepatic cycling in the manner employed in man; and a fourth study in which bile was collected, and not re-infused (surgical interruption of EHC). Finally, two studies were done using a third dog ("Ike"), one in which bile was continuously removed (cycling-interrupted), and a second in which bile was removed and re-infused (cycling-intact). A similar protocol was used in a fourth dog ("Blackie"); however, during the cycling-interrupted study, "clean", previously collected bile was re-infused in order to maintain hepatic biliary flow.

4.2.2 Analytical methods

HPLC analysis of non-radiolabelled lorazepam was identical to the methods described in section 3.2.2.3.1. Quantitation of radiolabelled drug was determined by collecting HPLC solvent fractions eluted during measurement of the nonlabelled drug. Ten mL of scintillation cocktail (Ready Micro, Beckman Instruments Inc., Mississauga Ont.) was added to each sample, and the radioactivity measured with a LS5801 Beckman scintillation counter using automated quench compensation (Beckman

Instruments Inc.). Measurements were corrected against the extraction efficiency of individual samples. An LKB 2212 Helirac fraction collector (LKB-Produkter, Bromma, Sweden) was used to collect radioactive samples.

4.2.3. Pharmacokinetic methods

Pharmacokinetic analysis of intravenously administered lorazepam was identical to that described in section 3.2.2.4. The apparent volume of distribution (V_{area}) was calculated by the following equation:

$$V_{area} = \text{Dose} / (\text{AUC} \bullet \lambda) \quad (4.1)$$

where λ is the terminal disposition rate constant. Biliary clearance (CL_B) of lorazepam and lorazepam glucuronide was calculated by measuring the amount excreted in the bile and the AUC of the concentration-time profile of the respective isolates, and applying the following equation:

$$CL_B = \text{Amount excreted in bile} / AUC_{plasma} \quad (4.2)$$

4.2.4. Statistical methods

Statistical analysis was identical to that described in section 3.2.2.5.

4.3. RESULTS

The pharmacokinetics of lorazepam and lorazepam glucuronide were studied in the first dog (Billy Rubin). Complications stemming from the surgery necessitated that the animal be euthanized following the initial kinetic study. This was a cycling-intact

situation, in which collected bile was re-infused through the catheter leading directly into the dog's gut. Although no measure of the extent of the EHC of lorazepam was obtained from this study, it provided useful pharmacokinetic parameters of lorazepam in dogs. Lorazepam was rapidly cleared from the systemic circulation of the dog; the elimination half-life of intravenous lorazepam (radiolabelled with ^{14}C) was 37 minutes. The corresponding systemic clearance of total lorazepam was 30.5 mL/min/kg, and the volume of distribution at steady-state was 1.53 L/kg (V_{area} = 1.19 L/kg). The apparent clearance of orally administered (non-radiolabelled) lorazepam was considerably greater (73.6 mL/min/kg) than systemic clearance, although the half-life of 38.5 minutes was similar. The hepatic extraction ratio of lorazepam was 0.58, based on a calculated oral availability of 0.42. Recovery of oral lorazepam as the glucuronide was estimated at 105.8% after 64 hours. Approximately 94% of the total radioactivity administered was recovered in the urine within the same time, with 58% as lorazepam glucuronide. The ratio of AUC of lorazepam glucuronide to the AUC of lorazepam in plasma was 34 for the intravenously administered drug and 153 for the orally administered drug, giving a ratio of oral to intravenous (R or/iv) of 4.4.

In the second dog (Gill), the pharmacokinetics of lorazepam were examined in four separate studies, all of which employed simultaneous iv and oral administration. In one of the studies, the enterohepatic circulation was intact, as bile was removed and re-infused hourly. Two of the studies used orally administered neomycin (500mg every 6 hours) and low (1 g every 2 hours) or high dose (4 g every 2 hours) cholestyramine to block enterocycling. In the fourth study, bile was removed hourly from the dog's gallbladder, and was not re-infused. The plasma concentration-time profiles (up to 12 hours) of lorazepam and lorazepam glucuronide following intravenous and oral lorazepam in the dog are shown in Figures 4.1 and 4.2. The pharmacokinetic parameters of lorazepam in the four studies are given in Table 4.1.

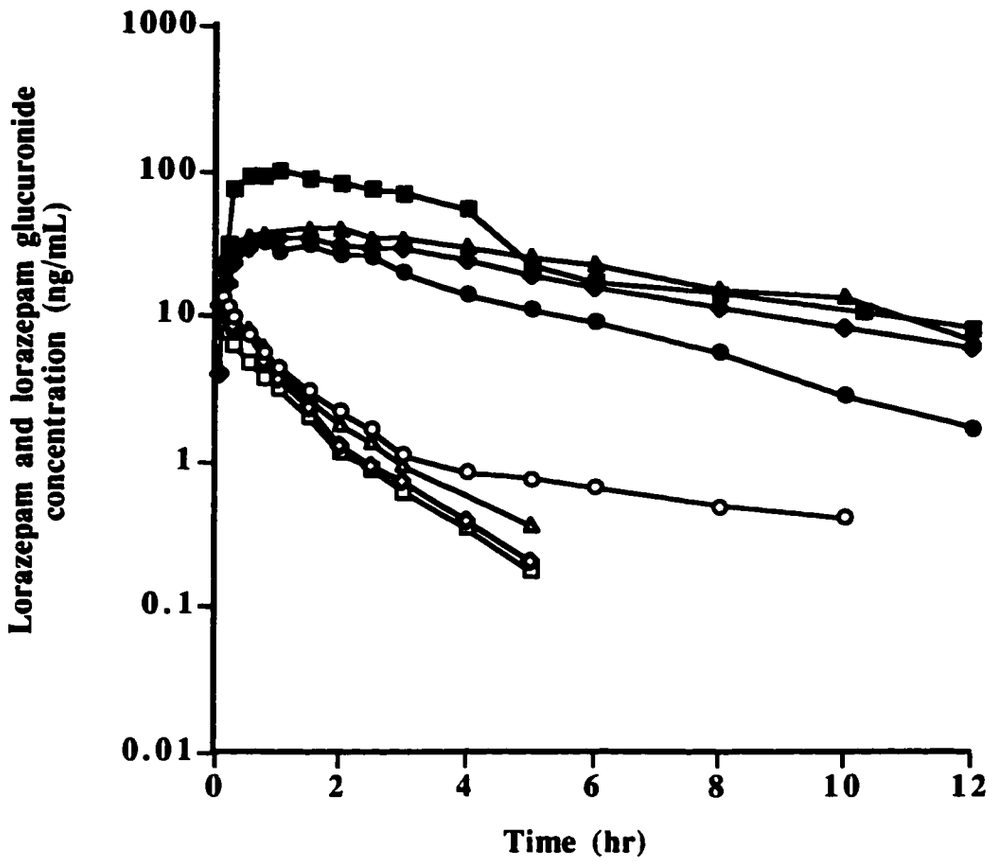


Figure 4.1. Concentration-time profiles of lorazepam and lorazepam glucuronide in a single dog following intravenous administration.
Open symbols denote lorazepam; solid symbols denote lorazepam glucuronide.
Circles indicate study where bile was collected and re-infused.
Diamonds indicate study using neomycin and low dose cholestyramine.
Triangles indicate study using neomycin and high dose cholestyramine
Squares indicate study where bile was collected and not re-infused.

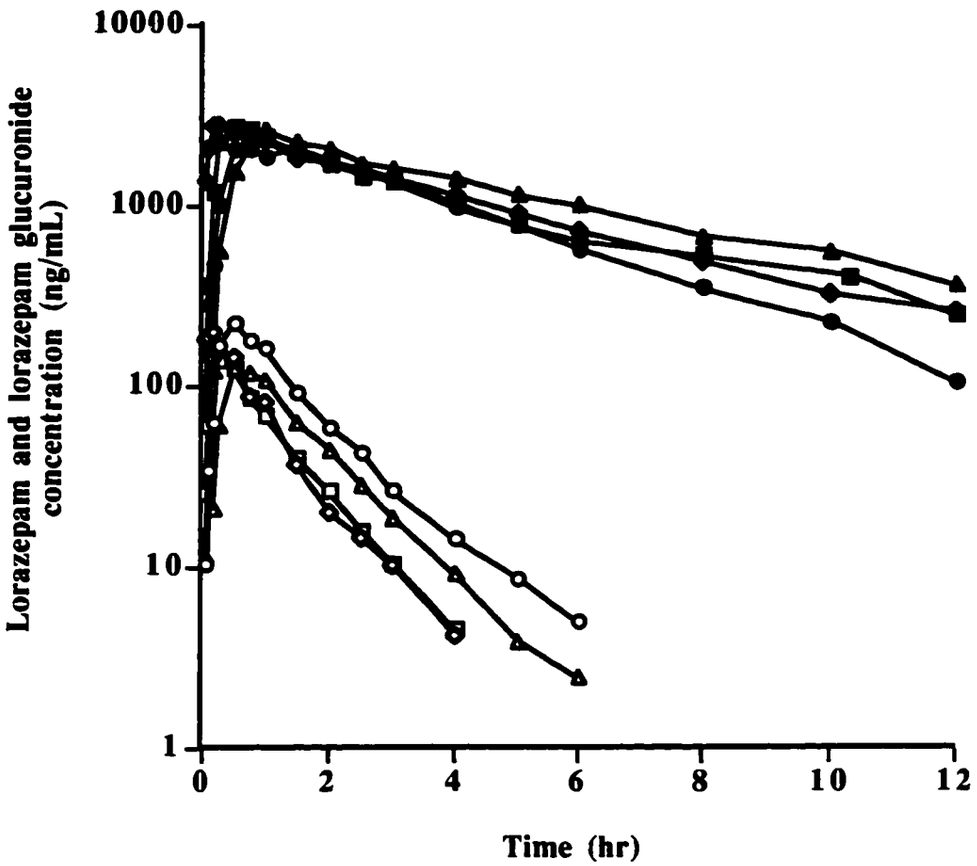


Figure 4.2 Concentration-time profiles of lorazepam and lorazepam glucuronide in a single dog following oral administration.
Open symbols denote lorazepam; solid symbols denote lorazepam glucuronide.
Circles indicate study where bile was collected and re-infused.
Diamonds indicate study using neomycin and low dose cholestyramine.
Triangles indicate study using neomycin and high dose cholestyramine
Squares indicate study where bile was collected and not re-infused.

Table 4.1. Pharmacokinetic parameters of intravenous and oral lorazepam in a single dog during 4 separate studies with intact and interrupted enterocycling.

Parameter	Study conditions			
	bile re-infused	low dose neo/chol	high dose neo/chol	bile removed
$Dose_{iv}$	310	314	319	373
$Dose_{oral}$	9128	9577	9568	9298
CL_{iv}	13.32	25.73	21.23	40.12
CL_{oral}	22.10	42.33	41.39	51.76
$T1/2_{iv}$	5.11	1.15	2.08	1.11
$T1/2_{oral}$	1.12	0.74	0.87	0.79
Vd_{ss}	72.0	39.0	23.2	55.7
Vd_{area}	117.9	48.9	69.4	66.5
F	0.60	0.61	0.51	0.78
$AUCG_{iv}$	162	255	331	423
$AUCG_{oral}$	9688	12225	14535	12825
$AUCG/AUC_{iv}$	8.0	23.2	23.6	54.2
$AUCG/AUC_{oral}$	28.6	65.9	73.0	76.6
Ratio oral/iv	3.6	2.9	3.1	1.4

$Dose_{iv}$	dose of intravenous lorazepam (μg).
$Dose_{oral}$	dose of oral lorazepam (μg).
CL_{iv}	total clearance of intravenous lorazepam (mL/min/kg).
CL_{oral}	apparent total clearance of oral lorazepam (mL/min/kg).
$T1/2_{iv}$	elimination half-life of intravenous lorazepam (hr).
$T1/2_{oral}$	elimination half-life of oral lorazepam (hr).
Vd_{ss}	volume of distribution at steady-state of lorazepam (L).
Vd_{area}	volume of distribution of lorazepam (L).
F	bioavailability of lorazepam.
$AUCG_{iv}$	area under the plasma concentration-time curve of lorazepam glucuronide following intravenous administration, normalized to 325 μg (ng/mL-hr).
$AUCG_{oral}$	area under the plasma concentration-time curve of lorazepam glucuronide following oral administration, normalized to 9 mg (ng/mL-hr).
$AUCG/AUC_{iv}$	ratio of area under the plasma concentration-time curve of lorazepam glucuronide to the area under the plasma concentration-time curve of lorazepam, for intravenously administered drug.
$AUCG/AUC_{oral}$	ratio of area under the plasma concentration-time curve of lorazepam glucuronide to the area under the plasma concentration-time curve of lorazepam, for orally administered drug.
R oral/iv	ratio of $AUCG/AUC_{oral}$ to $AUCG/AUC_{iv}$

The terminal disposition half-life of intravenous lorazepam from the dog when EHC was intact (bile re-infused) was 5.11 hours, the result of a slow terminal elimination phase (radiolabelled lorazepam was measured until disintegrations per minute (DPMs) reached twice background levels). In contrast, elimination rates were faster, and elimination half-lives (1-2 hours) considerably less in studies where enterohepatic circulation was interrupted either by neo/chol treatment or by removal of bile. The greater rate of elimination was due in part to changes in the systemic clearance of lorazepam. Interruption of enterohepatic circulation caused a 1.5 to 3-fold increase in lorazepam clearance compared to the cycling-intact study. Interruption of enterohepatic circulation also caused a 23-68% decrease in the volume of distribution at steady-state, and a 41-59% decrease in the volume of distribution as determined by the terminal disposition rate constant (Vd_{area}). Area under the plasma concentration-time curve of lorazepam glucuronide (AUCG) was 57-161% greater during studies where enterohepatic circulation was interrupted compared to the cycling-intact study, even after being corrected for differences in dose. During the first four hours of the experiment when bile was removed and not re-infused, lorazepam glucuronide levels were much higher than any other studies.

Similar patterns were observed with orally administered drug. Plasma levels rose quickly, with peak levels achieved within 10-30 minutes. The half-life of lorazepam was 22-34% less during experiments where enterocycling was interrupted. The half-life of oral drug was in all cases less than the half-life of lorazepam measured during intravenous studies, with the greatest difference observed during the cycling-intact protocol. Like the intravenously administered lorazepam, clearance of oral lorazepam was 2-2.5 times greater during cycling-interrupted studies. Oral clearance values were 22-48% greater than comparable intravenous studies. The bioavailability of lorazepam ranged from 0.51 (high dose neo/chol) to 0.78 (bile removed). Concentrations of lorazepam glucuronide peaked very rapidly (within 15-60 minutes)

during oral lorazepam administration. The AUCG of lorazepam glucuronide was 26-50% greater during cycling-interrupted studies than the cycling-interrupted study, after correcting for dose.

Ratios of the area under the plasma concentration-time curve of lorazepam glucuronide to the area under the plasma concentration-time curve of lorazepam (AUCG/AUC) were lowest during the cycle-intact study and highest during the bile-removed experiment following both intravenous and oral administration. These ratios were greater during orally administered drug. The ratio of oral to intravenous AUCG/AUC ranged from 1.4 (bile-removed) to 3.6 (bile re-infused).

Less than 1% of lorazepam was excreted in the urine as unchanged drug. Recovery of lorazepam as lorazepam glucuronide during the intravenous studies (Table 4.2) ranged from 56 (bile re-infused) to 90 (bile removed) percent of the total amount of drug given (During the high dose neo/chol treatment, the dog urinated ~60 mL on the floor. Therefore, urinary data from this study is considered incomplete). These values were greater for the orally administered lorazepam, where recovery ranged from 99-141%, with the exaggerated recovery found during the bile-removed experiment.

