

**ROLES OF AMINO ACIDS IN THE FOOD EFFECT:
METABOLIC AND PHARMACOKINETIC STUDIES ON
PROPAFENONE AND METOPROLOL**

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in the College of Pharmacy and Nutrition
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ABSTRACT

The bioavailability of some high hepatic first-pass drugs, such as propafenone and metoprolol, has been shown to increase substantially when they are orally administered with a protein-rich meal in humans. A change in hepatic metabolic capacity, induced by dietary amino acids, may be one of the mechanisms contributing to this food effect. In this project, an attempt was made to elucidate the possible mechanism by examining the kinetic and metabolic interaction of propafenone and metoprolol with amino acids in animal models including the isolated, perfused rat liver preparation and the intact rat.

Since the metabolism of propafenone in the rat has never been reported, kinetic and metabolic studies on propafenone were conducted in the isolated, perfused rat liver to ensure the feasibility of the interaction study. Firstly, a sensitive and convenient HPLC method for the analysis of propafenone enantiomers in rat liver perfusate and human plasma has been developed using Tetra-O-acetyl- β -D-glucopyranosylisothiocyanate as a chiral derivatizing reagent. A pilot study employing this assay in the isolated, perfused rat liver showed that propafenone was highly bound to hepatic tissue and its disposition was stereoselective. Secondly, by using HPLC/MS and tandem mass spectrometry with electrospray and atmospheric pressure chemical ionizations, phase I and phase II metabolites in rat liver perfusate were structurally identified and two novel glucuronides of hydroxylated propafenone derivatives were found. The levels of metabolites were determined simultaneously by a newly developed HPLC method with UV detection. The results revealed that a propafenone derivative

hydroxylated in the ω -phenyl ring (ω -OH-PF), which was not found in humans, was the major phase I metabolite in the rat liver, whereas N-despropylpropafenone and 5-hydroxypropafenone were of minor importance in quantity. Based on these findings, it was concluded that propafenone was not an appropriate model drug for studying the drug-food interaction in the isolated, perfused rat liver.

The interaction between metoprolol and amino acids was studied in the isolated, perfused rat liver and the *in vivo* rat. First of all, a modified and revalidated HPLC method for the simultaneous analysis of metoprolol and its metabolites, α -hydroxymetoprolol and O-demethylmetoprolol, in rat liver perfusate and rat plasma was described. Twenty-four livers from Sprague-Dawley rats were divided into three groups, and perfused in either antegrade or retrograde flow directions with 5.48 μ M of metoprolol in an erythrocyte-enriched Krebs bicarbonate buffer, and meanwhile coin fused with a balanced mixture solution of amino acids. A transient and reversible reduction in metoprolol metabolism was observed in the presence of amino acids. The magnitude of the reduction was dependant on the direction of perfusion, the hepatic oxygen delivery and the concentration of amino acids, consistent with the hypothesis that inhibition of metoprolol metabolism was partially due to oxygen deprivation in the pericentral zone of the liver. This finding is relevant to the food effect observed in humans. Finally, a parallel study was carried out, in which eighteen Sprague-Dawley rats were orally given metoprolol (10 mg/kg) with either water or a mixture solution of amino acids. The AUC_{oral} of metoprolol showed an increasing trend with amino acids, while the t_{\max} of metabolites was significantly prolonged. The changes in these pharmacokinetic parameters, probably consequent to a temporary inhibition of hepatic

metoprolol metabolism by amino acids, resembled the food effect in humans. In conclusion, the results from both *ex vivo* and *in vivo* studies strongly support the proposed mechanism.

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To my parents

for their love and support

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LIST OF ABBREVIATIONS

%O ₂ Sat	percentage of oxygen saturation of hemoglobin
α -OH-ML	α -hydroxymetoprolol
λ_z	2.303 times the slope of the terminal exponential phase of a plot of log concentration vs. time.
+ESI	positive ion electrospray ionization
4'-OH-3'-OCH ₃ -PF	4'-hydroxy-3'-methoxypropafenone
4'-OH-PF	4'-hydroxypropafenone
5-OH-4-OCH ₃ -PF	5-hydroxy-4-methoxypropafenone
5-OH-PF	5-hydroxypropafenone
AA	amino acid
AAs	amino acids
Ante	antegrade perfusion
APCI	atmospheric pressure chemical ionization
AUC	area under the plasma concentration versus time curve
AUC _{0-∞}	area under the concentration-time curve from time 0 to infinity
AUC _{0-60min}	area under the concentration-time curve from 0 to 60 min
AUC _{0-t*}	area under the concentration-time curve from time 0 to time t*
AUC _{oral}	area under the plasma concentration versus time curve after a single oral dose

$AUMC_{0-\infty}$	area under the first moment concentration-time curve from time 0 to infinity
$AUMC_{0-t^*}$	area under the first moment concentration-time curve from time 0 to time t^*
C^*	last measurable concentration
CID	collision-induced dissociation
C_{in}	steady-state concentration of drug entering the liver
CL	clearance
CL_f	metabolite formation clearance
CL_H	hepatic clearance
CL_I	apparent intrinsic hepatic clearance
CL_{int}	hepatic intrinsic clearance
CL_{oral}	oral clearance
C_M	effluent metabolite concentration at steady state
C_{max}	peak plasma concentration
C_{out}	steady-state concentration of drug leaving the liver
CV	coefficient of variation
CYP	cytochrome P-450
E	hepatic extraction ratio
ESI	electrospray ionization
f_u	unbound fraction of drug in the blood
GITC	tetra-O-acetyl- β -D-glucopyranosylisothiocyanate
Hb	hemoglobin content (g/dl)

HCl	hydrochloride salt
HPLC	high performance liquid chromatography
HPLC/MS	high performance liquid chromatography/mass spectrometry
IS	internal standard
LiverWt	weight of the liver
LNAAs	large neutral amino acids
LOD	limit of detection
LOQ	limit of quantitation
ML	metoprolol
MRT	mean residence time
MS	mass spectrometry
MS/MS	tandem mass spectrometry
N-des-PF	N-despropylpropafenone
O ₂ Csm	oxygen consumption rate (μmol/min/g liver)
O ₂ Ct	oxygen content (ml/dl)
O ₂ Del	oxygen delivery rate (μmol/min/g liver)
O ₂ Ext	oxygen extraction (%)
O-Dem-ML	O-demethylmetoprolol
OH-PF	hydroxylated propafenone derivative
PF	propafenone
pO ₂	partial pressures of oxygen (mmHg)
Q	perfusion flow rate

QC	quality control
Q_H	hepatic blood flow rate
r	correlation coefficient
Retro	retrograde perfusion
RSD	relative standard deviation
t^*	time of the last measurable concentration
$t_{1/2(z)}$	half-life of the terminal phase in the concentration-time curve
TEA	triethylamine
t_{max}	time to reach the peak concentration
UV	ultraviolet
V_d	apparent volume of distribution
V_{max}	maximum velocity of metabolism
ω -OH-PF	propafenone derivative hydroxylated in the ω -phenyl ring

CHAPTER 1

INTRODUCTION

1.1 General considerations of food-drug interactions

It has long been recognized that the coadministration of food with medication may alter drug bioavailability (Anderson, 1988; Melander, 1978; Welling, 1977). Food may have marked effects on drug disposition by increasing or decreasing the extent or rate of drug absorption, distribution, metabolism and/or excretion (Roberts and Turner, 1988; Williams et al., 1996; Welling, 1977). The mechanisms by which food modifies drug efficacy are numerous, including purely chemical interactions and a wide range of physiologically mediated interactions at one or more phases of drug action (Figure 1.1). Acknowledging the importance of this phenomenon, most regulatory agencies around the world now require information on the effects of concomitant intake of food for virtually all new chemical entities and new modified-release dosage forms as well (Salmonson, 1993). A good understanding of drug-food interactions is also needed to provide a basis for predicting the extent of these interactions.

Drug-food interactions refer to the instances in which food alters an individual's response to a drug or in which a drug interferes with an individual's nutrition (Hartshorn, 1977; Hathcock, 1985). This chapter will focus on the effects of food intake on the disposition of orally administered drugs.

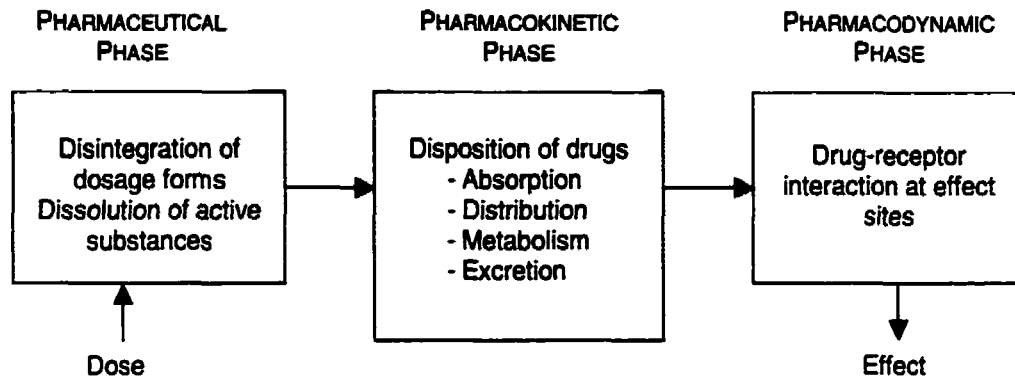


Figure 1.1. Phases of drug actions

1.1.1 Absorption

When a drug is administered by the oral route, it is absorbed from the gastrointestinal tract and then enters the systemic circulation, from which it is eventually delivered to the site of action. Absorption of drugs consists of numerous processes (Shargel and Yu, 1999). These processes include: 1) disintegration of the drug product and subsequent release of the drug; 2) dissolution of the drug in the acid gastric juice or in the more alkaline and biliary milieu of the small intestine; and 3) absorption across cell membranes of the gastrointestinal wall into the portal blood. Drugs may be absorbed by passive diffusion or by active transport from any part of the alimentary canal. For most drugs, the optimum site for drug absorption is the proximal region of the small intestine because its unique anatomical brush structure provides an immense surface area for the drug to diffuse. Some absorption, particularly that of acidic or neutral compounds that may be soluble and in an unionized state in gastric fluids, may occur efficiently from the stomach. Drugs may also be absorbed from distal regions of the intestinal tract.

Once they are absorbed into the splanchnic circulation, drugs are carried to the liver via the portal vein, and thence by the hepatic vein into the systemic circulation. During this passage, drugs are exposed to the metabolizing enzymes of the liver and may undergo first-pass hepatic metabolism or biliary excretion. First-pass metabolism may also occur when certain drugs pass through the gastrointestinal wall where they may be transformed by metabolic enzymes.

Probably the most important interaction between drugs and nutrients is the influence of food on the absorption phase of a drug. A number of studies indicate that food may enhance or decrease the bioavailability of a drug. Absorption of drugs may be reduced, increased, delayed or unaffected by the physiological changes that occur in the gastrointestinal tract in the fed state compared with the fasting state, or by direct physical or chemical interactions between drug products and food molecules (Welling, 1984). The magnitude of a food-drug interaction is dependent on the physical and chemical nature of the drug, the formulation in which the drug is administered, the type and size of a meal, and the time interval that elapses between eating and drug administration (Welling, 1996).

1.1.1.1 Influence of food on the gastrointestinal tract

Ingested food produces a number of physiological effects on the gastrointestinal tract, and thereby affects the absorption of drugs. These effects are well summarized by P.G. Welling (1989) in Table 1.1.

Table 1.1. Physiological interactions due to ingested food and fluid which may influence drug absorption

Physiological function	Effect of food	Possible effect on drug absorption
Stomach emptying	Decreased rate with solid meals, fats, high temperature, acids, solutions of high osmolarity. Increased rate with large fluid volumes	Absorption generally delayed, may be reduced with unstable compounds, may be increased due to drug dissolution in stomach. Absorption increased with large fluid volumes
Intestinal motility	Increased	Faster dissolution and decreased diffusional path promotes absorption. Shorter transit time may reduce absorption
Splanchnic blood flow	Generally increased, but may be decreased by ingestion of glucose	Absorption increased with faster blood flow. Variable effects on first-pass metabolism, depending on drug
Bile secretion	Increased	Absorption may increase due to faster dissolution or decrease due to complexation
Acid secretion	Increased	Increased absorption of basic drugs provided they are acid stable. Decreased absorption of acid labile compounds
Enzyme secretion	Increased	Increased or decreased absorption depending on drug characteristics
Active absorption process	Increased	Active drug absorption may be reduced by competitive inhibition

Taken from Welling, 1989.

I. Gastrointestinal motility

Gastric motility. Ingested food tends to inhibit gastric motility, i.e. the rate of stomach emptying, primarily due to feedback mechanisms from receptors situated in the proximal small intestine. Gastric emptying time is prolonged by hot food, fat and to a lesser extent, protein and carbohydrates, in the stomach (Welling, 1989). Large fluid volumes, on the other hand, accelerate stomach emptying.

As a consequence, the most commonly observed effect of food is to delay absorption of the drug due to its delayed presentation to its major site of absorption in the proximal small intestine. Delayed stomach emptying may delay absorption of acidic compounds or drugs in enteric-coated formulations by delaying drug transit from the acidic environment of the stomach to the relatively alkaline region of the small intestine. An excellent example is that absorption of aspirin enteric-coated tablets is markedly delayed by food with no drug appearing in plasma until 4 hr after dosing (Bogentoft et al., 1978). Delayed drug absorption means that the time for the drug to reach its peak blood level after a single dose is lengthened. It is generally considered not clinically important for the majority of drugs. For drugs such as antibiotics which may need to produce high serum levels quickly, however, a delay of absorption may be significant.

Moreover, prolonged gastric emptying time may promote absorption of some drugs because it permits more drug to be dissolved in the stomach before passing on to the small intestine to be absorbed. This is particularly true for drugs that have poor solubility at acidic gastric pH and slowly dissolving formulations. It has been shown that food enhances the bioavailability of nitrofurantoin in dosage forms that exhibit poor dissolution characteristics (Rosenberg and Bates, 1976). Increased nitrofurantoin

absorption may be attributed to better dissolution of the weakly acidic nitrofurantoin molecule by food-induced slow stomach emptying, as judged from studies employing anticholinergic pretreatment (Melander, 1978; Welling, 1977).

Prolonged stomach retention may also increase the absorption efficiency of drugs that are absorbed by saturable mechanisms or that exhibit an 'absorption window' effect, by prolonging the time during which the drug is in contact with the active site. Increased absorption of riboflavin (Levy and Jusko, 1966) and riboflavin-5'-phosphate (Levy and Jusko, 1967) in the presence of food, particularly after high drug doses, has been shown to be consistent with a site-specific saturable absorption mechanism. In fasted subjects, high doses of vitamins saturated the absorption site in the small intestinal tract, resulting in reduced absorption efficiency. When the vitamin was taken with food, the reduced gastric emptying rate decreased the rate of drug passing the active absorption site and facilitated complete absorption over a wide dose range.

Nevertheless, slow stomach emptying may decrease the absorption of acid labile compounds, such as penicillin (Welling, 1977), erythromycin (Melander, 1978) and didanosine (Knupp et al., 1993). This is due to prolonged residence in the acidic gastric fluids, leading to degradation of these acid-labile drugs.

Intestinal motility. In contrast to inhibition of gastric motility, ingested food stimulates intestinal motility. Increased intestinal motility after food intake may increase drug availability due to more drug dissolution and greater exposure of drug molecules to the intestinal epithelial surface, but may also decrease availability because of a reduced intestinal transit time.

II. Gastrointestinal secretions

Ingestion of food increases most gastrointestinal tract secretions, including digestive enzymes, hydrochloric acid and bile. All of those could increase drug availability depending on the acidic or basic nature and lipophilicity of the drug or drug dosage form.

Biliary secretion. Increased bile flow by dietary fat may enhance absorption of certain highly lipid-soluble drugs as a result of the solubilizing and emulsifying activity of bile acids. This is believed to be the mechanism that explains the increased bioavailability of griseofulvin (Welling, 1977), cefuroxime (Mackay et al., 1992) and fenretidine (Doose et al., 1992) with a meal containing fat. Griseofulvin is an antifungal drug with very low water solubility and hence poor bioavailability. Absorption of griseofulvin has been shown to be increased by high-fat meals but not by high-protein or high-carbohydrate meals (Welling, 1977). As griseofulvin is an extremely lipophilic molecule, its dissolution and hence absorption may be accelerated directly by the present of fat in the meal and indirectly by fat-stimulated bile secretion. A mechanistic study on cefuroxime showed that hyoscine butylbromide had no effect on cefuroxime absorption whereas cholecystokinin resulted in a 20% increase in cefuroxime C_{\max} and AUC values (Mackay et al., 1992). These results lead to the conclusion that bile release, but not gastric emptying, may be at least partially responsible for increased cefuroxime absorption in the presence of food. On the other hand, for very hydrophilic compounds, such as β -blocking agents, atenolol and sotalol, absorption is reduced by food-induced bile secretion (Barnwell et al., 1993). The mechanism by which food decreases atenolol absorption was addressed in a study conducted with healthy volunteers who received

single doses of a commercial tablet, or of a capsule containing bile acids. It is claimed that bile acids reduce systemic availability of atenolol to a similar or greater extent than that for ingested food.

Acid secretion. Increased acid secretion into the stomach may promote dissolution of basic drugs, but may prevent dissolution of acidic drugs. It may cause degradation of acid-labile compounds. Increasing gastric acidity may also affect drug absorption via interactions with the drug formulation. For most enteric-coated products, absorption may be considerably delayed, and possibly reduced in the presence of food, due to the combined effects of slow gastric emptying and increasing acid secretion.

Digestive enzyme secretion. Increased secretion of proteolytic enzymes into the duodenum and proximal small intestine may have a variable effect on drug absorption, depending on their interaction with particular characteristics of drugs and formulations.

III. Splanchnic blood flow

Food ingestion affects splanchnic blood flow, but the degree and direction of change vary with the type and size of meal (Welling, 1996). A high protein meal has been shown to cause a 35% increase in splanchnic blood flow, whereas a liquid glucose meal causes an 8% decrease (Svensson et al., 1983). In most cases after solid meals, one would expect splanchnic blood flow to increase. Increased postprandial splanchnic blood flow has been touted as one of the mechanisms involved in decreased first-pass metabolism, and hence increased systemic availability of some high hepatic first-pass drugs, such as propranolol and metoprolol (Axelson et al., 1987; Melander and Mclean, 1983; Olanoff et al., 1986).

1.1.1.2 Direct food-drug interactions

In addition to the indirect effects on gastrointestinal physiology, food may also affect drug absorption directly. Typically, food may act as a physical barrier inhibiting drug dissolution and preventing drug access to the mucosal surface of the gastrointestinal tract. Drug absorption may be reduced by chelation with specific ions or complexation with other substances in food. For drugs that are actively absorbed, a direct competition for active carriers may occur between food components and drug molecules, resulting in decreased systemic availability. The relative contributions of direct interactions and indirect interactions resulting from changes in gastrointestinal physiology are uncertain. Both may contribute to a varying degree depending on meal types, temporal relationships between eating and dosing, chemical and physical properties of the drug, and formulations.

Inactive complexes formed between drugs and nutrients by chemical interactions are the most common known phenomena of direct food-drug interactions. The reaction of tetracyclines with calcium in dairy products is an example of this type of interaction (Welling, 1977). Tetracyclines have a strong tendency to form chelates with divalent and trivalent cations. The complexes formed between these cations and tetracyclines are usually insoluble and not absorbed. Therefore, when tetracyclines are taken with milk or other dairy products containing calcium and magnesium, their absorption efficiencies are reduced. The extent of influence by milk differs among various tetracyclines, depending upon their binding affinities to calcium ions. Fluoroquinolones, such as ciprofloxacin (Neuvonen et al., 1991) and norfloxacin (Kivistö et al., 1992), are also

known to bind with heavy metal ions, including calcium, to form insoluble chelates. It has been demonstrated that coadministration with milk or yogurt greatly reduces the systemic availability of these drugs.

Some specific dietary components can result in a transient or net reduction in drug absorption by direct interactions with drugs. The absorption of acetaminophen is delayed and decreased when its intake is associated with pectin ingestion (Welling, 1977). Pectin acts as an adsorbent and protectant in the gastrointestinal tract and may delay drug absorption by adsorption, complexation, or increase in the viscosity of gastrointestinal contents. Dietary fiber components, which have anionic and cationic sites, may adsorb, combine or exchange with the drug, resulting in decreased availability of the drug for absorption. The combination with such fiber components might also serve as a depot for slow release of the drug.

L-Dopa presents an interesting example of how food may reduce drug absorption by competitive inhibition at the absorption site. Coadministration of L-dopa with a high protein meal reduces plasma levels of L-dopa (Roos et al., 1993) and inhibits the therapeutic effect (Welling, 1977). L-Dopa, a neutral amino acid precursor of dopamine, is absorbed and transported by a specific uptake system which also carries other large, neutral amino acids. L-Dopa absorption hence may be inhibited by the competition for absorption with dietary amino acids in the high protein meal.

1.1.2 Distribution

After a drug reaches the systemic circulation, it may be distributed into other body fluids and tissues. The most important pharmacokinetic parameter to express the

degree of drug distribution is the apparent volume of distribution (V_d). The value of V_d depends on such factors as: 1) the drug's binding to blood components, i.e. albumin and other serum proteins; 2) its binding to different tissues; 3) its solubility in body fluids and fats; and 4) its ability to penetrate barriers. A high V_d indicates a wide distribution of a drug, both in the blood and outside, whereas a low V_d indicates the confinement of a drug primarily in the blood. An increase in the V_d of a drug may reduce the peak plasma concentration of the compound. This may result in altered therapeutic responses and adverse reactions.

Most literature refers to the effects of chronic nutrition intake, which results in undernourishment and malnutrition, on drug distribution, but few report acute regulation of drug distribution by food ingestion. An example is L-dopa mentioned in Section 1.1.1.2. L-Dopa, a neutral amino acid precursor of dopamine, exerts its antiparkinsonian effect after delivery to the brain. It has been clearly shown that a high-protein diet can inhibit the therapeutic effect of L-dopa (Welling, 1977). Protein consumption increases the levels of large neutral amino acids (LNAAs) in the plasma, which share the same transport mechanism as L-dopa to cross the blood-brain barrier. Since all of the LNAAs in the plasma compete for the same uptake system, the amount of L-dopa entering the brain depends on the ratio of L-dopa concentration to the sum of the LNAAs. Pincus and Barry (1987) demonstrated that regular and high-protein diets resulted in marked elevations in the plasma concentrations of the LNAAs. Despite elevated plasma L-dopa levels, all patients in their study with elevated LNAA levels experienced Parkinsonian symptoms. When the LNAA levels dropped while L-dopa plasma levels remained elevated, the subjects experienced a relief of symptoms. On a low-protein diet, however,

the LNAAS plasma levels remained low and all subjects were consistently dyskinetic even though the mean plasma levels of L-dopa were lower than when the subjects consumed a high-protein diet. These results suggest that coadministration of the high-protein meal decreases the therapeutic response of L-dopa primarily by reducing the amount of L-dopa that penetrates into the brain.

Ingestion of a high-fat meal may potentially alter drug distribution. Digestion and absorption of a high-fat meal may lead to a substantial increase in plasma free fatty acid levels. These molecules bind to the same sites on albumin as many drugs, which results in competitive binding and competitive displacement when both drug and nutrient are present. However, in the normal range of free fatty acid concentrations, even with the variation introduced by meals, the amount of drug displaced ranges from almost none to about 30% (Hathcock, 1985). This slight effect of fatty acids suggests that it is possible that following high-fat meals some drugs will be displaced from albumin, but this food-nutrient interaction is enough to enhance the pharmacological and toxicological actions to only a small degree.

1.1.3 Metabolism

Many drugs are metabolized in the liver and sometimes also at extrahepatic sites, such as the gastrointestinal tract, lungs, etc. The capacity of hepatic metabolism is described as the hepatic clearance of drugs, depending on the activity of enzymes responsible for biotransformation, and hepatic blood flow, which determines the rate of delivery of drugs to the liver.

Biochemically, drug metabolism can be classified into two phases on the basis of biotransformation reactions: 1) phase I: oxidation reactions, including hydroxylation, epoxidation, peroxidation and oxidative dealkylation, reduction and hydrolysis; and 2) phase II: conjugation reactions, including glucuronidation, acetylation and sulfation. By far the most important enzymes involved in phase I metabolism are the cytochrome P-450 (CYP) system. Among various CYP isoenzymes, the CYP3A subfamily represents the predominant and most abundant enzymes in the liver (approximately 30% of total hepatic CYP content), and this family of enzymes is also expressed in the gastrointestinal tract (Shimada et al., 1994; Walter-Sack and Klotz, 1996).

Concurrent intake of food may affect drug metabolism in two ways. Firstly, food may transiently alter presystemic drug metabolism of moderate to high hepatic clearance drugs, such as propranolol, metoprolol and propafenone (Melandar and Mclean, 1983). This issue will be discussed thoroughly in Section 1.2. Secondly, some dietary macronutrients and micronutrients may exert a direct influence on drug metabolizing enzymes, such as CYP enzymes and conjugating enzymes. Generally, the amount and/or activity of most drug metabolizing enzymes can be altered by prolonged dietary changes, whereas some can be regulated acutely by specific food components or nutrients. It has been demonstrated that coadministration of grapefruit juice significantly increased the oral bioavailability of some drugs, of those most are CYP3A substrates, such as dihydropyridine calcium channel blockers, benzodiazepines and cyclosporine (Ameer and Weintraub, 1997). The predominant mechanism for the enhanced bioavailability is presumably inhibition of intestinal CYP3A4, but not hepatic CYP3A4

or colon CYP3A5, by bioflavonoid naringenin and furanocoumarins in grapefruit juice (Singh, 1999).

1.1.4 Excretion

The most important route for drug excretion is renal excretion. Drugs and their metabolites are excreted by the kidney via glomerular filtration, active tubular secretion, and passive reabsorption. Drugs that are effectively bound to plasma proteins are poorly filtered; and conversely, drugs that are not bound are cleared from the blood at a rate approximately equal to creatinine clearance. Some drugs are actively secreted by special mechanisms located in the mid-segment of the proximal convoluted tubule. Weakly acidic drugs, including many diuretic drugs, are secreted in this manner and may compete with endogenous acids such as uric acid for the carriers. Active secretion is a saturable process. Once in the tubular urine, the highly lipid-soluble, non-ionized drug molecules are rapidly and extensively reabsorbed; whereas polar compounds and ions are unable to diffuse across the renal epithelium and excreted in the urine. Since the metabolism of many drugs results in a less lipid-soluble product, the metabolite is more readily excreted than the parent compound.

Some drugs and their metabolites may be secreted by the liver cells into the bile and pass into the intestine. Some or most of the secreted drug may be reabsorbed from the lumen of the small intestines and undergo enterohepatic cycling; the rest is excreted in the feces. Biliary secretion is an active, capacity-limited process, subject to competitive inhibition. Other excretion pathways include salivary excretion and milk excretion.

Food-induced changes in absorption, distribution and metabolism of the drug lead to altered rates and patterns of drug excretion. There are, however, some direct effects of diet and nutrients on the excretory processes of drugs (Roberts and Tumer, 1988). For example, urinary pH has a major influence on the excretion of weakly acidic and basic drugs. All foods which tend to alkalinize or acidify the urine may influence the excretory pattern of drugs. High-fibre diets can cause loss of bile acids and more rapid biliary excretion of drugs that undergo extensive enterohepatic circulation, such as spironolactone and its metabolites (Roberts and Tumer, 1988).

1.1.5 Summary

The great number of articles published during the last 20 years involving interaction between drugs and ingested food illustrates elevating interest and demand in the area. Food ingestion can cause changes in pharmacokinetic patterns of drugs. The mechanisms involved in food-drug interactions are numerous, including effects of food on absorption, distribution, metabolism and excretion. Change in absorption is probably the most common mechanism responsible for food-drug interactions for orally administered drugs. In recent years, the influence of diet on drug metabolism has received more and more attention, in part due to the growing knowledge of drug metabolizing enzyme systems, such as cytochrome P-450 isoenzymes.

The above changes in drug pharmacokinetics may have a profound effect on the therapeutic effect. However, food may also potentiate or decrease the therapeutic effect of drugs, by directly altering drug pharmacodynamics. For example, the anticoagulant activity of warfarin is partially dependent on the presence or absence of vitamin K and

the effectiveness of warfarin may be decreased if an excessive amount of vitamin K is consumed. This would occur with the ingestion of such food as liver or leafy green vegetables. If the diet should change in this way, the action of warfarin would be antagonized and the clotting problems that were controlled by warfarin might reappear (Roberts and Turner, 1988). Because insufficient data exist, however, the clinical importance of the effects of food on various pharmacologic properties of drugs is still being investigated. It is only when an adverse drug reaction follows a food-drug interaction that the matter becomes of serious concern to the patient or clinician.

1.2 Food interaction with high hepatic first-pass drugs

1.2.1 Theoretical considerations of hepatic clearance

1.2.1.1 Hepatic clearance models

For a drug eliminated predominantly by the liver, the hepatic clearance approximates total body clearance. Based on mass balance principles, at steady state, the instantaneous rate of hepatic elimination is equal to the difference between the rate of drug delivery to the liver and the rate of exit from the liver. Accordingly, hepatic drug clearance (CL_H) is defined by

$$CL_H = \frac{Q_H \cdot (C_{in} - C_{out})}{C_{in}} = Q_H \cdot E \quad (1.1)$$

where Q_H represents the hepatic perfusion rate, E represents the extraction ratio across the liver, and C_{in} and C_{out} represent the concentrations of the drug entering and leaving the liver, respectively. Changes in hepatic clearance can only result from an alteration in Q_H and E .

Hepatic clearance can be thought of as a measure of the efficiency with which a drug is removed from the body by the liver. It depends on four main factors (Pang and Rowland, 1977): 1) hepatic blood flow rate (Q_H); 2) unbound fraction of drug in blood (f_u); 3) activity of hepatic drug-metabolizing enzymes, i.e. hepatic intrinsic clearance of the drug (CL_{int}); and 4) diffusion clearance of drug between the blood and hepatocytes. The diffusion clearance is dependent upon the dissociation of the drug-protein complex and the permeability of the hepatocyte to the drug. In general, the diffusion process is

assumed to be very rapid. Therefore, in the perfusion-limited case of hepatic elimination, the clearance is principally determined by the former three factors.

Several methods have been proposed to calculate the average hepatic concentration from hepatic inflow and outflow concentrations, each method leading to a different relationship between hepatic clearance and its three physiological determinants, so-called the 'hepatic clearance' models (Wilkinson, 1987). These developed clearance models include the venous equilibrium, undistributed sinusoidal perfusion, distributed sinusoidal perfusion, and dispersion models. All of the models provide similar predictions with respect to hepatic clearance. However, inter-model differences become remarkable in their predictions of how various pharmacokinetic parameters are influenced by changes in the determinants of hepatic clearance, particularly when hepatic clearance of the drug is high. The differences among various models of hepatic clearance can be best illustrated by considering the venous equilibrium and undistributed sinusoidal models (Pang and Rowland, 1977). In the venous equilibrium (or 'well-stirred') model, the liver is considered as a single well-stirred compartment in which the concentration of substrate is assumed to be uniform and equal to the concentration exiting from the liver. This is the same assumption as is made in linear compartmental analysis. Accordingly, hepatic clearance is defined by

$$CL_H = \frac{Q_H \cdot CL_{int} \cdot f_u}{Q_H + CL_{int} \cdot f_u} \quad (1.2)$$

In the undistributed sinusoidal (or 'parallel tube') model, the liver is regarded as a series of parallel identical tubes with enzymes distributed evenly around the tubes. It is assumed that the concentration of drug declines exponentially along the tubes in the

direction of flow, and the logarithmic average of inflow and outflow concentration is the concentration used in the Michealis-Menten equation. Under first-order conditions, hepatic clearance is described by

$$CL_H = Q_H \cdot (1 - e^{-CL_{int} \cdot f_a / Q_H}) \quad (1.3)$$

For drugs of low hepatic clearance relative to hepatic blood flow, i.e. low hepatic extraction ratio drugs, the differences between the two models are minimal. With increasing hepatic clearance, however, the differences increase and may become considerable for high hepatic extraction ratio drugs.

1.2.1.2 Regulation of hepatic first-pass effect

After oral administration, drugs with a high hepatic extraction ratio undergo a significant first-pass effect, which is manifested by an extremely low oral bioavailability even though the drug is completely absorbed (Gibaldi and Perrier, 1982). A high hepatic first-pass effect is seen with a wide range of clinically important drugs, including certain β -adrenoceptor antagonists, vasoactive agents and antidepressants (Pond and Tozer, 1984). The majority of these drugs are lipophilic bases which are extensively bound to plasma proteins and α_1 -acid glycoprotein.