The renal clearance of lorazepam glucuronide varied from 0.67-0.95 mL/min/kg for intravenous lorazepam, and from 0.71-0.95 for oral lorazepam, and appeared to be the same irrespective of experimental conditions and the route of administration. The percent of total radioactivity recovered from the urine ranged from 77% for the cycle-intact study to 114% for the bile-removed experiment. The amount of radioactivity accounted for by lorazepam glucuronide was near 75% for all four studies. The percentage of orally administered drug recovered as lorazepam glucuronide in the urine over 12 hours ranged from 98.5-141.1%. Recoveries greater than 100% of total radioactivity and lorazepam glucuronide from nonlabelled drug indicate a possible methodological problem in the bile-removed cycling-interrupted study.

Table 4.2. Lorazepam glucuronide following intravenous and oral administration of lorazepam in a single dog during 4 separate kinetic studies.

Study conditions	$LzGl_{urine}$ iv	$LzGl_{urine}$ oral	CL_R iv	CL_R oral	% of RA recovered	% of RA as $LzGl_{urine}$
bile re-infused[1]	56.7	98.5	0.95	0.76	76.8	73.8
low dose neo/chol	62.6	111.2	0.70	0.71	81.3	77.0
high dose neo/chol[2]	36.6	68.9	0.33	0.39	45.9	79.7
bile removed	90.2	141.1	0.67	0.95	114.1	79.1

[1] at 10 hours urinary catheter pulled out temporarily
[2] at 6 hours, dog urinated ~60mL on floor which could not be recovered

$LzGl_{urine}$	% of dose recovered as lorazepam glucuronide in urine over 12 hours.
CL_R	renal clearance of lorazepam glucuronide (mL/min/kg).
% of RA recovered	% of radioactivity recovered in urine over 24 hours.
% of RA as $LzGl_{urine}$	% of radioactivity recovered in urine over 24 hours as lorazepam glucuronide.

The pharmacokinetics of intravenous and oral lorazepam were studied in a third dog (Ike) during two separate experiments: a cycling-intact study in which bile was collected and re-infused; and a cycling-interrupted study in which bile was collected but not re-infused. Clearance of intravenous lorazepam was 49% greater when EHC was interrupted (bile not reinfused) (7.08 vs. 10.56 mL/min/kg). However, the volume of distribution at steady state increased by 38% (41.7 vs. 68.4 L), such that no change was observed in elimination half-life. Indeed, the elimination half-lives of intravenous lorazepam were identical between cycle-intact and the cycle-interrupted states for both the intravenously (3.08 vs. 3.01 hr.) and orally (1.43 vs. 1.41 hr.) administered drug, although intravenous values were twice those of the oral route. Oral availability of lorazepam measured 0.71. The area under the curve of lorazepam glucuronide for

intravenous and oral lorazepam was greater during the study where bile was re-infused, by 5% and 76%, respectively. Bile flow during the cycle-intact study averaged 21 mL/hour, compared to a bile flow of only 10.4 mL/hour during the cycle-interrupted study.

A similar protocol was used in a fourth dog (Blackie), however, during the cycling-interrupted study, bile was collected hourly, and an equal volume of "clean" bile, which had been collected the week prior to any of the studies was re-infused in order to maintain hepatic biliary flow. In addition, the oral dose of lorazepam was decreased by one-half to approximately 4 mg and the intravenous dose was reduced by about 25% compared to previous studies.

The elimination of intravenously administered lorazepam showed a smooth and steady decline during the study where clean bile was re-infused into the dog's gut (cycling-interrupted). However, the study in which the enterohepatic circulation was maintained, the drug's elimination profile was much less stable, with a large peak occurring between 2 and 5 hours (Figure 4.3). The two profiles were identical during the final three hours, producing similar terminal disposition rate constants and half-lives (Table 4.3). The clearance of intravenously administered lorazepam was slightly greater, and the elimination half-life slightly less during the cycle-intact study. Surprisingly, the Vd_{ss} of intravenous lorazepam was 24% less during the study where enterohepatic circulation was intact. The AUC of lorazepam glucuronide was 42% greater during the cycling-interrupted study.

The rapid elimination of orally administered lorazepam, coupled with the lower dose, resulted in low plasma concentrations such that the plasma concentration-time curve, and hence the pharmacokinetic parameters for oral drug could not be estimated. Lorazepam glucuronide levels of oral lorazepam were measured, and as with the intravenous lorazepam, the area under the curve of lorazepam glucuronide was 19% greater when enterohepatic circulation was interrupted.

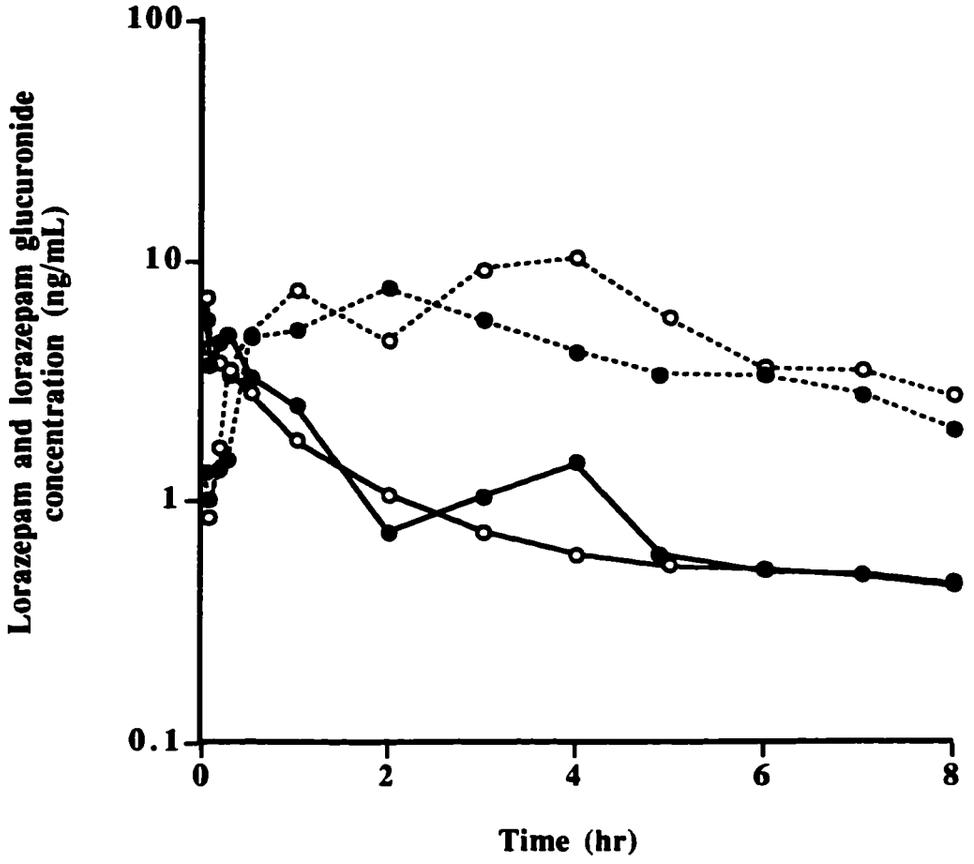


Figure 4.3. Concentration-time profile of lorazepam and lorazepam glucuronide in a dog with an intact and interrupted enterohepatic circulation
Solid circles indicate study with bile re-infused (intact enterohepatic circulation); open circles indicate study where "clean" bile was re-infused (interrupted EHC)
Solid lines indicate lorazepam; dashed lines indicate lorazepam glucuronide

Table 4.3. Pharmacokinetic parameters of intravenous and oral lorazepam in a dog (Blackie) during an intact (bile re-infused) and interrupted ("clean" bile re-infused) enterohepatic circulation.

Study	CL_{iv}	$T_{1/2iv}$	Vd_{ss}	$AUCG_{iv}$	$AUCG_{oral}$	AUCG/ AUC_{iv}
cycle-intact	10.81	8.04	141	34.8	2134	3.6
cycle-interrupted	10.22	9.97	185	49.5	2543	5.8

CL_{iv}	systemic clearance of intravenous lorazepam (mL/min/kg).
$T_{1/2iv}$	elimination half-life of intravenous lorazepam (hr).
Vd_{ss}	volume of distribution at steady-state of lorazepam (L).
$AUCG_{iv}$	area under the plasma concentration-time curve of lorazepam glucuronide following intravenous administration, normalized to 250µg (ng/mL-hr).
$AUCG_{oral}$	area under the plasma concentration-time curve of lorazepam glucuronide following oral administration, normalized to 250µg (ng/mL-hr).
AUCG/AUC	ratio of the areas under the plasma concentration-time curve of lorazepam glucuronide to lorazepam, for intravenous lorazepam.

Lorazepam concentrations in bile were approximately 10-fold greater than the corresponding concentrations in plasma when the bile was re-infused back into the animal, and 100-fold greater than plasma levels when previously collected "clean" bile was re-infused (Figure 4.4). Similarly, concentrations of lorazepam glucuronide in bile were approximately 100 times greater than the levels in plasma following both intravenous and oral drug administration (Figure 4.5). Infusion of previously collected "clean" bile maintained a constant rate of bile production as biliary flow was the same in both the cycling and interrupted study. Moreover, the dramatic increase in plasma concentration of lorazepam glucuronide noted in previous interruption experiments was not noted. Although AUCG values were higher, the increase was modest, especially for oral drug. This was in spite of a reduced renal clearance of lorazepam glucuronide with interruption of EHC, which was also noted in the other studies.

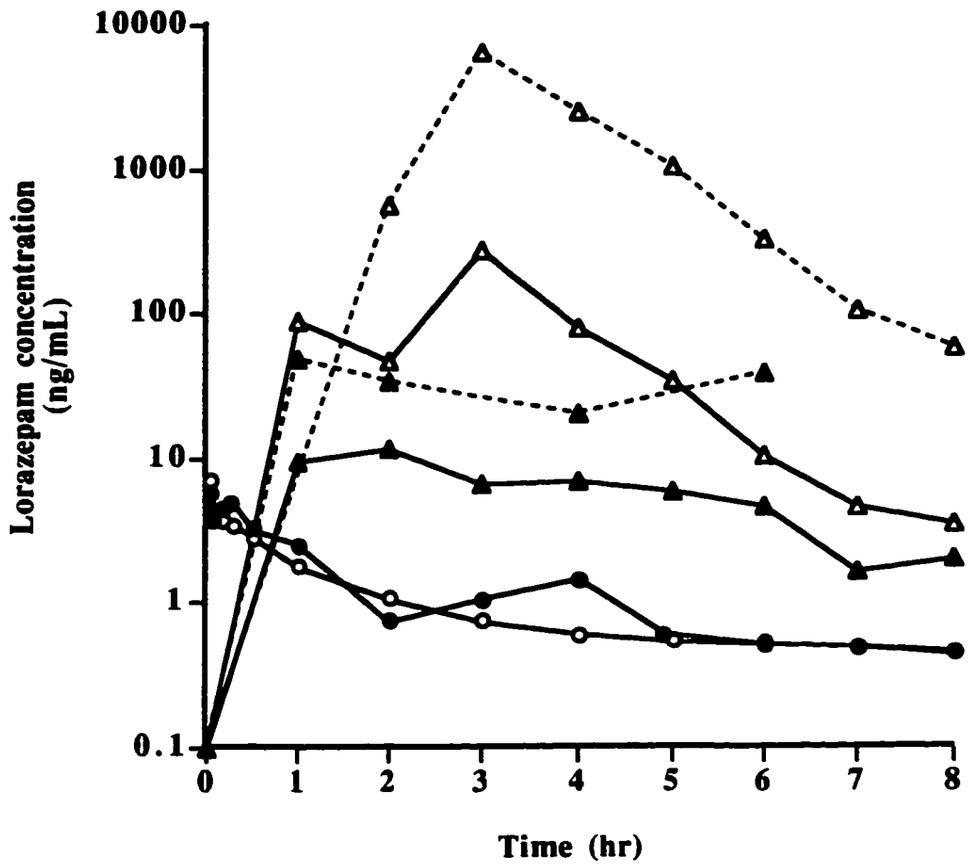


Figure 4.4. Concentration-time profiles of lorazepam in plasma and bile in a dog with an intact and an interrupted enterohepatic circulation.
Circles indicate measurements in plasma; triangles indicate measurements in bile.
Solid symbols indicate cycling-intact study; open symbols indicate cycling-interrupted study.
Solid lines indicate intravenous lorazepam; dashed lines indicate oral lorazepam.

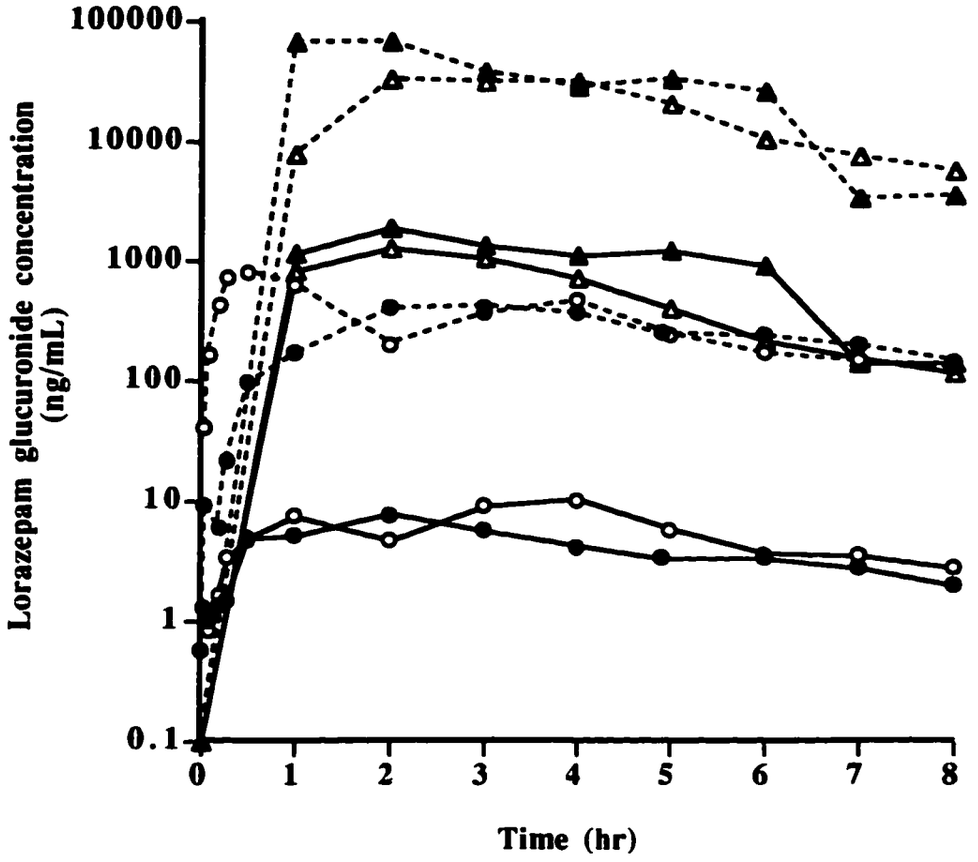


Figure 4.5. Concentration-time profiles of lorazepam glucuronide in plasma and bile in a dog with an intact and an interrupted enterohepatic circulation.
Circles indicate measurements in plasma; triangles indicate measurements in bile.
Solid symbols indicate cycling-intact study; open symbols indicate cycling-interrupted study.
Solid lines indicate intravenous lorazepam; dashed lines indicate oral lorazepam.