For a drug that is completely absorbed and eliminated only by hepatic metabolism, the oral bioavailability of the drug, i.e. the fraction of the total oral dose that reaches the system circulation, is given by

$$F = 1 - E \quad (1.4)$$

where F symbolizes the bioavailability. The total area under the blood drug

concentration-time curve following a single oral dose of this drug (AUC_{oral}) is defined by

$$AUC_{oral} = \frac{F \cdot Dose}{CL_H} \quad (1.5)$$

According to the venous equilibrium clearance model [Eq. (1.2)], AUC_{oral} can be defined as

$$AUC_{oral} = \frac{Dose}{CL_{int} \cdot f_u} \quad (1.6)$$

whereas the undistributed sinusoidal model describes AUC_{oral} as

$$AUC_{oral} = \frac{Dose \cdot e^{-CL_{int} \cdot f_u / Q_H}}{Q_H \cdot (1 - e^{-CL_{int} \cdot f_u / Q_H})} \quad (1.7)$$

From Eq. (1.6) and (1.7), we are able to predict quantitatively the effect of changes in one or more of the three major physiological determinants of hepatic clearance on the oral bioavailability of a drug. Figure 1.2 demonstrates simulations (Maple® V computer algebra program, Release 4 Student Edition, Brooks/Cole Publishing Company, Pacific Grove, CA, USA) of the model-dependent effects of changes in intrinsic clearance, hepatic blood flow and unbound fraction in blood on the area under the plasma concentration versus time curve after oral administration (AUC_{oral}) of a high extraction ratio drug, which is almost totally bound to plasma protein and eliminated only by the liver. The simulations are based on a hepatic blood flow of 1.5 L/min, an unbound fraction of 0.1, a hepatic clearance of 0.85 L/min and a dose of 100 mg. It is shown that AUC_{oral} can be increased or decreased by changing CL_{int} and /or f_u in both models. However, a striking difference exists between the two models regarding the effect of

Q_H . In the venous equilibrium model, AUC_{oral} is independent of Q_H , whereas a marked dependence of AUC_{oral} on Q_H is predicted in the undistributed sinusoidal model.

In addition to the three determinants (CL_{int} , Q_H , f_u), the drug input rate is another important factor affecting the oral bioavailability of high hepatic first-pass drugs in the case of a saturable first-pass effect (Wagner, 1988). Many drugs with a large first-pass effect exhibit Michaelis-Menten kinetic characteristics, due to saturation of the drug-metabolizing enzymes by the relatively high drug concentrations reaching the liver when the drug is absorbed from the gastrointestinal tract. According to the venous equilibrium and undistributed sinusoidal models, an increase in drug input rate will increase AUC_{oral} more than proportionately.

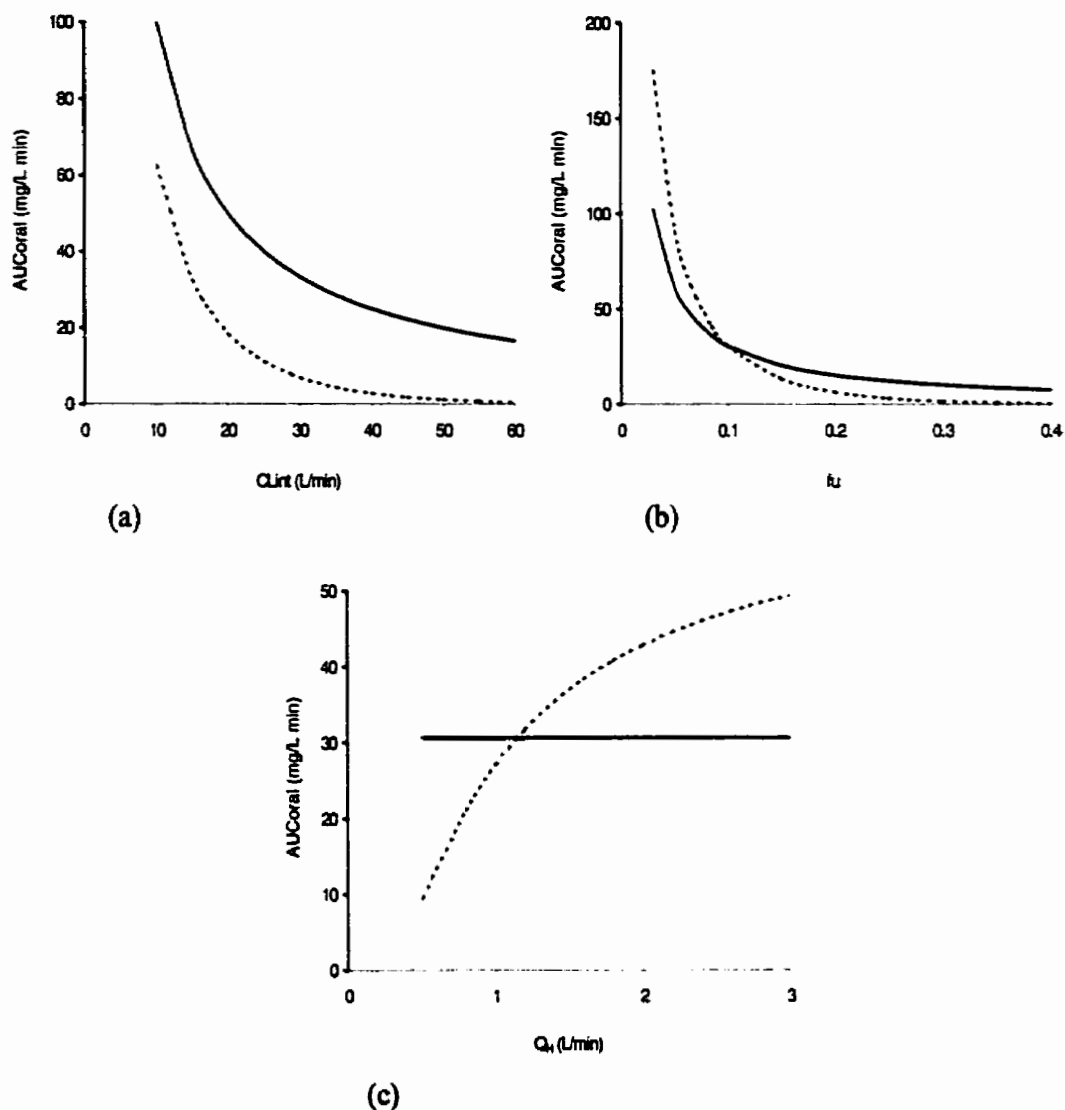


Figure 1.2. Simulations showing the relationship between the area under the plasma concentration versus time curve after oral administration (AUC_{oral}) and (a) intrinsic clearance (CL_{int}); (b) hepatic blood flow rate (Q_H); and (c) unbound fraction (f_u), according to the venous equilibrium model (—) and undistributed sinusoidal model (····). Assuming that hepatic clearance = 0.85 L/min, Q_H = 1.5 L/min, f_u = 0.1, and dose = 100 mg. Simulations were performed by using Maple® V computer algebra program.

1.2.2 High hepatic first-pass drugs and the food effect

1.2.2.1 Phenomenon of the food effect

It has been found that concomitant food intake causes a marked increase (an average of approximately 50%) in the oral bioavailability of certain high hepatic first-pass drugs, including propranolol (Melander et al., 1977), metoprolol (Melander et al., 1977), labetalol (Daneshmend et al., 1982), dicyrazine (Liedholm et al., 1985), zuclopenthixol (Aaes-Jorgensen et al., 1982), propafenone (Axelson et al., 1987) and diprafenone (Koytchev et al., 1996). This phenomenon has been referred to as the food effect. Not all high first-pass drugs are affected by food in this way (Melander and McLean, 1983; Melander et al., 1988), and so far no criterion has been found to distinguish drugs exhibiting the food effect from others which do not.

Propranolol is the model drug that has been the most extensively studied for the food effect on first-pass metabolism. Its increased bioavailability by food has been reproduced in human studies by a number of investigators (Melander et al., 1977; McBride et al., 1980; McLean et al., 1981; Walle et al., 1981; Liedholm and Melander, 1986; Olanoff et al., 1986; Liedholm et al., 1990). The results of all these studies have shown that the mean increase in AUC_{oral} with food is about 50% and the individual change varies greatly from a decrease to an increase exceeding 300%. It has been noticed that the type of meal seems to be a factor in the food effect. High protein meals unquestionably enhance the bioavailability of propranolol and appear to cause the largest observed increase in AUC_{oral} (Melander et al., 1977; McLean et al., 1981; Walle et al., 1981; Liedholm and Melander, 1986; Olanoff et al., 1986; Liedholm et al., 1990), whereas there is no or less effect following the intake of carbohydrate-rich meals

(McLean et al., 1981; Walle et al., 1981; Jackman et al., 1981; Liedholm et al., 1990). Unlike human studies, however, studies in rats and dogs demonstrated ambiguous effects of food/nutrients on the AUC_{oral} of propranolol. In dogs, ingestion of protein-rich food does not change the AUC_{oral} of propranolol (Bai et al., 1985; Power et al., 1995). On the contrary, a high protein diet slightly increases propranolol AUC_{oral} in rats (Ogiso et al., 1994), but ingestion of fatty acids and glucose causes decreases in AUC_{oral}, which is the opposite of the 'food effect' (Chow and Lalka, 1993; Ogiso et al., 1994).

1.2.2.2 Mechanisms of the food effect

The mechanisms of the food interaction with high hepatic first-pass drugs have been investigated in many studies, but our understanding of this phenomenon remains poor. As all the drugs exhibiting the food effect are completely absorbed from the gastrointestinal tract in the fasting state and undergo extensive hepatic first-pass metabolism, the increase in AUC_{oral} cannot be attributed to an increase in the extent of absorption, but rather to a reduction in first-pass metabolism (Melander et al., 1988). A number of hypotheses, which rely on the use of hepatic clearance models and incorporate the known physiological sequelae of food ingestion, have been proposed for mechanisms involved in the food effect. The most likely causes are thought to be food-induced increase in splanchnic blood flow, inhibition of hepatic metabolism by food components, alterations in plasma protein binding of the drug, changes in intrahepatic tissue binding of the drug, increase in the absorption rate of the drug and/or others.

I. Splanchnic blood flow

As mentioned in Section 1.1.1.1, ingested food may induce an increase in splanchnic blood flow, which makes up part of hepatic blood flow. According to the undistributed sinusoidal model of hepatic clearance, the AUC_{oral} of drugs with moderate to high extraction ratio can increase with a food-induced increase in hepatic blood flow, but the venous equilibrium model indicates that AUC_{oral} could not change since it is independent of hepatic blood flow. In fact, however, a transient increase in hepatic blood flow may increase AUC_{oral} even according to the venous equilibrium model. This is because food-induced elevated hepatic blood flow during the gastrointestinal absorption of the drug, followed by a return to normal after absorption is complete, will cause a decrease in the hepatic first-pass effect (increased F) but no effect or much less effect on overall systemic clearance (unchanged CL) and hence an increase in AUC_{oral} (Eq. 1.5). Since McLean et al. (1978) first proposed that transient changes in hepatic blood flow could explain much of the increase in AUC_{oral} by food and provided a computer simulation to demonstrate this hypothesis, a number of theoretical and experimental results have been reported to support this view (Elvin et al., 1981; Walle et al., 1981; Heinzow et al., 1984; Olanoff et al., 1986).

Continuing investigations uncovered potential defects of this blood flow hypothesis. Firstly, high carbohydrate/low protein meals increased propranolol AUC_{oral} (McLean et al., 1981; Jackman et al., 1981), but had little effect on Q_H in humans (Svensson et al., 1984). Furthermore, the observed time course of the change in Q_H caused by the consumption of a high-protein meal did not appear to be sufficient to account for the observed magnitude of the increase in propranolol AUC_{oral} , in that only

14% and 30% increase was calculated from the venous equilibrium model and the undistributed sinusoidal model, respectively (Svensson et al., 1983). A computer simulation predicted that changed Q_H caused even less effect on AUC_{oral} when nonlinear hepatic first-pass metabolism was assumed during the drug absorption phase (Semple et al., 1990). Moreover, manipulation of posture, designed to produce changes in Q_H with a similar magnitude to that observed after food consumption (20 to 50%), increased Q_H by 15 to 40% but failed to change the AUC_{oral} of propranolol (Modi et al., 1988). These observations suggest that the food-related changes in Q_H may be only a minor cause of the increase in AUC_{oral} and other causes of decreased first-pass metabolism secondary to food consumption should be involved.

II. Inhibition of hepatic metabolism

Besides hepatic blood flow, intrinsic clearance (CL_{int}) is one of the main determinants of hepatic clearance. A reduction in CL_{int} will cause an increase in AUC_{oral} , according to either the venous equilibrium model (Eq. 1.6) or the undistributed sinusoidal model (Eq. 1.7). For non-linear hepatic first-pass elimination, changes in the maximum velocity of metabolism (V_{max}) can cause a large increase in AUC_{oral} based on Michaelis-Menten kinetics. Therefore, the acute inhibition of hepatic metabolism by macro/micronutrients from food becomes a plausible hypothesis for the mechanism of the food effect. A number of animal studies have demonstrated that dietary components such as glucose and protein can act as modulators of hepatic drug metabolism (Chow and Lalka, 1992; Yang and Yoo, 1988; Ogiso et al., 1994). Data from studies in isolated perfused rat liver preparations demonstrated that direct addition of amino acids into the

perfusate resulted in a transient, reversible, global reduction in propranolol and metoprolol metabolism (Semple and Xia, 1995; Wang and Semple, 1997), indicating that transient inhibition of hepatic metabolism by amino acids, derived from the dietary protein, could contribute to the mechanism of the increase in AUC_{oral} .

Although previous studies unequivocally demonstrated an increase in propranolol AUC_{oral} after coadministration of food, no significant change in oxidative metabolite AUC_{oral} was observed in studies where plasma concentrations of the metabolites were also examined (Walle et al., 1981; Liedholm et al., 1990). Only one study showed that the food-induced increase in plasma propranolol concentration was accompanied by a reduction of the AUC_{oral} of conjugated propranolol (Liedholm and Melander, 1986), but could not account for the magnitude of increase in AUC_{oral} of propranolol. Despite no change in the propranolol metabolite AUC_{oral} , the above studies did show a delay in the appearance of the oxidative metabolites. These results are consistent with the hypothesis that food-induced transient, reversible inhibition of hepatic drug metabolism may cause a reduction in metabolite formation during the absorption phase, but no change in subsequent hepatic metabolism of systemic propranolol. To understand the possibility and significance of inhibition of hepatic metabolism in the food effect, more animal studies on the effect of food components on hepatic drug metabolism are necessary.

III. Alterations in plasma protein binding

According to the venous equilibrium model (Eq. 1.6) and the undistributed sinusoidal model (Eq. 1.7), a decrease in the fraction of unbound drug (f_u), i.e. increased

plasma protein binding, may increase AUC_{oral} . It is well known that some food components, such as fatty acids, can compete with the drug for binding sites and displace the drug from albumin and other plasma proteins. Food-induced competitive displacement results in an increase in f_u , which should cause reduced AUC_{oral} , an opposite consequence to the food effect. Furthermore, high protein and high carbohydrate meals did not change the plasma protein binding of propranolol (Naranjo et al., 1982; Feely et al., 1983; Chow and Lalka, 1993). Thus, changes in plasma protein binding are not a plausible mechanism of the food effect.

IV. Other mechanisms

Drug input rate becomes an important determinant of the oral bioavailability of high hepatic first-pass drugs, such as propranolol, when we consider that saturated first-pass metabolism occurs during the absorption phase. An increase in drug input rate leads to more drug escaping the first-pass metabolism and thereby a non-linear increase in AUC_{oral} (Wagner et al., 1985; Wagner, 1985). It has been shown that the first-pass metabolism of propranolol is a saturable process resulting in dose-dependent bioavailability (Silber et al., 1983) and that the AUC_{oral} for the sustained-release product is lower than that for the same dose in a conventional rapid-release form (McAinsh and Gay, 1985; Ohashi et al., 1984; Garg et al., 1984). Moreover, concomitant food intake does not enhance the bioavailability of sustained-release propranolol, indicating the importance of drug input rate in the food effect (Byrne et al., 1984; Liedholm and Melander, 1986). An increase in drug input rate could be caused by a food-induced increase in the gastrointestinal absorption rate. However, drugs that exhibit the food

effect are mostly lipophilic, weakly basic compounds. Theoretically, their absorption from the gastrointestinal tract should be delayed by food ingestion because of delayed gastric emptying. Unless hard evidence showing that food intake promotes the absorption of propranolol or other drugs becomes available, it would be difficult to sustain a hypothesis that an increase in the absorption rate could be a contributing mechanism to the food effect.

Hepatic clearance is also dependent on the dissociation of the drug-protein complex and diffusion of drug from blood into the hepatocytes. Usually, these processes are assumed to be very rapid and not to be the limiting steps in hepatic metabolism, but rarely is experimental evidence available to justify this assumption. Food intake could cause changes in the rate of dissociation and/or diffusion via altering the intrahepatic tissue binding, for example, and therefore change AUC_{oral} .

Hormonal and neural regulation due to food ingestion can not be ignored in considering potential mechanisms of the food effect (Semple and Xia, 1994; Power et al., 1995). Power et al (1995) demonstrated an increase in AUC_{oral} in dogs and man when propranolol administration followed a teasing protocol that only allowed subjects to see and smell a meal but without eating it. The results suggested that the mechanism of the food effect might involve physiological responses to sensory exposure to food.

1.3 Metoprolol

1.3.1 Chemistry

Metoprolol ($C_{15}H_{25}NO_3$, M.W. 267.38), 1-isopropylamino-3-[p-(2-methoxyethyl) phenoxy]-2-propanol, belongs to the group of β -blocking agents known as aryloxypropanolamines (Figure 1.3). It contains an asymmetric carbon atom at the 2-propanol position. Like atenolol and propranolol, metoprolol is a basic drug with a pK_a of about 9.6 (Benfield et al., 1986). It possesses intermediate lipophilicity among the β -blocking agents, which is considerably lower than that of propranolol but higher than practolol (Ablad et al., 1975).

Metoprolol tartrate [$(C_{15}H_{25}NO_3)_2 \cdot C_4H_6O_6$] (Figure 1.3) is the marketed form of metoprolol. Metoprolol tartrate is a white crystalline solid with solubility of >1g/ml in water and >0.5g/ml in methanol. It is chemically stable in both solid state and solution. Both the solid and aqueous solution can be stored at room temperature for more than five years without physical and chemical changes (Luch, 1983). A solution of metoprolol tartrate in water or methanol exhibits ultraviolet absorption maxima at 224 nm and 275 nm, and affects fluorescence with an emission maximum wavelength at 298 nm when excited with ultraviolet light.

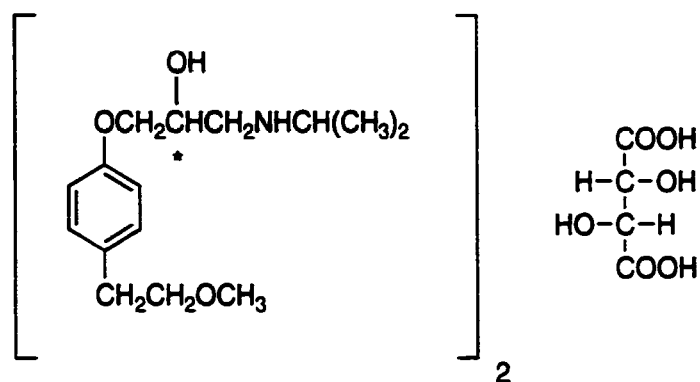


Figure 1.3. Chemical structure of metoprolol tartrate

1.3.2 Pharmacology

Metoprolol is a competitive β -adrenoceptor antagonist used in the treatment of various cardiovascular disorders, including hypertension and ischemic heart disease (Benfield et al., 1986). It acts preferentially on β_1 -adrenoceptors and lacks intrinsic sympathomimetic activity (partial agonist activity). Compared with propranolol, metoprolol is approximately equipotent in inhibiting stimulation of β_1 -adrenoceptors in the heart but 50- to 100- fold less potent in blocking β_2 -receptors (Benowitz, 1998). Thus, it has a weak inhibiting effect on isoprenaline-mediated tachycardia, greatly slows the heart and rapidly reduces cardiac output and systolic blood pressure.

Metoprolol is a racemic compound with two enantiomeric forms, S and R. As with other propanolamine-type β -blockers, the S-enantiomer possesses far greater β -blocking potency than the corresponding R-enantiomer (Hanna, 1991; Wahlund et al., 1991).

1.3.3 Pharmacokinetics

1.3.3.1 Absorption and bioavailability

Human studies have shown that metoprolol is rapidly and completely absorbed from the gastrointestinal tract in the fasting state (Regardh and Johnsson, 1980). About 95% of the oral dose is recovered from the urine. The rate of absorption is related to the type of formulation. The maximum concentration in plasma (C_{\max}) is attained 1 to 2 hr after oral administration of an aqueous solution, and 1 to 3 hr after an ordinary tablet (Regardh et al., 1974, 1975). Approximately two-thirds of metoprolol leaving the stomach is absorbed in the duodenum, and the rest is absorbed from the jejunum, ileum and colon with decreasing absorption extent in anatomical order. Only negligible amounts are absorbed in the stomach. The various regions of the intestines exhibit similar capacities for first-order absorption (Benfield et al., 1986).

In the rat, metoprolol is also rather well absorbed after oral administration, about 70% of the given dose being recovered from the urine while very little is found in the faeces (Borg et al., 1975a). The bioavailability after an oral dose is about 4%. The maximum concentration after oral administration of an aqueous solution is reached within 0.5 hr.

Despite complete gastrointestinal absorption, the bioavailability is only 50% in humans (Benfield, et al., 1986) because of extensive first-pass hepatic metabolism. Considerable inter- and intra-individual variability exists in the area under the plasma concentration-time curve (AUC), peak plasma concentration (C_{\max}) and time to peak plasma concentration (t_{\max}) after oral administration. Long term treatment with

metoprolol results in an increase in its system availability (Regardh and Johnsson, 1980; Benfield et al., 1986).

1.3.3.2 Distribution

Metoprolol is rapidly and extensively distributed to various tissues, such as lung, liver and kidney, after reaching the systemic circulation (Benfield et al., 1986; Bordin et al., 1975). The apparent volume of distribution ($V_{d\beta}$) is high, about 5.5 L/kg in man, and 6.3 L/kg in rats (Ablad et al., 1975), indicating that only a small amount of the drug in the body is localized in the blood.

Unlike propranolol and some other β -blockers, metoprolol shows insignificant binding to plasma and serum proteins. It is about 12% bound to human serum albumin, and negligibly bound to other serum proteins (Regardh and Johnsson, 1980). The concentration of metoprolol in erythrocytes is 20% higher than that in plasma (Benfield et al., 1986).

Due to its moderate lipid solubility and low plasma protein binding, metoprolol readily penetrates the central nervous system (CNS) after reaching the systemic circulation (Benfield et al., 1986; Regardh and Johnsson, 1980).

1.3.3.3 Elimination

Metoprolol is eliminated mainly via hepatic oxidative metabolism. In humans, urinary recovery of the dose reaches about 95% within 72 hr after oral or intravenous administration, of which only 5-10% is unchanged metoprolol and 85-90% is

metabolites (Borg et al., 1975b). In healthy subjects, the renal clearance is 0.11 L/min, and the total body clearance ranges between 0.72 L/min and 1.54 L/min, showing that metoprolol is a high hepatic clearance drug (Benfield et al., 1986). In the majority of healthy individuals, the elimination half-life ($t_{1/2(\beta)}$) varies between 3 and 4 hr, although extremes of 2.1 and 9.5 hr have been reported (Benfield et al., 1986). Within the therapeutic range, half-life is independent of dose.

In the rat, metoprolol is eliminated by biotransformation and excretion in the urine mainly as metabolites (Borg et al., 1975b). About 75% of the oral dose is excreted in the urine, while about 15% is excreted in the feces. Unchanged metoprolol in the urine accounts for <10% (Borg et al., 1975a; 1975b). The $t_{1/2(\beta)}$ is 0.6 hr in the rat (Borg et al., 1975a). Dose-dependent hepatic metabolism has been demonstrated in the isolated perfused rat liver preparation (Shen et al., 1993).

Metoprolol is metabolized by three main pathways in humans and rats without noticeable conjugation (Regardh and Johnsson, 1980): (1) O-demethylation with subsequent oxidation, (2) α -hydroxylation (aliphatic hydroxylation of the methoxyethyl substituent), and (3) oxidative deamination of the long side chain. The biotransformation of metoprolol in man and the rat is summarized in Figure 1.4 (Arfwidsson et al., 1976). However, the relative abundance of the metabolites from these three pathways in humans differs from those in rats. In man, there are three main urinary metabolites, which together account for 85% of the total urinary excretion (Borg et al., 1975b). Metoprolol acid, a secondary metabolite formed from O-demethylmetoprolol, accounts for about 60% of the total urinary excretion, whereas α -hydroxymetoprolol and H104/83 (a metabolite from oxidative deamination) account for only 10% each. In rat

urine after oral administration and rat liver microsomes (Arfwidsson et al., 1976), the two major metabolites are metoprolol acid and α -hydroxymetoprolol, accounting for 62% and 25%, respectively. The other two metabolites, O-demethylmetoprolol and H104/83, account for only 3.5% and 1%, respectively. It is interesting that in rat microsomal incubations the relative amount of metoprolol acid increases with time, while those of α -hydroxymetoprolol and O-demethylmetoprolol remain constant.

Among the metabolites of metoprolol, only O-demethylmetoprolol and α -hydroxymetoprolol show β_1 -adrenoceptor blocking activity, but are substantially less potent than the parent drug (Regardh and Johnsson, 1980). Therefore, no pharmacologically important metabolite is produced.

In humans, cytochrome P-450 2D6 (CYP2D6), the same cytochrome P450 enzyme responsible for debrisoquine oxidation, which is absent or inactive in poor metabolizers, mediates exclusively α -hydroxylation and partially O-demethylation (Belpaire et al., 1998; Lennard et al., 1986; Otton et al., 1988), and CYP2D2 is the corresponding isoenzyme in rats (Schulz-Utermoehl et al., 1999). It is not surprising that, therefore, metoprolol metabolism exhibits the debrisoquine-type of genetic polymorphism in humans (Lennard, et al., 1982; 1986).

Moderate enantioselectivity of metoprolol clearance is found in humans, mainly in extensive metabolizers (Lennard, et al., 1983). After an oral dose of racemic metoprolol, the R-enantiomer has a higher clearance than the S-enantiomer, resulting in a 35% higher AUC_{oral} for the S-enantiomer in extensive metabolizers. The stereoselectivity in the O-demethylation pathway is mainly responsible for the difference in the clearance of metoprolol enantiomers (Murthy et al., 1990; Kim, 1993),

and the genetic polymorphism causes differences in enantiomer metabolism between extensive and poor metabolizers (Lennard et al., 1989). In the rat, however, there is only a small difference in metabolism between R and S-metoprolol (Vermeulen et al., 1993). After oral administration of the racemic metoprolol, AUC_{oral} is slightly higher for the R-enantiomer, while clearance is slightly lower.

1.3.3.4 Effect of food

Metoprolol is a high hepatic first-pass drug which exhibits increased bioavailability after concomitant intake of food. It has been shown that a high protein meal causes a mean increase in AUC_{oral} of about 40% with a range from a slight decrease to an increase of 132% after oral administration of metoprolol in healthy subjects (Melander et al., 1977). In the isolated, perfused rat liver, amino acids cause a transient, partially reversible, global reduction in metoprolol metabolism (Wang and Semple, 1997), suggesting that inhibition of metabolism by amino acids could contribute to the mechanism of the food effect on metoprolol bioavailability.

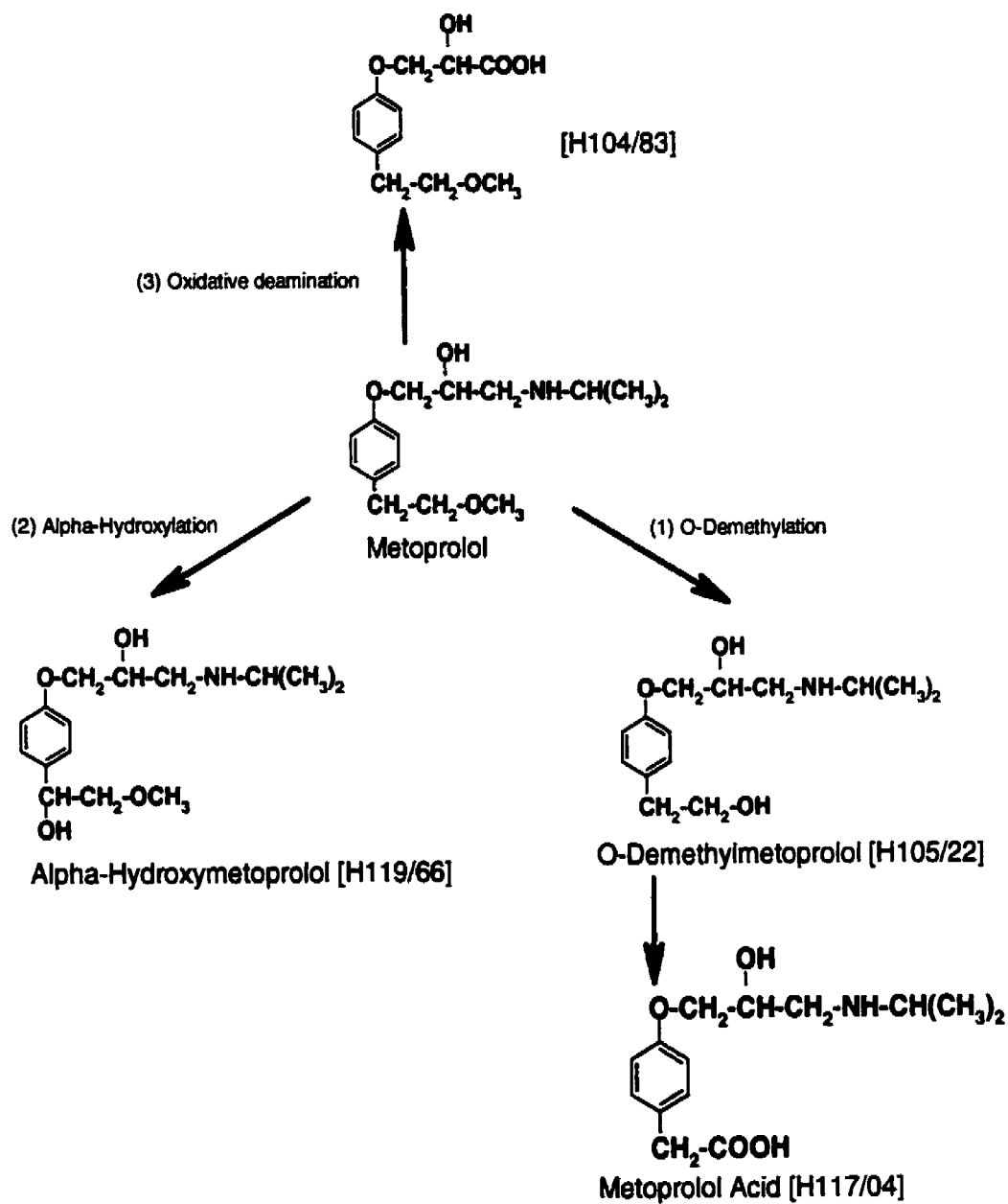


Figure 1.4. Major pathways of metoprolol metabolism in man and the rat.

1.4 Propafenone

1.4.1 Chemistry

Propafenone ($C_{21}H_{27}NO_3$, M.W. 341.5), 1-[2-[2-hydroxy-3-(propylamino)propoxy] phenyl]-3-phenyl-1-propanone, was synthesized in 1970 by Helopharm of Germany and later licensed by Knoll AG, Germany. It is structurally similar to the β -blocking agents. Propafenone has a chiral carbon atom on the propanolamine side chain, and thus occurs as a racemic mixture of R (-)- and S (+)-enantiomers. It is a lipophilic base with a pK_a of 9.0 (Chow, et al., 1988). Propafenone is available as the hydrochloride salt ($C_{21}H_{27}NO_3 \cdot HCl$, M.W. 377.9), which is slightly soluble in cold water (Merck Index, 1996).

1.3.2 Pharmacology

Propafenone is a potent and effective antiarrhythmic drug widely used in the treatment of ventricular and supraventricular arrhythmias (Chow et al., 1988). It possesses Class IC electrophysiologic properties according to the Vaughan Williams classification scheme (Harron and Brogden, 1987). Typically for the IC group of antiarrhythmic agents, propafenone has slow kinetics of interaction with fast cardiac sodium channels in normal and ischemic Purkinje fibers, and therefore markedly depresses the rate of depolarization of phase 0 of the action potential. Its sodium channel blocking potency is similar to that of flecainide, and is both frequency- and voltage-dependent (Hii, et al., 1991).

In addition to its Class IC antiarrhythmic effect, propafenone exhibits both mild β -adrenoceptor blocking (Class II) activity and weak calcium channel blocking (Class IV) activity (Harron and Brogden, 1987). Unlike flecainide, propafenone has non-selective β -adrenoceptor blocking activity, and its potency is 1/20 to 1/50 that of propranolol (Faber and Camm, 1996). Because the plasma concentrations of propafenone may be 50 or more times greater than those of propranolol, the β -blocking effect of propafenone may be clinically relevant. Clinical data show that, however, this effect varies between undetectable to significant (Faber and Camm, 1996). Propafenone also inhibits the slow calcium influx at high concentration, but this action is weak (approximately 1/20 of verapamil) and does not contribute to the clinical importance (Harron and Brogden, 1987).

Both the R- and S-enantiomers of propafenone are equally potent in blocking sodium channels in a dose-frequency-dependent manner. However, the affinity of the S-enantiomer for the human β -adrenoceptors is 100-fold greater than that of its antipode (Kroemer, et al., 1989a). The two major metabolites of propafenone, 5-hydroxypropafenone and N-despropylpropafenone, also have sodium channel blocking activity. The potency of 5-hydroxypropafenone is comparable with that of the parent compound, while N-despropylpropafenone is less potent (Thompson et al., 1988; Oti-Amoako et al., 1990).

1.4.3 Pharmacokinetics

1.4.3.1 Absorption and Bioavailability

Propafenone is rapidly and almost completely absorbed after oral administration in the fasting state. More than 90% of the oral dose is absorbed and its peak plasma concentration (C_{\max}) is usually reached one to three hr after administration (Hege, et al., 1984a; Hollman, et al., 1983). Generally, the bioavailability of propafenone ranges from 5-50%, reflecting high first-pass clearance (Hii et al., 1991).

1.4.3.2 Distribution

Following intravenous administration, propafenone distributes rapidly into various tissues. The highest concentration of propafenone is found in the lung, followed by the liver and heart. The concentration of 5-hydroxypropafenone is similar to that of parent drug in the heart, but lower in the lung (Latini et al., 1987).

By fitting with the two compartment pharmacokinetic model, the half-life of the distribution phase ($t_{1/2(\alpha)}$) is estimated to be 4.7 min, indicating rapid distribution after intravenous administration. The volume of the central compartment (V_c) is 0.7 to 1.1 L/kg and the apparent volume of distribution at the steady state [$(V_d)_{ss}$] is about 1.9-3 L/kg (Hii, et al., 1991).

Propafenone is highly (>95%) bound to plasma protein (Hii et al., 1991). Two proteins, albumin and α_1 -acid glycoprotein, have been identified to be responsible for binding propafenone. S-Propafenone (free fraction of 4.9%) shows greater protein binding than that of R-propafenone (free fraction of 7.6%) (Hii et al., 1991).

1.4.3.3 Elimination

Propafenone undergoes extensive hepatic metabolism via hydroxylation and conjugation in humans and dogs (Hege et al., 1984a; 1984b;1986). The major metabolic pathways are shown in Figure 1.5. After an oral dose in man, less than 1% of unchanged propafenone is excreted in the urine and feces (Hege et al, 1984b). The metabolites in the urine and feces are almost exclusively excreted as conjugates with glucuronic acid and sulphuric acid, mainly via the feces (53% of the dose within 48 hours). The major metabolites are propafenone glucuronide and conjugates of 5-hydroxypropafenone, N-despropylpropafenone, and 5-hydroxy-4-methoxypropafenone (Hege et al., 1984b; Kates et al., 1985). The most extensively studied metabolites include two pharmacologically active compounds, 5-hydroxypropafenone and N-despropylpropafenone. In the dog, the major metabolites are 4'-hydroxypropafenone and 5-hydroxypropafenone; each of them accounting for about 15% of the dose (Hege et al., 1984a; 1986).

In human, the formation of 5-hydroxypropafenone is mediated by CYP2D6 (Kroemer et al., 1989b), while the formation of the N-despropyl metabolite is catalyzed by CYP3A4 and CYP1A2 (Botsch et al., 1993). As the metabolism of propafenone is dependent on the debrisoquine metabolizer phenotype, its elimination half-life varies from 5.5 hr in extensive metabolizers to 17.2 hr in poor metabolizers (Bryson et al., 1993). Non-linear (saturable dose-dependent) pharmacokinetic characteristics are observed in extensive metabolizers following an oral dose of the drug, while in poor metabolizers propafenone shows linear kinetics (Hii et al., 1991).

The enantiomers of propafenone undergo stereoselective disposition and elimination (Bryson et al., 1993). In both extensive and poor metabolizers, a higher rate of clearance and lower AUC_{oral} are observed for the R- than S-enantiomer after oral administration of the racemic mixture of propafenone (Kroemer et al., 1989a). In contrast, R-propafenone has a lower oral clearance and larger AUC_{oral} than S-propafenone in extensive metabolizers following two separate doses of each enantiomer (Brode et al., 1988). It is concluded that, when the enantiomers are coadministered, each inhibits the metabolism of the other so as to reverse pharmacokinetic differences between enantiomers and also reduce clearance rates (Bryson et al., 1993). The stereoselective pharmacokinetics of propafenone has also been demonstrated in the rat. After intravenous, intraperitoneal, and oral administration, the AUC for R-enantiomers is higher than that for S-enantiomers (Mehvar, 1990).

1.4.3.4 Effect of Food

Coadministration of propafenone with a high protein meal increases its bioavailability (Axelson et al., 1987). This phenomenon represents the most prominent documented interaction between food and high hepatic first-pass drugs. It is interesting that such enhancement of propafenone bioavailability could only be demonstrated in subjects with the extensive metabolizer phenotype. The mean increase in AUC_{oral} was 120% with a maximum of 638% when all 24 healthy subjects were used in the calculation, and 147% when only extensive metabolizer phenotype subjects were incorporated. Beside the change in AUC_{oral} , C_{max} is more rapidly attained in the presence of food.

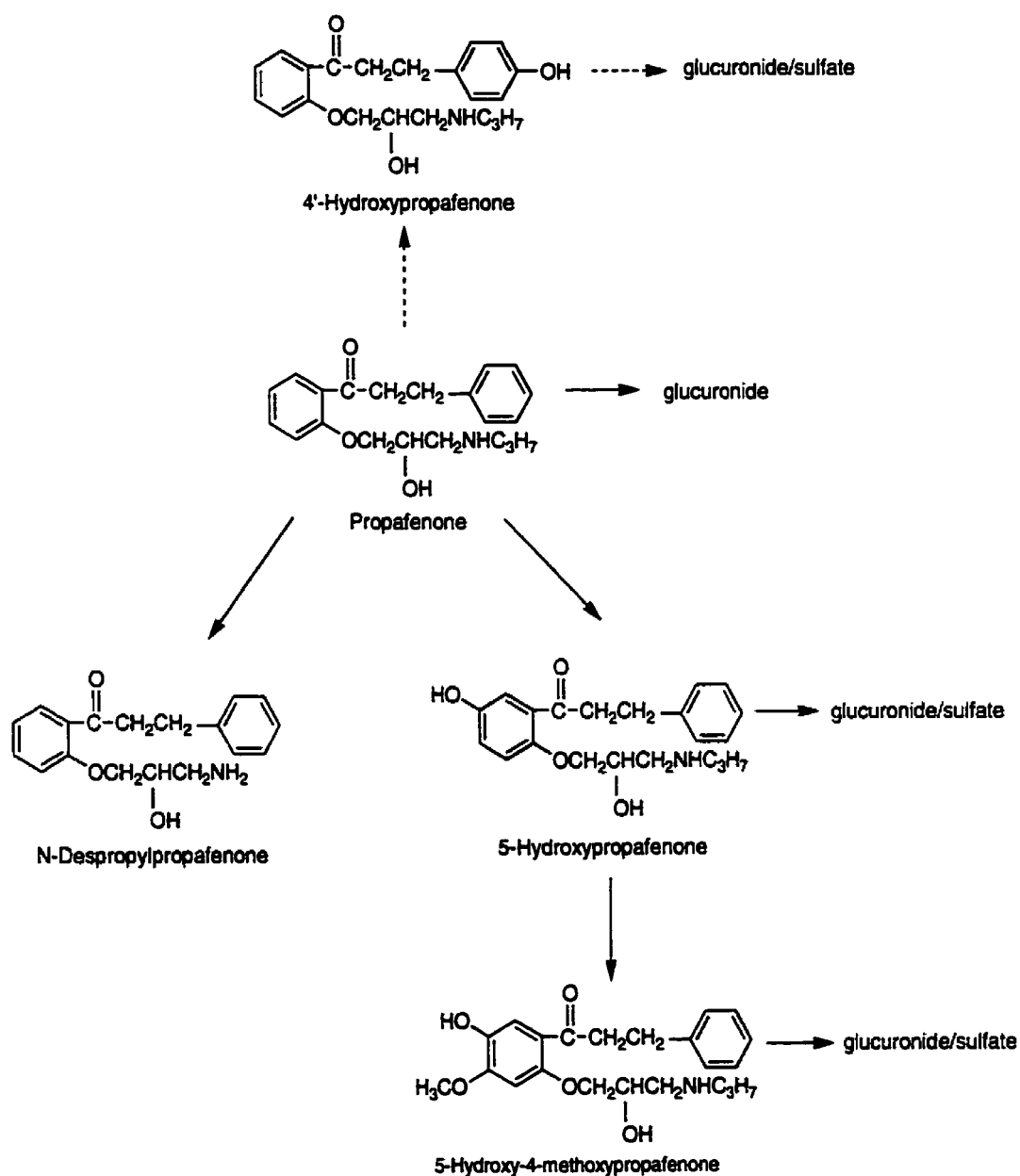


Figure 1.5. Main pathways of propafenone in man and the dog.

\longrightarrow In man and the dog
 $\cdots\longrightarrow$ In the dog

CHAPTER 2

RATIONALE, HYPOTHESES AND OBJECTIVES

2.1 Rational

2.1.1 Food effect and its mechanisms

Ingestion of food has been found to enhance the oral bioavailability of certain drugs subject to extensive hepatic first pass metabolism, such as propranolol, metoprolol and propafenone (Melander et al., 1988). The effect of food can be profound. When these drugs are administered orally along with a meal, increases in the area under the concentration-time curve as large as 600% can occur. The alteration of systemic drug levels may result in unexpected ineffective or toxic pharmacological responses for individual subjects. High intra-individual variability limits the effective use of some high first pass drugs. Therefore, understanding the cause of the food effect is warranted.

Although the food effect has been studied by many investigators for over two decades, its mechanisms remain unclear. The most reasonable explanation for the increased bioavailability is a reduction in hepatic first-pass metabolism since gastrointestinal absorption of the affected drugs seems to be virtually complete in the fasting state. According to the concept of hepatic clearance models, hepatic extraction is dependent on hepatic metabolic enzyme activity (intrinsic clearance), hepatic blood flow and drug plasma protein binding. Much effort has been expended to define the role of transient changes in hepatic blood flow in the food effect, which is now believed to be

minor. Moreover, studies on drug plasma protein binding have revealed that food is unlikely to cause an increase in the bound fraction of propranolol. Thus, inhibition of hepatic metabolism, i.e., decreased intrinsic clearance, by dietary nutrients becomes a possible mechanism of the food effect. Although it is generally agreed to be a possibility, it remains to be experimentally elucidated.

2.1.2 Interaction between amino acids and drug metabolism related to the food effect

Most human diets are complex mixtures of macro-nutrients such as carbohydrates, fats and proteins, which are digested and transported into the portal vein as micro-nutrients, such as glucose, fructose, free fatty acids and amino acids. These micro/macro-nutrients are widely recognized to be modulating factors for hepatic drug metabolizing enzymes (Anderson, 1988; Yang and Yoo, 1988). Of these components in the diet of humans, however, only protein appears to be responsible for the 'food effect'. Human studies have demonstrated that protein-rich meals unquestionably enhance the AUC_{oral} of propranolol, metoprolol and propafenone, whereas there is no or less effect of carbohydrate-rich meals.

Based on the importance of protein in the food effect, amino acids, derived from the gastrointestinal digestion of protein, may be a key food component involved in the 'food effect'. Nevertheless, there is no report that relates to acute interaction between amino acids and drugs exhibiting the 'food effect', except the perfused rat liver studies in our laboratory. In the isolated perfused rat liver preparation, a mixture of amino acids can reversibly inhibit all pathways of propranolol and metoprolol metabolism (Semple

and Xia, 1995; Wang and Semple, 1997). Since it is unknown why amino acids cause the inhibition of drug metabolism and how the inhibition would be relevant to the 'food effect', further investigation into the amino acid-drug interaction is necessary.

The observed interaction may be due to direct or indirect inhibition of drug metabolizing enzymes. Direct inhibition seems unlikely because amino acids at physiological levels have been shown not to inhibit metoprolol metabolism in a study of rat liver microsome preparations (Alcorn, 1997). Therefore, indirect mechanisms should be involved, such as limiting the supply of cosubstrates, oxygen and/or NADPH. In parallel with inhibition of metoprolol metabolism, amino acids also cause an acute and dramatic increase in hepatic oxygen consumption (Wang and Semple, 1997). This implies that the oxygen supply could become limiting in the liver. It is well known that differences in the distribution of enzymes and an oxygen/nutrient gradient exist along the hepatic acinus from the periportal to the perivenous area. The metabolism of amino acids, an oxygen-consuming process, is preferentially localized in the periportal zone of the acinus (Häussinger and Gerok, 1986), and the cytochrome P450 enzymes and glucuronosyl transferases, which are the enzymes most commonly involved in xenobiotic metabolism, are mainly distributed in the pericentral region of the acinus (Thurman et al., 1986). When both amino acids and the drug are infused into the portal vein, the amino acid metabolizing enzymes have the priority to utilize oxygen, and thus a transient oxygen deficiency may be suffered by the drug metabolizing enzymes which are distributed downstream (Wang and Semple, 1997). Many drug metabolizing enzymes are impaired by reductions in oxygen supply (Angus et al., 1990; Woodroffe et al., 1995). For instance, the activity of cytochrome P450 isozymes responsible for

propranolol metabolism is very sensitive to changes in the hepatic oxygen concentration (Elliott et al., 1993; Woodrooffe et al., 1995). Consequently, inhibition of metoprolol metabolism could be attributed to the amino acid-mediated pericentral oxygen deprivation in the hepatic sinusoids.

2.1.3 Experimental animal models

The Sprague-Dawley strain rat has been chosen as the experimental animal. It is considered to be an extensive metabolizer in term of hepatic debrisoquine 4-hydroxylase activity. The isolated perfused liver preparation appears to be an appropriate *ex vivo* model to investigate the effect of amino acids on hepatic drug metabolism because the architecture of the liver remains undisturbed and the zonal distribution of enzymes is maintained. As it is not physiologically complete, however, studies with an *in vivo* rat model are also required in order to link the 'food effect' with any proposed mechanism delineated from the *in vitro* investigations.

2.1.4 Selection of model drugs

Two high hepatic first pass drugs that exhibit the 'food effect' in humans, propafenone and metoprolol, are considered appropriate model drugs to investigate mechanisms of the 'food effect'.

Propafenone is widely used in the clinical setting as a class IC antiarrhythmic drug. Food ingestion caused an average 147% increase in oral bioavailability in 20 subjects (Axelson et al., 1987), which is the largest reported effect of food. It should be a potential model drug and can be expected to be sensitive to the effect of amino acids.

The major pathways of propafenone metabolism in man are 5-hydroxylation and N-dealkylation as well as glucuronidation of both parent drug and phase I metabolites. Nevertheless, no information is yet available on the metabolism of propafenone in the rat. To evaluate whether propafenone could be a useful model drug, identification of its metabolites in the rat is needed. In addition to racemic propafenone metabolism, the disposition of propafenone enantiomers should be taken into consideration because stereoselectivity has been shown in the pharmacokinetics of propafenone.

Metoprolol is a selective β_1 -adrenergic blocking drug. In humans, its AUC_{oral} was increased by an average of 40% when concomitantly given with a protein-rich meal (Melander et al., 1977). In the isolated, perfused rat liver, the V_{max} values of metoprolol and the two metabolites, α -hydroxymetoprolol and O-demethylmetoprolol were reversibly reduced by about 50% after coinfusion with a balanced mixture of amino acids (Wang and Semple, 1997). The metabolic pathways of metoprolol are similar in humans and rats, where metoprolol is metabolized to α -hydroxymetoprolol, O-demethylmetoprolol and metoprolol acid without any significant primary phase II conjugation. The low hepatic tissue binding (Wang, 1995) is an important advantage of metoprolol as a model drug in the isolated, perfused rat liver. The steady state can be reached within a short time even at non-saturated inlet concentrations. Therefore, metoprolol could also be a good model drug to study in the isolated, perfused rat liver.

2.2 Hypotheses

- (1) Inhibition of hepatic drug metabolism by dietary amino acids is one of the mechanisms involved in the 'food effect'.
- (2) Propafenone and metoprolol are appropriate model drugs for investigating the drug-food interaction in the isolated, perfused rat liver.
- (3) Amino acids inhibit the metabolism of the model drugs by reduction of the oxygen supply to the pericentral zone of the hepatic acinus.
- (4) Ingestion of amino acids can increase the bioavailability of the model drugs after an oral dose in the *in vivo* rat.

2.3 Objectives

- (1) To establish the appropriateness of the isolated perfused rat liver preparation system for studying the metabolism of propafenone and metoprolol.
- (2) To develop and validate sensitive HPLC methods for the analysis of propafenone enantiomers and metoprolol, and their metabolites.
- (3) To identify the metabolic pathways of propafenone in rat liver.
- (4) To determine whether the effect of amino acids on propafenone and/or metoprolol metabolism is dependent on the oxygen delivery rate and the direction of flow in the isolated, perfused rat liver.
- (5) To determine whether the ingestion of amino acids can cause a food-like effect on propafenone and/or metoprolol kinetics in the intact rat in order to find a possible link between the amino acid-drug interaction *ex vivo* and the 'food effect' *in vivo*.

CHAPTER 3

ISOLATED PERFUSED RAT LIVER PREPARATION

3.1 Introduction

Although it has been over 100 years since Claude Bernard first reported the use of the isolated perfused rat liver (Miller, 1973), it is still a valuable and commonly used intact organ model for examining the hepatobiliary disposition of drugs. The isolated perfused rat liver technique and its applications have been extensively elaborated by many distinguished researchers (Meijer et al., 1981; Pang, 1984; Gores et al., 1986; Wolkoff et al., 1987; Powell et al., 1989; Brouwer and Thurman, 1996). Compared with *in vivo* models, the use of the isolated perfused rat liver rules out complications due to many factors that are difficult to control such as neuronal and hormonal influences on hepatic metabolism, as well as absorption, distribution, metabolism and excretion by nonhepatic routes. In contrast to *in vitro* models like liver slices, hepatocytes and microsomes, the perfused rat liver preserves hepatic architecture, enzyme heterogeneity, and bile flow.

In order to investigate the disposition characteristics of propafenone and metoprolol, which are almost completely eliminated via hepatic metabolism in the body, we used the perfused rat liver preparation technique throughout most of the present work in recognition of its advantages. Two systems were applied for different purposes. One system employed was the isolated perfused rat liver preparation in the single pass

mode, by which the hepatic metabolic pathways of propafenone were examined. The other system employed was the *in situ* perfused rat liver system facilitated with the switch of antegrade and retrograde flow directions for studying the amino acid-metoprolol interaction. Although the techniques of these two systems had common features, different devices, perfusion media and surgical procedures were employed. They are thus separately described in the following text.

3.2 Liver perfusion system for propafenone metabolism study

3.2.1 Apparatus

The schematic representation of the apparatus for single-pass experiments (manufactured by the University of California, San Francisco Research and Development Laboratory, San Francisco, CA, USA) is shown in Figure 3.1. The perfusion medium was passed from polypropylene containers, in which they were bubbled through gas dispersion tubes with 95% O₂/5% CO₂, through a switching valve (model 5011, Rheodyne, Cotati, CA, USA), peristaltic pump (Ismatec Vario with a six-roller pump head, Cole Parmer, Niles, IL, USA), and bubble trap/manometer, and then into the liver. A portion of the effluent was passed through the microelectrode flow-through cell of an oxygen monitor (YSI model 5300, Fisher Scientific, Edmonton, Alberta, Canada), while the major portion was collected for sample analysis. The entire perfusion circuit was housed in a 37°C temperature-controlled cabinet.

After each experiment, the perfusion system was rinsed thoroughly with distilled water.

37 °C Perfusion Chamber

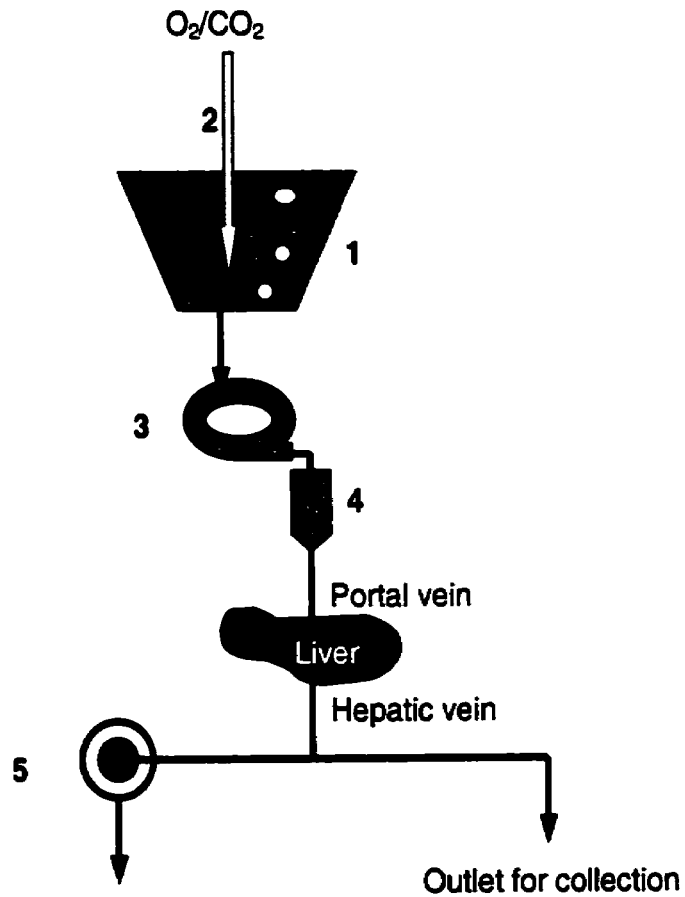


Figure 3.1. Single-pass rat liver perfusion apparatus.

(1) Main reservoir with perfusion medium; (2) gas dispersion tube; (3) peristaltic roller pump; (4) bubble trap; (5) microelectrode flow-through cell of an oxygen monitor.

3.2.2 Perfusion medium

The perfusion medium was oxygenated with Krebs-Henseleit bicarbonate buffer containing 11 mM glucose, which served as an energy source. The ample supply of glucose (normally 5.5 mM) was for providing sufficient NADPH via the pentose phosphate shunt to support cytochrome P-450 activity. Alternatively, sodium lactate and pyruvate might be included. The composition of the perfusion medium is shown in Table 3.1.

Only fresh perfusion medium was used in each experiment. Typically, to make 10 L of perfusion buffer, the solution containing all ingredients, except CaCl_2 , NaHCO_3 and glucose, was prepared in 9.5 L distilled, deionized water. This solution was used as a stock solution. On the day of each experiment, glucose and NaHCO_3 were added to the stock solution and stirred until dissolved. The CaCl_2 solution in 500 ml water was added last with stirring to avoid precipitation of calcium as the phosphate salt. The prepared perfusion buffer was then transferred to the containers and placed in the perfusion cabinet after warming up to 37°C. The drug was added to a particular container containing blank perfusion buffer to make a certain concentration for the metabolism study. The perfusion medium was saturated directly with a mixture gas of 95% O_2 /5% CO_2 through a glass gas dispersion tube, and buffered to pH 7.4. To sustain adequate oxygenation in the liver, a flow rate of 3–4 ml/min/g liver was applied.

Table 3.1. The composition of hemoglobin-free perfusion medium

Compound	Concentration (mM)	Amount (g/10 L)
Krebs-Henseleit bicarbonate buffer		
<i>Sodium chloride</i> (NaCl)	118	69.0
<i>Potassium chloride</i> (KCl)	4.7	3.50
<i>Calcium chloride dihydrate</i> (CaCl ₂ •2H ₂ O) *	2.5	3.67
<i>Potassium dihydrogen orthophosphate</i> (KH ₂ PO ₄)	1.2	1.63
<i>(Magnesium sulphate heptahydrate</i> (MgSO ₄ •7H ₂ O)	1.2	2.96
<i>Sodium hydrogen carbonate</i> (NaHCO ₃)	25	21.0
Sodium lactate (CH ₃ CHOHCOONa)	2.95	3.31
Sodium pyruvate (CH ₃ COCOONa)	0.307	0.338
Dextrose (C ₆ H ₁₂ O ₆)	11	20

* To prevent precipitation, CaCl₂•2H₂O is dissolved in a small amount of water and added slowly with stirring into the mixture solution of the other compounds.

3.2.3 Surgical procedure

1. Following an overnight fast, anesthesia was introduced in a male Sprague-Dawley rat by inhalation of 4.5% halothane (MTC Pharmaceuticals, Cambridge, Ontario, Canada) and the condition was maintained with 1.5% halothane through a mask.
2. An abdominal midline incision and a T section were made without damaging the diaphragm. The intestines of the animal were covered with a saline-wetted gauze and then displaced to the right of the operator to expose the portal area of the liver.
3. The pancreaticoduodenal veins close to the portal vein were ligated. The celiac artery, which branches to the hepatic artery, was tied to ensure unidirectional flow. Double loose ligatures (surgical silk) were placed around the portal vein and the inferior *vena cava* proximal to the right renal vein.
4. The portal vein was cannulated with a 16G Jelco™ intravenous catheter (Critikon, Inc., Tampa, FL, USA), which consisted of an inner needle and an outer sheath for placement. After insertion, the inner needle was withdrawn, leaving the outer catheter in place for moving forwards to the hilus of liver. The perfusate with a slow flow rate of 1 ml/min/ g liver was immediately connected to the portal catheter once the head of catheter was filled with the back flow of blood. Introduction of an air bubble into the liver was carefully avoided at this stage. The inferior *vena cava* close to the right renal vein was severed at once to allow blood and perfusate to exit before a buildup of pressure in the liver occurred.

5. After ligatures around the portal vein were closed, the thorax was opened and the right atrium was cut. The rat died during these procedures. The perfusion flow was quickly increased to a sustained rate of 3 ml/min/g liver. A 3 cm polyethylene tube (Intramedic®, 1.67 mm i. d. × 2.42 mm o.d., Clay Adams, Division of Becton Dickinson Company, Parsippany, NJ, USA) was inserted through the incision of the right atrium into the thoracic inferior *vena cava* and shifted until its tip was close to the hepatic veins. The cannula was then secured with double closed ligatures, and served as the perfusate outlet for sample collection in the experiment.
6. Another 3 cm polyethylene tube was placed into the abdominal *vena cava* to allow a small portion of the effluent to pass an oxygen monitor electrode for measurement of the oxygen tension in the effluent.
7. The liver was rapidly dissected from the body cavity, and transferred to the perfusion cabinet. The position of liver was adjusted so that a steady flow of perfusate (3 ml/min/g liver) was achieved. The liver was allowed to stabilize for about 20 min before commencement of the study protocol.

3.2.4 Liver viability assessment

The duration of liver perfusion was within 2 hr. The viability of the isolated perfused liver was assessed throughout the experiment: 1) the gross appearance of the liver; 2) the weight of the liver at the end of perfusion; 3) the oxygen consumption of the liver; and most importantly, 4) the hepatic clearance of the drug. The weight of liver should remain at 3-4% of total body weight. The hepatic oxygen consumption and drug

clearance should be constant throughout the perfusion. Normally, the oxygen consumption was maintained at 2-3 $\mu\text{mol}/\text{min}/\text{g}$ liver.

3.3 Liver perfusion system for amino acid-metoprolol interaction

3.3.1 Apparatus

Normal flow through the perfused liver is from the portal vein to the hepatic vein (antegrade perfusion). In order to investigate the role of enzyme heterogeneity in the liver, the flow direction can be reversed to create the so-called 'retrograde perfusion'. The device described here is specially designed for this purpose.

The perfusion apparatus (Figure 3.2) was modified from that used in the propafenone metabolism study as described above. The perfusion system, maintained in a temperature-controlled (37°C) cabinet, included two reservoir units, a peristaltic pump (Ismatec SA, Vario-pump system, Cole-Parmer, Niles, IL, USA), a silastic tubing oxygenator, a bubble trap/filter device and two three-way stopcocks which were used to facilitate the switch between antegrade and retrograde flow to the liver during perfusion (Pang and Terrell, 1981). A pH meter (PHM84 Research pH meter, Radiometer A/S Copenhagen, Denmark) and a biological oxygen monitor (YSI model 5300, YSI, Inc., Yellow Springs, OH, USA) were interconnected with the flow-line before and after the liver, respectively, to monitor the pH of inlet perfusate and the oxygen content of the liver effluent. A syringe pump (Model 975, Harvard Apparatus, South Natick, MA, USA) was attached to the perfusion line one centimeter from the entry of the liver to coadminister a mixture solution of amino acids.

After each experiment, the whole apparatus was cleaned by rinsing with a chlorhexidine (Savlon®) solution. The tubings and containers were then disassembled, and were sterilized in an autoclave.

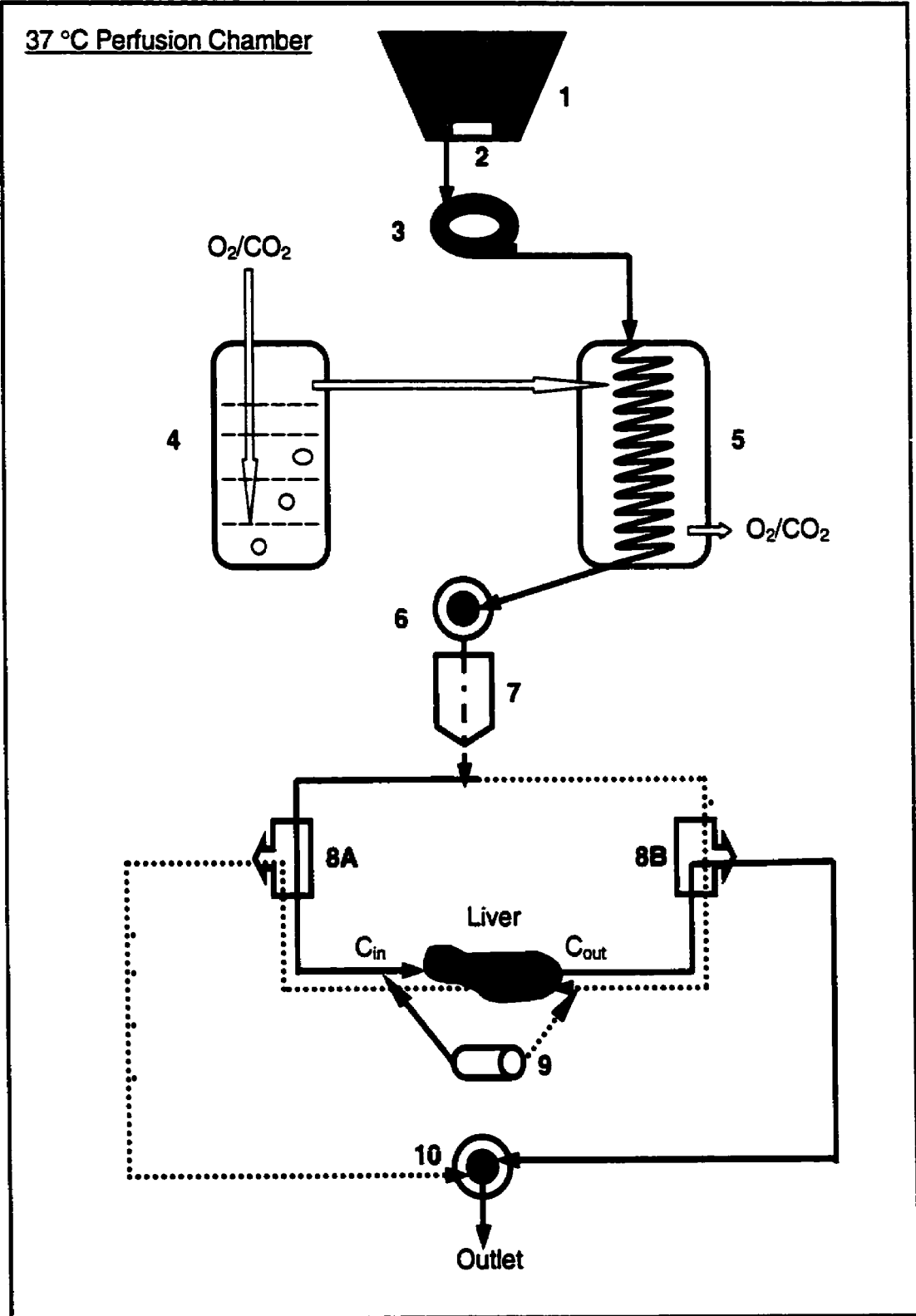


Figure 3.2. Rat liver perfusion apparatus for antegrade (solid line) and retrograde

(dashed line) perfusion.