Table 4.4. Bile flow and biliary clearance of lorazepam and lorazepam glucuronide following intravenous and oral administration of lorazepam in a dog with an intact and an interrupted enterohepatic circulation.

Study	bile flow	CL_B		
		Lz_{iv}	$LzGl_{iv}$	$LzGl_{oral}$
cycle-intact	9.75	0.02	1.42	0.79
cycle-interrupted	11.90	0.27	0.75	0.42

bile flow — average bile flow during the 8 hour study (mL/hr).
CL_B — biliary clearance (mL/min/kg).
Lz_{iv} — intravenous lorazepam.
$LzGl_{iv}$ — lorazepam glucuronide formed from intravenous lorazepam.
$LzGl_{oral}$ — lorazepam glucuronide formed from oral lorazepam.

The biliary clearance of intravenous lorazepam was 14 times greater during the cycling-interrupted experiment and the biliary clearance of lorazepam glucuronide during the cycling-intact study was double the values found in the interrupted study. In both studies, biliary clearance of lorazepam glucuronide was two-fold greater for intravenous lorazepam compared to orally administered drug. The significance of these findings is unknown.

During the cycling-intact study, 20% of the intravenous dose, and 39% of the oral dose was recovered as lorazepam glucuronide within the 8 hour study period (Table 4.5). These values were reduced in half during the cycling-interrupted study. Renal clearance of lorazepam glucuronide formed from intravenous lorazepam was nearly 3-fold greater in the study in which enterohepatic circulation was maintained, and a similar pattern was observed for oral lorazepam. Renal clearance of lorazepam glucuronide was greater for metabolite formed from the intravenous dose during both studies.

Table 4.5. Urinary recovery and renal clearance of lorazepam glucuronide following intravenous and oral lorazepam in a dog with an intact and an interrupted enterohepatic circulation.

Study	$LzGl_{urine\text{-}iv}$	$LzGl_{urine\text{-}oral}$	$CL_{R\text{-}iv}$	$CL_{R\text{-}oral}$
cycle-intact	20.3	38.5	0.96	0.53
cycle-interrupted	11.3	22.2	0.36	0.24

$LzGl_{urine\text{-}iv}$ percent of intravenous lorazepam recovered in urine as lorazepam glucuronide over the 8 hour study interval.
$LzGl_{urine\text{-}oral}$ percent of oral lorazepam recovered in urine as lorazepam glucuronide over the 8 hour study interval.
$CL_{R\text{-}iv}$ renal clearance of lorazepam glucuronide from intravenous lorazepam (mL/min/kg).
$CL_{R\text{-}oral}$ renal clearance of lorazepam glucuronide from oral lorazepam (mL/min/kg).

4.4. DISCUSSION

The purpose of this study was to establish the dog as a suitable model for studying the enterohepatic circulation (EHC) of drugs, and to determine if lorazepam enterocycled in this animal. The dog was chosen as a candidate species to study EHC not only for practical reasons (cost, availability and ease of care), but for scientific reasons as well. Biliary excretion appears to be species dependent. The mouse, rat, hen and dog are good biliary excretors, whereas the rabbit, guinea pig, and rhesus monkey are relatively poor excretors. The cat and sheep fall somewhere in between (Fleck and Bräunlich, 1991). Although rats are good biliary excretors, they do not have a gallbladder, and bile flow in rodents tends to be 10-20 fold greater than in mammals (Siegers, 1991). Bile flow in dogs and humans is very similar (Klaassen and Watkins, 1984). In addition, the choice of a dog was particularly suited for the present

investigation. Lorazepam is extensively metabolized to lorazepam glucuronide in the dog, while lorazepam glucuronide is a minor metabolite in the rat (Ruelius, 1978).

The pharmacokinetic parameters of lorazepam in all four dogs are in good agreement with other work found in the literature. In a study examining the hepatic and extrahepatic metabolism of lorazepam in mongrel dogs, Gerkens *et al.* (1981) reported dispositional parameters for lorazepam that were similar to dogs with intact EHC in this study. Clearance, volume of distribution, and half-life values were comparable between the two studies. Lorazepam disappeared quickly from the systemic circulation, whereas lorazepam glucuronide levels rose quickly, but disappeared at a slower rate than the parent drug. Recovery of lorazepam glucuronide in urine was also similar.

EHC could only be studied in three of the dogs. However, evidence supporting the presence of a significant EHC of lorazepam was found in each animal. In the second dog (Gill) in which four separate lorazepam kinetic studies were performed, plasma concentrations of i.v. and oral lorazepam were distinctly lower when EHC was blocked, whether the interruption was by neomycin and cholestyramine treatment, or by complete removal of bile from the gallbladder (Figures 4.1, 4.2). In the plasma concentration-time profiles of another dog (Blackie) (Figure 4.3), the elimination phase was marked by several secondary peaks. By comparison, the decrease in plasma concentrations followed a steady decline with no secondary peaks when EHC was blocked. A third effect on the concentration-time profiles was the effect on metabolite levels. Interruption of EHC produced higher levels of lorazepam glucuronide when compared to studies in which EHC was intact in all studies, although the effect on orally administered drug in the fourth dog, when "clean" bile was re-infused, was modest.

Another effect of interruption of EHC is an increase in a drug's clearance rate. Cycling reduces the effective clearance of a drug, by returning drug back to the

systemic circulation after it has been excreted in the bile (Herman *et al.*, 1989). Increases in the clearance of both intravenous and oral lorazepam were observed when EHC was interrupted in two of the three dogs. In the dog in which four separate lorazepam kinetic studies were performed (Gill), treatment with neomycin and cholestyramine caused an approximate doubling of the clearance for both i.v. and oral lorazepam, while removal of bile resulted in a 2.5-fold increase in oral clearance, and a 3-fold increase in i.v. clearance. Changes in clearance were also observed with the interruption of EHC in the other two dogs; however, the effects were not as conclusive. In the third dog (Ike), clearance of i.v. lorazepam increased by 49% when EHC was interrupted, while that of oral lorazepam decreased by 6%. In the fourth dog (Blackie), clearance of i.v. lorazepam actually decreased 5.5% when cycling was blocked. No measurements could be made for orally administered drug as the concentrations were below the limit of detection for the assay.

Volume of distribution also is susceptible to change with interruption of EHC. This is because diversion of drug through EHC, into a non-eliminating or poorly-eliminating enteric loop changes an excretory process into a distributing process, adding to the effective volume of distribution of the drug. Again, the most consistent changes were observed in the second dog with the 4 separate studies. Blocking the EHC of lorazepam caused a substantial decrease in the volume of distribution of lorazepam, regardless of whether the volume was determined as Vd_{ss} or as Vd_{area}. With the other two dogs, the volume of distribution increased by 74% and 25% when the bile was removed and not re-infused.

Considering the disparate effects of blocking EHC on clearance and volume of distribution between the three dogs, it is not surprising that the effect on the elimination half-life showed no consistency. In Gill, elimination half-life was reduced for both oral and i.v. lorazepam whenever EHC was interrupted. Conversely, with the other two dogs, no change in half-life was observed. Bioavailability of lorazepam was similar

between all three the studies, indicating that interruption of EHC had no effect on the absorption of the oral drug.

Consistent changes were noted among other measured variables with the interruption of EHC. As mentioned earlier, the area under the curve of the glucuronide metabolite of lorazepam (AUCG) was increased when enterocycling was interrupted, as were ratios of AUCG/AUC. This was noted for both oral and intravenous routes of administration. In addition, the elimination half-life of orally administered drug was always lower, often by a factor of one-half, than that of lorazepam given by the intravenous route. Unfortunately, AUCG and AUC was accurately measured for all oral and intravenous drug in only one dog (Gill). In that study, the ratio of oral/iv of AUCG/AUC was the greatest in the study where cycling was intact, and was lower in the other EHC interrupted studies.

Data from urine also revealed evidence that lorazepam may enterocycle in dogs. In the analysis of urine from two dogs (Tables 4.2, 4.5) the recovery of lorazepam glucuronide and the renal clearance of lorazepam glucuronide was lower when EHC was interrupted. This was not observed in the study with Gill where bile was removed; however, analysis of the data indicate that plasma and urine recovery levels were considerably higher than expected. Further evidence of EHC of lorazepam in dogs was suggested by the analysis of bile from one of the dogs (Table 4.4, Figures 4.4, 4.5). Concentrations of lorazepam were 10-100 fold greater in the bile during both studies, and concentrations of lorazepam glucuronide were 100 times greater in bile compared to plasma. This suggests that both lorazepam and its conjugated metabolite are actively secreted into bile in the dog. Estimates of biliary clearance of lorazepam glucuronide were comparable to renal clearance values.

Although there are no reported studies of the EHC of lorazepam in dogs, support for this model can be found in the literature. Lorazepam has been shown to enterocycle in ponies (Greenblatt and Engelking, 1988). Lower plasma concentrations

of lorazepam as well as lower urinary recoveries of lorazepam glucuronide were observed in the ponies when EHC was interrupted, as was noted for the dogs in the present study. The AUC of the glucuronide, however, was lower in ponies during cycling-interrupted studies, unlike the results obtained with our dogs. Oxazepam, a benzodiazepine closely related to lorazepam, has been shown to enterocycle in beagles (Alván *et al.*, 1977) and also in guinea pigs, where 35% of administered drug is recovered in bile within 3 hours (Bertangni *et al.*, 1972; 1978). Like lorazepam (Ruelius, 1978), the dominant metabolite of oxazepam in dog and man is the glucuronide, which accounts for 95% of the dose (Alván *et al.*, 1977). Interruption of oxazepam enterocycling produced lower blood and plasma levels of the intact drug, as well as lower urinary recoveries. Again, oxazepam glucuronide levels were lower in both studies when EHC was interrupted. This is also seen when the EHC of lorazepam is interrupted in man (Herman *et al.*, 1989).

Why the glucuronide levels were consistently higher in the dogs when EHC was interrupted is not known, yet this effect was observed in all cases. This was unexpected, since interruption of EHC should increase elimination of lorazepam glucuronide. Part of the reason may be due to a decreased biliary excretion of the glucuronide during the EHC interrupted studies, leading to an increase in plasma levels. During the studies with the ponies and guinea-pigs, bile was re-infused during the interrupted studies in order to maintain bile flow, which is a major factor in controlling biliary clearance. In two of the three dogs (Gill, Ike), no bile was re-infused during the interruption of EHC. This could shut down bile flow in an attempt to retain bile salts, leading to a shunting of glucuronide conjugates into the systemic circulation. This cannot be a full explanation, however, since an increase in plasma glucuronide levels was still noted in the third dog (Blackie), even when clean bile was re-infused, although difference between the AUC of lorazepam glucuronide between the two studies in that dog were reduced considerably. The increased AUC of lorazepam

glucuronide during cycling-interrupted studies is in contrast to similar studies done in humans, in which glucuronide levels were reduced by interruption of EHC (Herman *et al.*, 1989) as well as in the studies of oxazepam in beagles (Alván *et al.*, 1977).

Increased AUCG/AUC levels during cycling-interrupted studies may have been the result of deceased renal clearance of the metabolite. Indeed, this was a consistent finding throughout these studies. However, how interruption of EHC might affect renal clearance of lorazepam glucuronide is not known at this time.

Except in the first dog (Billy Rubin), the elimination half-life of orally administered drug was consistently less than the value obtained for drug administered through the intravenous route. Indeed, the elimination of oral drug from one dog (Blackie) was so quick that an elimination profile could not be constructed. The reason for this discrepancy between the two routes is not clear, however, it is consistent with results found in human studies employing a similar simultaneous i.v/oral dosing protocol (Herman *et al.*, 1989, Chaudhary *et al.*, 1993). These were initially believed to represent extrahepatic metabolism in man (Herman *et al.*, 1989); however, more recent work suggest that these ratios are the result of saturation of renal elimination mechanisms. Although extrahepatic metabolism of lorazepam has been shown in dogs, the contribution to overall lorazepam metabolism is minor.

Taken together, the data from the three dogs in which both cycling-intact and cycling-interrupted studies were performed indicate that lorazepam undergoes EHC in the dog, and suggests that this model would be useful in the study of EHC of drugs. Admittedly, the model is not simple, in that it requires an intricate and complex surgical preparation. However, once established, the benefits of this model are apparent. Because the measurement of EHC within animals requires multiple studies, it is important that the model be stable. Although complications in the first dog required that the animal be euthanized, subsequent dogs were extremely healthy. Since all the catheters were externalized in a single location towards the dog's back, opportunistic

infections were minimized. The placement of the catheters in this location also allowed the animals to move freely between studies, which undoubtedly enhanced their chances of good health. Daily flushing with heparinized saline kept the catheters virtually blockage-free. When blockage did occur, it could usually be freed with persistent flushing. The use of an occludable cuff around the common bile duct also helped to maintain hepatobiliary function, since pressure was placed around the duct only during experimental studies. Thus, one of the main benefits of this animal model is its longevity; indeed, these animals were healthy up to one year following the surgical preparation.

Several important findings can be taken from these studies. Firstly, studies in the second dog (Gill) point to the effectiveness of neomycin and cholestyramine in blocking the enterocycling of lorazepam. Treatment with neo/chol produced similar effects in lorazepam disposition when compared to the surgically-interrupted EHC study. In these studies, there did not appear to be any differential effects of the cholestyramine doses used for EHC-interruption. Unfortunately, only two such studies were performed. Further studies should be done to strengthen these findings.

The studies with the fourth dog (Blackie) shows the importance of maintaining bile flow during studies in which EHC is interrupted. One of the surprising, but consistent observations throughout the studies in the dogs was the fact that the AUC of lorazepam glucuronide (AUCG) was increased in studies in which EHC was interrupted. Although renal clearances of lorazepam glucuronide were similarly decreased during these studies, a second possibility for the elevated AUCG levels was that removal of bile during the EHC-interrupted studies caused biliary secretion to shut down. Since the driving force behind biliary secretion is the secretion of bile salts, it is quite likely that this would occur. The effect of a decreased biliary secretion would be to shunt lorazepam glucuronide into the systemic circulation, which would lead to elevated plasma concentrations. Support for this explanation can be taken from the

studies with Blackie, in which "clean" bile was reinfused during the cycling-interrupted study. This resulted in AUCG levels that were only modestly increased over cycling-intact studies, despite a decrease in renal clearance of the glucuronide metabolite.

Although lorazepam appears to undergo EHC in the dog, differences in lorazepam disposition between this dog model and man are readily apparent. The total clearance of lorazepam and the elimination half-life are considerably greater in the dog than in man. In addition, plasma levels of lorazepam glucuronide tend to increase with interruption of EHC in the dog model, in contrast to the effect observed in man. Nonetheless, the stability of this model, as well as the comparable biliary function between the dog and man, indicate that the dog may be a useful model with which to study enterohepatic circulation of drugs.

5. EFFECTS OF FEEDING ON THE DISPOSITION OF DIAZEPAM IN MAN

5.1. Introduction

Peaks occur in the concentration-time profile of diazepam that coincide closely in time with the ingestion of food (Linnoila *et al.*, 1975; Kortilla *et al.*, 1976; Naranjo *et al.*, 1980a; 1980b). Several investigators have suggested that enterohepatic circulation of diazepam is the mechanism of these post-prandial peaks (Van de Kleijn *et al.*, 1971; Baird and Hailey, 1972; 1973; Sellman *et al.*, 1975). Others have reported that post-prandial diazepam peaks are due to changes in plasma protein binding (Naranjo *et al.*, 1980a; 1980b). However, not all investigators have observed meal-induced changes in diazepam binding to plasma proteins (Klotz *et al.*, 1977a; Korttilla and Kanges, 1977). The purpose of this investigation was to study the effects of feeding on the disposition of diazepam in acute and steady-state conditions, and to evaluate the influence of enterohepatic circulation and plasma protein binding on post-prandial diazepam peaks.