(1) Main reservoir with erythrocyte-enriched perfusion medium; (2) magnetic stirring bar; (3) peristaltic roller pump; (4) flask with saline for humidification of O₂/CO₂ gas mixture; (5) silastic tube oxygen exchanger; (6) flow cuvette with pH electrode; (7) bubble trap and filter; (8) three-way stopcock A and B facilitating the change of flow direction; (9) syringe pump for coinfusion of a mixture solution of amino acids; (10) microelectrode flow-through cell of an oxygen monitor. For antegrade perfusion, the opening c of stopcock A is closed to establish flow into the portal vein while the opening a of stopcock B is closed to shuttle all reservoir buffer through stopcock A to the liver and to allow perfusate to exit. For retrograde perfusion, the procedure for stopcock A and B is reversed.

3.3.2 Perfusion medium

The perfusion medium consisted of 20% (v/v) washed outdated human red blood cells, 1% bovine serum albumin and 0.1% dextrose in a Krebs-Henseleit bicarbonate buffer (Table 3.2). Human red blood cells were washed with equal volumes of physiological saline three times, followed by equal volumes of Krebs-Henseleit bicarbonate buffer three times. After each wash, the red cells were collected by centrifugation at $1000 \times g$ for 10 min at 4°C , and the supernatant together with protein deposits and ghost cells were removed by aspiration. The washed red blood cells were used immediately. The prepared red cells were filtered through a thin mesh prior to their addition to the Krebs-Henseleit buffer containing glucose and albumin.

The perfusate was buffered to pH 7.2 - 7.4, and oxygenated by equilibration with a flow of 95% O_2 /5% CO_2 at 1 L/min through a silastic tubing gas exchanger. Alternatively, in certain experiments, the perfusate was equilibrated with a mixture of 95% O_2 /5% CO_2 and 95% N_2 /5% CO_2 to achieve a reduced level of oxygen delivery. The proportion of oxygen to nitrogen determined the oxygen level in the perfusate. The liver was perfused at a constant rate of about 15 ml/min/liver.

Table 3.2. Preparation of 1 L liver perfusate containing red blood cells

Composition	Amount added
Krebs-Henseleit buffer (Table 3.1)	800 ml
Washed human red blood cells	200 ml
Bovine serum albumin	10 g
Glucose	1 g

3.3.3 Surgical procedure

The surgical procedure was similar to that described in 3.1.3, except for the following changes. The inferior *vena cava* proximal to the right renal vein was ligated, so that the thoracic inferior *vena cava* was cannulated for both oxygen measurement and sample collection. After the surgery, the rat was transferred to the perfusion system where the liver was perfused *in situ*.

3.3.4 Liver viability assessment

The duration of liver perfusion was about 150 min, while the retrograde perfusion was performed for no more than 75 min. The viability of the isolated perfused liver was assessed during the experiment according to the methods described in Section 3.2.4.

CHAPTER 4

HPLC ASSAY FOR PROPAFENONE ENANTIOMERS IN RAT LIVER PERFUSATE AND HUMAN PLASMA

4.1 Introduction

Propafenone (PF) is a class IC antiarrhythmic agent which is administered as a racemate of S (+)- and R (-)-enantiomers (Harron and Brogden, 1987). Although both the R- and S- enantiomers are equally potent in blocking sodium channels, the S-enantiomer is a more potent β -antagonist than the R-enantiomer. The enantiomers also display stereoselective disposition characteristics, the R-enantiomer being cleared more rapidly than the S-enantiomer (Hii et al., 1991). In order to study the mechanism of interactions between propafenone and nutrients in isolated rat livers and human subjects, a stereospecific assay method was required to determine the individual enantiomers of PF in biological samples.

Like most of the β -blocking agents, PF contains an isopropanolamine side-chain (Figure 4.1). Therefore, chiral derivatizing reagents can be employed for this drug. Tetra-O-acetyl- β -D-glucopyranosyl isothiocyanate (GITC, Figure 4.1) is a chiral reagent which has been used for derivatization and separation of the enantiomers of several β -blockers (Egginger and Lindner, 1993). Kroemer et al (1989) applied this reagent for high performance liquid chromatographic (HPLC) analysis of the propafenone enantiomers in plasma after multiple-dose administration of the racemate. The reported

HPLC assay, however, utilized multi-step extraction and incomplete GITC derivatization processes which resulted in a detection limit of 100 ng/ml. The low sensitivity of the assay limits its utilization when lower concentrations are expected.

In the present paper, we report a sensitive and convenient HPLC method for measurement of the propafenone enantiomers in rat liver perfusate and human plasma, based on a modification of Kroemer's method.

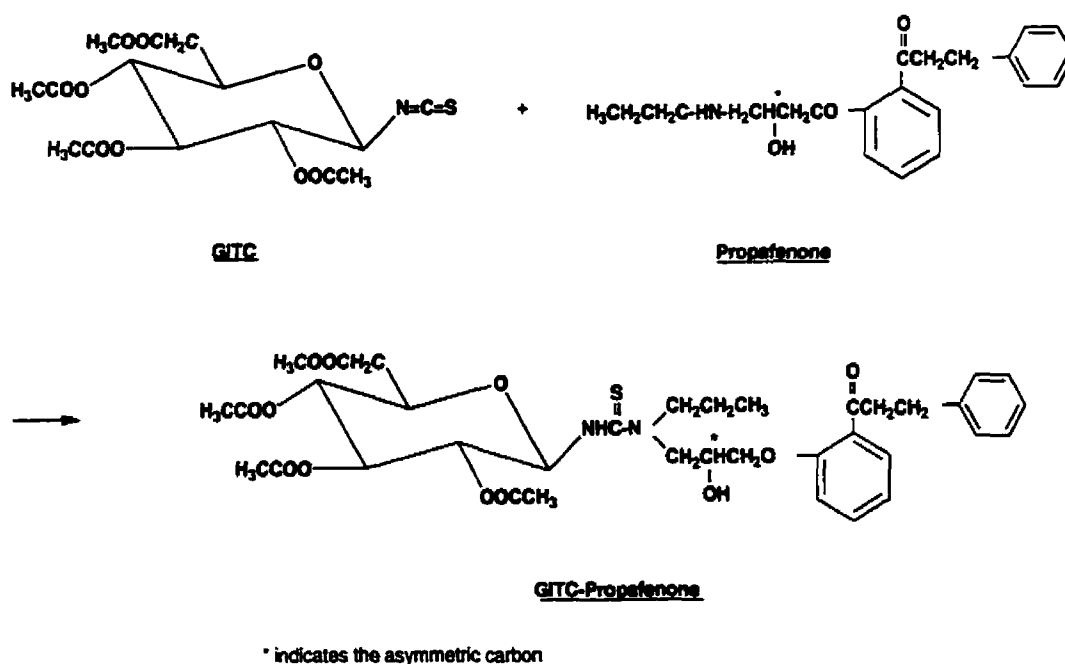


Figure 4.1. Derivatization of propafenone with GITC to form the diastereomeric thiourea products.

4.2 Experimental

4.2.1 Chemicals

Racemic propafenone hydrochloride (HCl) and GITC were purchased from Sigma (St. Louis, MO, USA). Authentic standards of racemic propafenone-HCl (Lu 29007) and the internal standard (Li 1115-HCL, the N-ethyl analogue of PF) were kindly supplied by Knoll AG, Ludwigshafen, Germany. The structures of propafenone and the internal standard (IS) are presented in Figure 5.1. Other chemicals used were commercial analytical grade purchased from BDH. All solvents were HPLC grade obtained from BDH Chemicals Canada (Edmonton, AB, Canada).

4.2.2 Standard solutions and reagents

Stock solutions of racemic PF (100 µg/ml) and IS (10 µg/ml) were prepared separately in deionized, distilled water. Appropriate dilutions of the stock solution of PF were made to generate a series of working standard solutions (10, 1 µg/ml). All stock and working standard solutions were stored at 4°C for no more than 3 months. Varying volumes of the PF working solutions were added to blank perfusate or plasma to produce final concentrations of 200, 400, 1000, 1750 and 2500 ng/ml for liver perfusate and 25, 200, 400, 600 and 1000 ng/ml for human plasma, respectively.

A 2 mg/ml solution of GITC in either acetonitrile or toluene was prepared. The solution was further diluted with either acetonitrile or toluene to give a final desired concentration. The solution was prepared fresh daily just before use.

4.2.3 Sample preparation

To 1 ml of rat liver perfusate or human plasma were added 50 μ l IS solution (10 μ g/ml) and 2 ml of 1M carbonate buffer (pH 11.0). Following addition of 5 ml of a hexane-2-propanol (90:10, v/v) mixture, the samples were vortex mixed for 10 min, and then centrifuged for 10 min at $1200 \times g$. The organic layer was then transferred to a clean borosilicate glass tube and evaporated to dryness under a nitrogen stream at room temperature. The residue was dissolved in a mixture composed of 40 μ l of the GITC solution (0.5 mg/ml) and 60 μ l of a triethylamine (TEA) solution (5 μ l/ml) in acetonitrile. The samples were slightly shaken at room temperature for 20 min. After evaporation under a nitrogen stream, the residue was reconstituted in 200 μ l of HPLC mobile phase, 20 μ l of which was injected into the HPLC column.

4.2.4 HPLC system

The HPLC system consisted of a Waters 510 pump, a Waters 490 programmable multiwave length detector set at a wavelength of 254 nm (Millipore-Waters, Mississauga, ON, Canada), a Model 7125 syringe loading sample injector with 50 μ l loop (Rheodyne, Cotati, CA, USA) and a Chromatopac C-R3A integrator (Shimadzu, Kyoto, Japan). Separations were carried out on a 250×4 mm I.D., 5 μ m particle size Lichrospher RP-18 column (E. Merck, Darmstadt, Germany). The mobile phase of 0.03% triethylamine, 0.1% H_3PO_4 in water-acetonitrile (40:60 v/v) was pumped at a flow rate of 1 ml/min.

4.2.5 Extraction efficiency

Perfusate or plasma samples ($n = 5$, respectively) containing 100, 2500 and 5000 ng/ml of PF were extracted according to the extraction procedure described as above. Without derivatization with GITC, the extracted residues were reconstituted and injected into the HPLC system. The peak heights of the racemic PF in these samples were then compared to those obtained from PF standard solutions with comparable concentrations.

4.2.6 Accuracy and precision

Rat liver perfusate samples. Three sample pools containing 245, 1275 and 2550 ng/ml of PF were prepared by adding appropriate volumes of standard solutions to drug-free liver perfusate. The samples were stored at -20°C until analysis. Five replicate samples from each pool were extracted and analyzed on five separate days. Concentrations were determined by comparison with a calibration curve prepared on the day of analysis. From the data obtained, intra- and inter- day relative standard deviations (RSD) and mean accuracy were calculated.

Human plasma samples. Three pools of plasma samples containing 24.5, 510 and 935 ng/ml of PF were prepared and stored at -20°C . On three different days, five replicate samples of each concentration were analyzed. The intra- and inter-day precision and accuracy were calculated in the same way as that for liver perfusate samples.

4.2.7 Application to isolated perfused rat liver study

The present assay for PF enantiomers was applied in a pilot study, in which the effects of insulin and glucagon on the hepatic disposition of PF enantiomers were tested in the isolated perfused rat liver. The study was designed for examining whether insulin and/or glucagon are involved in the food effect since the portal concentrations of insulin and glucagons are known to be dramatically changed after a meal. The technique of the isolated perfused rat liver is described in the section 3.2 of Chapter 3. Racemic PF (20 µg/ml) in Krebs-Henseleit bicarbonate buffer containing 4 mM glucose was infused into a single-pass isolated perfused rat liver for 130 min at a flow rate of 25 to 30 ml/min. Insulin or glucagon at 2×10^{-9} was introduced from 60 to 90 min. The effluent samples were collected every 5 min during the perfusion, and immediately stored at -20°C. The assay was conducted on the next day after the experiment.

4.3 Results and discussion

In the present study, a stereospecific HPLC assay for the enantiomers of PF in rat liver perfusate and human plasma has been developed using precolumn derivatization with GITC. Under the stated conditions, the formed diastereomers of IS and the diastereomer S (+)- and R (-)-PF, eluted at 10.3, 11.2, 13.1 and 14.2 min, respectively. The HPLC chromatograms are shown in Figure 4.2a for rat liver perfusate samples and Figure 4.2b for human plasma, respectively.

GITC is widely used for precolumn derivatization of secondary amines in the development of stereospecific HPLC methods for the separation of the enantiomers of a number of β -adrenoceptor antagonists (Egginger and Lindner, 1993). GITC reacts rapidly with primary and secondary amines, and it is stereochemically stable and available in a highly pure form. The isothiocyanate group of GITC reacts with the secondary amino group of the PF enantiomer under mild conditions to form a corresponding stable thiourea derivative of PF (Figure 4.1). The stereoselective mechanism responsible for the good separation of the formed diastereomers presumably contributes to the formation of rigid conforms via intra molecular hydrogen bonding between the acetyl groups and the hydroxyl group in the β -position of the derivatized amino group (Egginger and Lindner, 1993). By applying this reagent in an HPLC method, Kroemer et al. (1989) successfully separated PF enantiomers in plasma. However, the derivatization yield was very low under the conditions of Kroemer's method, in which PF reacted with GITC alone in a toluene solution.

To optimize derivatization, GITC was dissolved in two organic solvents, acetonitrile and toluene, each in the presence and absence of different concentrations of the basic modifier, triethylamine (TEA). The results are shown in Figure 4.3. Among the solvents tested, the most satisfactory result was obtained when GITC was dissolved in acetonitrile with the addition of 0.3% (v/v) triethylamine. GITC concentration is another key factor in the extent of derivatization. The derivatization yield of PF enantiomers reached its maximum at a GITC concentration of 0.1 mg/ml or higher (Figure 4.4). In our final method, the concentration of GITC at 0.2 mg/ml was added for the derivatization. The reaction of PF enantiomers with GITC under these conditions was rapid even though it was at room temperature. After 15 min, the reaction yield achieved its maximum (Figure 4.5); therefore the reaction time was 20 min in the final method. Using the optimum conditions stated in the Experimental section, the derivatization yield, based on residual analysis of propafenone, was 93.0%, which was much higher than that obtained from Kroemer's method (71.3%).

To increase the extraction efficiency from human plasma, different organic solvents, including dichloromethane, diethylether, toluene-n-butanol, toluene-diethylether, hexane-n-butanol and hexane-n-propanol, and different basic solutions, including carbonate buffer, NaOH solution and Na₂CO₃ solution, were selected as extraction solvents. Among the solvents and basic solutions tested, a solvent of hexane-2-propanol (90:10, v/v) and a basic solution of 1M carbonate buffer (pH 11.0) were used to extract the drug from the biological samples. The extraction efficiencies (mean \pm SD) of PF from human plasma were $81.5 \pm 7.2\%$ and $80.1 \pm 3.5\%$ at concentrations of 100

and 2500 ng/ml, respectively (Table 4.1). This is contrast to the low extraction yield of 40% which was reported by Kreomer et al. (1989) after multi-step extraction of PF.

Using 1 ml of perfusate or plasma, the limits of detection (signal-to-noise ratio = 3:1) were 3.2 ng/ml for both S (+)-PF and R (-)-PF in rat liver perfusate, and 4.3 ng/ml for S (+)-PF and 5.0 ng/ml for R (-)-PF in human plasma. The relationship between S (+)- or R (-)-PF/IS peak height ratios and PF plasma concentrations were linear ($r > 0.9998$). The typical equations describing the relationship were

$$\text{S (+)-PF: } y = 8.839 \times 10^{-3} x - 0.3066;$$

$$\text{R (-)-PF: } y = 8.410 \times 10^{-3} x - 0.2324$$

in rat liver perfusate samples (Figure 4.6a), and

$$\text{S (+)-PF: } y = 7.592 \times 10^{-3} x - 3.692 \times 10^{-3};$$

$$\text{R (-)-PF: } y = 7.417 \times 10^{-3} x - 1.761 \times 10^{-2}$$

in human plasma samples (Figure 4.6b), where y and x are the peak height ratios and plasma concentrations, respectively.

The assay precision and accuracy for perfusate and plasma samples are reported in Tables 4.2 and 4.3, respectively. The interday RSD and error values ranged from 3.8% to 14.9% and -1.4% to 5.5% for perfusate samples, respectively; for plasma samples, the interday RSD and error values ranged from 7.6% to 16.9% and -2.1% to 16.1%, respectively.

The assay was successfully utilized for the analysis of rat liver perfusate samples obtained from the isolated rat liver perfused with 20 $\mu\text{g/ml}$ PF. The time courses of PF

enantiomers after coinfusion of insulin and glucagon are shown in Figure 4.7. The time to reach steady state was about 50 min, indicating that the tissue binding of PF was very high. The disposition of PF in the rat liver was stereoselective, but no insulin and glucagon interaction with PF was observed. The assay for human plasma samples was not employed in the present work due to the discontinuation of the study protocol.

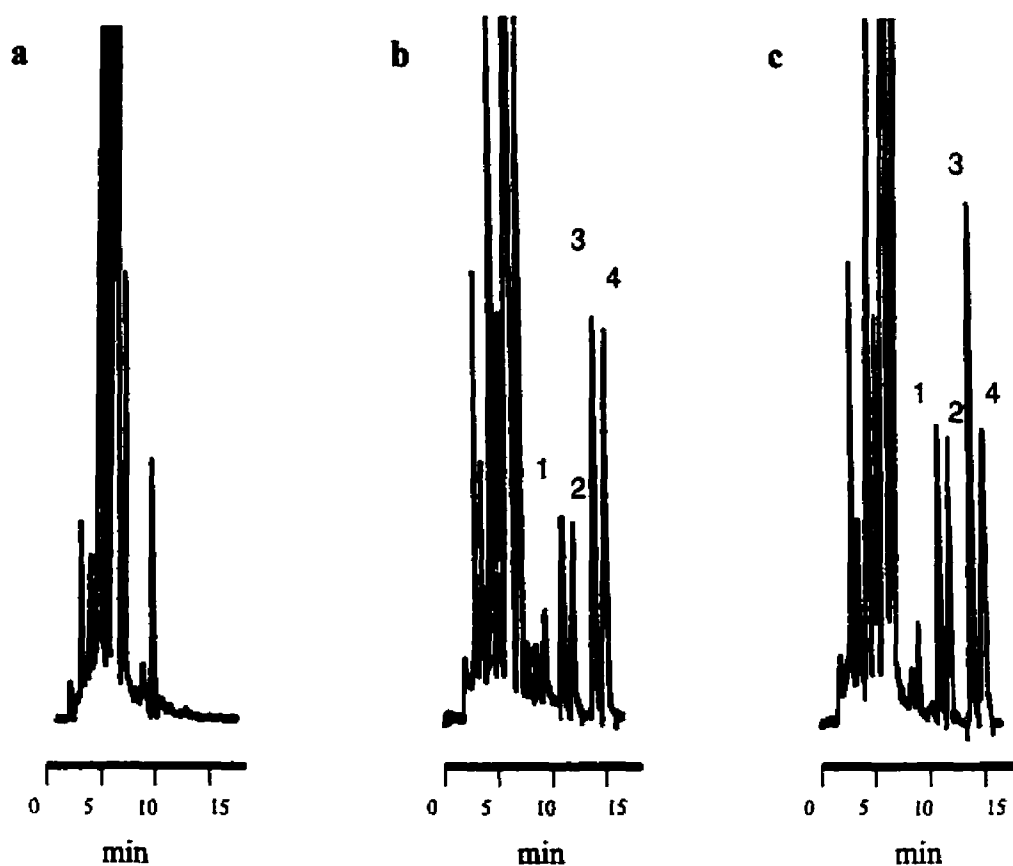


Figure 4.2a. Chromatograms of extracts from (a) a blank liver perfusate sample, (b) a blank liver perfusate sample spiked with racemic PF and IS, and (c) a liver perfusate sample taken at 70 min, after derivatization with GITC.

Peaks 1 and 2 represent the diastereomers formed with IS; Peaks 3 and 4 represent the S (+)- and R (-)- PF, respectively.

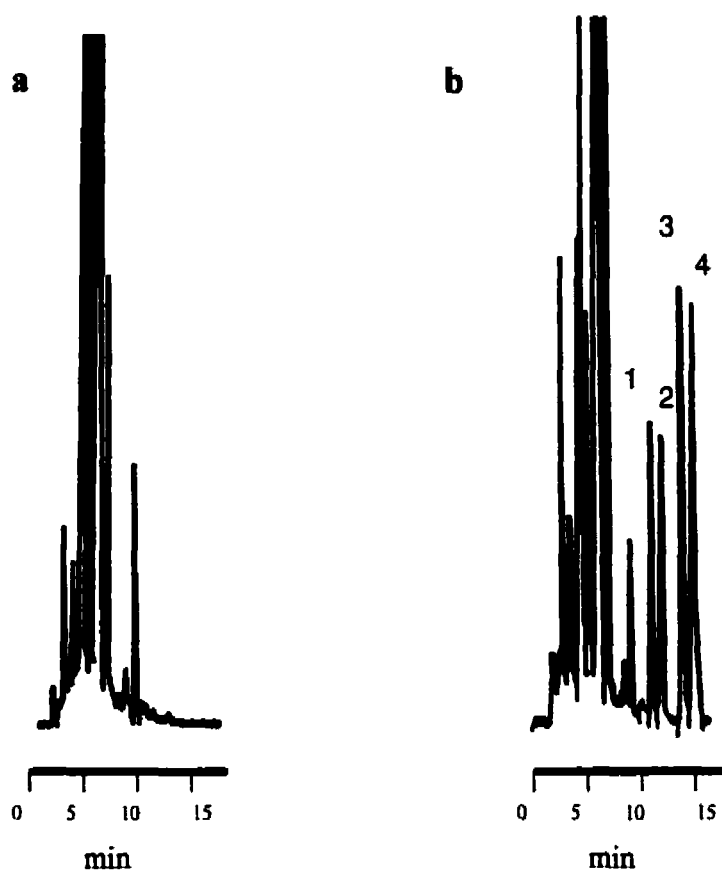


Figure 4.2b. Chromatograms of extracts from (a) a blank plasma sample and (b) a blank plasma sample spiked with PF and IS, after derivatization with GITC. Peaks 1 and 2 represent the diastereomers formed with IS; Peaks 3 and 4 represent the S (+)- and R (-)- PF, respectively.

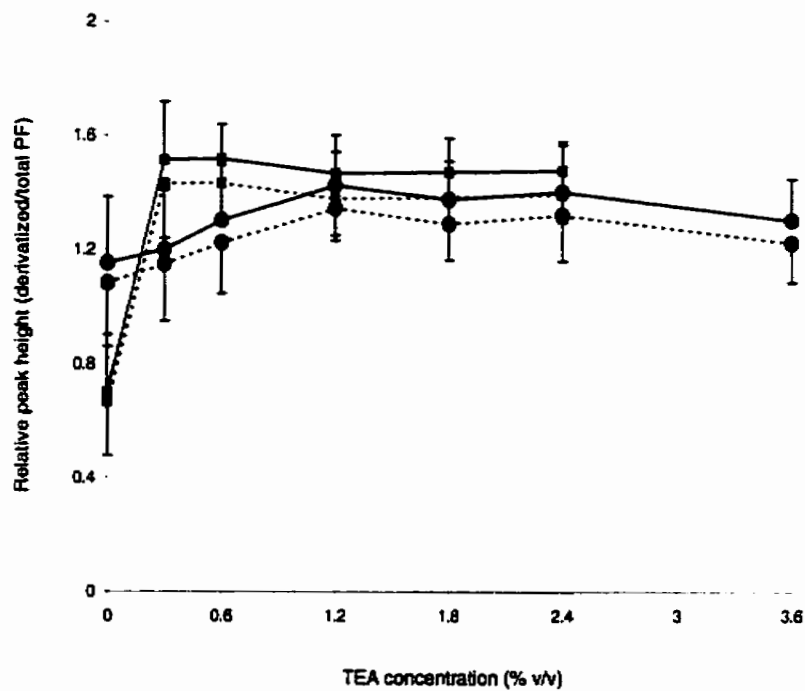


Figure 4.3. Effect of triethylamine concentration on the derivatization yield at 60 min after derivatization of S (+)-PF (solid line) and R (-)-PF (dashed line) in a 0.4 mg/ml GITC solution in either acetonitrile (square symbol) or toluene (circle symbol).

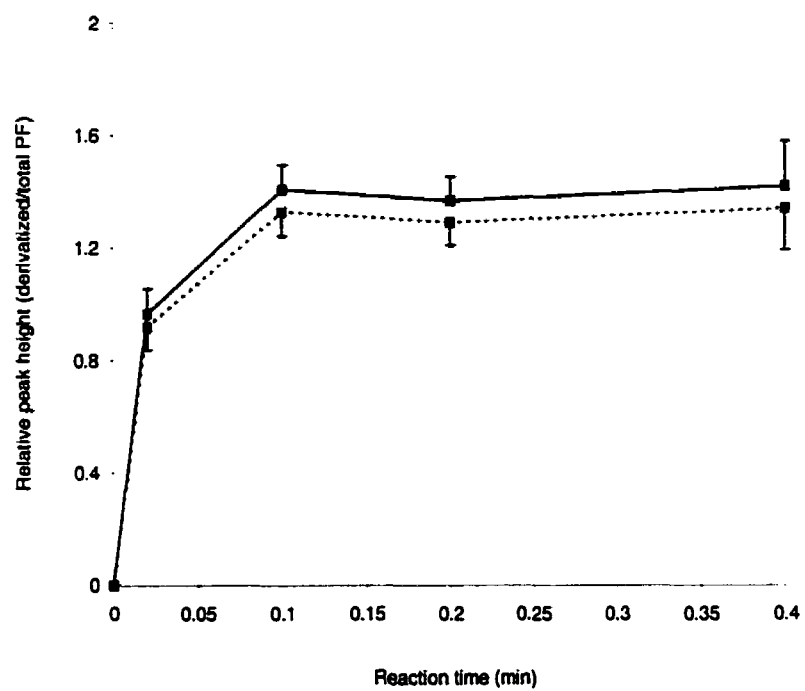


Figure 4.4. Effect of GITC concentration on the derivatization yield at 30 min after derivatization of S (+)-PF (solid line) and R (-)-PF (dashed line) in 0.3% triethylamine acetonitrile solution.

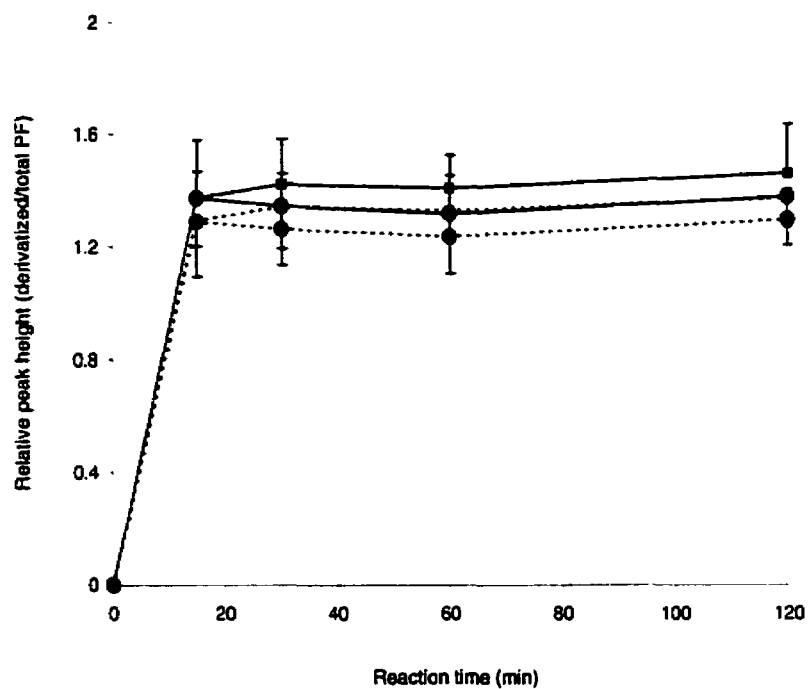


Figure 4.5. Effect of reaction time on the derivatization yield of S (+)-PF (solid line) and R (-)-PF (dashed line) after reaction with 0.4 mg/ml GITC in 0.3% triethylamine solution in either acetonitrile (square symbol) or toluene (circle symbol).

Table 4.1. Extraction recovery of propafenone from rat liver perfusate and human plasma (n = 5)

Concentration	Extraction Recovery (% \pm RSD)	
	Liver perfusate	Plasma
100 ng/ml	95.3 \pm 6.1%	81.5 \pm 7.2%
2500 ng/ml	94.4 \pm 1.5%	80.1 \pm 3.5%
5000 ng/ml	94.4 \pm 1.5%	78.0 \pm 4.2%

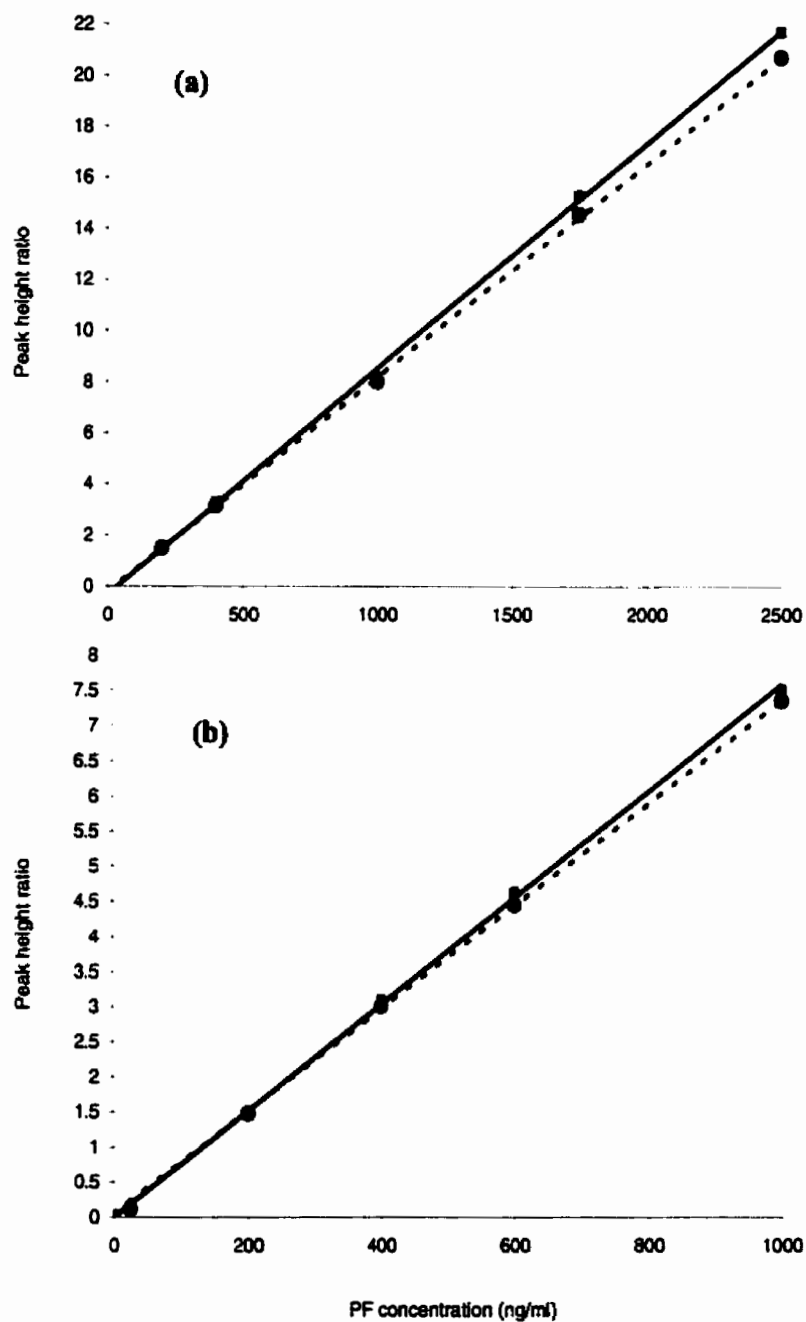


Figure 4.6. Standard curves of S (+)-PF (solid line) and R (-)-PF (dashed line) for rat liver perfusate samples (a) and human plasma samples (b).