5.2 MATERIALS AND METHODS

5.2.1 Experimental methods

5.2.1.1 Subjects

Four volunteers were recruited to participate in the study through advertisements posted throughout the Royal University Hospital (RUH), Saskatoon,

Saskatchewan and the University of Saskatchewan. All were healthy, drug-free Caucasian males between the ages of 21-23 years. Each was required to pass a complete medical evaluation including a history, physical examination and routine screening of general metabolic, renal and hepatic function. Potential volunteers with known medical or surgical disease were excluded from participating. All subjects were non-smokers and abstained from alcohol and use of other medications one week before and throughout the study interval. All were required to sign a consent form which was approved by the University Advisory Committee on Ethics in Human Experimentation.

5.2.1.2. Pharmacokinetic studies

The disposition of diazepam was studied in each individual in six separate parts.

5.2.1.2.1. Single-dose diazepam study

Subjects presented themselves to the Clinical Investigation Unit (CIU) of the RUH at 7:30 a.m. in the fasted state. An intravenous infusion of 0.9% saline, with a 4-way stopcock for sampling blood, was established in one arm and baseline blood and urine samples were collected. At precisely 8:00 a.m., each patient was given a 2 mg dose of diazepam (Frank W. Horner Inc. Montreal, Canada) by mouth, after which blood samples were taken at 15, 30, and 45 minutes, and at 1, 1.5, 2, 2.5, 3, 4, 4.5, 5, 6, 7, 8, 8.5, 9, 10, 11, 12, 16, 24, 36, 48 and 72 hours. Separate blood samples were taken at 0, 4, 5, 6, 8, 9, and 10 hours for plasma protein binding analysis. In addition, all urine produced by the patient over the entire study interval was collected in 12 hour intervals. Blood samples were collected in 14 mL polypropylene tubes containing 100 µL diluted heparin (Hepalean, Ayerst Laboratories, Toronto, Canada) solution (approximately 30 units of heparin per tube), and immediately placed in ice. Blood samples were centrifuged at 800 x g for 20 minutes at 4°C (Sorvall RC-3 general purpose automatic refrigerated centrifuge; Ivan Sorvall Inc., Norwalk, Conn.), and the

plasma separated and stored at -20°C until analysis. Subjects remained fasted until the 4 hour sample (12:00 p.m.), at which time they were given a standard low-fat meal. A second low-fat meal was given four hours later at the 8 hour sample (4:00 p.m.) Patients remained in the CIU until the 12-hour sample, at which time the intravenous infusion set was removed, and the volunteers were allowed to go home, returning periodically thereafter for collection of further blood and urine samples.

5.2.1.2.2. Multiple-dose diazepam studies

Following the single-dose diazepam study, 5 additional studies were performed on each individual after chronic administration of diazepam to steady-state levels. Steady-state was achieved by giving 2 mg of diazepam every 12 hours by mouth, with the dosing timed to precisely 8:00 a.m. and 8:00 p.m. using individual alarm watches. This dosing schedule was maintained for at least four weeks before any of the studies were undertaken. Between each study, a minimum 2 week washout period was observed to ensure that there was no carry over of the intervention protocol from one experiment to the next.

5.2.1.2.2.1. 24 hour diazepam study with meals

This study involved collection of data over consecutive night-time (8:00 p.m. to 8:00 a.m.) and day-time (8:00 a.m. to 8:00 p.m.) dosing intervals. Volunteers arrived at the CIU at 7:30 p.m. after their usual evening meal, but prior to their next regularly scheduled evening steady-state dose of diazepam. An intravenous saline infusion was established in one arm, and blank blood and urine samples were obtained. At 8:00 p.m., 2 mg of diazepam was administered by mouth, and bloods and urine were collected over the following 12 hour interval. At 8 a.m., a second 2 mg dose of diazepam was given, and blood and urine samples were collected over the ensuing 8:00

a.m. to 8:00 p.m. dosing interval. Separate blood samples were taken for plasma protein binding analysis at the same times as the single-dose study. Subjects remained fasted (from about 6 p.m.) overnight, but were given standard low-fat meals at 12:00 p.m. and 4 p.m. during the day, as in the single-dose study.

5.2.1.2.2.2. 24 hour diazepam study with meals, with neomycin and cholestyramine

Subjects were started on neomycin (Mycifradin, Upjohn Company of Canada, Don Mills, Ontario) 1 g approximately every 6 hours (4 doses/day during usual waking hours), and cholestyramine (Questran, Bristol Laboratories of Canada, Montreal, Que.), 4 g approximately every 4 hours, (6 doses/day during usual waking hours), both by mouth, to block the putative enterocycling of diazepam. One week later, and while still receiving the diazepam and neomycin and cholestyramine, they returned for a second steady-state study of oral diazepam with blood and urine sampling over consecutive night-time and day-time dosing intervals. Neomycin and cholestyramine (neo/chol) were continued throughout the entire sampling interval.

5.2.1.2.2.3. 12 hour diazepam study, fasted

Subjects presented themselves to the CIU at 7:30 a.m., after a 12 hour overnight fast. An i.v. infusion was established, and at precisely 8 a.m. (the timing of the next regularly scheduled dose of the drug) 2 mg of diazepam was given by mouth. Bloods and urine were collected over the following 12 hour (8 a.m. to 8 p.m.) dosing interval. Patients remained completely fasted, although they were allowed to drink water. Blood samples for plasma protein binding were taken at the 0, 4, 5, 8, and 9 hour collection times.

5.2.1.2.2.4. 12 hour diazepam study, fasted with heparin infusions

Study volunteers came to the CIU at 7:30 a.m. after an overnight fast, and were studied over a 12 hour day-time dosing interval, similar to the fasting study, except that patients received intravenous bolus infusions of heparin (Hepalean, Ayerst Laboratories, Toronto, Canada), 1000 and 2000 units, at the 4 and 8 hour sampling times, respectively. Additional blood samples were taken at 0, 4, and 8 hours, as well as 5, 10, 15 and 30 minutes after each heparin infusion for plasma protein binding measurements.

5.2.1.2.2.5. 12 hour diazepam study, fasted with fat emulsion infusions

This study was similar to the fasted protocol, except that at the 4 hour sampling time (12:00 p.m.) subjects were started on a continuous infusion of a fat emulsion (Liposyn II 20%, Abbott Laboratories, Montreal, Canada) into an arm vein, delivered by an infusion pump (IVAC Corporation, San Diego, CA) at a constant rate of 120 mL/hr. The infusion was stopped after 3.5 hours, and blood samples and urines were collected until the end of the 12 hour dosing interval. Additional blood samples for plasma protein binding were taken at 0, 4, 6, 8, and 10 hours.

5.2.2. Analytical methods

5.2.2.1. HPLC assay of diazepam

The HPLC assay of diazepam in plasma and urine employed an identical to the methods described for lorazepam in section 3.2.2.3.1., with only minor changes. The internal standard was 100 ng of chromatographically pure lorazepam (a gift from Wyeth Laboratories, Philadelphia, PA). Calibration curves were prepared in the range of 5-

200 ng/mL, and were linear in this range. The limits of detection for diazepam and desmethyldiazepam were approximately 3 ng/mL for each. Intra-assay and inter-assay coefficients of variation were 4.9% and 6.2%, respectively, for low range (25 ng/mL) quality controls and 2.3% and 2.8%, respectively, for high range (170 ng/mL) quality controls.

5.2.2.2. Plasma protein binding measurements

Measurement of plasma protein binding of diazepam employed identical methods to those described for lorazepam in section 3.2.2.3, with minor changes. Approximately 7 x 10^{-2} μCi (approximately 370 ng) of ^{14}C-diazepam (specific activity 54 mCi/mmol, Amersham Corp.) was used as a radiolabel. Diazepam free fraction was also measured in 'fat-free' plasma samples in studies of diazepam disposition with parenteral infusions of fat emulsion (section 5.2.1.2.2.5). Separation of fat in plasma samples was attained by centrifugation at 10000 x g for 30 minutes at 4° C, and subsequently removing the 'fat-free' fraction through a perforation in the bottom of the centrifuge tube.

5.2.3. Pharmacokinetic methods

Pharmacokinetic analysis employed identical methods to those described for lorazepam (section 3.2.3), with a few changes. The apparent volume of distribution (V_{area}) for studies of single-dose diazepam given by mouth was calculated by the following equation:

$$V_{area} = \text{Dose} / (\text{AUC} \bullet \lambda) \qquad (5.1)$$

where λ is the terminal disposition rate constant. Theoretically, apparent volume of distribution can only be accurately determined after intravenous administration; however, V_{area} can be calculated following oral data (Gibaldi and Perrier, 1982c), if

complete absorption and complete systemic availability can be assumed (Greenblatt *et al.*, 1980).

Apparent total body clearance (CL/F) was calculated by the equation:

$$CL/F = Dose / AUC\ (0\text{-}\infty) \quad (5.2)$$

where F is the bioavailability of the drug. For diazepam, it is assumed that bioavailability is unity (F=1), therefore:

$$CL_{or} = Dose / AUC\ (0\text{-}\infty) \quad (5.3)$$

For studies in which drug disposition was studied at steady-state, clearance was determined by:

$$CL = (F \bullet Dose) / AUC\ (0\text{-}\tau) \quad (5.4)$$

where AUC (0-τ) is the area under the curve during one dosing interval.

5.2.4. Statistical methods

Statistical analysis employed methods identical to those described in section 3.2.4.

5.3. RESULTS

The characteristics of the four subjects who participated in this study are shown in Table 5.1. All were healthy, non-smoking male subjects, between the ages of 21-23 years at the start of the study. All were within 25% of their ideal body mass index and none of the patients were taking other drugs prior to, or during any of the various study protocols.

Table 5.1 Baseline characteristics of normal subjects in diazepam disposition study.

Subject	Age (years)	Weight[1] (kg)	Height (cm)	BMI (kg/m^2)
GP	21	83.5	177.4	26.5
DS	23	73.9	184.5	21.7
AS	21	78.5	179.1	23.1
MK	21	86.0	174.3	28.3
Mean±S.D.	21.5 ± 1.0	80.5 ± 5.4	178.8 ± 4.3	24.9 ± 3.0

[1] Weight shown is the weight of each subject the morning of the single dose study.
BMI body mass index.

Figure 5.1 shows the plasma concentration-time profiles of diazepam and its major metabolite, desmethyldiazepam, (means ± standard deviation) in the 4 study subjects following single-dose administration of 2 mg of diazepam by mouth. Concentrations of diazepam were detected in the plasma at the 15 minutes sample and reached peak concentrations within the first collection hour. Thereafter, plasma concentrations decreased rapidly until the 4 hour sample, after which time they began to rise in all 4 subjects, coincident with the ingestion of the first, end-of-fast, meal.

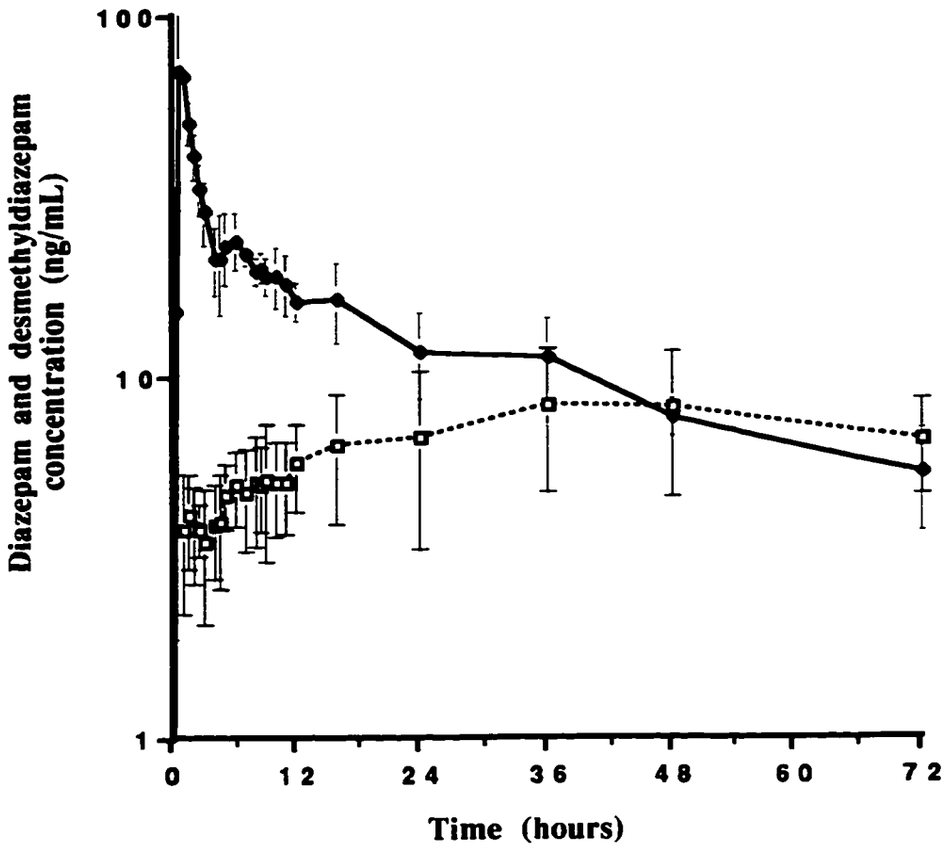


Figure 5.1. Mean plasma concentration-time profile of diazepam (solid diamonds) and desmethyldiazepam (open squares) in 4 normal subjects following a single 2 mg oral dose of diazepam.
Error bars represent standard deviations

Diazepam concentrations increased over the next 1-2 hours before declining again. The increase in plasma concentrations averaged 24 ± 13% (range 10-38%) of the levels measured immediately before the peak. Since diazepam levels would have continued to decline over the 1-2 hour period in the absence of a meal, this may slightly underestimate the extent of the change. A small additional peak was observed in the plasma concentration-time profile between the 8 and 10 hour sample collection times, coincident with the second meal. Diazepam plasma concentrations declined in a relatively smooth log-linear manner during the rest of the sample collection interval.

Desmethyldiazepam concentrations were detected within 30-60 minutes of diazepam administration, and increased slowly to peak levels of 4-13 ng/mL between 36-48 hours, after which they began to decrease, at a rate slower than the parent drug. No corresponding post-prandial peaks were identified in the desmethyldiazepam concentration-time profiles, although levels were low and fluctuations difficult to discern from random variations.

The mean pharmacokinetic parameters calculated for diazepam from the concentration-time profiles were an apparent oral clearance of 0.361 ± 0.059 mL/min/kg; an apparent oral volume of distribution of 1.08 ± 0.12 L/kg; and a terminal elimination half-life of 35.0 ± 5.0 hr. (Table 5.2). These values are consistent with the published literature on diazepam (Bertilsson *et al.*, 1989, Greenblatt *et al.*, 1980). Insufficient data were available from the single-dose study to confidently characterize the desmethyldiazepam elimination profiles.

Table 5.2. Pharmacokinetic parameters for diazepam in 4 normal subjects following a single oral 2 mg dose.

Subject	AUC_{or}	CL_{or}	V_{area}	$T_{1/2oral}$
GP	911.0	0.438	1.24	32.7
DS	1351.0	0.314	1.02	37.4
AS	1203.5	0.375	0.95	29.3
MK	1225.6	0.316	1.11	40.6
Mean±S.D.	1172.8±186.2	0.361±0.059	1.08 ± 0.12	35.0 ± 5.0

AUC_{or} area under the curve of diazepam following oral administration (ng/mL-hr).
CL_{or} apparent clearance of diazepam following oral administration (mL/min/kg).
V_{area} apparent volume of distributionof diazepam following oral administration (L/kg).
$T_{1/2oral}$ elimination half-life of diazepam following oral administration (hr).

The mean concentration-time profiles of diazepam and desmethyldiazepam over consecutive night-time (8 p.m. to 8 a.m.) and day-time (8 a.m. to 8 p.m.) dosing intervals in the same 4 subjects during multiple-dose studies with meals (control) and multiple-dose neo/chol (cycling-interrupted) studies are shown in Figure 5.2.

During the night, diazepam concentrations peaked with absorption about 2 hours after the evening dose, and then declined in a log-linear fashion. Desmethyldiazepam levels were approximatlely 50% greater compared to diazepam concentrations at the start of the night-time dosing interval. The night-time desmethyldiazepam concentration-time curves fell steadily until the middle of the dosing-interval , at which time they began a modest rise, such that the levels of the metabolite were approximately 1.8-fold greater than diazepam at the start of the day-time dosing interval.

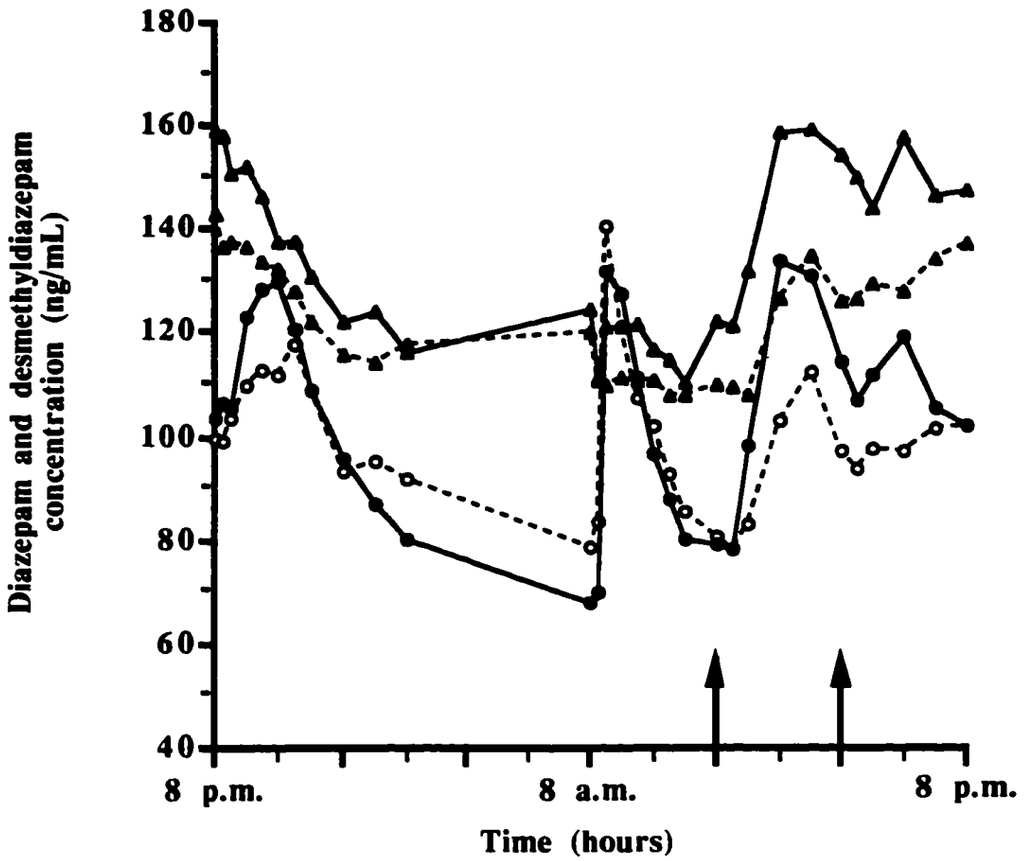


Figure 5.2. Mean plasma concentration-time profiles of diazepam (circles) and desmethyldiazepam (triangles) in 4 normal subjects during consecutive night-time and day-time dosing intervals in multiple-dose diazepam studies.
Solid symbols/solid lines indicate studies with meals; open symbols/dashed lines indicate studies with neo/chol treatment.
Arrows indicate times of meals.

During the day, in addition to the absorption peak, there were two large peaks in the elimination phase of both diazepam and desmethyldiazepam coincident with the 4 hour and 8 hour meals, respectively. Concentrations of both diazepam and its metabolite began to rise within the first 30 minutes following the ingestion of the meal, reached a peak approximately 1.5 - 2 hours later, and then gradually fell over the next several hours. Diazepam concentrations during the first of these peaks changed from a mean pre-prandial concentration of 79 ng/mL to a peak concentration of 133 ng/mL, representing an average increase of 77% (range 48-128%) above pre-meal levels. Corresponding changes for desmethyldiazepam were from 122 ng/mL to 158 ng/mL, for an average increase of 32% (range 21-58%). Concentrations of diazepam and desmethyldiazepam had not returned to baseline by the time of the second (8 hour) meal 4 hours later, and as such, the late afternoon/early evening peak appeared to be superimposed on the first, early afternoon peak. Diazepam and desmethyldiazepam concentrations remained elevated over the remainder of the dosing interval, appearing to carry over into the subsequent night-time dosing interval. Indeed, mean concentrations of both substrates were considerably greater at 8 p.m. than those measured at 8 a.m. (diazepam, 102.3 ± 26.6 vs 68.1 ± 19.0 ng/mL, $p = 0.003$; desmethyldiazepam, 147.4 ± 45.7 vs 124.6 ± 35.3, $p = 0.03$).

The AUCs and apparent oral clearances of total and free diazepam for all multiple-dose diazepam studies appear in Table 5.3. As a result of post-prandial diazepam peaking, day-time dosing interval AUCs were generally higher, and day-time diazepam oral clearances generally lower, than corresponding night-time estimates; however, these differences were not statistically significant ($p=0.11$). There were no differences between day-time and night-time AUCs and clearances compared to values determined following single-dose diazepam administration. Indeed, cumulative 24 hr diazepam clearances (0.372 ± 0.098 mL/min/kg) were similar to single-dose clearance estimates (0.362 ± 0.59 mL/min/kg).

Table 5.3. Mean AUC and apparent oral clearance (total and free) of diazepam in 4 normal subjects for all multiple-dose studies

	Multiple-dose studies						
	meals (night)	meals (day)	neo/chol (night)	neo/chol (day)	fasting	heparin	fat emulsion
AUC_{or}	1079 (297)	1281[1] (315)	1129 (423)	1180[1] (327)	869 (171)	872 (281)	869 (201)
CL_{or}	0.407 (0.112)	0.343[1] (0.089)	0.405 (0.115)	0.378[1] (0.092)	0.472 (0.078)	0.494 (0.142)	0.537 (0.171)
FCL_{or}	*	16.76[1] (4.53)	*	18.73 (5.07)	19.09 (3.31)	16.76[1] (4.58)	17.16 (4.15)

All values are means. Numbers in parenthesis indicate standard deviations.

[1] significantly different from fasting as determined by analysis of variance with repeated measures ($p<0.05$)

AUC_{or} area under the curve of diazepam following oral administration (ng/mL-hr).

CL_{or} apparent oral clearance (mL/min/kg).

FCL_{or} apparent free oral clearance (mL/min/kg).

* FCL_{or} was not calculated for night-time dosing intervals, as no plasma protein binding determinations were made during this period

Measurement of diazepam free fraction in plasma samples collected at the start of each study and immediately before the first meal were considered to be fasting specimens, whereas those collected after the 4 hour meal, including the specimen at 8 hours, just before the second meal, were considered to be post-meal plasma samples, since the second meal was only four hours after the first. As can be seen (Table 5.4), diazepam free fraction was significantly lower in post-meal plasma, compared to plasma collected before meals ($p=0.0001$). The meal effect was consistent in all timed measurements and was significant in the single-dose study ($p=0.0004$), as well as the two multiple-dose studies with meals and neomycin/cholestyramine treatment; ($p=0.002$).

Table 5.4. Diazepam free fraction (%) in 4 normal subjects, before and after meals in single-dose and multiple-dose studies with meals and with neo/chol treatment.

Sampling time	single-dose study	multiple-dose studies	
		meals	neo/chol
Pre-meal			
0 hr	2.45 ± 0.35	2.23 ± 0.18	2.14 ± 0.92
4 hr	2.39 ± 0.20	2.27 ± 0.40	2.17 ± 0.19
Post-meal[1]			
5 hr	2.12 ± 0.17	2.04 ± 0.16	1.93 ± 0.11
6 hr	2.18 ± 0.41	1.90 ± 0.17	1.90 ± 0.94
8 hr	2.17 ± 0.20	2.05 ± 0.13	1.96 ± 0.19
9 hr	2.26 ± 0.23	1.92 ± 0.10	1.96 ± 0.09
10hr	2.21 ± 0.14	1.92 ± 0.17	1.99 ± 0.11

Data are mean ± S.D.
Meals were given after the 4 and 8 hour sampling times
[1] Repeated measures ANOVA with planned contrasts showed significant differences between pre-meal (0 and 4 hr) and post-meal (5,6,8,9 and 10 hr) samples in all three studies (single dose, p=0.0004; meals, p=0.0001); neo/chol, p=0.002).

However, the drop in diazepam free fraction with feeding was not as great in the magnitude of increase in total diazepam concentrations. Thus, free concentrations of diazepam were observed to increase after food intake (Figure 5.3). Following the first meal, concentrations of free diazepam rose by about 60% (range 41-84%) compared to pre-prandial values. A second, smaller, increase in free diazepam levels was also observed following the 8 hour meal. The increase in free diazepam concentrations during the study with meals resulted in a decrease in the clearance of unbound diazepam. Indeed, compared to fasting studies, total diazepam clearance decreased 27% with meals ($p = 0.0033$); however, the clearance of free diazepam during the day-time dosing interval decreased 12% with feeding ($p = 0.04$) (Table 5.3).

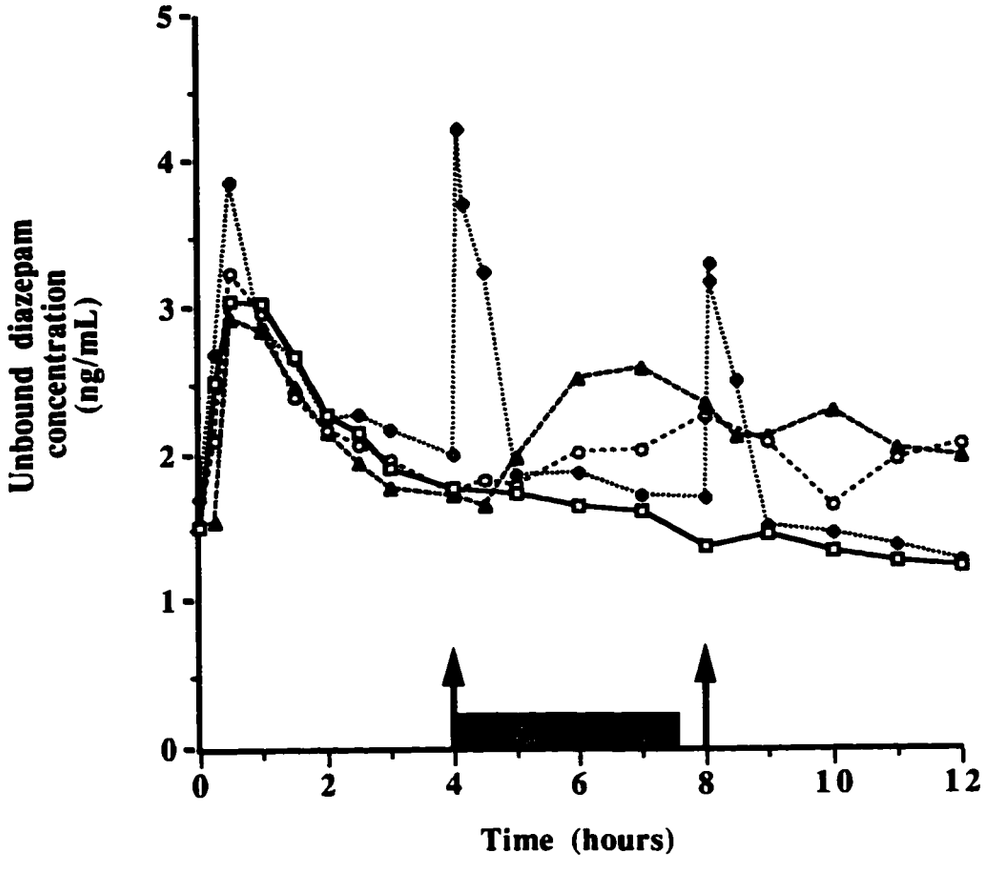


Figure 5.3. Mean unbound (free) diazepam concentration-time profiles in 4 normal subjects during multiple-dose diazepam administration with meals (triangles), fasting (squares), heparin injections (diamonds), and fat emulsion infusion (circles).
Arrows indicate times of meals and heparin injections; bar indicates time of fat emulsion infusion.

Treatment with neo/chol blunted and, in some cases, delayed the appearance of the post-prandial diazepam and desmethyldiazepam peaks, but did not prevent their occurrence. As with the control study, post-prandial peaking appeared to be associated with a carry-over of drug concentration from the day-time into the night-time dosing interval, with higher mean diazepam (102±42 vs. 79±28 ng/mL, p=0.05) and desmethyldiazepam (137±52 vs. 120±50 ng/mL, p=0.10) concentrations at 8 p.m. than at 8 a.m.. Similarly, day-time AUCs were slightly greater and diazepam oral clearances slightly lower than corresponding night-time measurements, but again, these differences were not statistically significant. Diazepam free fractions were about 4% lower in pre-prandial plasma samples from neo/chol-treated patients compared to controls (not significant), although the same effects of feeding on reducing diazepam free fraction and the same absolute post-prandial diazepam free fractions were observed for both. As with the control study, clearances of total diazepam on neo/chol were slightly, but not significantly greater during the night than during the day and were identical with control measurements during both the night-time and day-time dosing intervals.

The effect of fasting on the concentration-time profiles of steady-state diazepam in the four normal subjects is shown in Figure 5.4. Figures 5.5 and 5.6 show the corresponding effects of two bolus intravenous heparin injections and a 3.5 hour intravenous infusion of fat emulsion, both of which were also done during fasting conditions. The mean concentration time-profiles for these studies are shown in Figure 5.7, which also contains the mean concentration-time profiles of diazepam during meals with and without neo/chol treatment during the day-time dosing intervals. Plasma desmethyldiazepam concentrations, although measured, are left out of the figures for purposes of clarity.