Table 4.2. Accuracy and precision for S (+)- and R (-)- PF in rat liver perfusate
(5 days; n = 5 in each day)

Parameter	Concentration in rat liver perfusate					
	245 ng/ml		1275 ng/ml		2550 ng/ml	
	S (+)-	R (-)-	S (+)-	R (-)-	S (+)-	R (-)-
Intra-day RSD ^a (%)	4.1	4.3	6.6	6.9	4.5	4.1
Inter-day RSD ^b (%)	14.9	11.7	3.8	4.9	8.8	7.1
Mean accuracy (%)	98.6	100.9	101.7	105.5	101.3	103.4

^a Mean of the daily RSD values.

^b RSD values of daily means.

Table 4.3. Accuracy and precision data for S (+)- and R (-)- PF in human plasma
(3 days; n = 5 in each day)

Parameter	Concentration in human plasma					
	24.5 ng/ml		510 ng/ml		935 ng/ml	
	S (+)-	R (-)-	S (+)-	R (-)-	S (+)-	R (-)-
Intra-day RSD ^a (%)	4.7	3.1	5.8	4.8	3.5	2.9
Inter-day RSD ^b (%)	12.9	16.9	11.2	10.5	8.8	7.6
Mean accuracy (%)	97.9	100.8	113.3	116.1	112.3	115.2

^a Mean of the daily RSD values.

^b RSD values of daily means.

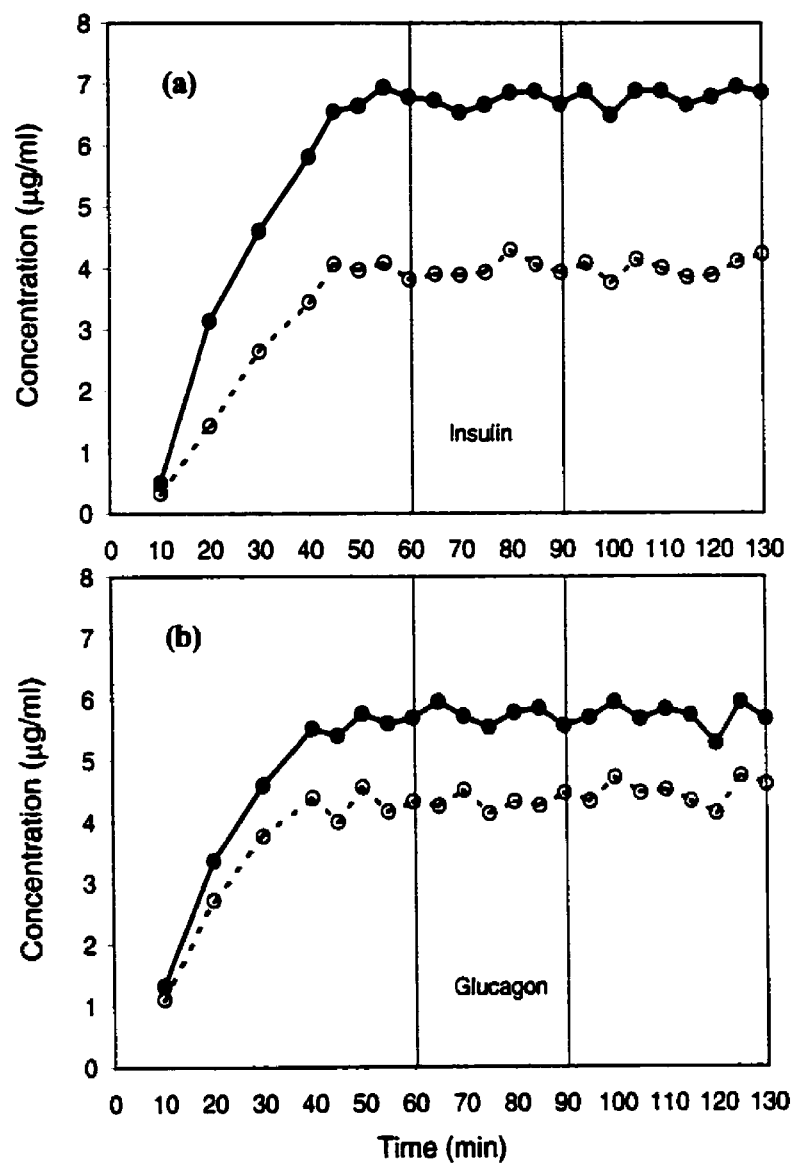


Figure 4.7. Time courses of concentration of S (+)-PF (solid line) and R (-)-PF (dashed line) after coinfusion with insulin (a) and glucagon (b) in the isolated rat liver perfused with 20 µg/ml of racemic PF.

CHAPTER 5

IDENTIFICATION AND DETERMINATION OF PHASE I METABOLITES OF PROPAFENONE IN RAT LIVER PERFUSATE ^{1,2}

5.1 Introduction

Propafenone (PF), 2'-(2-hydroxy-3-propylamino-propoxy)-3-phenylpropionephene, is a potent antiarrhythmic drug widely used in the treatment of ventricular and supraventricular arrhythmias (Harron and Brogden, 1987). Like some other high hepatic first-pass drugs, e.g. metoprolol and propranolol, propafenone shows dramatically increased bioavailability when given with food, even though it is completely absorbed after an oral dose (Melander and Lalka, 1988; Axelson et al., 1987). In order to carry out studies on this "food effect", which may be related to the interaction between nutrients and drugs in the liver, the metabolism of propafenone was studied in the isolated, perfused rat liver.

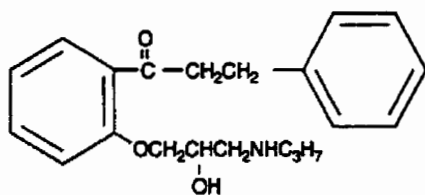
Propafenone undergoes extensive hepatic oxidative metabolism in the dog and man. Less than 1% of the dose was recovered as unchanged substance in urine and bile (Hege et al., 1984a; 1984b). After oral doses of deuterated propafenone in man, two hydroxylated propafenone derivatives, 5-hydroxypropafenone (5-OH-PF) and 5-

¹ Presented at the Tenth Annual American Association of Pharmaceutical Scientists Meeting, October 1996, Seattle, Washington, USA.

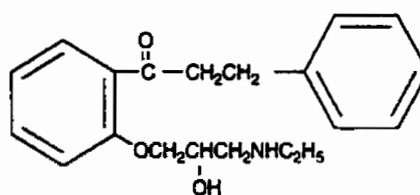
² Published in 1998 (Tan, W., Li, Q., McKay, G. and Semple, H. A. Identification and determination of phase I metabolites of propafenone in rat liver perfusate. *J. Pharm. Biomed. Anal.* 16: 991-1003).

hydroxy-4-methoxypropafenone (5-OH-4-OCH₃-PF) were described as the two major phase I metabolites in the samples of urine, bile, feces and plasma (Hege et al., 1984a; 1986). Moreover, N-despropylpropafenone (N-des-PF), an N-dealkylated metabolite, was found to accumulate in the plasma of patients during chronic therapy with the parent drug (Kates et al., 1985). All of these three major metabolites have activity comparable to that of propafenone (Hege et al., 1984a; Oti-Amoako et al., 1990; Thompson et al., 1988). In dog urine and bile samples, the major phase I metabolites, 5-hydroxypropafenone and 4'-hydroxypropafenone (4'-OH-PF), accounted for about 15% of the dose for each (Hege et al., 1984a; 1986), whereas 5-hydroxy-4-methoxypropafenone and N-despropylpropafenone are quantitatively only of minor importance. The structures of propafenone and its major phase I metabolites in dog and man are shown in Figure 5.1.

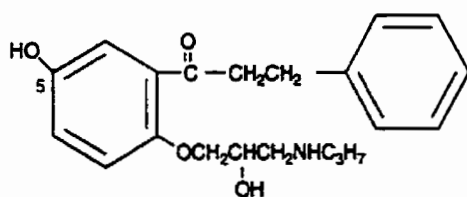
Although the major phase I metabolites of propafenone have been identified in humans and dogs, no metabolic studies have been reported in rats or other animals. The present paper describes the identification of the phase I metabolites of propafenone in the effluent samples from the isolated, perfused rat liver by high performance liquid chromatography/mass spectrometry (HPLC/MS) and tandem mass spectrometry (MS/MS) methods. Finally, the levels of these metabolites were determined simultaneously by a simple and convenient HPLC method.



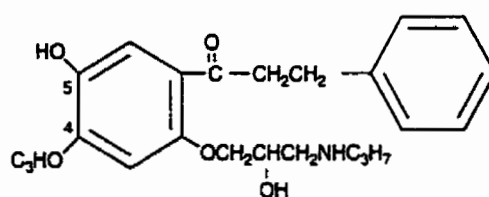
Propafenone



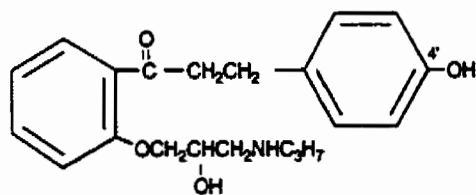
Internal standard



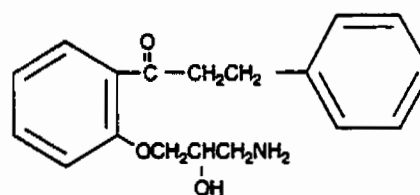
5-Hydroxypropafenone



5-Hydroxy-4-methoxypropafenone



4'-Hydroxypropafenone



N-Despropylpropafenone

Figure 5.1. Structural formulae of propafenone and its metabolites, and internal standard.

5.2 Experimental

5.2.1 Chemicals

All solvents used for extraction and preparation of the HPLC mobile phase were HPLC grade obtained from BDH Chemicals Canada Ltd. (Edmonton, AB, Canada). Other chemicals used were commercial analytical grade purchased from BDH. Propafenone hydrochloride (HCl) and Type H-1 β -glucuronidase (from *Helix pomatia*) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Authentic standards of propafenone HCl (Lu 29007), 5-hydroxypropafenone HCl (Lu 40545), N-despropylpropafenone fumarate (Lu 48686) and the internal standard (Li 1115-HCl, the N-ethyl analogue of PF) were kindly supplied by Knoll AG, Ludwigshafen, Germany. The structures of these compounds are shown in Figure 5.1.

5.2.2 Liver perfusion

The surgical procedure and the perfusion apparatus were identical to those previously described (Chapter 3, Section 3.2). Following an overnight fast, male Sprague-Dawley rats weighing 220 to 250 g were anesthetized by halothane inhalation, the *vena cava* and hepatic portal vein were catheterized, and the livers were isolated and placed in a perfusion cabinet. The rat livers were perfused via the hepatic portal vein with oxygenated perfusate (5% CO₂ and 95% O₂) at a flow rate of 25 to 30 ml/min/liver. The perfusate contained 2 g/l of glucose and 5 or 20 μ g/ml of propafenone in Krebs bicarbonate solution, buffered to pH 7.4. A portion of the effluent from the *vena cava*

was passed through the microelectrode flow-through cell of an oxygen monitor (YSI model 5300, Fisher Scientific, Edmonton, AB, Canada), while the major portion was collected for analysis. The entire circuit was temperature controlled at 37°C. Perfusate samples were collected during the time interval of 60 - 100 min after perfusion with drug, in which propafenone was known to be at steady state (Chapter 4, Figure 4.7). Aliquots of perfusate samples were stored at -20°C until analysis.

5.2.3 Sample preparation

The phase I metabolites were extracted with diethyl ether. Perfusate samples (1 ml) were vortex mixed with 5 ml of diethyl ether for 10 min. After centrifugation at $1200 \times g$ for 10 min, the organic layer was transferred to a clean borosilicate glass tube and evaporated to dryness under a stream of N_2 at room temperature. The residues were reconstituted in 200 μ l of HPLC mobile phase, and injected into the HPLC system or HPLC/MS system.

Each peak obtained from the HPLC with ultraviolet (UV) detection was purified by a Spe-ed® cartridge packed with solid phase C_{18} (Applied Separations Inc, Bethlehem, PA, USA). The cartridge was conditioned by eluting with 2 ml of acetonitrile, 2 ml of methanol, and 2 ml of distilled water. Each fraction collected from the HPLC was treated with a flow of nitrogen to evaporate organic solvents, and then lyophilized. The residue was reconstituted in 1 ml of water, and applied on the conditioned cartridge. After the samples had adsorbed to the solid phase, the cartridge was washed with 3 ml of water to remove salts contained in the samples. Finally the

metabolite fraction was eluted with 1 ml of methanol, which was evaporated under a stream of nitrogen. The residue was reconstituted in 1 ml of methanol for injection into the MS/MS spectrometer.

5.2.4 Chromatographic methods

5.2.4.1 HPLC conditions

Propafenone and its phase I metabolites were separated by an HPLC system with a mobile phase of ammonium acetate (0.005 M)-acetonitrile-methanol (50:15:35, v/v/v), apparent pH (pH*) 2.90 adjusted with trifluoroacetic acid, pumped at a flow rate of 1 ml/min. The HPLC system consisted of a Waters 510 pump, a Waters 490 programmable multiwavelength detector set at 210 nm (Millipore-Waters, Mississauga, Ont., Canada), a Model 7125 syringe loading sample injector with 200 µl loop (Rheodyne Inc., Cotati, California, USA), and a 250×4 mm I.D., 5-µm particle size Lichrospher RP-18 column (E. Merck, Darmstadt, Germany). The data were recorded using a Chromatopac C-R3A integrator (Shimadzu Co., Kyoto, Japan).

5.2.4.2 HPLC/MS and MS/MS

HPLC/MS and MS/MS spectra were obtained using a VG Quattro-II triple quadrupole mass spectrometer equipped with an electrospray ion source (Micromass, Altrincham, UK). HPLC/MS was performed with an atmospheric pressure chemical ionization (APCI) interface in the positive ion mode. The probe temperature and corona discharge pin were maintained at 500°C and 3.5 kV, respectively. Product ion spectra

were obtained by positive ion electrospray (+ESI) MS/MS with collision-induced dissociation (CID). The collision energy, E_{lab} , was 20 eV, and the argon pressure was set at 1.0×10^{-3} mBar. The HPLC solvent delivery system utilized a model 140A dual syringe pump (Applied Biosystems, Mississauga, Ont., Canada) fitted with a Rheodyne 7125 valve loop injector equipped with a 20 μ l sample loop. The separation column and HPLC conditions used in HPLC/MS system are described as above.

5.2.5 Quantitation of propafenone and its metabolites in rat liver perfusate

To 1 ml of rat liver perfusate were added 30 μ l of internal standard solution (100 μ g/ml). The drug, metabolites and internal standard were extracted with 5 ml of diethyl ether and 2 ml of sodium bicarbonate buffer (0.05M, pH 10.0). The extracted samples were reconstituted in 200 μ l of mobile phase, and 20 μ l aliquots were injected into the HPLC system with a mobile phase of water-acetonitrile-methanol (45:20:35, v/v/v), containing 0.03% (v/v) triethylamine and 0.05% (v/v) of concentrated phosphoric acid, apparent pH 3.10. Standard samples were prepared in 1 ml of blank rat liver perfusate at concentrations of 50, 100, 200, 400, 800 and 1000 ng/ml of 5-hydroxypropafenone and N-despropylpropafenone, and 5, 6, 8, 10, 12 and 15 μ g/ml of propafenone. Calibration curves were obtained by plotting peak height ratios of drug/internal standard vs. drug concentrations in standard samples.

1 ml samples of liver perfusate collected during 100 - 105 min after infusion with 20 μ g/ml propafenone were incubated with 5000 units of β -glucuronidase (contains 338,000 units/g of β -glucuronidase and 16,000 units/g of sulfatase) at 37°C for 4 hr, and

the levels of propafenone and its phase I metabolites were determined. Quality control (QC) samples duplicated at two different concentrations (close to the highest and lowest standard curve concentrations) of PF and its metabolites in the range of the calibration curves were prepared with the analyst blinded, and incorporated into each batch.

5.3 Results

5.3.1 Identification of metabolites

A typical HPLC-UV chromatogram of an extract from rat liver perfusate after infusion of PF (Figure 5.2C) gave four major peaks which were not present in the chromatogram of a blank liver perfusate extract (Figure 5.2A). Of the four peaks, assigned as peaks 1, 2, 3 and 4, peaks 2, 3 and 4 had the same retention time values as those obtained from authentic 5-OH-PF, N-des-PF and PF (Figure 5.2B). The liver perfusate extract was then analyzed by HPLC/MS. The HPLC/MS total ion chromatogram scanned from m/z 200-450 (Figure 5.3) demonstrated similar chromatographic peaks labeled 1, 2, 3, and 4 as those previously obtained in the HPLC-UV analysis. The positive ion background-subtracted mass spectra of these peaks are reported in Figure 5.4. During APCI ionization, very little fragmentation was observed, and intense probable protonated molecular ions (MH^+) were evidenced, respectively, at m/z 358, 358, 300 and 342, together with apparently natriurated molecular ions (MNa^+). On the basis of their molecular weights and HPLC retention time values, peaks 2, 3 and 4 were tentatively identified as 5-OH-PF, N-des-PF and PF, respectively. Peak 1, however, did not correspond to any authentic compounds, although it is suggestive of a hydroxylated analogue of PF because of the inferred molecular weight of 357 Da, 16 Da above that of parent drug and the same nominal m/z as that of 5-OH-PF.

In order to obtain structural information about Peak 1, the appropriate fractions corresponding to each peak from the HPLC chromatograms were collected, desalted,

and then analyzed by direct loop injection into the mass spectrometer operated in the MS/MS mode. Pre-selection of the precursor ions, m/z 358, 300 and 342, was carried out in each peak and the corresponding CID product ion spectra were recorded. The CID product ion spectra of peaks 2, 3, and 4, which are shown in Figure 5.5A, 5.6 and 5.7, respectively, gave further confirmation of the identities to 5-OH-PF, N-des-PF and PF, respectively, since they were practically superimposable with the spectra of the authentic compounds. The product ion spectrum of peak 1 (Figure 5.8A), however, showed distinctive differences from that of peak 2, indicating that these two hydroxylated PF metabolites with pseudomolecular ions at m/z 358 are structural isomers. The product ion observed at m/z 107, could be explained if hydroxylation occurred at the terminal phenyl ring. Similarly the product ions at m/z 234 and 175, could be generated from the unhydroxylated disubstituted phenyl ring (Figure 5.8B), strongly suggesting that the site of hydroxylation in the material eluting under peak 1 must be on the ω -phenyl ring. This derivative hydroxylated in the ω -phenyl ring (ω -OH-PF) should correspond to 4'-OH-PF, according to Hege et al (1986). In contrast to the product ion spectrum of peak 1, 5-OH-PF showed diagnostic ions at m/z 91 generated from the unhydroxylated terminal phenyl ring and m/z 281 generated from the hydroxylated middle phenyl ring (Figure 5.5A), and no product ions at m/z 234, 175 and 107.

5.3.2 Determination of propafenone and its metabolites

The HPLC chromatograms under the conditions used to quantitate PF and its metabolites are shown in Figure 5.9. Because no standard reference compound for peak

1 was available, the fraction collected from HPLC and identified by MS/MS was used as a standard reference to confirm the retention time of ω -OH-PF after the mobile phase was modified. The quantity of the metabolite from the collected fraction was insufficient to prepare a standard solution for a calibration curve, therefore the peak height ratios of drug/internal standard were used to reflect the levels of this metabolite in the rat liver perfusate.

The extraction efficiency of diethyl ether at pH 10.0 was evaluated by comparing the peak height ratios of the extracted spiked samples to the unextracted samples which were directly injected into the HPLC. The mean recoveries ($n=5$) after extraction were 96.6% for 5 μ g/ml of PF, 95.4% for 500 ng/ml of 5-OH-PF and 93.6 % for 500 ng/ml of N-des-PF, respectively. The calibration curves for these three analytes are summarized in Table 5.1. All the determined concentrations of QC samples were within 12% error of the nominal concentrations.

The total concentrations of PF, 5-OH-PF and N-des-PF in rat liver perfusate after infusion of 20 μ g/ml (53.0 μ M) of PF were determined after cleavage of conjugates, which were 12657 ng/ml (33.5 μ M) \pm 4.5%, 948.6 ng/ml (2.41 μ M) \pm 3.8% and 632.3 ng/ml (1.52 μ M) \pm 1.3% (mean \pm RSD, $n=3$), respectively. Peak height ratios of peak 1 and 5-OH-PF are $2.145 \pm 2.2\%$ and $0.429 \pm 3.6\%$, respectively.

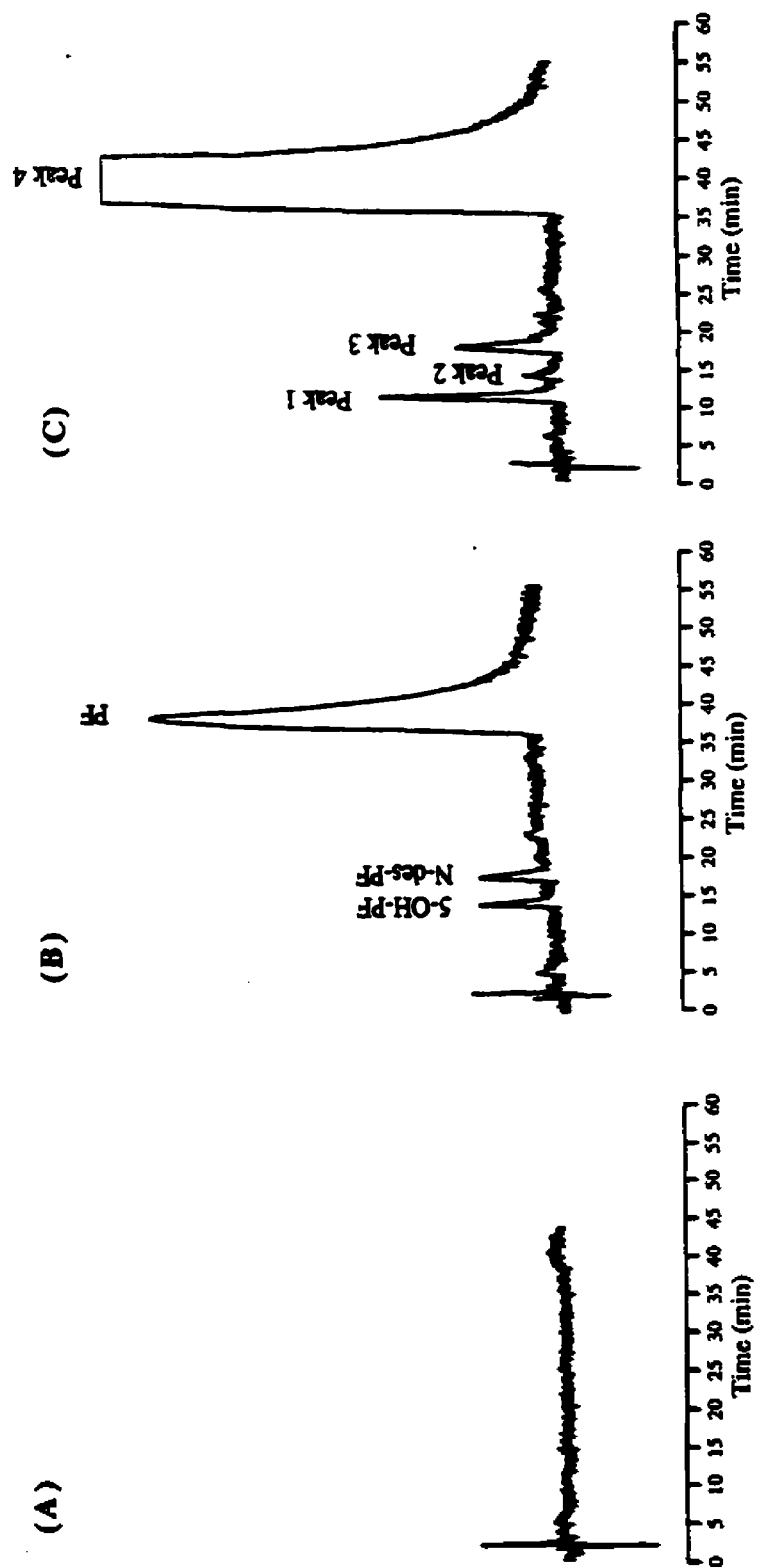


Figure 5.2. HPLC-UV chromatograms of extracts from (A) blank rat liver perfusate; (B) blank rat liver perfusate spiked with reference standards; (C) rat liver perfusate after infusion of 50 µg/ml PF.

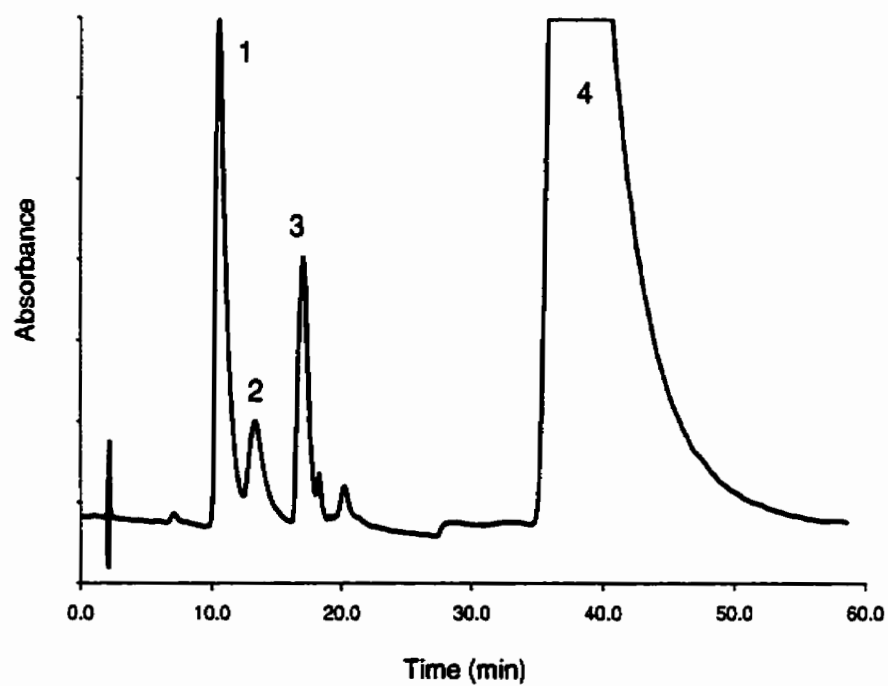


Figure 5.3. HPLC/MS total (positive ion APCI) ion chromatogram (m/z 200-450) of extract from rat liver perfusate after infusion of 50 $\mu\text{g/ml}$ PF.

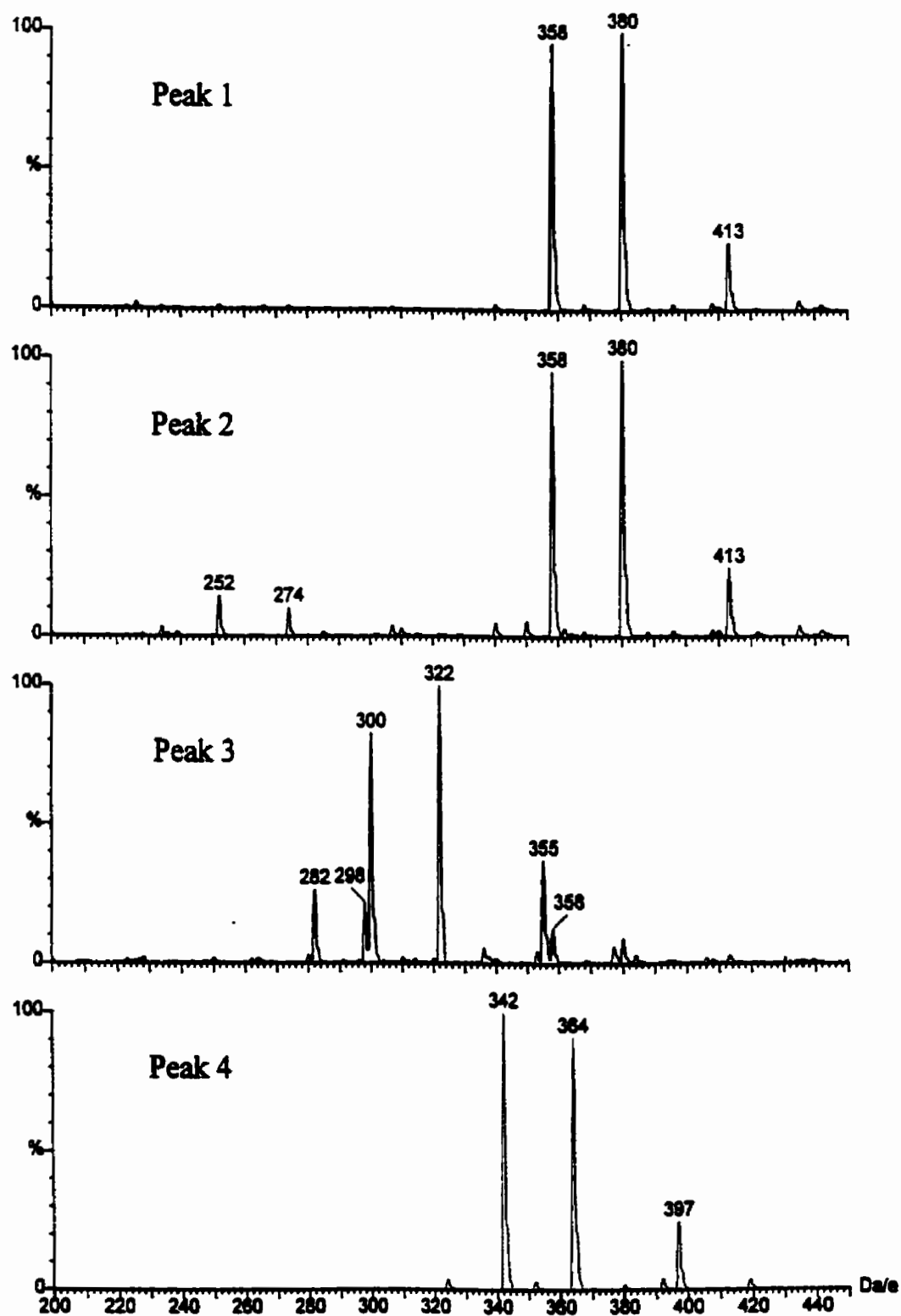


Figure 5.4. Positive ion background-subtracted mass spectra of peaks 1, 2, 3 and 4.