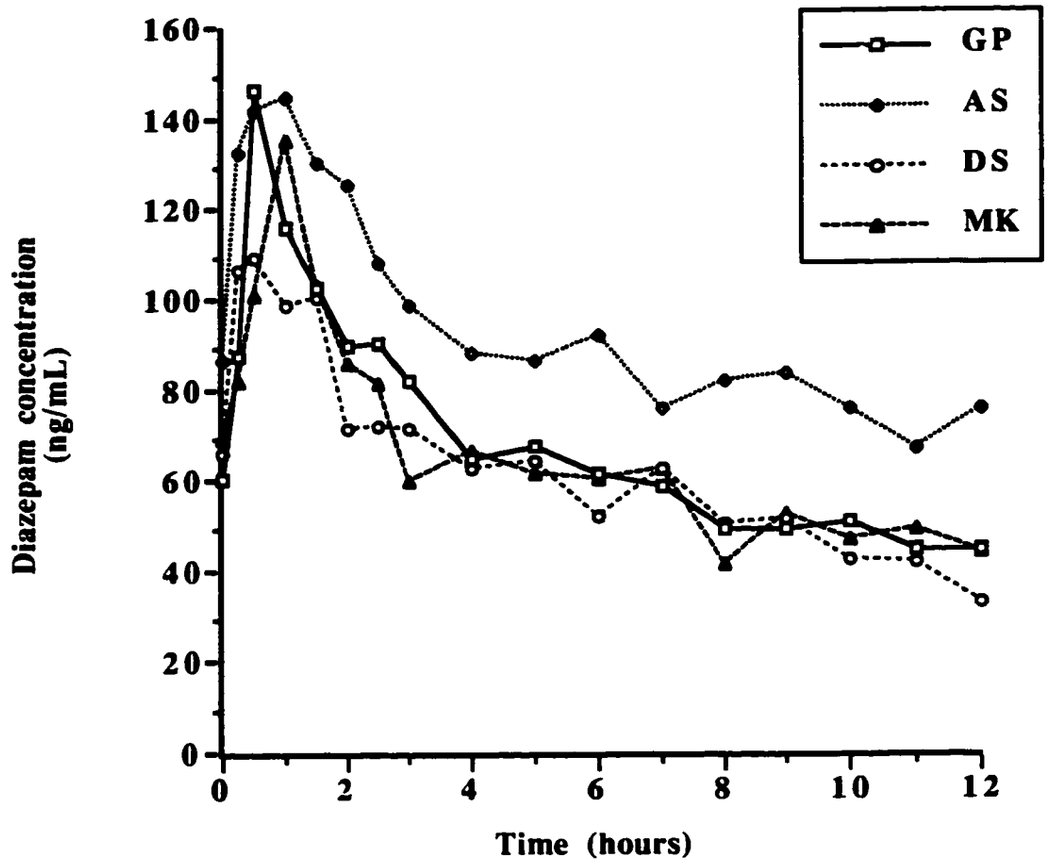


Figure 5.4. Concentration-time profiles of diazepam at steady-state in 4 normal subjects during a single fasted day-time dosing interval.

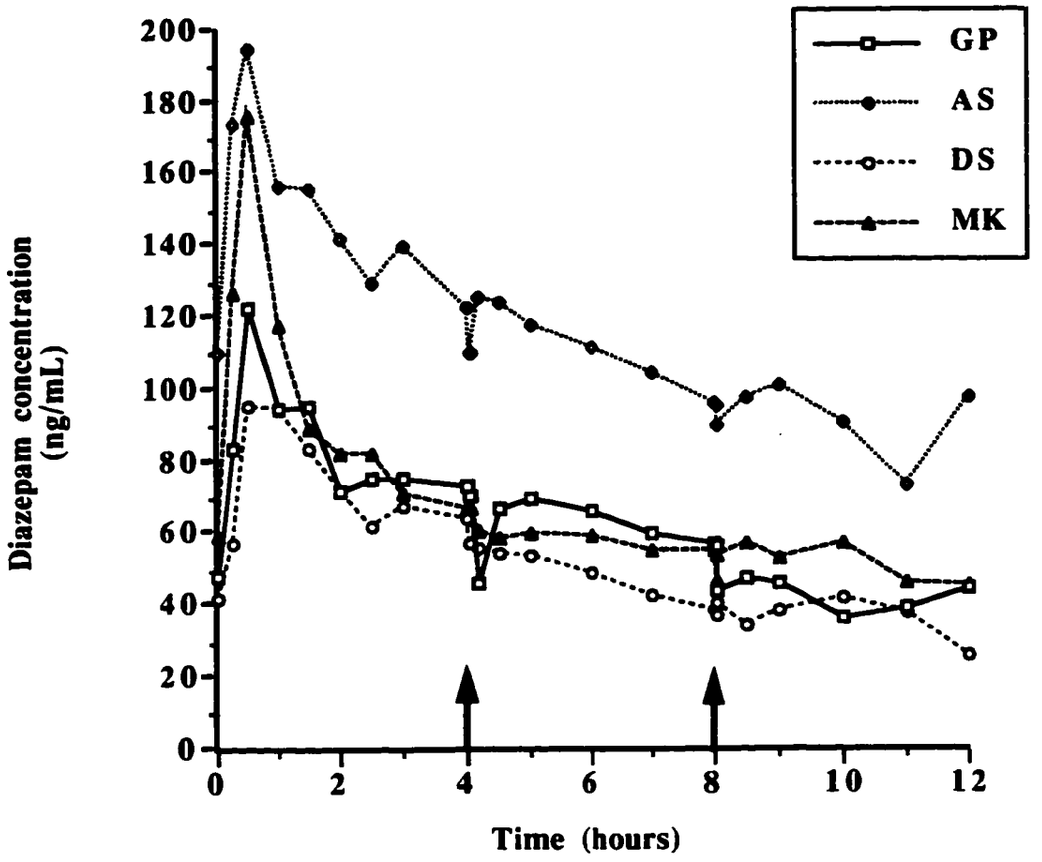


Figure 5.5. Effects of bolus heparin injections on the concentration-time profiles of diazepam at steady-state in 4 normal subjects.
Arrows indicate the times of heparin injections (1000 and 2000 units at 4 and 8 hours, respectively.

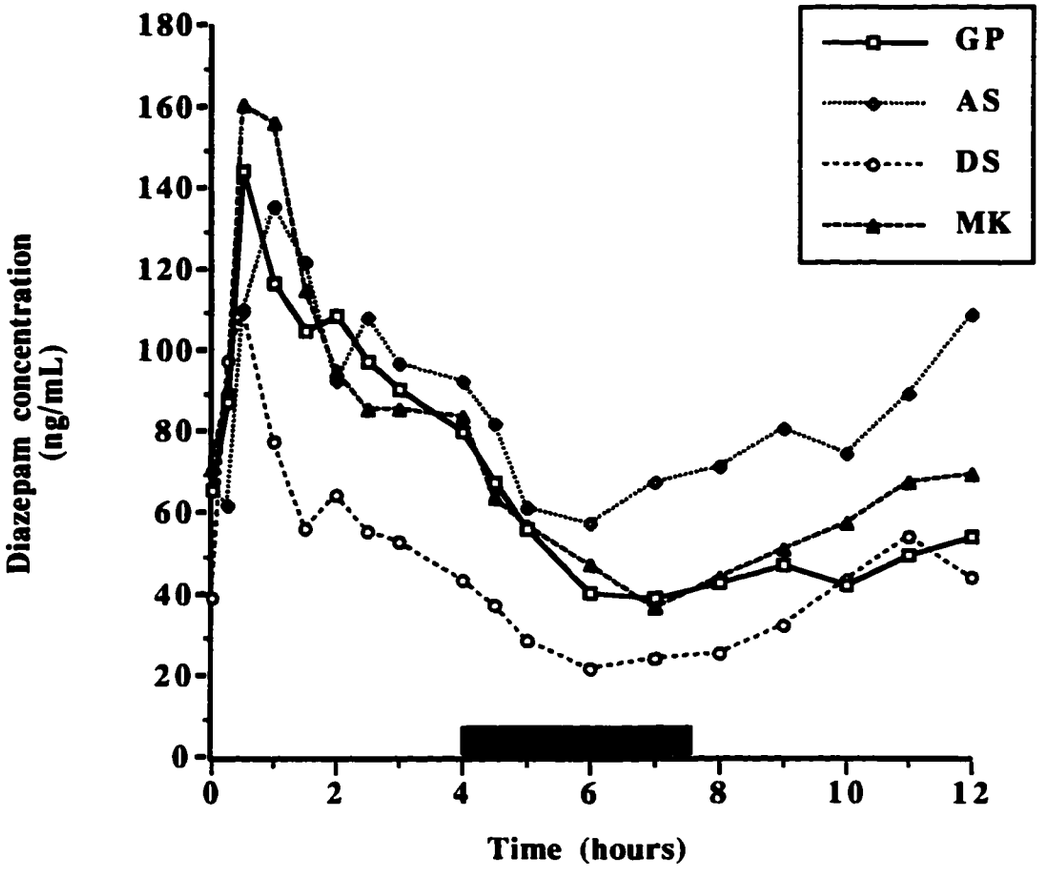


Figure 5.6 Effect of a fat emulsion infusion on steady-state diazepam levels in 4 normal subjects during a single day-time dosing interval. The bar indicates the time period of the infusion, during which fat emulsion (Liposyn II 20) was infused at a rate of 120 mL/hr.

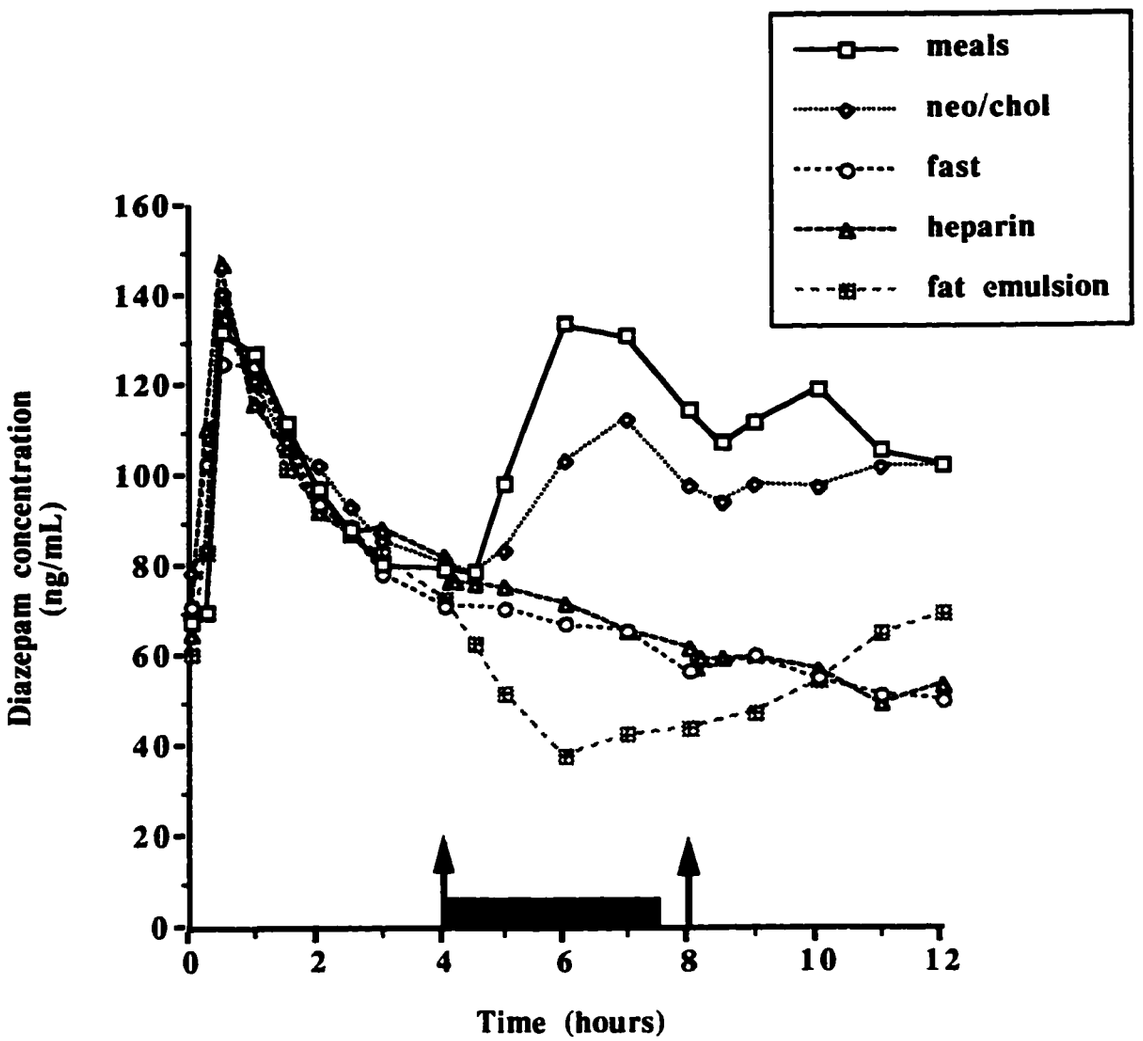


Figure 5.7. Mean concentration-time profile of steady-state diazepam in 4 normal subjects during all day-time multiple-dose studies. Arrows indicate times of meals and heparin injections (1000 and 2000 units at 4 and 8 hours, respectively. The bar indicates the time period of the fat emulsion infusion.

With fasting, diazepam concentrations peaked within 30-60 minutes and declined in a smooth log-linear fashion, with no indication of secondary peaking (Figure 5.4). Desmethyldiazepam levels increased slowly reaching peak concentrations 2-3 hours after diazepam dosing, and declined smoothly, thereafter, at a slower rate than diazepam (data not shown). In contrast to the feeding studies, concentrations of both diazepam and desmethyldiazepam were lower at 8 p.m. versus 8 a.m. (diazepam, 50.2 ± 18.5 vs. 71.1 ± 11.1 ng/mL, p = 0.0082, desmethyldiazepam, 83.8±33.0 vs. 105.3±24.2 ng/mL, p=0.005). No change was observed in diazepam free fraction in plasma collected at identical sampling times during the fasting study.

Intravenous bolus injections of heparin caused sharp, immediate declines in total diazepam concentrations in all study subjects (Figure 5.5) (18.0±13.0%, range 8.8-36.8% for 1000 units at 4 hours; 14.4±6.4%, range 6.2-22.0% for 2000 units at 8 hours). (This effect is partially obscured in Figure 5.7 due to the discordant nature of the timing of the decreases among the four individuals). However, the effect was short-lived, with concentrations returning to near pre-infusion levels within approximately 10-30 minutes. Similar transient declines were seen in desmethyldiazepam concentration profiles. Coincident with these changes were large reciprocal increases in diazepam free concentrations, averaging 2-fold in magnitude (Figure 5.8). Clearances of total (bound + unbound) diazepam were similar to fasted, and greater than fed total diazepam clearances (p = 0.0009). Free clearances of diazepam showed an opposite response (Table 5.3).