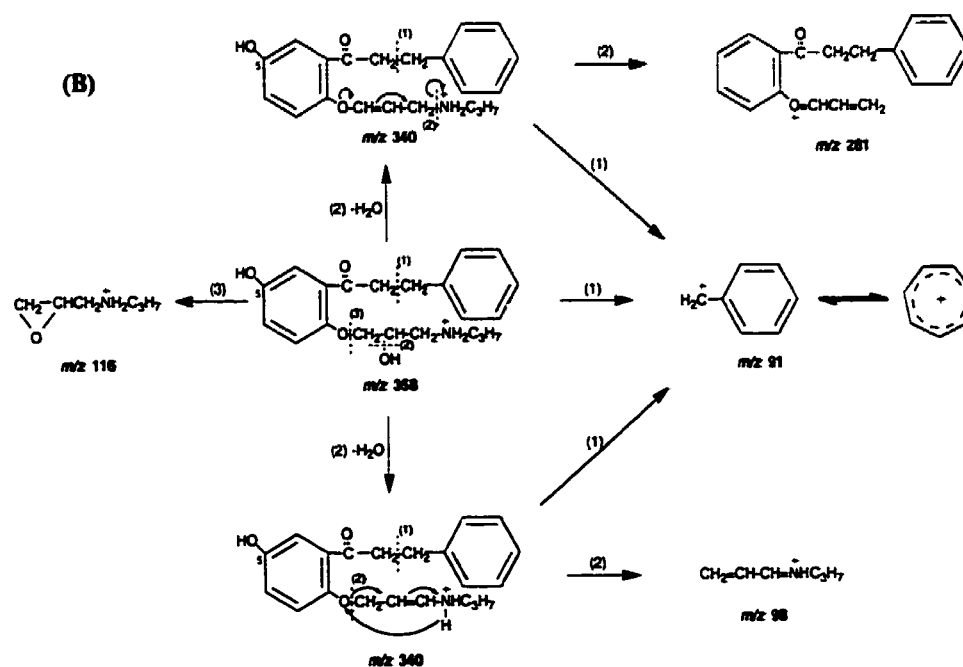
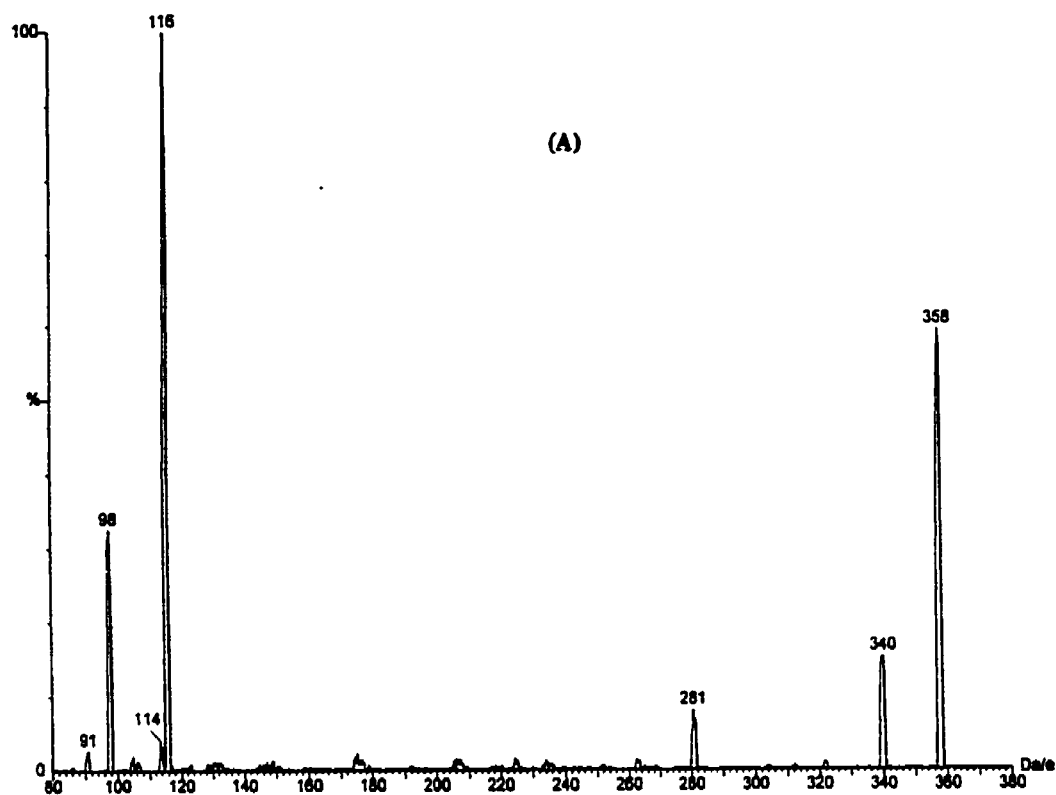


Figure 5.5. Product ion analysis for peak 2. (A) product ion mass spectrum of the protonated molecule at m/z 358; (B) proposed fragmentation pattern.

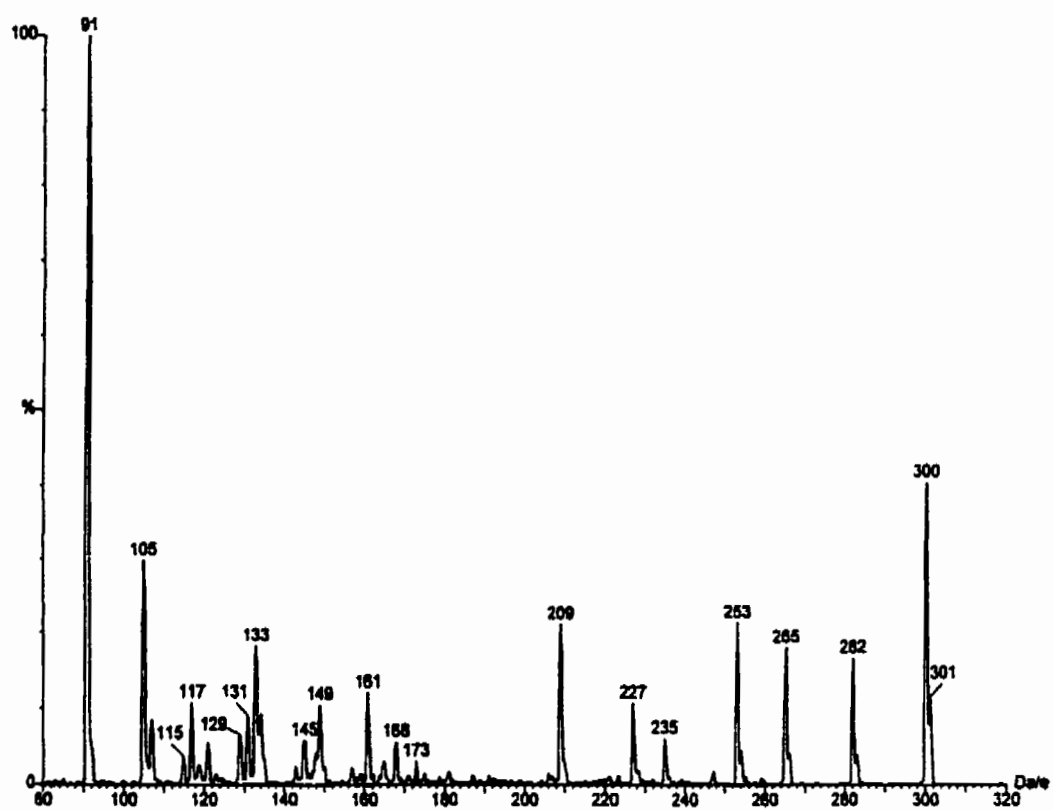


Figure 5.6. Product ion mass spectrum of the protonated molecule of peak 3 at m/z 300.

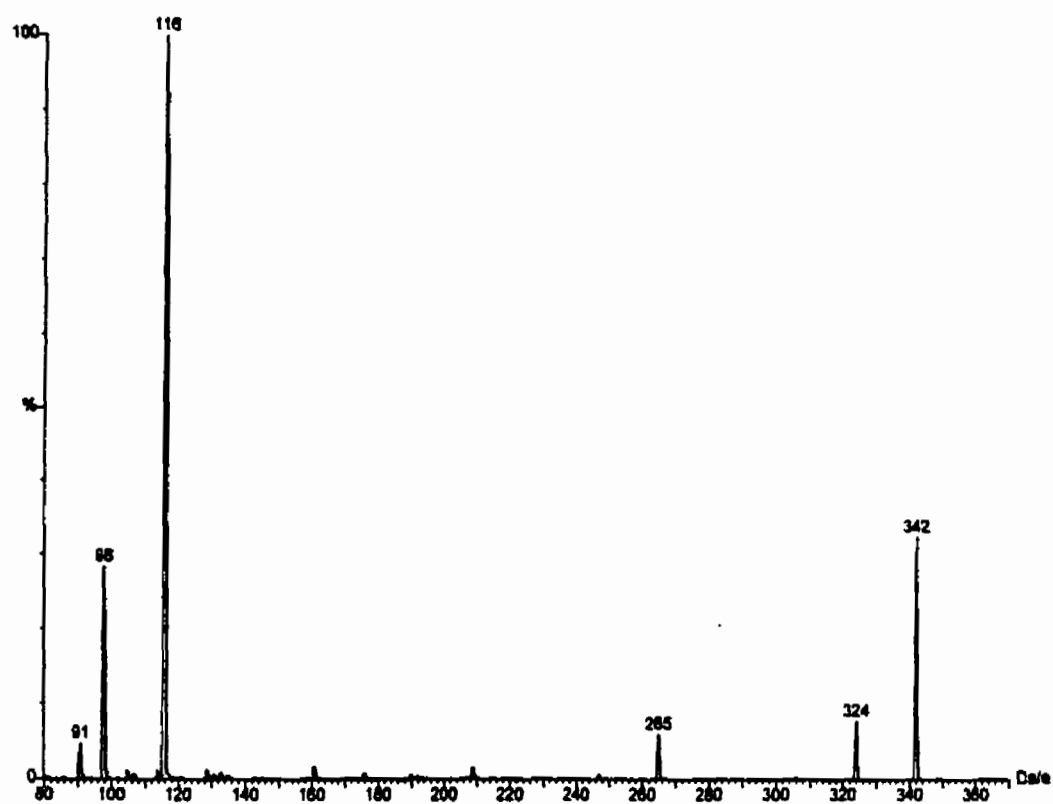


Figure 5.7. Product ion mass spectrum of the protonated molecule of peak 4 at m/z 342.

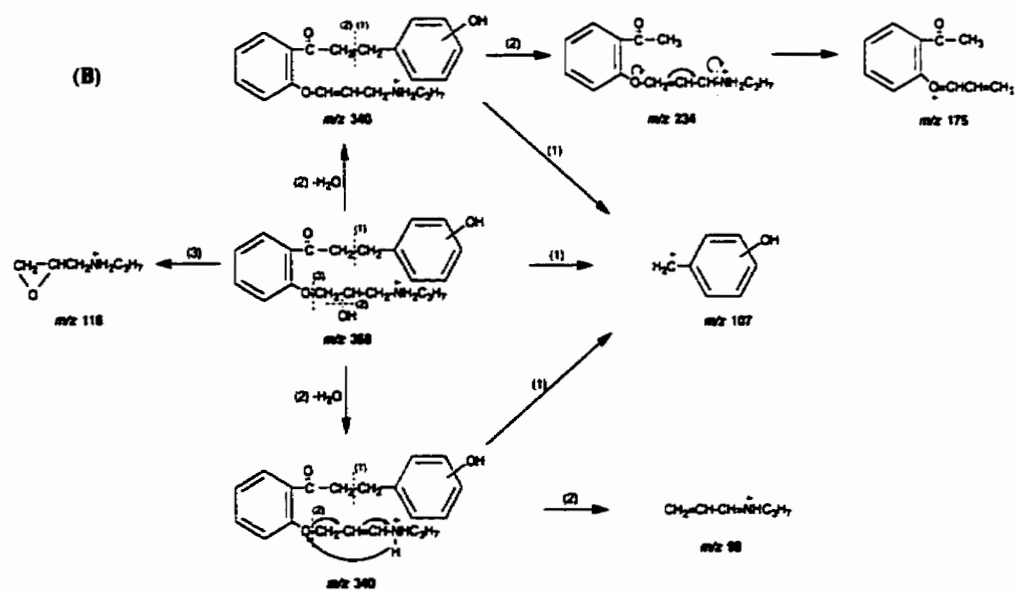
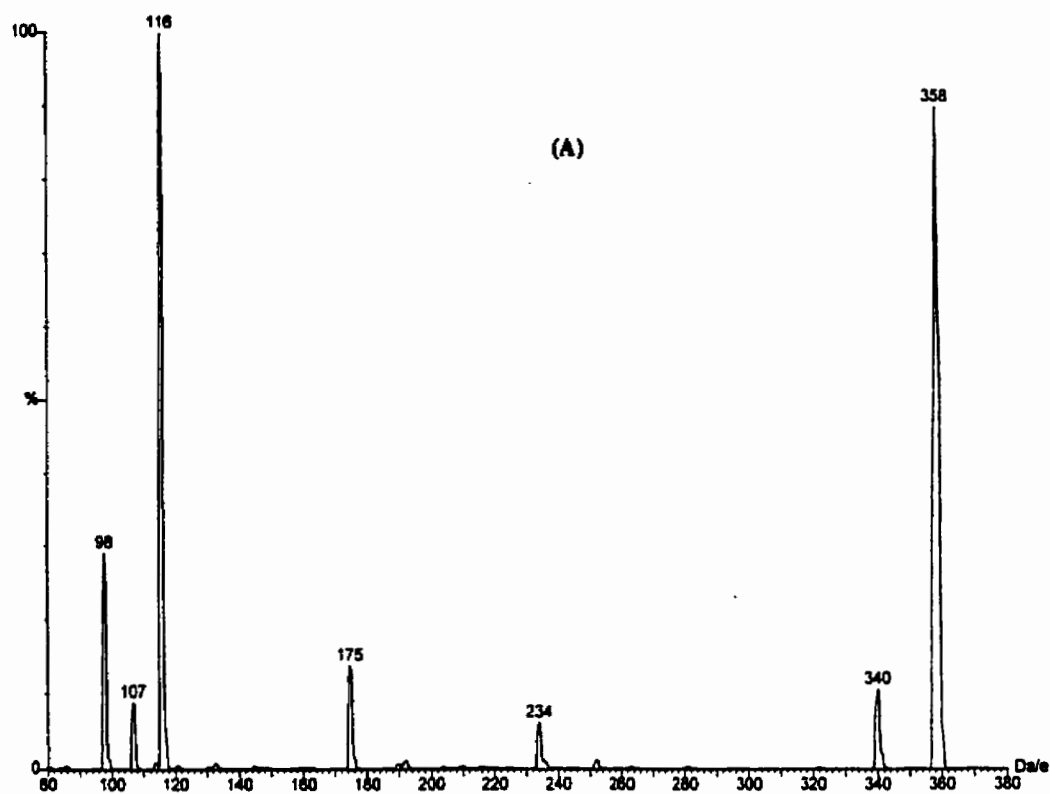


Figure 5.8. Product ion analysis for peak 1. (A) product ion mass spectrum of the protonated molecule at m/z 358; (B) proposed fragmentation pattern.

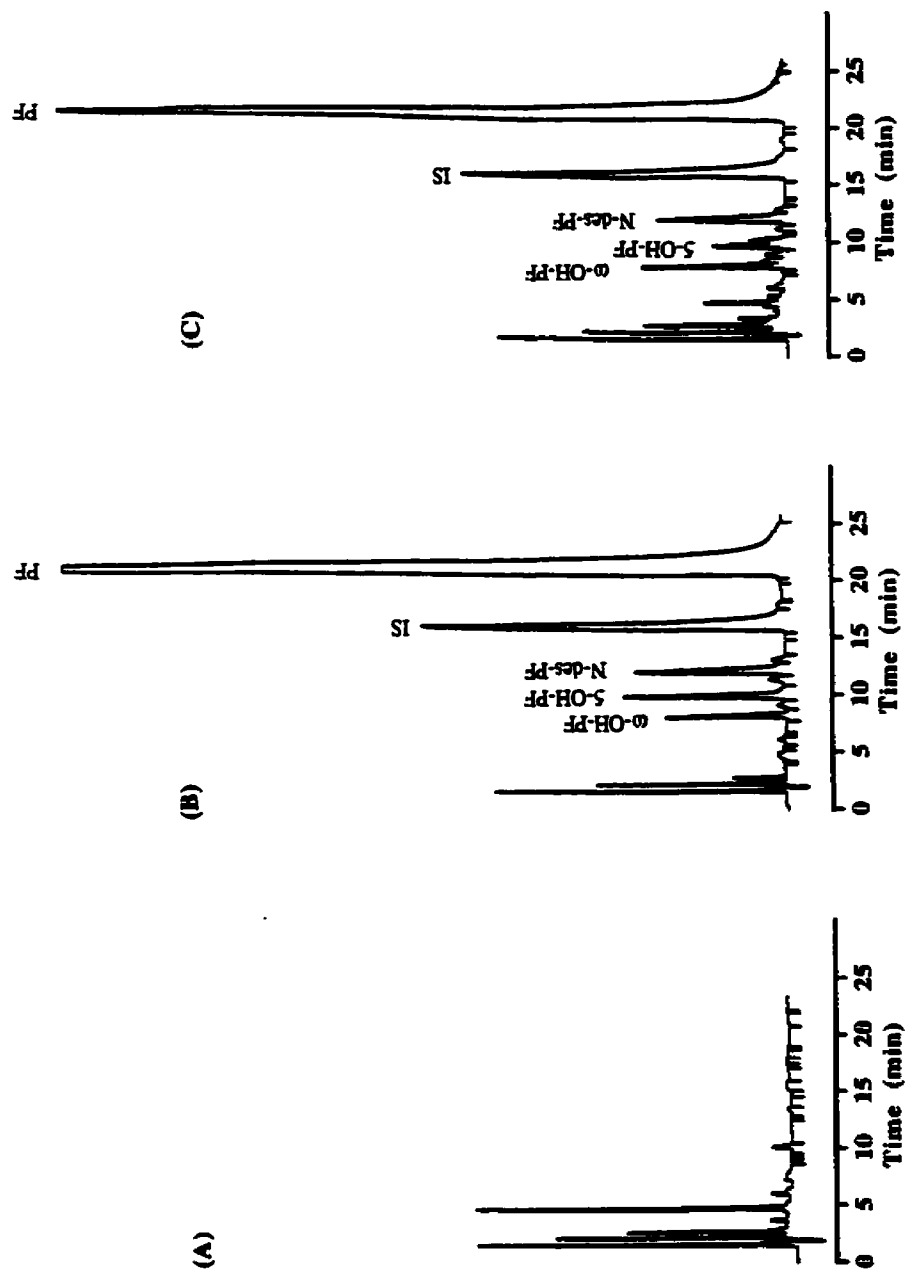


Figure 5.9. HPLC chromatograms for determination of PF and metabolites. (A) blank liver perfusate; (B) blank liver perfusate spiked with standard references, internal standard (IS) and the collected fraction of peak 1; (C) liver perfusate after infusion of 20 μg/ml PF.

Table 5.1. Calibration curve parameters for quantitation of PF, 5-OH-PF and N-des-PF in rat liver perfusate using HPLC/UV

Analyte	Retention time (min)	Range (ng/ml)	Slopes ($\times 10^{-4}$)	Intercepts	r	Mean accuracy (%) (\pm RSD)
PF	21.3	5000-15000	1.551	0.0646	0.999	101.1 \pm 5.23%
5-OH-PF	10	50-1000	4.353	0.1607	0.999	103.2 \pm 1.62%
N-des-PF	12.2	50-1000	3.807	0.0333	0.998	102.0 \pm 3.82%

5.4 Discussion

Studies of propafenone metabolism in humans (Hege et al., 1984b; 1986; Kates et al., 1985) have revealed that 5-OH-PF, N-des-PF and 5-OH-4-OCH₃-PF are the three major phase I metabolites in plasma. In dogs (Hege et al., 1984a; 1986), 5-OH-PF and 4'-OH-PF were found to be the two major phase I metabolites, while other metabolites, such as 5-OH-4-OCH₃-PF, 4'-hydroxy-3'-methoxypropafenone (4'-OH-3'-OCH₃-PF) and N-des-PF were present in lower quantities. We have demonstrated that at least three phase I metabolites, ω -OH-PF, 5-OH-PF and N-des-PF were produced in isolated, perfused rat livers, and ω -OH-PF was the main metabolite. In all these three species, aromatic ring hydroxylation commonly dominates the phase I metabolism, whereas N-dealkylation reactions are of secondary importance. Most drugs containing aromatic moieties are susceptible to aromatic oxidation, so the ring hydroxylation of propafenone is not surprising. These species, however, showed different regioselectivity of hydroxylation in the aromatic rings. Compared to humans, in which hydroxylation is only favored in the disubstituted phenyl ring, the rat and dog have broader spectra of hydroxylated metabolites as they are capable of hydroxylating both phenyl rings, and the rat metabolic enzymes appear more selective for the ω -phenyl ring. The metabolism of diprafenone (Figure 5.10), a new antiarrhythmic agent with a chemical structure analogous to PF, has also been studied in Sprague-Dawley rats after intravenous and oral administration (Brunner et al., 1989). A major metabolite other than 5-hydroxydiprafenone was found but not identified. This unknown abundant metabolite of diprafenone may be a derivative hydroxylated in the terminal phenyl ring based on our

studies on the metabolism of PF, which potentially could be parallel to that of diprafenone.

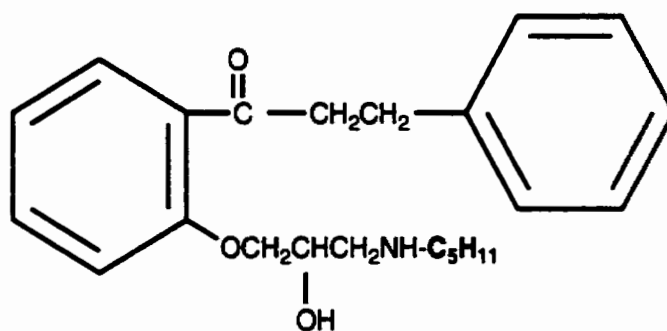


Figure 5.10. Structural formula of diprafenone.

Different regioselectivity of hydroxylation between species has also been demonstrated in some other drugs containing aromatic ring(s), e.g. propranolol (Figure 5.11). One major metabolic pathway of propranolol is naphthalene ring hydroxylation, which gives rise to several regioisomeric hydroxylated metabolites in humans and rats (Walle et al., 1982). Of these hydroxylated metabolites, 4-hydroxypropranolol and 5-hydroxypropranolol are the two major metabolites in humans, while 4-hydroxypropranolol and 7-hydroxypropranolol are predominant in rats (Walle et al., 1982). This observed positional difference in naphthalene ring hydroxylation between rats and humans may be attributed to interspecies variation in the intrinsic stereochemical preference of CYP2D enzymes (Narimatsu et al., 1999). CYP2D6 is responsible for 4, 5, and 7-hydroxylation in humans (Masubuchi et al., 1994) and the CYP2D subfamily is responsible in rats (Masubuchi et al., 1993), likely including CYP2D2 (Schulz-Utermoehl et al., 1999). Similarly to the situation with propranolol, it

was demonstrated that the 5-hydroxylation of PF in humans was mediated via CYP2D6 (Kroemer et al., 1989), a debrisoquine 4-hydroxylase which does not exist in rats. Therefore, the differences in the CYP2D subfamily between rats (or dogs) and humans may explain the positional difference in the phenyl ring hydroxylation of PF.

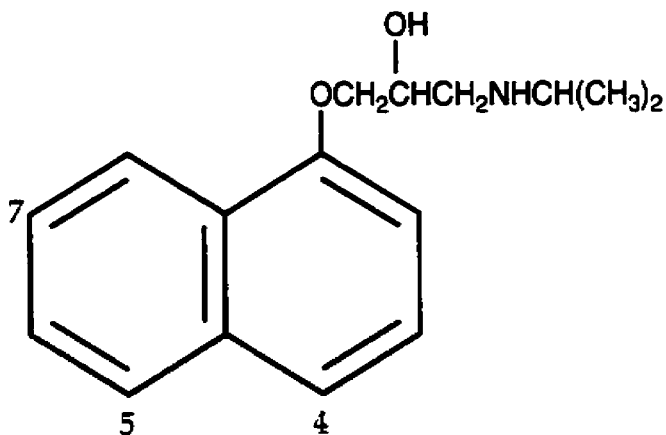


Figure 5.11. Structural formula of propranolol.

The metabolites for identification were extracted into diethyl ether directly from liver perfusate of pH 8 to 9. This implies that only basic and neutral metabolites, but probably not acidic metabolites were extracted. Since the acidic metabolites of PF are secondary metabolites, present in very small quantities in dog (Hege et al., 1984a) and human (Hege et al., 1984b), it is postulated that acidic metabolites would be formed in much lower quantities in the single-pass isolated rat liver. In addition to the monohydroxylated compounds, dihydroxylated and other metabolites could also be produced in rat livers, but in small quantities.

Although we could not find direct evidence to confirm the position(s) of hydroxylation in the ω -phenyl ring, because no standard reference was available, 4'-OH-PF is proposed as the most plausible chemical structure for the metabolite hydroxylated on the ω -phenyl ring. This proposition is based on (1) the report by Hege (1986) in which the possibility of hydroxylation on 2', 3'-positions of the ω -phenyl ring was ruled out; and (2) the well established general preference for the para position in metabolic arene oxidation (Low et al., 1991). Nevertheless, hydroxylation at other positions could not be excluded for certain.

Based on the identification of propafenone and its metabolites, the levels of these compounds in liver perfusate can be determined simultaneously by a rapid and convenient conventional HPLC method. The HPLC method is sensitive and accurate for determining the concentrations of PF and its metabolites, although the concentration range chosen was quite high, due to the high concentrations of PF used in our experiments. Unfortunately, no standard reference for ω -OH-PF was available, so we used peak height ratios to record changes in its concentrations in rat liver perfusate. Extraction with diethyl ether at pH 10.0 provided high recoveries of PF, 5-OH-PF and N-des-PF from liver perfusate. The mobile phase was modified by the addition of triethylamine and phosphoric acid. This modified mobile phase provided better peak shapes, shorter retention time values, and therefore higher sensitivity for all peaks (Figure 5.9) than the mobile phase used for metabolite identification, where triethylamine and phosphoric acid were inappropriate in the mass spectrometric analysis.

The quantitation of PF and its metabolites after conjugate cleavage showed that the total phase I metabolites accounted for about 36.8% of the 20 µg/ml of PF perfused through rat liver. Compared to dogs and humans, the smaller proportion of phase I metabolism may have been the result of saturation of hepatic uptake or metabolism at the extremely high inlet concentrations employed. Assuming that ω-OH-PF would give a similar absorbance value to that of 5-OH-PF at 210 nm, the calibration curve for 5-OH-PF was adapted to estimate concentrations of ω-OH-PF in rat liver perfusate. The results showed that ω-OH-PF, 5-OH-PF and N-des-PF accounted for about 63.6%, 12.4% and 7.7% of total phase I metabolites, respectively, showing that ω-OH-PF is the major phase I metabolite in rat liver perfusate, whereas other phase I metabolites only accounted for a small percentage. It was observed that the ratio of these three metabolites changed with sample collection time and with the PF inlet concentration in perfused rat livers, but ω-OH-PF was consistently eluted at higher levels than those of the other two metabolites.

5.5 Conclusions

The metabolism of propafenone in rats resembles that in dogs, but not that in humans. N-Dealkylation and hydroxylation in the middle and terminal phenyl rings are the major metabolic pathways in isolated, perfused rat livers. The ω -phenyl ring hydroxylated metabolite is the most predominant metabolite. The difference in phase I metabolism of PF in the rat to that of human may limit the usefulness of this species as a model of human PF metabolism. The position of hydroxylation in the ω -phenyl ring needs to be further elucidated because no standard references were available.

CHAPTER 6

CHARACTERIZATION OF PHASE II METABOLITES OF PROPAFENONE IN RATS USING ELECTROSPRAY MASS SPECTROMETRY^{1,2}

6.1. Introduction

Propafenone (PF) is a class IC antiarrhythmic agent. Like some other lipophilic, high hepatic first-pass drugs, such as propranolol and metoprolol, propafenone shows a dramatic increase in bioavailability when given with food, even though it is completely absorbed by fasting subjects after an oral dose (Axelson et al., 1987). The interaction between food and these drugs has been shown to be located in the liver but the mechanism has not been completely elucidated. Our aim was to investigate the mechanisms that might contribute to PF interaction with food/nutrients. Such a study can be facilitated through the use of the isolated, perfused rat liver model system. A thorough understanding of the metabolism of propafenone in the rat liver is thus required.

Our previous study on the metabolism of propafenone (chapter 5) has revealed that propafenone undergoes extensive oxidative metabolism in the isolated, perfused rat liver. Three phase I metabolites, ω -hydroxypropafenone (ω -OH-PF), N-despropylpropafenone (N-des-PF), and 5-hydroxypropafenone (5-OH-PF) have been

¹ Presented at the Tenth Annual American Association of Pharmaceutical Scientists Meeting, October 1996, Seattle, Washington, USA.

² Manuscript prepared for submission to *J. Chromatogr.*

identified in rat liver perfusate. Among these metabolites, the ω -phenyl ring hydroxylated metabolite was shown to make up the largest proportion after enzymatic conjugate cleavage. In addition to phase I metabolism, the parent drug and hydroxylated metabolites are subjected to a considerable amount of phase II conjugation. As enzymatic cleavage does not give direct indication of the structures of the conjugates, uncertainty remains with respect to the phase II metabolism of propafenone. By using high performance liquid chromatography (HPLC) and subsequent tandem mass spectrometry (MS/MS) with electrospray ionization (ESI), we have therefore examined the non-cleaved conjugate metabolites of propafenone in effluent samples from the isolated, perfused rat liver, and have characterized their structures. This report is an extension of our previous study on the metabolism of propafenone in the rat.

The information about the phase II metabolism of propafenone is limited. Glucuronidation of propafenone and its hydroxylated metabolites is known to be the major phase II metabolism pathway in humans (Hege et al., 1984b; Fromm et al., 1995) and dogs (Hege et al., 1984a; 1986). The major phase II metabolites in man were indicated to be propafenone glucuronide and the conjugates of 5-hydroxypropafenone and hydroxy-methoxy-propafenone with glucuronic acid and sulphuric acid (Hege et al., 1984b; Fromm et al., 1995). But none of these conjugates has been unequivocally structurally identified. In the dog, only conjugates with glucuronic acid have been found and their structures were characterized by mass spectrometry. These conjugates include propafenone glucuronide and hydroxylated propafenone derivatives conjugated to a hydroxyl function in the different aromatic rings (Hege et al., 1986).

6.2. Experimental

6.2.1. Chemicals

All the solvents used for extraction and preparation of the HPLC mobile phase were HPLC grade obtained from BDH Chemicals Canada Ltd. (Edmonton, AB, Canada). Other chemicals used were commercial analytical grade purchased from BDH. Propafenone hydrochloride (HCl) and Type H-1 β -glucuronidase (containing 338,000 units/g of β -glucuronidase and 16,000 units/g of sulfatase from *Helix pomatia*) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Authentic standards of propafenone HCl (Lu 29007), 5-hydroxypropafenone HCl (Lu 40545), N-despropylpropafenone fumarate (Lu 48686) and internal standard (Li 1115-HCl, the N-ethyl analogue of PF) were kindly supplied by Knoll AG, Ludwigshafen, Germany. The structures of these compounds are shown in Figure 5.1.

6.2.2. Liver perfusion

The perfusion apparatus, surgical procedure and sample collection were identical to those previously described (Chapter 5, Section 5.2.2).

6.2.3. Sample preparation

Perfusate samples (1 ml) were vortex mixed with 5 ml of diethyl ether for 10 min. After centrifugation at 1200 x g for 10 min, the organic layer containing most of the parent drug and phase I metabolites was removed, and the aqueous layer was

lyophilized to dryness. The residues were dissolved in a small amount of methanol. After removing the undissolved salts by centrifugation at 1200 x g for 5 min, the methanol solution was transferred to a clean borosilicate glass tube and evaporated to dryness under a stream of N₂ at room temperature. The residue was reconstituted in 1 ml of water, and applied on a Spe-ed® cartridge packed with solid phase C₁₈ (Applied Separations Inc, Bethlehem, PA, USA), which was previously conditioned with 2 ml of acetonitrile, 2 ml of methanol, and 2 ml of distilled water. After the samples had adsorbed to the solid phase, the cartridge was washed with 3 ml of water to remove salts contained in the samples. Finally the phase II metabolite fraction was eluted with 1 ml of methanol, which was evaporated under a stream of nitrogen. The residue was reconstituted in the mobile phase or methanol for injection into the HPLC system with UV detection (HPLC/UV) or mass spectrometry system.

The eluate fractions corresponding to appropriate HPLC/UV peaks were collected. The fractions were treated under nitrogen to evaporate organic solvents, and then lyophilized. The residue of each fraction was dissolved in 1 ml of water, and purified on a conditioned Spe-ed® C₁₈ cartridge according to the procedure described as above. The final eluate was dried under a stream of nitrogen, and the residue was reconstituted in methanol and injected into the MS/MS spectrometer.

6.2.4 Chromatographic methods

6.2.4.1. HPLC conditions

The phase II metabolites were separated by an HPLC system with a mobile phase of ammonium acetate (0.005 M)-methanol (50:50, v/v), pumped at a flow rate of

1 ml/min. The HPLC system consisted of a Waters 510 pump, a Waters 490 programmable multiwavelength detector set at 210 nm (Millipore-Waters, Mississauga, Ont., Canada), a Model 7125 syringe loading sample injector with a 200 μ l loop (Rheodyne Inc., Cotati, California, USA), and a 250 \times 4 mm I.D., 5 μ m particle size Lichrospher RP-18 column (E. Merck, Darmstadt, Germany). The data were recorded using a Chromatopac C-R3A integrator (Shimadzu Co., Kyoto, Japan).

6.2.4.2. HPLC/MS and MS/MS

MS and MS/MS were carried out on a VG Quattro-II triple quadrupole mass spectrometer equipped with an electrospray ion source (Micromass, Altrincham, UK). MS spectra were obtained by direct loop injection electrospray ionization in either positive or negative ion mode. Product ion spectra were obtained by positive ion electrospray (+ESI) MS/MS with collision-induced dissociation (CID). The collision energy was 20 eV, and the argon pressure was set at 1.0×10^{-3} mBar.

6.2.5. Conjugation cleavage

The rat liver perfusate samples (1 ml, n = 5) collected at steady state were incubated with 5000 units of β -glucuronidase at 37 °C for 4 hr at pH 4.6. The levels of propafenone and its phase I metabolites, ω -hydroxy-propafenone, 5-hydroxy-propafenone and N-despropylpropafenone were determined as described in Chapter 5.

6.3. Results and discussion

The conjugate cleavage results are shown in Table 6.1. Incubation of the perfusate samples with β -glucuronidase/sulfase showed that conjugation of ω -OH-PF and 5-OH-PF accounted for about 80% and 75%, respectively, of total 4'-OH-PF and 5-OH-PF. However, only 2.4% of PF was subject to conjugation. The low percentage of conjugated PF may result from saturation of hepatic enzymes, since very high concentrations of PF were employed to infuse into the isolated, perfused rat livers.

Table 6.1. The data of conjugate cleavage

Compound	Concentration (ng/ml)		Conjugated %
	Before cleavage	After cleavage	
PF	12357	12657	2.40
ω -OH-PF	0.3844*	2.145*	82.1
5-OH-PF	223.8	948.6	76.5
N-des-PF	580.5	632.3	8.2

* value is indicated as peak height ratio due to no standard reference available.

Figure 6.1B shows a typical HPLC/UV chromatogram of the aqueous extract from liver effluent collected from an isolated rat liver perfused with 20 μ g/ml PF in Krebs buffer. In comparison to a blank liver perfusate sample (Figure 6.1A), four major additional peaks, assigned as peaks 1, 2, 3 and 4, were present. The liver perfusate extract was then analyzed by direct loop injection electrospray ionization spectrometry

in both positive and negative ion modes. The positive ion mass spectrum of the aqueous extract (Figure 6.2A) clearly showed two probable protonated molecular ions $[M + H]^+$ at m/z 518 and 534, and their natriurated molecular ions. The inferred molecular weights of 517 and 533 Daltons (Da), 176 Da above those of propafenone and hydroxylated PF derivatives (OH-PF), respectively, suggest that glucuronide conjugates of PF and OH-PF could be present in the liver perfusate extract. The negative ion mass spectrum (Figure 6.2B) also demonstrated two quasi molecular ions $[M - H]^-$ at m/z 516 and 532, corresponding to the two protonated molecular ions present in the positive ion mass spectrum. The amphoteric characteristics of these molecules further confirmed the presence of PF and OH-PF glucuronides in the liver perfusate extract.

The appropriate fractions corresponding to the HPLC/UV peaks 1 – 4 were collected, and each individual fraction was analyzed by MS/MS spectrometry. After the positive ion ESI MS spectra of each fraction were observed, the precursor ions at m/z 518 or 534 were selected and their corresponding CID product ion spectra were recorded.

Peak 1. The positive ion MS spectrum of peak 1 (Figure 6.3A) gave a protonated molecular ion $[M + H]^+$ at m/z 534 and its corresponding sodium adduct ion $[M + Na]^+$ at m/z 556. The CID product ion mass spectrum of the quasi molecular at m/z 534 is displayed in Figure 6.3B. It showed a parent ion at m/z 534 and main fragment ions at m/z 358, 144, 116, 107 and 98. The abundant fragment ion at m/z 358 arose from a loss of the glucuronic acid moiety (176 Da) from the quasi molecular ion at m/z 534, strongly suggesting that the peak 1 fraction contains a glucuronide conjugate of OH-PF. The diagnostic ions at m/z 116, 107 and 98 were in agreement with the fragment pattern of

ω -OH-PF (Chapter 5). The fragment ion at m/z 144 corresponded to the propoxyamine side chain with a $-\text{CHO}$ group, derived from the cleavage of the C^5-O and C^1-C^2 bonds on the glucuronyl group. Accordingly, the metabolite in the peak 1 fraction was assigned to ω -OH-PF glucuronide conjugated to the hydroxyl group of the propoxyamine side chain.

Peak 2. The positive ion MS spectrum of peak 2 (Figure 6.4A) also exhibited a protonated molecular ion $[\text{M} + \text{H}]^+$ of m/z 534 with high intensity, together with two weak natriated molecular ions, $[\text{M} + \text{Na}]^+$ at m/z 556 and $[\text{M} - \text{H} + 2\text{Na}]^+$ at m/z 578. An abundant fragment ion of m/z 358 was observed in the product ion spectrum (Figure 6.4B), indicating that the peak 2 metabolite is a glucuronide conjugate of OH-PF. The observed diagnostic fragment ion at m/z 144 suggests that the glucuronide moiety should be conjugated to the aliphatic hydroxyl group. However, the fragment ions at m/z 281, 116 and 98 were inconsistent with the fragmentation pattern of ω -OH-PF, but in agreement with that of 5-OH-PF (Chapter 5). The peak 2 metabolite was thus characterized as 5-OH-PF glucuronide on the propoxyamine side chain.

Peak 3. Similarly as for peaks 1 and 2, the positive ion MS spectrum of peak 3 (Figure 6.5A) exhibited an intense protonated molecular ion at m/z 534, which produced a fragment ion at m/z 358 (Figure 6.5B) by MS/MS, indicating that the peak 3 metabolite was a OH-PF glucuronide isomer. The fragment ions at m/z 236, 175, 116, 107 and 98 showed a characteristic fragment ion pattern of ω -OH-PF. Inconsistent with that of peak 1, however, the product ion spectrum of peak 3 (Figure 6.5B) did not yield a diagnostic ion of m/z 144, suggesting that the location of the glucuronic acid was not on the propoxyamine chain but on the ω -phenyl ring.

Peak 4. The protonated molecular ion at m/z 518 (Figure 6.6A) and its product ion at m/z 342 (Figure 6.6B) indicate that the metabolite of peak 4 corresponded to PF glucuronide.

6.4 Conclusions

The analysis of the phase II extract revealed that a considerable amount of glucuronides of the parent drug and the hydroxylated metabolites are present in rat liver effluent after perfusion with PF. By ESI MS and MS/MS spectrometry, four intact glucuronides have been detected and characterized: 1) propafenone glucuronide; 2) 5-OH-PF glucuronide conjugated to the aliphatic hydroxyl group; and 3) a pair of isomeric ω -OH-PF glucuronides, one being conjugated to the aliphatic hydroxyl group and the other to the aromatic hydroxyl group. Compared with the published findings for the phase II metabolites of PF in dog (Hege et al., 1986), both ω -OH-PF glucuronide and 5-OH-PF glucuronide conjugated on the aliphatic side chain were identified. This represents the first report to the identification of OH-PF glucuronides conjugated to the hydroxyl group of the aliphatic side chain.

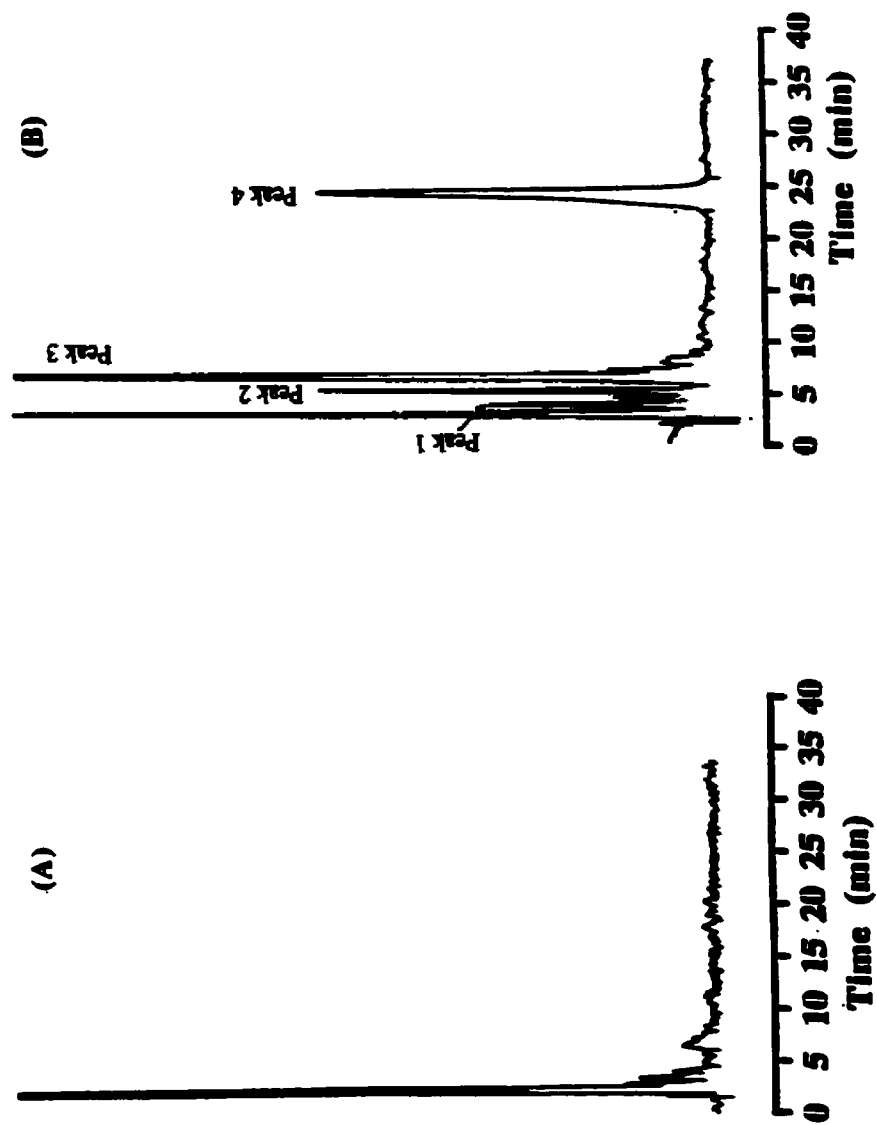


Figure 6.1. HPLC-UV chromatograms of aqueous extracts from (A) blank rat liver perfusate; (B) rat liver perfusate after infusion of 20 µg/ml PF.

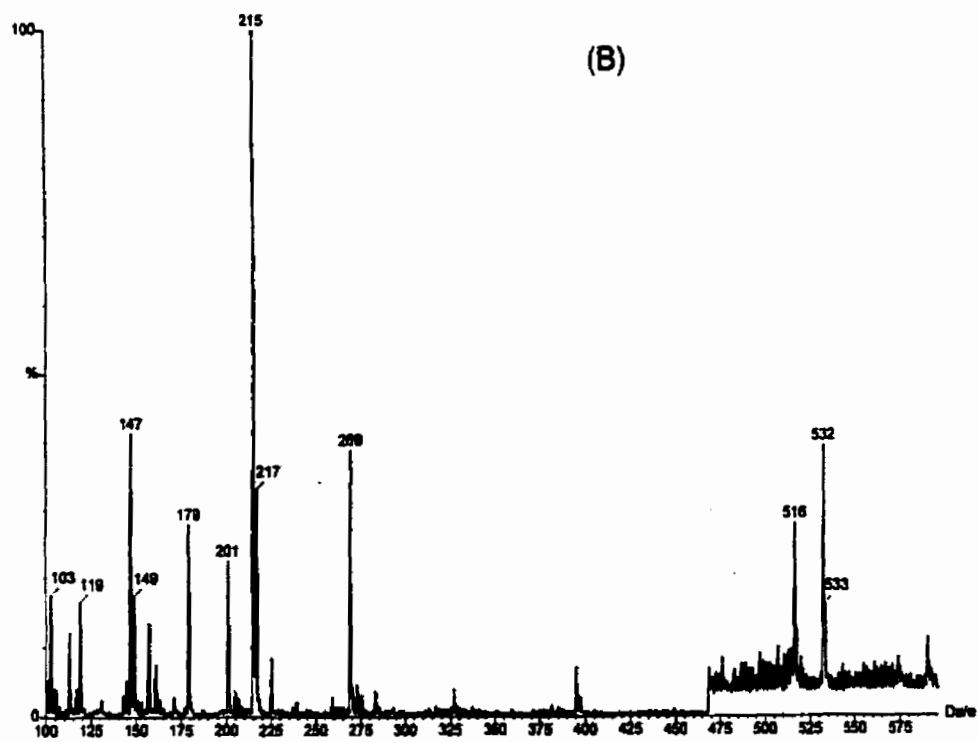
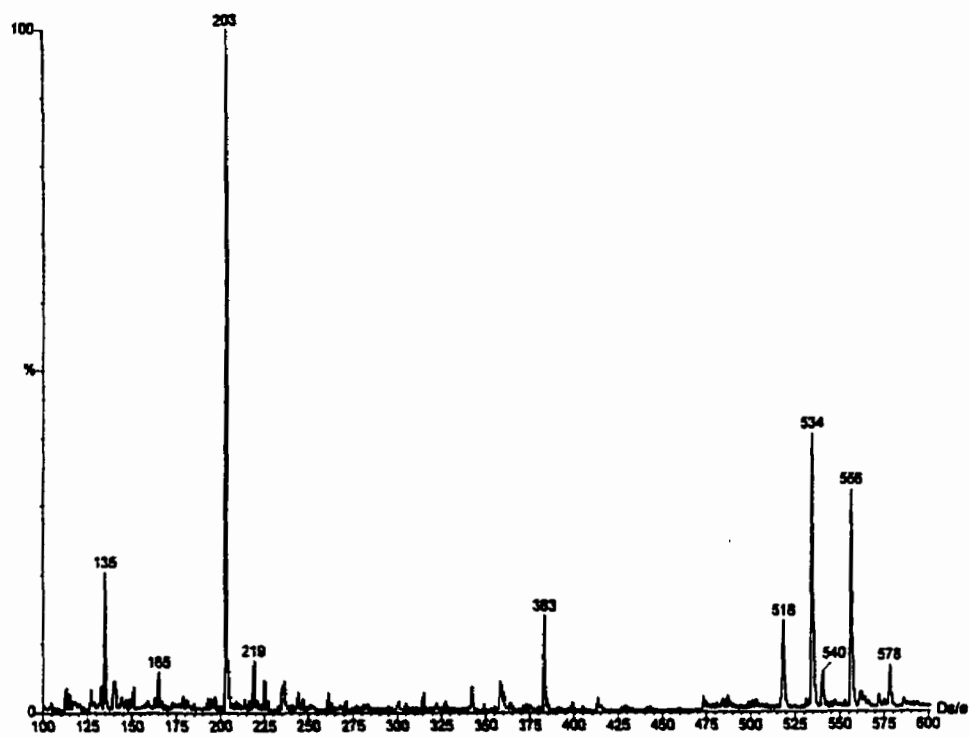


Figure 6.2. Positive ion (A) and negative ion (B) mass spectra of aqueous extracts from liver perfusate.

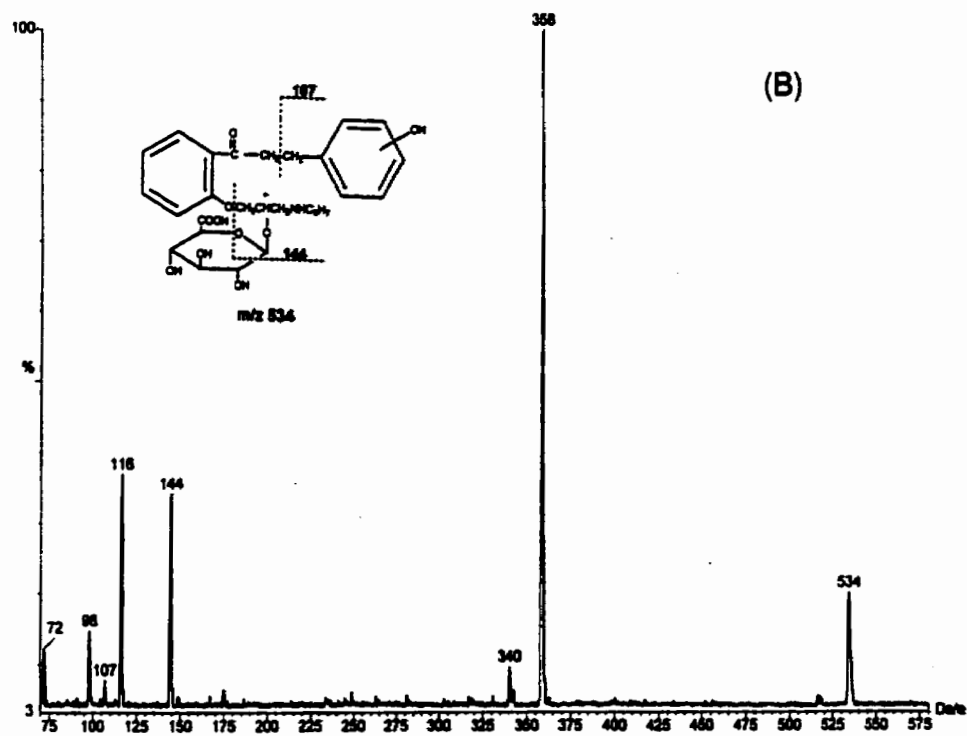
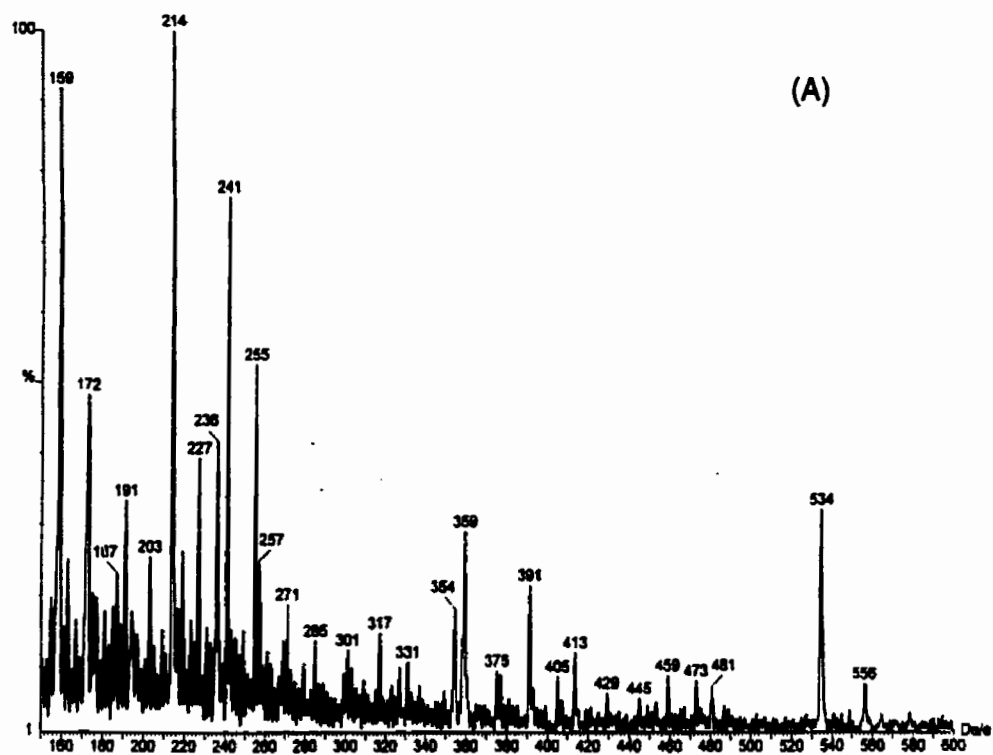


Figure 6.3. Positive ion (A) and product ion (B) mass spectra of peak 1.

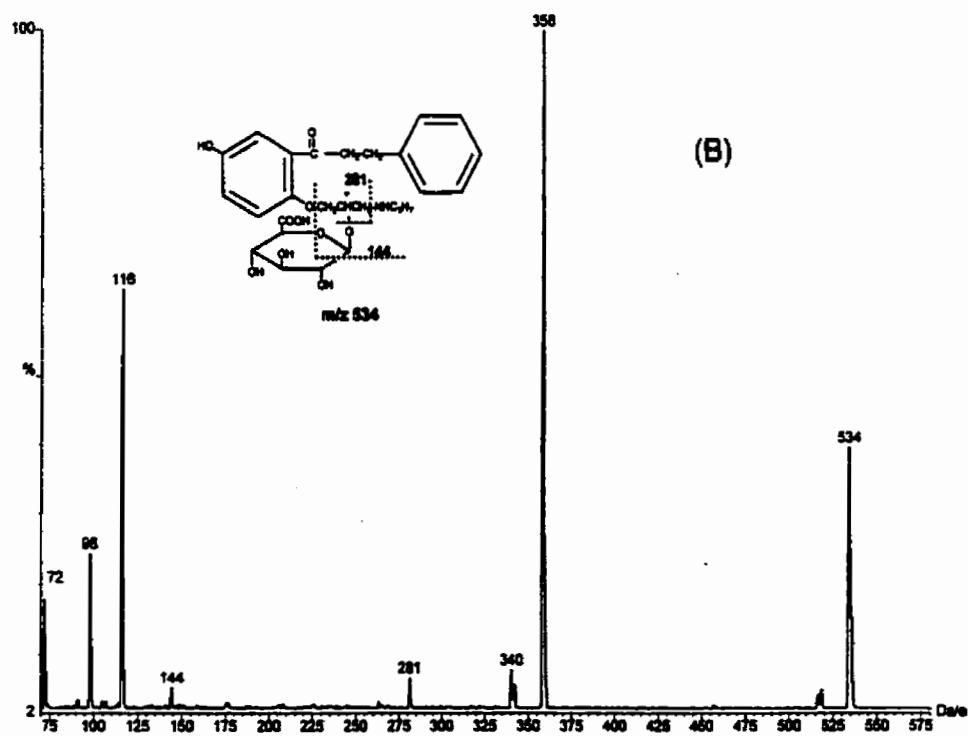
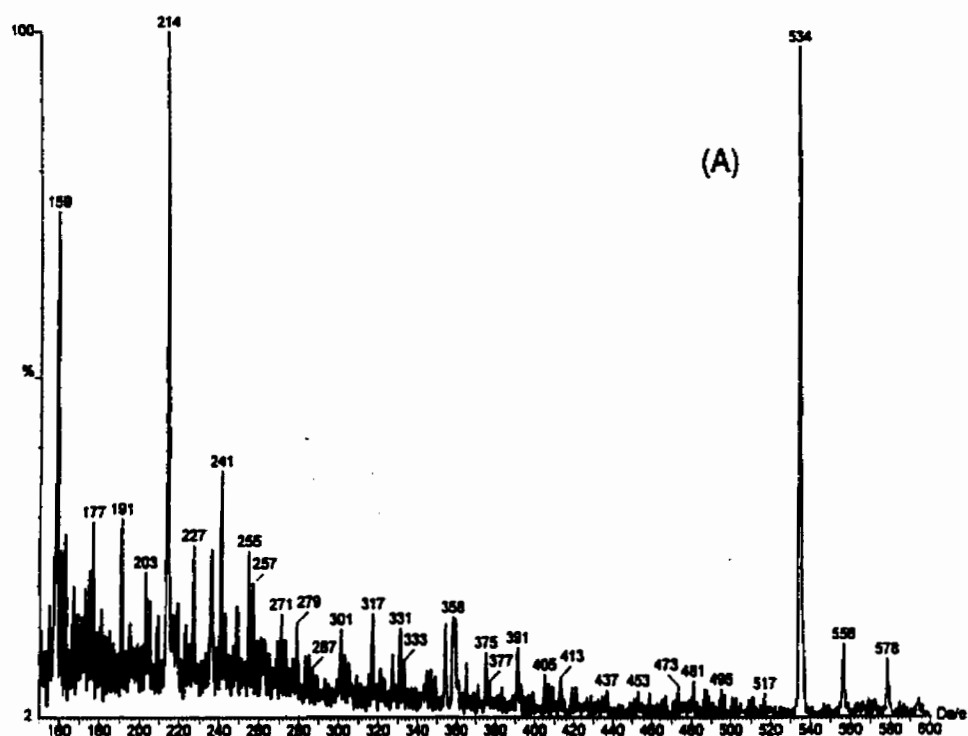


Figure 6.4. Positive ion (A) and product ion (B) mass spectra of peak 2.

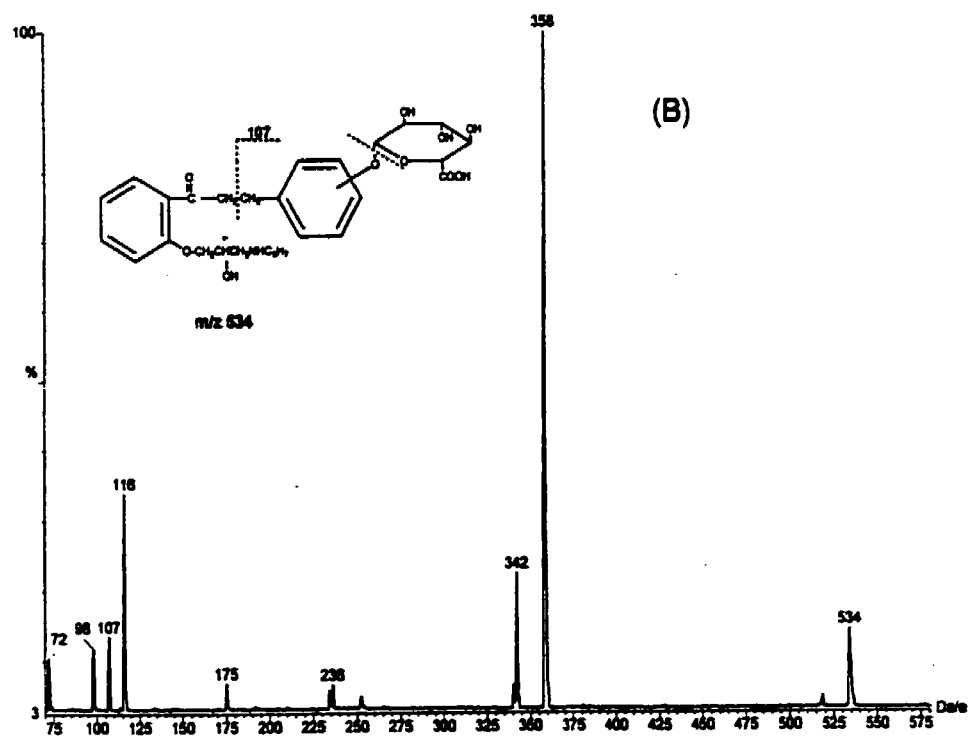
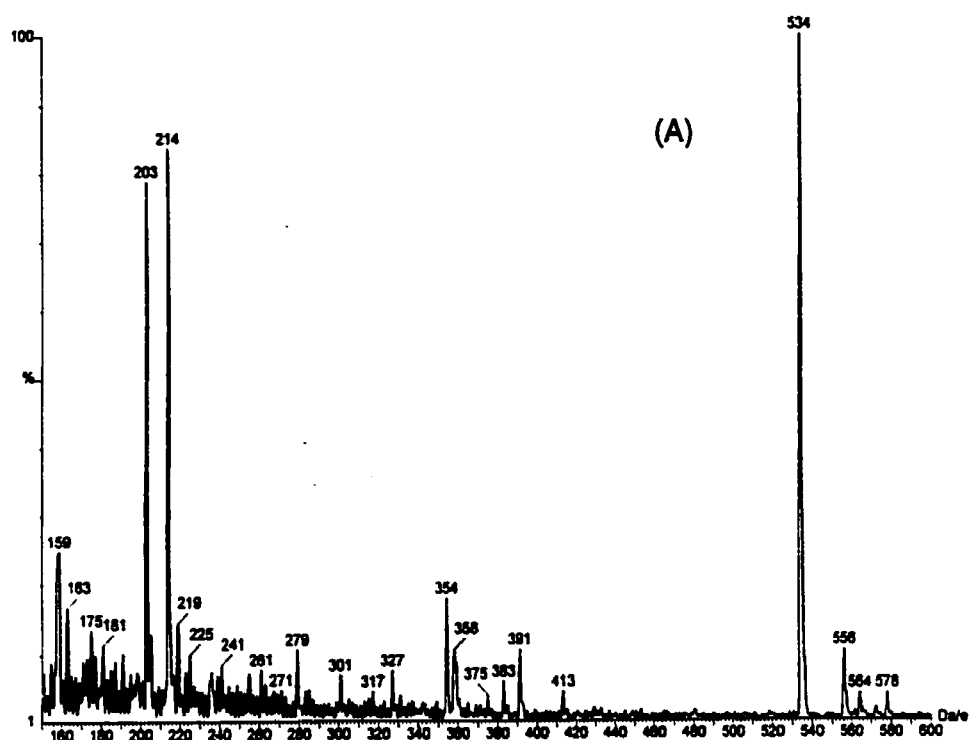


Figure 6.5. Positive ion (A) and product ion (B) mass spectra of peak 3.

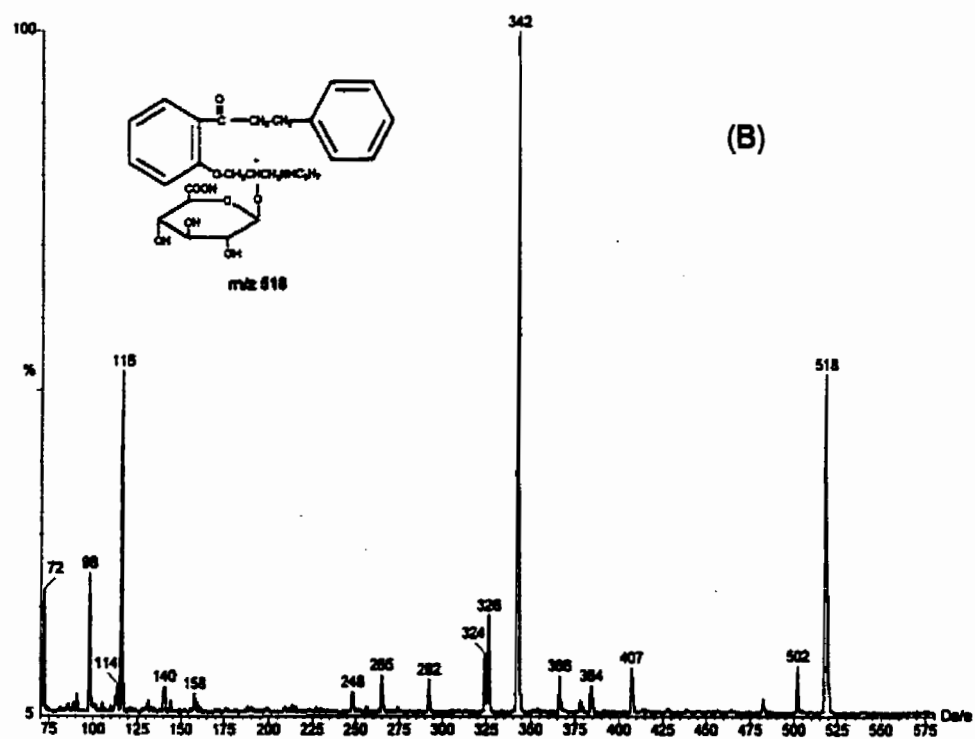
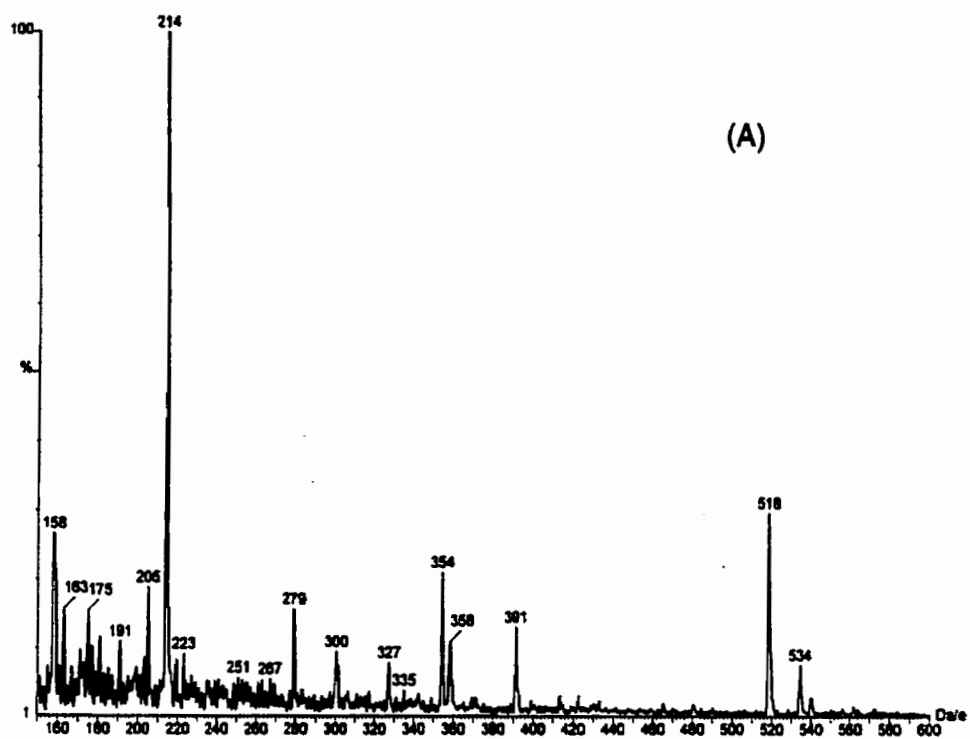


Figure 6.6. Positive ion (A) and product ion (B) mass spectra of peak 4.