Continuous infusion of fat emulsion produced effects similar to heparin, but over a longer time interval. Deep depressions were seen in the concentration-time profiles of total diazepam and desmethyldiazepam, which then rebounded to higher than

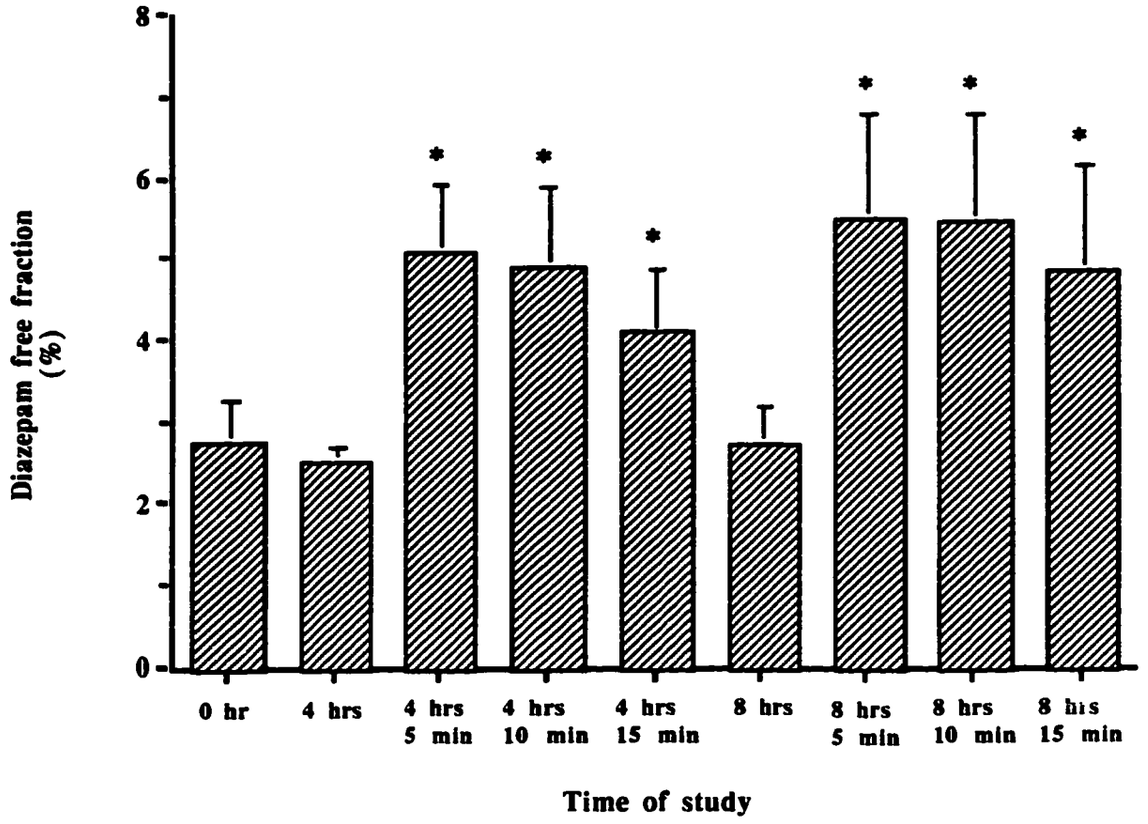


Figure 5.8. Diazepam free fraction in plasma from multiple-dose diazepam studies with heparin injections. Heparin injections were given intravenously at 4 hours (1000 units) and 8 hours (2000 units). Bars represent means of 4 normal subjects Error bars represent standard deviations.

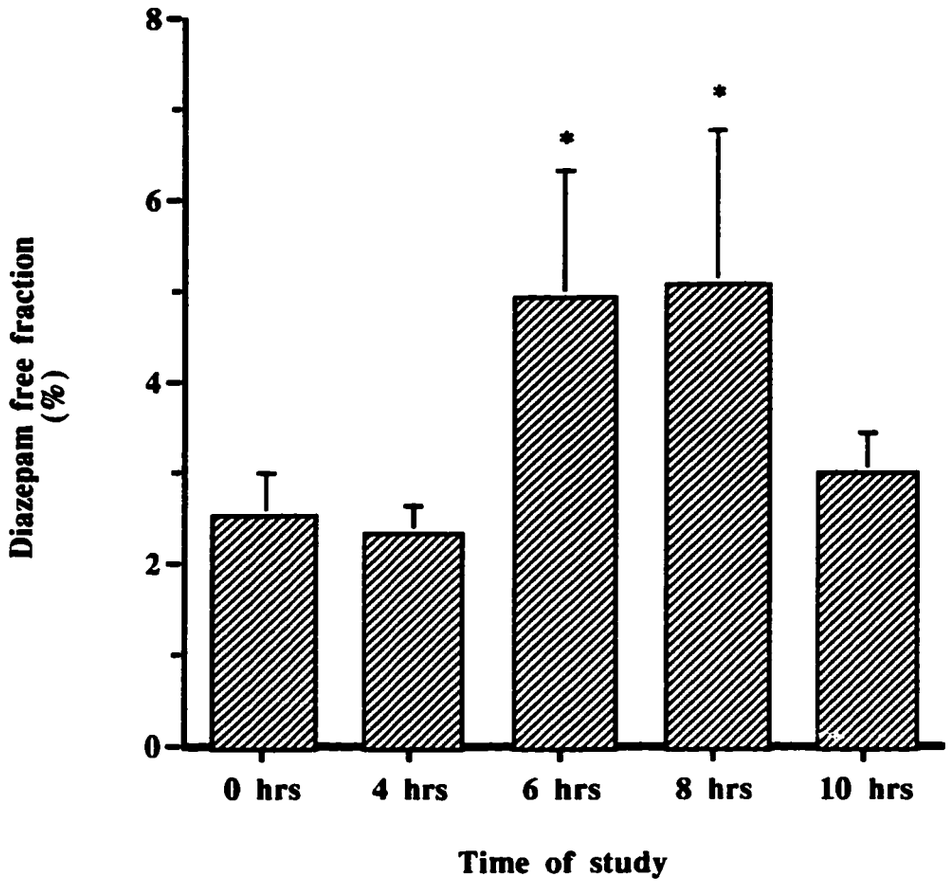


Figure 5.9. Diazepam free fraction in plasma from multiple-dose diazepam studies with fat emulsion infusion.
Fat emulsion (Liposyn II 20) was infused from 4 to 7.5 hours at a rate of 120 mL/hr.
Bars represent means of 4 normal subjects.
Error bars represent standard deviations.

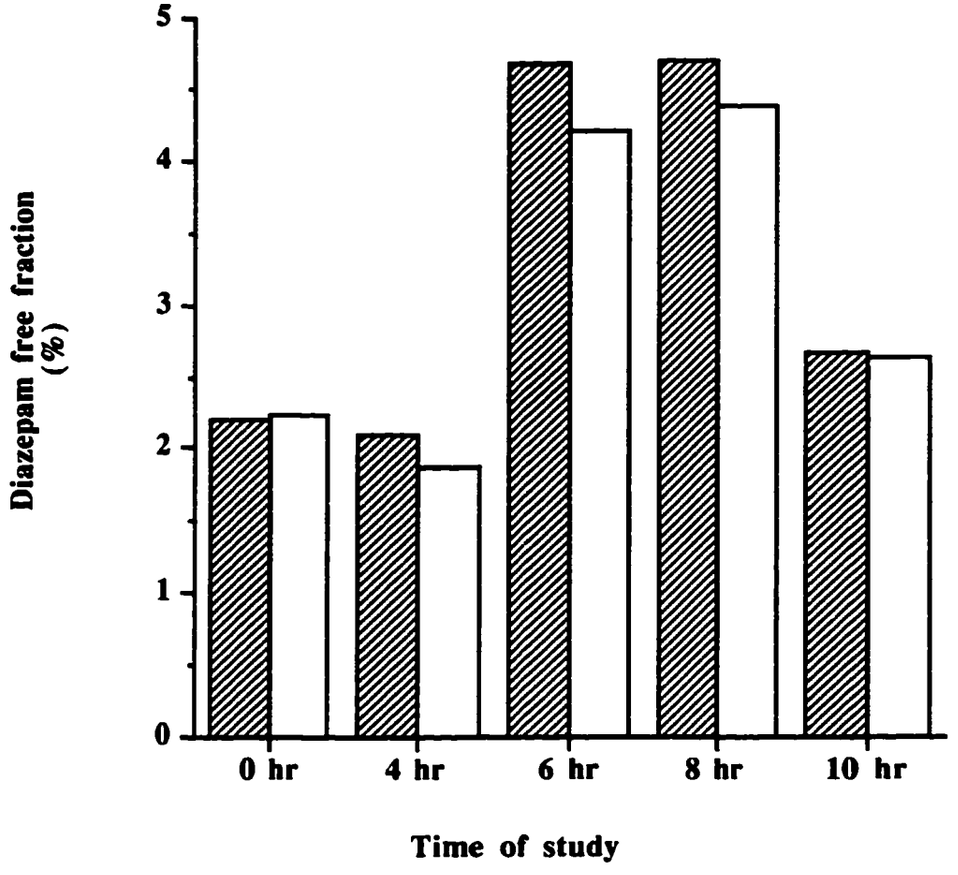


Figure 5.10.Diazepam free fraction in plasma collected during intravenous fat emulsion infusion in a single subject. Shaded bars represent plasma with fat; open bars represent plasma without fat.

expected levels after the infusion was stopped. Diazepam free fraction (Figure 5.9) and free diazepam concentrations were markedly increased. This effect continued to be observed for some time after the infusion of fat emulsion was terminated. Plasma protein binding of diazepam was identical whether binding was performed on lipemic or non-lipemic, "fat-removed", plasma (Figure 5.10). Clearances of total diazepam were slightly, but not significantly, greater than fasted clearances (with or without injections of heparin), and were higher than those of patients who were fed ($p=0.0001$) or fed and given neo/chol ($p=0.005$). Clearances of free diazepam were less than those obtained fasting ($p=0.059$), but similar to fed and heparin-treated values.

5.4. DISCUSSION

This study shows that feeding has dramatic effects on the disposition of diazepam. Post-prandial peaking is clearly demonstrated for diazepam and its major metabolite, desmethyldiazepam. The peaks are physically and temporally related to the ingestion of the meal, and do not occur when subjects are fasted. During multiple-dosing, which is the usual manner of drug use by patients, these peaks become quite large, indeed, as large or larger than the original absorption peak. However, similar considerations are in order following single-dose diazepam administration. The abruptness and magnitude of change in both total and free diazepam and desmethyldiazepam concentrations under these circumstances should be considered both clinically and pharmacokinetically important.

Large, random fluctuations in plasma concentrations introduce complexities in estimating diazepam pharmacokinetic parameters, particularly since they tend to occur late in the sampling interval where collection is often less frequent. Systematic errors

may arise in the determinations of half-life, volume of distribution, and clearance, and large variabilities may obfuscate the intended measurement of small treatment or disease effects. Moreover, the changes that occur in free diazepam and total diazepam concentrations are often discordant and are necessarily predictable. For example, the decrease in systemic free fatty acids (FFAs) as a consequence of feeding causes an increase in diazepam plasma protein binding. This results in an increase in total diazepam concentrations, as well as a decrease in total diazepam clearance, but no change in free concentrations or free clearance. The observation that both measures were affected by feeding in the present study suggests that there must be an independent effect of feeding on free clearance. This becomes even more complex when one considers that since the half-life of diazepam is long, the effects may be shifted into the subsequent dosing interval. In the case of the infusion of fat emulsion, the free clearance is less than the fasted free clearance, as would be expected for a feeding study, but this is not reflected in the marked increase in free concentrations resulting from the effects of feeding on binding. Moreover, total clearance during the fat infusion is greater than the total clearance in fed studies, presumably because of the redistribution of drug into tissue with its return only late in the sampling interval. Measurement of total diazepam concentrations could lead to completely erroneous conclusions regarding the effects of feeding. There are many studies of the effects of perturbations in plasma protein binding. However, few studies have demonstrated the effects of altered tissue binding as nicely as this experiment.

More pressing than these issues, however, is the question not addressed by these studies as to whether or not drug effect is also altered during post-prandial diazepam peaking. This would be anticipated given the magnitude of the changes, and the fact that both free diazepam and free desmethyldiazepam are involved. Also, substrate shifts from peripheral to circulatory compartments are likely to have some

impact on pharmacodynamic response. Certainly, further studies are needed in this area.

The mechanisms of post-prandial diazepam peaking have been debated. However, clearly neomycin and cholestyramine did not prevent the occurrence of post-prandial diazepam peaking in our study, nor were there differences in the AUCs and diazepam clearances between day-time and night-time dosing intervals as has been described with other cycling drugs (Herman *et al.*, 1989). With only four subjects, the sample size would seem too small to completely rule out the possibility of a Type II error. However, if the post-prandial peaks were due to EHC, interrupting the cycle would eliminate the peaks and produce total clearance values similar to fasted studies. Accordingly, to detect a 38% increase in the clearance of diazepam, which is the magnitude of difference between fasting and studies with meals only 3.7, or 4 subjects are needed, using the formula:

$$n = (Z_\alpha + Z_\beta)^2 \bullet \sigma^2 / \Delta^2 \qquad (5.1)$$

with α=0.05 and β=0.2 (power of 80%), where n the is number of subjects, σ is the standard deviation, and Δ is the difference between the studies (Pope and Bellamy, 1995). The power of our study to detect a 38% difference was 82%.

Reports within the literature also argue against an enterohepatic circulation for diazepam. Compounds which are actively secreted in the bile in man are usually polar and have a molecular weight exceeding 500 (Dobrinska, 1989). Diazepam satisfies neither criteria. Although lorazepam, a related benzodiazepine, possesses a significant EHC, it does so through a glucuronide conjugate. However, diazepam is not metabolized to a glucuronide directly. Diazepam disposition studies using biliary T-tubes in patients with normal liver function (Eustace *et al.*, 1975; Klotz *et al.*, 1975) and in patients with liver disease (Hepner *et al.*, 1977) concluded that biliary diazepam

and desmethyldiazepam concentrations were insufficient to have an effect on drug disposition. Theoretically, diazepam could cycle through a metabolite, such as oxazepam, which is directly glucuronidated. However, for this to occur, oxazepam glucuronide secreted into the bile would have be reduced back to diazepam within the gut before being reabsorbed. Such a pathway seems unlikely. Since there was no statistically significant effect of blocking EHC, and the power associated with the study was high, in combination with other evidence which points to the mechanism of post-prandial peaking, we can confidently exclude the presence of an EHC for diazepam.

The rate of rise and extent of fluctuation with the peaks is blunted slightly by the presence of neo/chol. This appears to be a result of a delay in absorption, and perhaps a reduction in the amount of the diazepam absorbed. Cholestyramine is a nonabsorbable, strongly basic anion-exchange resin containing quaternary ammonium functional groups (Witztum, 1996). Cholestyramine has been shown to interact with a number of drugs that exist as anions at physiological pH, although the amount adsorbed and the stability of the complex formed could not be related to the acidity of the solute (Gallo *et al.*, 1965). Given that diazepam is a weak base, such an interaction would appear to be less likely. However, there are other mechanisms through which an interaction may occur. Cholestyramine is used clinically to bind bile acids and interrupt the EHC of cholesterol. Large doses of the drug as employed in our study can induce maldigestion, and malabsorption. Anion exchange resins, such as cholestyramine, have been reported to decrease the absorption of a number of different drugs, including thyroxine, digitalis glycosides, anticoagulants, some thiazides, propranolol, tetracycline, and furosemide (Witztum, 1996).

Neomycin has also been reported to have effects on drug absorption. It is well-documented that neomycin reduces digoxin absorption. Lindenbaum *et al.* (1976) showed that neomycin, in 1 or 3 g doses, markedly impairs the absorption of digoxin in normal subjects. *In vitro*, neomycin did not interfere with movement of digoxin

across dialysis membranes and did not precipitate digoxin from human bile of intestinal fluid. Neomycin also has been reported to reduce the absorption of spironolactone (Bartle *et al.*, 1979), penicillin (Cheung and White, 1962), and a variety of nutrients (Dobbins, 1968; Green and Tall, 1979). Although high doses of neomycin (6 g/day) have been reported to induce morphologic alterations of the intestinal mucosa that may cause a malabsorptive syndrome (Dobbins *et al.*, 1968), the exact mechanism by which neomycin interferes with the absorption of drugs remains unclear.

In our study, post-prandial diazepam peaks were associated with marked increases in the binding of diazepam to plasma proteins, together with reciprocal decreases in the clearance of total (bound and unbound) diazepam. This increase in binding has been previously observed (Naranjo *et al.*, 1980a, Naranjo *et al.*, 1980b), Other investigations have failed to detect changes meal-related changes in diazepam free fraction (Klotz *et al.*, 1977; Kortilla and Kangas, 1977); however, the free fractions were unusually high during these studies, and accordingly, the discrepancies may be related to the precision and sensitivity, as well as the timing of the plasma protein binding determinations (Naranjo *et al.*, 1980a; Naranjo *et al.*, 1980b).

The change in diazepam binding with meals is believed to be due to an effect of feeding on free fatty acids (Naranjo *et al.*, 1980a). The largest caloric reservoir in humans is triacylglycerol, also known as triglycerides, stored in adipose tissue. Triglycerides are broken down to glycerol and free fatty acids (FFAs), and their degradation is increased during starvation and other situations (e.g. exercise) in which glucose cannot meet the fuel requirements of the body (Ruderman *et al.*, 1995). The breakdown of triglycerides is regulated by a hormone-sensitive lipase within the adipocyte. Catecholamines, glucagon, and several other hormones that increase cAMP by activating adenylate cyclase, activate adipose lipase by this means (Khoo *et al.*, 1973; Stryer, 1988). The increase in intracellular cAMP activates a protein kinase, and the lipase is subsequently activated through phosphorylation. The enzyme is

inactivated by dephosphorylation. Insulin can inactivate of the lipase by either decreasing cAMP, or by increasing the activity of a phosphoprotein phosphatase (Ruderman *et al.*, 1995). When FFAs enter the circulation, they bind to albumin, which facilitates their transport and diminishes their potential toxicity.

The principal fatty acids found in human plasma are oleic (43%), palmitic (24%), stearic (13%), linoleic (10%), and palmitoleic (5%) (Ruderman *et al.*, 1995). The binding of benzodiazepines at site II of albumin is generally inhibited when FFAs are complexed with albumin. The degree of inhibition is dependent upon the length of the FFAs, as middle-chain FFAs with 10 or 12 carbon atoms have the strongest inhibitory effect (Menke *et al.*, 1989; Wilkinson, 1983). The mechanism of the competition is also dependent upon the length of the fatty acid chain. Short chain FFAs (chain length smaller than C10) appear to bind to the benzodiazepine binding site on albumin and produce a direct competitive inhibition. Long chain FFAs (chain length greater than C10) bind to sites that are distant from the benzodiazepine binding site and inhibit binding through allosteric inhibition (Menke *et al.*, 1989). The molar ratio of FFAs:albumin may also be important for FFAs which allosterically inhibit benzodiazepine binding. Wong and Sellers (1979) found that with palmitic acid, molar ratios of 2 or greater decreased diazepam binding to albumin, whereas ratios of 0.5 and 1 increased binding. Oleic acid caused decreased binding at all studied molar ratios. The authors concluded that the increased binding observed with low amounts of palmitic acid was the result of a stabilization of the albumin tertiary complex.

Following a meal, plasma levels of FFAs fall, due to a decrease in the activity of hormonally-controlled adipocyte lipases. As plasma FFA levels drop, the inhibition of diazepam binding lessens, such that each molecule of albumin is able to bind quantitatively more diazepam. This leads to a re-distribution of diazepam out of peripheral tissues and into the systemic circulation with the result that large peaks occur in the plasma concentration-time profile. This explanation is also supported by the

large concordant peaks in desmethyldiazepam concentrations. Like diazepam, desmethyldiazepam free fraction increases as plasma FFA concentrations increase (Routledge *et al.*, 1980). On the other hand, the free fraction of lorazepam, a benzodiazepine which does not show large post-prandial peaks during multiple-dosing, is not affected by feeding (Chaudhary *et al.*, 1993) or heparin-induced changes in FFAs (Desmond *et al.*, 1980). The change in circulating FFAs is a gradual process, with the maximal decreases appearing approximately two hours after a meal (Golay *et al.*, 1986, Owen *et al.*, 1980). This is consistent with the time course of the post-prandial diazepam and desmethyldiazepam peaks observed in the present study, and may explain why no further changes in binding occurred after the second meal, which was given four hours after the first. It should be noted that the appearance of the post-prandial peaks may be complicated by the type of meal consumed; the intrinsic contribution of fat from high-fat meals can prevent any decrease in total plasma FFA levels (Karpe *et al.*, 1992) or may increase circulatory FFAs (Heimberg *et al.*, 1974).

If post-prandial diazepam peaks were due to increases in plasma protein binding as a result of a fall in plasma FFAs, then an increase in FFAs should produce an opposite effect. This was observed in this study with both the heparin infusions and the infusion of fat emulsion, although the overall effect on the diazepam concentration-time profile differed between the two treatments. Heparin infusions caused sharp, precipitous drops in total diazepam concentrations, accompanied by a dramatic increase in diazepam free fraction, both of which returned to baseline levels within 30 minutes. Infusion of fat emulsion also produced a drop in the total diazepam concentrations, attendant upon an increase in diazepam free fraction. However, the effect of the fat emulsion was more sustained, and lasted for several hours after the infusion was stopped, whereupon total diazepam concentrations rebounded to higher than expected (fasted) levels.

Both heparin and fat emulsion infusions mediate their effects through lipoprotein lipase (LPL). LPL hydrolyzes triglycerides in chylomicrons (formed in the intestinal epithelium) and very low density lipoproteins (produced in the liver), releasing FFAs and monoglycerides, into the circulation which move easily along and across cell membranes and aqueous spaces in the tissue (Olivecrona *et al.*, 1993). LPL is produced by the parenchymal cells of many tissues, most notably the adipose tissue, heart and skeletal muscles, and lactating breast tissue (Eckel, 1989). It is transferred to the luminal surface of vascular endothelial cells, where it is tethered to heparan sulfate chains. Binding of apolipoprotein C-II is necessary for LPL activation. LPL is active in a dimeric configuration, a state which is stabilized by binding to glycosaminoglycans (heparan sulfate) and lipid (with or without apolipoprotein C-II).

Heparin competes with endothelial binding sites for LPL (Eckel, 1989). The activity of the LPL that is subsequently released is also markedly increased, presumably through an effect of heparin enhancing the stability of the active form of the lipase. Heparin does not increase the activity of the purified enzyme (Eckel, 1989). Following intravenous administration, the release and actions of LPL are rapid. Studies with rats show that plasma LPL activity is maximal within 30 seconds after heparin administration (Hultin *et al.*, 1992), with FFAs returning to baseline levels over 15-30 minutes (Desmond *et al.*, 1980). Since the post-prandial changes in diazepam and desmethyldiazepam concentrations, and changes in free fractions had the same general time frame, and the mechanism with respect to the role of FFAs was well worked out in the feeding experiments, a cause and effect can be established. Thus, heparin caused marked increases in diazepam free concentrations coincident with a large, but transient, re-distribution effect. The finding that heparin had no effect on the clearance of total (bound and unbound) or free diazepam is not surprising. The changes are too short-lived; diazepam would be expected to rapidly re-distribute back to the tissues, and have little overall effect on the AUCs.

Circulating FFAs are also increased following infusions of fat emulsions. Intravenous fats are metabolized in the same manner as natural chylomicrons. Liposyn II (20%) is a fat emulsion solution, of which the major fatty acids are linoleic (65.5%), oleic (17.7%), palmitic (8.8%), linolenic (4.2%), and stearic (3.4%) (Bennett, 1991). Like heparin, infusions of fat emulsion cause the release of LPL from endothelial binding sites, although the release is slower with fat emulsion and is believed to occur through a different mechanism, possibly through release of FFAs during metabolism of the fat particles (Peterson *et al.*, 1990, Hultin *et al.*, 1992). Once activated by fat emulsion, LPL activity remains elevated until its substrate, the fat emulsion particles, are cleared from the circulation. In the present study, the changes (depression) in the concentration-time profiles of diazepam started with the infusion of the fat emulsion, but continued for some time after. The observation that the levels rebounded to higher than expected levels again suggests that there is a redistribution of drug from the systemic circulation to a peripheral compartment, which is then returned once the fat emulsion was cleared from the blood. The fat infusions caused a 2-3-fold increase in diazepam free fraction, which was not due to an increase partitioning into the lipemic portion of the plasma, as measured by binding experiments with 'fat-free' plasma. Also, the free clearance of diazepam appeared to be decreased, in the range of the values observed in the feeding studies.

Another explanation for the increases in the plasma diazepam concentrations is a reduction in its hepatic metabolism. This also contributes to the feeding effect, since the clearance of free diazepam was also decreased. If ingestion of the meal caused only a displacement of diazepam from its binding site on albumin, one would have expected a decreased free fraction, but no effect on either free concentrations or free clearance. However, both the clearance of free diazepam and the ratios of metabolite to parent drug (1.54 pre-meal vs. 1.35 post-meal) decreased by 12-15%. Since diazepam is a

low clearance drug in man, this is strongly suggestive of a decrease in hepatic oxidative metabolism of the drug.

Many investigations have examined the effect of dietary components on drug metabolism, however the relationship is complex. A significant body of literature indicates that FFAs can inhibit a number of metabolic processes. Addition of unsaturated fatty acids to an incubation mixture of substrate with microsomal enzymes inhibited the metabolism of the drug substrate (DiAugustine and Fouts, 1969). Similarly, microsomes from rats treated intraperitoneally with unsaturated fatty acids had decreased rates of drug oxidation and glucuronidation reactions (Lang, 1976). A significant positive correlation between FFA levels and the decrease in intrinsic clearance of valproic acid was observed in rhesus monkeys infused with a fat emulsion (Johno *et al.*, 1982). The changes in free clearance of diazepam in the present study during feeding, and with infusion with fat emulsion are in keeping with these data.

Effects on drug metabolism have been seen with other dietary components. Co-infusion of amino acids with propranolol at steady-state in isolated perfused rat livers resulted in an increase in steady-state levels of the drug, and a decrease in propranolol metabolites, suggesting an inhibition of propranolol metabolism (Semple and Xia, 1995).

The reason for the decrease in hepatic metabolism during meals may be due to co-substrate inhibition. Reduced nicotinamide adenine dinucleotide phosphate (NADPH) is the major electron donor in reductive biosyntheses, and is an essential co-factor in the synthesis of fatty acids (Styrer, 1988). NADPH is also an essential co-factor in the oxidative metabolism of drugs through the CYP450 system and is an important rate-controlling factor for drug metabolism in intact cells (Qu *et al.*, 1994). It appears from this and other work that treatments which promote fatty acid synthesis (carbohydrate feeding) direct the utilization of NADPH or other co-factors toward FFA in preference to other functions. On the other hand, factors which depress fatty acid

synthesis (starvation, fat ingestion) may free NADPH for utilization in drug metabolism reactions. An alternate suggestion has been made by Qu *et al.* (1994), who found that O-demethylation of a saturating concentration of *p*-nitroanisole was significantly increased in perfused livers of food-restricted rats. However, no effect was observed in isolated microsomes in which excess NADPH was added. The authors concluded that the increase in drug metabolism was the result of an increase in NADPH levels secondary to a increase in β-oxidation of free fatty acids.

The effects of heparin and infusion of fat emulsions on free clearance are likewise explained by the high levels of circulatory FFAs. With heparin infusions, some of the effect on free clearance may be artifactual. Free diazepam clearances were calculated based on free diazepam concentrations, which in turn are dependent upon total diazepam concentrations and free fraction measurements. During the heparin infusion, diazepam free fractions were markedly elevated; however, it has been shown that some of the effects of heparin on the release of FFAs from in human plasma occur *in vitro* (Giacomini *et al.*, 1980; Brown *et al.*, 1981). Since heparin was also used as an anticoagulant in sample collection tubes and no inhibitor of lipoprotein lipase were added to the blood samples, some of the decrease in diazepam binding could indeed be experimental artifacts. Why the clearances of free diazepam appear to be increased with neo/chol treatment during the day is unknown at this time. Treatment with lipid lowering agents have been shown to cause proliferation of endoplasmic reticulum and to induce CYP450 enzymes (Orton and Parker, 1982). It is also possible that neo/chol decreased the availability of diazepam, and there was a confounding influence on diazepam free fraction and total clearance.

In summary, changes in the binding of diazepam and desmethyldiazepam to plasma proteins induced by FFAs very likely explain post-prandial peaking in drug concentration, as well as the effects of heparin and fat emulsion. These produce rapid, transient increases in free diazepam concentration, which are then buffered by extensive

redistribution into peripheral tissues. However, feeding also appears to cause a more sustained increase in free diazepam concentrations, suggesting a concomitant decrease in hepatic oxidative metabolism, or a possible redistribution from peripheral tissues. This is a well known effect of FFAs in animals and is shown here to occur in humans during fasting or infusion of soluble fat.

According to receptor occupancy theory, an increase in free drug concentrations in drug responsive tissues causes an increase in drug effect. Miller *et al.* (1986) demonstrated that benzodiazepine receptor occupancy is predictably related to brain and plasma drug concentrations over a wide range of doses and at various times after drug administration. This is particularly the case with diazepam where single intravenous dose studies have shown only a small lag-time between concentration and effect, owing to its high lipid solubility. Several studies have attempted to establish pharmacokinetic-pharmacodynamic relationships for benzodiazepines; however, the results of these studies are complicated by the objectivity of the measurements, as well as by differences in study design (Laurijssens and Greenblatt, 1996). In a multiple-dose study of diazepam in patients with cirrhosis and age- and sex-matched controls, plasma concentrations of diazepam and desmethyldiazepam correlated well with self-rated sedation in both groups; however, unbound drug concentrations were not determined (Ochs *et al.*, 1983). Free diazepam concentrations were measured in the present study, and it was shown that they increase markedly following a meal. Drug effect was not measured. However, what is important is the magnitude and rapidity with which these large movements of drug from one location in the body to another occur. Whether the pharmacokinetic changes related to post-prandial diazepam peaks result in pharmacodynamic changes in drug response is unknown, and further study is warranted in this area.

6. CONCLUSIONS

1. The glucuronidation of lorazepam may be increased by ingestion of an exogenous carbohydrate load. This is shown in patients with IDDM where insulin and glucose levels can be strictly controlled. However, these patients had no evidence of diabetic organ disease and are believed to represent the effects of glucose in normal healthy human subjects. This suggests that under certain conditions, co-substrate availability is a rate-determining step in the glucuronidation of compounds in man. The clearance of lorazepam glucuronide is similarly increased, suggesting that glucuronide transport into the glomerular filtrate by the kidney is either directly glucose dependent or indirectly affected by the energy-state of the individual, or that glucuronidation is occurring in the kidney, and that this is linked to renal glucuronide excretion. Lorazepam undergoes an enterohepatic circulation in patients with insulin-dependent diabetes mellitus. However, the extent to which lorazepam participates is dependent upon the rate of hepatic glucuronidation. Patients who are fasting, or in a low energy-state have low rates of glucuronidation and correspondingly low cycling fractions.

2. No effect of insulin, or insulin species (human vs. beef/pork) on the glucuronidation of lorazepam in IDDM patients could be demonstrated. Similary, an effect of glucagon on the disposition of lorazepam in IDDM patients was not observed..

3. Insulin has a direct effect on urine production in patients with IDDM. High levels of insulin depress urine production, whereas insulin deficiency may produce large volumes of urine, irrespective of the blood glucose level. This suggests that the

relative lack of insulin, along with hyperglycemia, may be important in the polyuria that is observed in this disease.

4. Lorazepam undergoes EHC in the dog. Treatment with neomycin and cholestyramine is effective in blocking the enterohepatic circulation of lorazepam. The animal model described in this investigation is suitable for studying the enterohepatic circulation of drugs.

5. Post-prandial diazepam peaks are not caused by enterohepatic circulation. Rather, they are the result of changes in plasma protein binding brought about by decreases in FFAs and redistribution of drug from peripheral stores. However, feeding is also associated with an inhibition of metabolism of diazepam which contributes to the size of the post-prandial diazepam peaks. These peaks can be quite large, particularly with chronic dosing of diazepam, and since free diazepam concentrations are likewise increased, it is logical to assume that a concomitant increase in pharmacological effect may occur. Pharmacodynamic testing should be done to see if any change occurs in the activity of the drug with the post-prandial peaks.

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