CHAPTER 7

VALIDATION OF AN HPLC METHOD FOR DETERMINATION OF METOPROLOL AND ITS METABOLITES IN RAT LIVER PERFUSATE AND RAT PLASMA

7.1 Introduction

Metoprolol is a lipophilic cardioselective β_1 -adrenoceptor antagonist commonly used in the treatment of hypertension and angina pectoris. It is a weak base (pKa 9.6) with a molecular weight of 267. The partition coefficient of metoprolol for an n-octanol-water system is 93. Similar to humans, metoprolol undergoes extensive hepatic first-pass metabolism in rats. The major metabolites are α -hydroxymetoprolol, O-demethylmetoprolol and its secondary metabolite, metoprolol acid (Figure 1.4). Previous studies using an HPLC assay established in our laboratory (Wang and Semple, 1997) have demonstrated that metoprolol metabolism is inhibited by a mixture of amino acids in the isolated rat liver perfused with a hemoglobin-free bicarbonate buffer; an observation worthy of further investigation as a mechanism of the 'food effect' observed in humans. In further studies on the interaction between metoprolol and amino acids in the *in vivo* rat (Chapter 9) and the isolated rat liver perfused with an erythrocyte-enriched medium (Chapter 8), however, the previously used HPLC method was found not applicable, due to the direct injection method employed. Under the conditions used in these studies, direct injection caused column blocking and a lack of sensitivity. An extraction procedure therefore needed to be developed, and the chromatographic

conditions required some modifications. As some specific validation parameters were likely affected by the change, revalidation of the modified assay was necessary prior to its routine use.

This chapter presents a convenient HPLC method for the simultaneous analysis of metoprolol and its metabolites, α -hydroxymetoprolol and O-demethylmetoprolol, in rat liver perfusate and rat plasma, modified according to the previously published methods (Lennard, 1985; Wang and Semple, 1997; Alcorn, 1997). The method was then validated according to the procedures described by Bressolle et al. (1996), including specificity, sensitivity, extraction recovery, linearity of calibration curves, accuracy and precision.

7.2 Experimental

7.2.1 Chemicals

R, S-Metoprolol tartrate and nadolol (internal standard) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The *p*-OH benzoate salts of α -hydroxymetoprolol (H119/66) and O-demethylmetoprolol (H105/22) were gifts from Astra (Hässle, Sweden). All solvents were HPLC grade obtained from BDH Inc. (Toronto, Ontario, Canada).

7.2.2 Standard solutions

Stock standard solutions of metoprolol, α -hydroxymetoprolol and O-demethylmetoprolol were prepared separately by dissolving 10 mg of each analyte in deionized, distilled water in a 100-ml volumetric flask to give a final concentration of 100 μ g/ml. The stock solutions were stable for at least three months when stored at 4°C. Appropriate portions of the stock solutions were mixed and diluted with water to make a series of working solutions of the three analytes. The working standard solutions were stored at 4°C for no more than four weeks.

The standard solutions in the biological matrix were prepared freshly by addition of varying volumes of the working solutions to blank rat liver perfusate or blank plasma to generate desired concentrations. Rat liver perfusate and rat plasma was obtained from male Sprague-Dawley rats. The liver perfusate was from the supernatant of the liver effluent, collected as described in Section 3.3, after centrifugation at 1000 \times g.

7.2.3 Extraction procedure

Extraction from rat liver perfusate. An aliquot of liver perfusate (1 ml), containing metoprolol and its metabolites as a standard or as an unknown, was mixed with 50 μ l of nadolol solution (5 μ g/ml, internal standard) in a 100 \times 13-mm I.D. screw-capped Pyrex borosilicate tube. Following addition of 2 ml of 1 M sodium carbonate buffer solution (pH 10.5), diethyl ether-dichloromethane (4:1, v/v, 5 ml) was added to the tube. The contents of the tube were vortex-mixed for 10 min and centrifuged at 2500 \times g for 10 min. The organic layer was then transferred to a clean glass tube and evaporated to dryness under nitrogen at room temperature. The residues were reconstituted in 300 μ l of mobile phase, and 100 μ l was injected into the HPLC system.

Extraction from rat plasma. The procedure of extraction from rat plasma was the same as above except for minor changes. To 100 μ l of standard or unknown rat plasma samples were added 50 μ l of nadolol (1 μ g/ml, internal standard) and 2 ml of 1 M sodium carbonate buffer (pH 10.5). The parent drug, metabolites and internal standard were extracted with 5 ml of diethyl ether-dichloromethane (4:1, v/v) after vortex-mixing for 8 min and centrifugation at 2500 \times g for 10 min. The organic layer was transferred to a clean glass tube and evaporated to dryness under nitrogen at room temperature. The residues were reconstituted in 120 μ l of mobile phase, and 100 μ l was injected into the HPLC system.

7.2.4 High-performance liquid chromatography

The HPLC instrumentation consisted of a Waters 510 pump, Waters 710B WISP automatic injector, Waters Baseline data system (Millipore-Waters, Mississauga, ON), and a Spectroflow 980 fluorescence detector (Applied Biosystems, Ransey, NJ, USA) set at an excitation wavelength of 224 nm. The separation of individual components was carried out on a 250 × 4 mm I.D., 5 µm particle size Lichrospher 60 RP-select B column (E. Merck, Darmstadt, Germany) with a pre-column. The mobile phase was water-acetonitrile (87:13, v/v), containing 0.3% (v/v) triethylamine, adjusted to apparent pH 3.0 with orthophosphoric acid, and was pumped through the column at a flow rate of 1 ml/min.

7.2.5 Validation procedure

7.2.5.1 Specificity

The specificity was determined by chromatographic analysis of blank rat liver perfusate and blank rat plasma from a number of different rats. The specificity of this method was established through a lack of effective responses in both blank matrices. A mixture of amino acids, which would be coadministered with metoprolol in the latter studies (Chapter 8 and 9), was also included in the blank matrices to screen for its possible interference in the assay.

7.2.5.2 Sensitivity

The ability to assay low concentrations is termed sensitivity, expressed as the limit of detection (LOD) and the limit of quantification (LOQ). The determination of the LOD of metoprolol and the two metabolites was carried out using six replicate standard solutions in rat liver perfusate or rat plasma. The LOD was defined as a signal-to-noise ratio of 3:1. The LOQ was the estimate of the lowest concentration that could serve on the calibration curve. The LOQ of metoprolol and its metabolites were determined by using five replicate samples of each standard in rat liver perfusate or plasma. The accuracy of LOQ was defined to be within $\pm 20\%$ of the nominal concentration with a coefficient of variation (CV) $\leq 20\%$.

7.2.5.3 Calibration curve

A set of 1-ml liver perfusate or 0.1-ml plasma calibration standard solutions was prepared from working solutions containing metoprolol and the two metabolites. The calibration concentrations (metoprolol: α -hydroxymetoprolol:O-demethylmetoprolol) were 50:50:20, 100:100:50, 200:200:100, 400:400:200, 700:700:300 and 1000:1000:500 ng/ml in liver perfusate and 15:10:10, 30:30:15, 60:100:30, 100:200:50, 150:500:75 and 200:1000:100 ng/ml in human plasma. The peak height ratios of each analyte to the internal standard were weighted by $1/y$ and plotted against the corresponding concentrations. Linear regression analysis gave calibration curves that were used to calculate the concentrations of metoprolol and its metabolites in spiked control or unknown samples.

7.2.5.4 Accuracy and precision

On three separate days, spiked liver perfusate or plasma samples were prepared at three different concentration sets of metoprolol: α -hydroxymetoprolol:O-demethylmetoprolol (60:60:30, 500:500:250 and 900:900:450 ng/ml in liver perfusate, and 15:10:10, 100:500:50 and 200:1000:100 ng/ml in plasma, respectively) in five replicates ($n = 5$). The samples were assayed, and the concentrations were determined from the standard calibration curves prepared on the same day of analysis. The mean accuracy was calculated by the ratio of the actual to nominal concentration ($n = 5 \times 3$ days). The precision was evaluated by the intra- (within-) and inter- (between-) day coefficient of variation (CV). The intra-day CV was calculated as the mean of the daily CV ($n = 5 \times 3$ days). The inter-day CV was calculated as the CV of the daily means of measured concentrations ($n = 3$ days).

7.2.5.5 Extraction recovery

Perfusate or plasma samples containing three different concentration sets of metoprolol: α -hydroxymetoprolol:O-demethylmetoprolol (50:50:20, 500:500:250 and 1000:1000:500 ng/ml in liver perfusate; and 30:20:15, 100:500:50 and 200:1000:100 in plasma) were extracted according to the procedure described in Section 7.2.3, prior to injection into the HPLC system. The recovery was determined by comparing the peak heights of each analyte in the extracted samples with those obtained from direct injection of extracted blank matrix to which standards were added at the same nominal

concentrations. Five replicate determinations were made at each concentration of all drug and metabolites.

7.2.6 Application of method

The method was applied in the isolated perfused rat liver and *in vivo* rat studies of amino acid-metoprolol interactions, which are presented in the following chapters. Typically, perfusate samples from two rat livers or the plasma samples from two to four rats were arranged in one analytical run. In each run, all samples including blank matrix, unknown samples, two sets of calibration standard samples and six QC samples were extracted and analyzed under the same conditions. Calibration standard samples were prepared freshly on the day of analysis, and the resulting calibration curves were used to determine the concentrations of metoprolol and the two metabolites in unknown and QC samples within the run. QC samples of each analyte duplicated at three concentrations (one near the low end, one near the center and one near the upper boundary of the standard curve) were prepared on the day of sampling, and stored under the same conditions as unknown samples to be analyzed. The six QC samples were randomly located amongst the unknown samples in the run. To accept a run, at least four of the six QC samples were within $\pm 20\%$ of their respective nominal values, where any two outside the $\pm 20\%$ range were not at the same concentration.

7.3 Results and discussion

7.3.1 Specificity

Typical chromatograms of extracts from liver perfusate samples and plasma samples are shown in Figures 7.1 and 7.2, respectively. Parent drug, metabolites and the internal standard were completely resolved in both biological matrices with retention times of 4.9 ± 0.2 , 5.8 ± 0.2 , 7.2 ± 0.4 and 17.0 ± 1.1 min for α -hydroxymetoprolol, O-demethylmetoprolol, internal standard, and metoprolol, respectively. The addition of amino acids in blank matrices did not produce any interfering peaks, ensuring the feasibility of the method in the amino acid-metoprolol interaction studies.

7.3.2 Sensitivity

Using 1 ml of rat liver perfusate, the limits of detection were 1.5 ng/ml for metoprolol and 1 ng/ml for both α -hydroxymetoprolol and O-demethylmetoprolol, whereas the limit of quantification was 5 ng/ml for all three analytes. Since sensitivity was not critical in the analysis of perfusate samples, higher concentrations than the LOQ were used for the standard curves.

Using 0.1 ml of rat plasma, the limits of detection were 12 ng/ml for metoprolol and 7.5 ng/ml for both α -hydroxymetoprolol and O-demethylmetoprolol. The limits of quantification were 15 ng/ml for metoprolol and 10 ng/ml for both α -hydroxymetoprolol and O-demethylmetoprolol. The LOQ served as the lowest concentrations of the calibration curves.

7.3.3 Calibration curves

In determining concentrations in rat liver perfusate samples, the calibration curve of metoprolol was linear over the concentration range of 50 to 1000 ng/ml with a correlation coefficient (r) of ≥ 0.998 . The calibration curves of α -hydroxymetoprolol and O-demethylmetoprolol were also linear over the range of 50 to 1000 ($r \geq 0.999$) and 20 - 500 ng/ml ($r \geq 0.999$), respectively.

The calibration curves for rat plasma samples were linear ($r \geq 0.99$) over the concentration range of 15-200 ng/ml for metoprolol, 10-1000 ng/ml for α -hydroxymetoprolol and 10-100 ng/ml for O-demethylmetoprolol.

7.3.4 Accuracy and precision

The assay precision and accuracy for perfusate and plasma samples are presented in Tables 7.1 and 7.2, respectively. In the analysis of liver perfusate samples, the intra- and inter-day coefficients of variation were less than 10% for all drug and metabolite concentrations. In the case of plasma, the intra- and inter-day coefficients of variation were less than 10% for all concentrations of the parent drug and metabolites, except at their limits of quantitation (LOQ) where the intra- and inter-run CV were less than 15%.

7.3.5 Extraction recovery

The extraction recoveries from liver perfusate and plasma of metoprolol and the metabolites are reported in Table 7.3. The recovery of metoprolol was $\geq 80\%$ for both

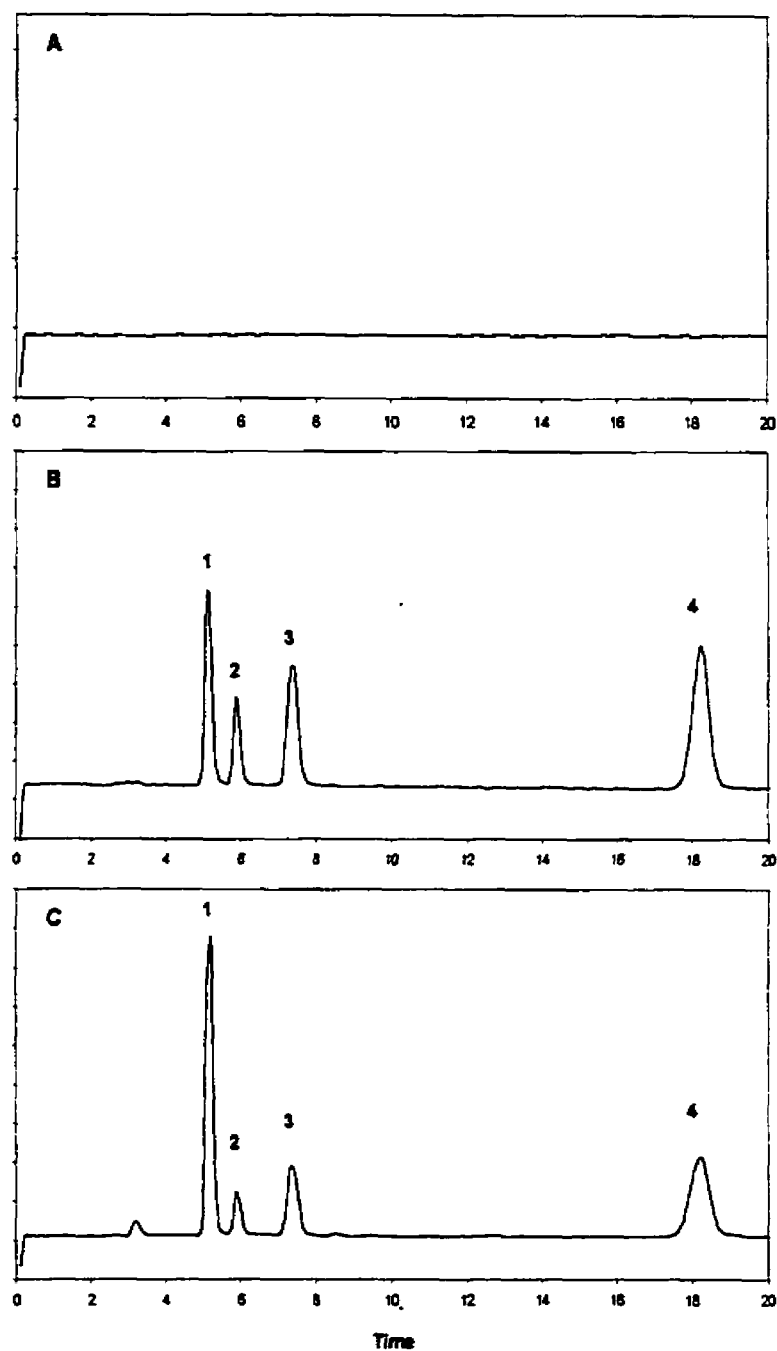
biological matrixes, whereas the recoveries of α -hydroxymetoprolol and O-demethylmetoprolol were less (in the 60 – 80% range). However, another major metabolite, metoprolol acid was not recovered using the present extraction procedure, due to its zwitterionic nature.

7.3.6 Application of method

The assay was successfully employed for the analysis of rat liver perfusate samples and plasma samples obtained in the *in vivo* and *ex vivo* studies of the amino acid-metoprolol interaction. Under the described conditions, the system worked appropriately during all these studies.

7.4 Conclusions

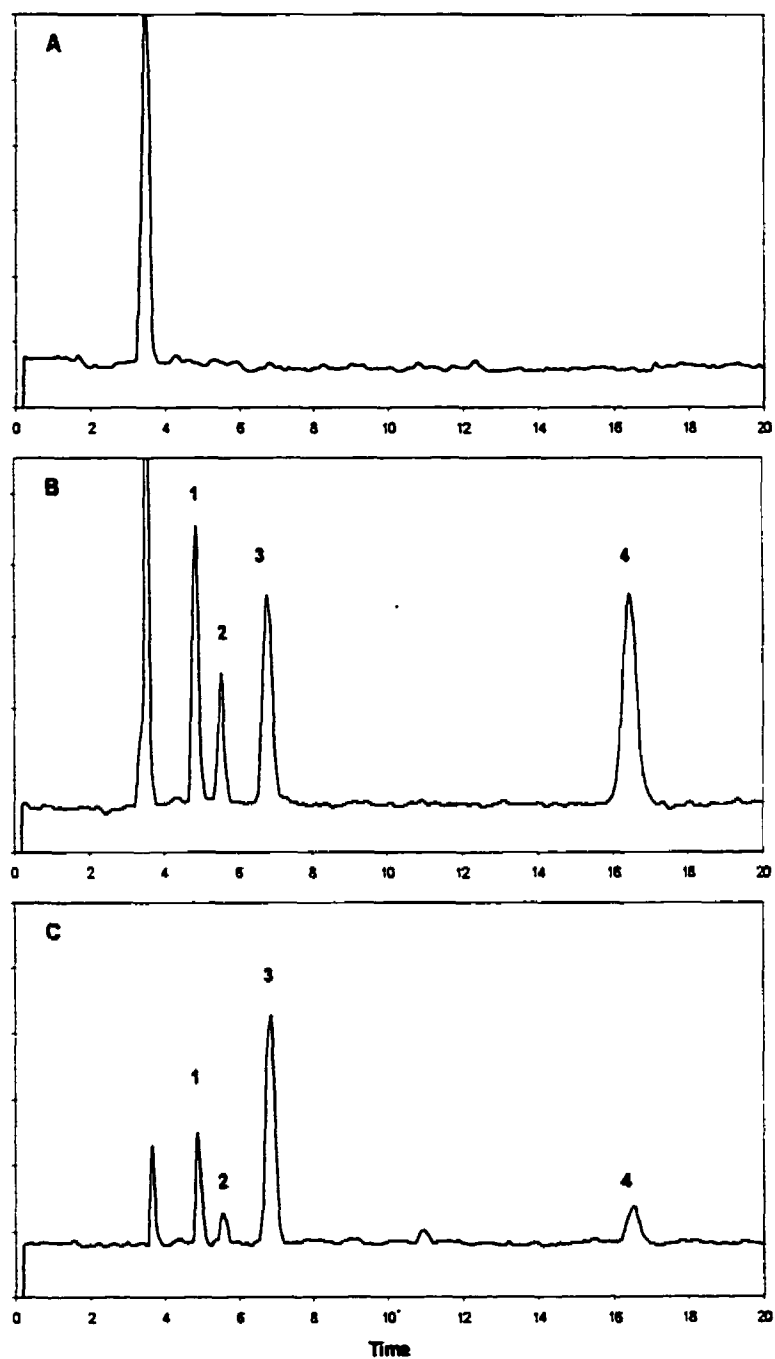
This chapter describes a specific, sensitive and quantitative HPLC assay modified from a method established previously in our laboratory. Validation of this assay has revealed that it is a reliable analytical method for the simultaneous determination of metoprolol, α -hydroxymetoprolol and O-demethylmetoprolol in rat liver perfusate and plasma.



1, α -hydroxymetoprolol; 2, O-demethylmetoprolol; 3, internal standard; 4, metoprolol

Figure 7.1. HPLC chromatograms for analysis of metoprolol and metabolites in rat liver perfusate.

(A) blank liver perfusate sample, (B) blank liver perfusate sample spiked with standard references, and (C) liver perfusate sample taken at 100 min after perfused with 2 $\mu\text{g}/\text{ml}$ of metoprolol and a mixture solution of amino acids.



1, α -hydroxymetoprolol; 2, O-demethylmetoprolol; 3, internal standard; 4, metoprolol

Figure 7.2. HPLC chromatograms for analysis of metoprolol and metabolites in rat plasma.

(A) blank plasma sample, (B) blank plasma sample spiked with standard references, and (C) plasma sample collected at 10 min after a single oral dose of 10 mg/kg.

Table 7.1. Accuracy and precision for metoprolol and metabolites in rat liver perfusate
(3 days, n = 5 in each day)

Added concentration (ng/ml)		Measured concentration (ng/ml) (Mean \pm SD, n = 5)	Within-day CV (%)	Between-day CV (%)	Mean accuracy (%)
Metoprolol					
60	Day 1	59.92 \pm 2.13	5.4	1.8	101.8
	Day 2	61.24 \pm 3.74			
	Day 3	62.09 \pm 4.10			
500	Day 1	510.0 \pm 16.2	4.4	0.4	102.3
	Day 2	510.4 \pm 36.6			
	Day 3	513.8 \pm 14.6			
900	Day 1	912.9 \pm 15.0	2.7	2.8	98.4
	Day 2	881.2 \pm 17.7			
	Day 3	863.8 \pm 37.3			
α-Hydroxymetoprolol					
60	Day 1	59.42 \pm 1.05	4.1	3.5	102.2
	Day 2	60.87 \pm 2.26			
	Day 3	63.70 \pm 4.41			
500	Day 1	513.6 \pm 17.5	3.2	1.4	101.3
	Day 2	499.0 \pm 6.71			
	Day 3	507.6 \pm 24.3			
900	Day 1	926.0 \pm 28.2	3.3	3.2	101.3
	Day 2	931.5 \pm 43.5			
	Day 3	877.7 \pm 19.4			
O-Demethylmetoprolol					
30	Day 1	31.00 \pm 1.36	5.7	1.9	103.4
	Day 2	31.64 \pm 2.25			
	Day 3	30.46 \pm 1.74			
250	Day 1	254.6 \pm 5.25	3.4	1.3	101.7
	Day 2	257.4 \pm 12.1			
	Day 3	251.0 \pm 8.86			
450	Day 1	461.6 \pm 17.4	4.1	3.6	101.3
	Day 2	468.1 \pm 29.9			
	Day 3	437.2 \pm 9.57			

Table 7.2. Accuracy and precision for metoprolol and metabolites in plasma
(3 days, n = 5 in each day)

Added concentration (ng/ml)		Measured concentration (ng/ml) (Mean \pm SD, n = 5)	Within-day CV (%)	Between-day CV (%)	Mean accuracy (%)
Metoprolol					
15	Day 1	13.50 \pm 1.65	12.4	14.3	107.3
	Day 2	16.89 \pm 2.01			
	Day 3	17.91 \pm 2.35			
100	Day 1	101.4 \pm 10.3	8.8	5.5	98.5
	Day 2	101.8 \pm 7.20			
	Day 3	92.20 \pm 8.50			
200	Day 1	198.2 \pm 3.20	2.1	3.3	99.7
	Day 2	206.4 \pm 3.71			
	Day 3	193.3 \pm 5.79			
α-Hydroxymetoprolol					
10	Day 1	10.74 \pm 1.56	13.9	8.7	104.8
	Day 2	9.470 \pm 1.65			
	Day 3	11.23 \pm 1.09			
500	Day 1	518.6 \pm 26.1	3.1	1.4	103.2
	Day 2	521.1 \pm 9.01			
	Day 3	507.9 \pm 12.6			
1000	Day 1	1033 \pm 19	2.1	1.5	103.2
	Day 2	1047 \pm 25			
	Day 3	1016 \pm 20			
O-Demethylmetoprolol					
10	Day 1	10.17 \pm 1.06	9.6	8.9	101.9
	Day 2	11.11 \pm 0.94			
	Day 3	9.292 \pm 0.93			
50	Day 1	51.67 \pm 3.27	5.4	4.2	102.8
	Day 2	49.17 \pm 2.42			
	Day 3	53.42 \pm 2.59			
100	Day 1	106.8 \pm 3.28	4.0	4.7	101.3
	Day 2	99.47 \pm 3.74			
	Day 3	97.77 \pm 5.14			

Table 7.3. Extraction recovery of metoprolol and metabolites from rat liver perfusate and plasma (n=5)

Compound	Liver Perfusate		Plasma	
	Concentration (ng/ml)	Recovery (%)	Concentration (ng/ml)	Recovery (%)
Metoprolol	50	87.5	30	85.8
	500	82.6	100	80.0
	1000	86.3	200	84.5
α -Hydroxy- metoprolol	50	59.8	20	67.5
	500	63.3	500	68.8
	1000	59.0	1000	70.0
O-Demethyl- metoprolol	20	69.1	15	81.9
	250	64.6	50	75.4
	500	65.8	100	71.8

CHAPTER 8

METABOLIC INTERACTION BETWEEN AMINO ACIDS AND METOPROLOL DURING ANTEGRADE AND RETROGRADE PERFUSION IN THE ISOLATED, PERFUSED RAT LIVER ^{1,2}

8.1 Introduction

Concomitant intake of food enhances the bioavailability of some high hepatic first-pass drugs, such as propranolol, metoprolol (ML) and propafenone (Melander et al., 1988). Because these drugs are almost completely absorbed from the gastrointestinal tract after oral administration, it has been generally agreed that a food-induced reduction in hepatic first-pass metabolism is responsible for this 'food effect'. Although it has been investigated for more than two decades, the exact mechanisms of the food effect remain unclear. The hepatic extraction of a drug is mainly dependent on hepatic metabolic enzyme activity, hepatic blood flow rate and drug plasma protein binding. It was first postulated that the most likely mechanism for the increased oral bioavailability is a transient increase in hepatic blood flow, resulting in the decreased hepatic extraction of the drug during the absorption phase after coadministration with food (McLean et al., 1978). However, it has been proposed that alteration in hepatic blood flow plays only a minor role in the food effect because *in vivo* experiments showed that flow changes could not account for the magnitude of the increase in AUC_{oral} (Svensson et al., 1983;

¹ Presented at the Twelfth Annual American Association of Pharmaceutical Scientists Meeting, November 1998, San Francisco, California, USA.

² Manuscript prepared for submission to *Drug Metab. Dispos.*

Modi et al., 1988). Furthermore, food has not been shown to influence the unbound fraction of propranolol in plasma (Feely et al., 1983). Therefore, transient inhibition of hepatic metabolic activity caused by one or more food components may contribute to the mechanisms of the food effect.

Human studies have revealed that the protein content of the meal appears to be an important factor in the food effect (McClean et al., 1981; Wall et al., 1981; Axelson et al., 1987). Ingested protein is digested and absorbed into the portal circulation in the form of amino acids (AA), so that any dietary protein-induced change in hepatic metabolic activity would be caused by the absorbed amino acids interacting with drug metabolizing enzymes in the liver. Previous studies in isolated perfused liver preparations (Semple and Xia, 1995; Wang and Semple, 1997) have demonstrated that a balanced mixture of amino acids infused into rat livers resulted in a transient and reversible reduction in the metabolism of propranolol and metoprolol by inhibiting the formation of all of their measured metabolites. Since no direct inhibition of metoprolol metabolism by physiological levels of amino acids was observed in the study of rat liver microsome preparations (Alcorn, 1997), the inhibition of drug metabolism may be caused by other mechanisms involving indirect regulation by amino acids, such as oxygen and/or NADPH limitation. In addition to the decrease in metoprolol metabolism, a tremendous increase in hepatic oxygen consumption also occurred after coinfusion of amino acids in the isolated, perfused rat liver (Wang and Semple, 1997). The increased oxygen consumption, due to the intensely oxygen-consuming metabolic process of amino acids, results in severe oxygen depletion in the liver, which may be the mechanism contributing to the inhibition of metoprolol metabolism. Within the hepatic

acinus large differences in distribution of hepatic enzymes exist from the periportal zone to the pericentral zone. The metabolism of amino acids is preferentially localized in the periportal zone (Häussinger and Gerok, 1986), whereas the cytochrome P-450 enzymes, mediating drug oxidative metabolism, are mainly distributed in the pericentral zone (Thurman et al., 1986). When both amino acids and metoprolol are infused into the hepatic portal vein, therefore, amino acid metabolizing enzymes may utilize oxygen preferentially so that metabolizing enzymes for metoprolol may suffer a transient oxygen deficiency and thus their activities may be temporarily impaired. We therefore hypothesize that the inhibition of metoprolol metabolism may be attributed to the amino acid-mediated pericentral oxygen depletion in the hepatic sinusoids (Wang and Semple, 1997). This hypothesis can be tested by using a single-pass rat liver perfusion technique in antegrade and retrograde directions. It would be expected that if the pericentral oxygen limitation caused by the metabolism of amino acids did contribute to the interaction between metoprolol and amino acids, metoprolol metabolism would be less inhibited by amino acids during retrograde than antegrade perfusion.

Metoprolol is a β -adrenoceptor antagonist with a high hepatic extraction ratio. It exhibits in human an average 40% increase in AUC_{oral} when coadministered with a high-protein meal even though it is completely absorbed (Melander et al., 1977). Similar to humans, metoprolol is metabolized in rats mainly via hepatic oxidation into α -hydroxymetoprolol, O-demethylmetoprolol and metoprolol acid, a secondary metabolite from O-demethylation (Arfwidsson et al, 1976). Also metoprolol has been found to be an appropriate model drug for mechanistic studies in the isolated, perfused rat liver preparation due to its minor tissue binding characteristics (Wang and Semple, 1997). As

an extension of our previous studies, this report presents the results of serial experiments in which the effect of different levels of amino acids on hepatic oxygen consumption and metoprolol metabolism has been examined during antegrade and retrograde perfusion of the isolated rat liver under different oxygenation conditions of the perfusion medium. We hereinafter try to answer these questions: 1) whether the inhibition of metoprolol metabolism by amino acids would likely be the result of pericentral oxygen depletion; and 2) whether and how the amino acid-metoprolol interaction and its mechanisms would be relevant to the food effect observed in human studies.

8.2 Materials and Methods

8.2.1 Chemicals

R, S-Metoprolol tartrate and nadolol (internal standard) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). α -Hydroxymetoprolol (H119/66) p-OH benzoate and O-demethylmetoprolol (H105/22) p-OH benzoate were gifts from Astra (Hässle, Sweden). Aminosyn® II 10% Amino Acids Injection came from Abbott Laboratories Ltd. (Montreal, Quebec, Canada). Bovine serum albumin Fraction V was obtained from Sigma Chemical Co. The outdated human red blood cells in Citrate Phosphate Dextrose Adenine Solution USP (CPDA-1) pack units were supplied by the Red Cross, Saskatoon, Canada. All solvents were HPLC grade obtained from BDH Inc. (Toronto, Ontario, Canada). All other chemicals used were analytical grade from BDH Inc.

8.2.2 Isolated Rat Liver Perfusion

Male Sprague-Dawley rats (Charles River, St. Constant, Quebec, Canada), weighing 180-280 g, were used as liver donors. The animals were maintained on standard laboratory chow and water *ad libitum* in accordance with the guidelines of the Canadian Council on Animal Care.

The surgical procedure reported by Pang (1984) was adopted with minor modifications. Following an overnight fast, rats were anesthetized by inhalation of halothane (MTC Pharmaceuticals, Cambridge, Ontario, Canada). The portal vein was

cannulated with a 16G Jelco™ intravenous catheter (Critikon, Inc., Tampa, FL, USA), which was used to provide the inlet perfusion medium. The outflow from the liver was collected via another cannula inserted through the right atrium into the thoracic inferior vena cava. The hepatic artery and inferior vena cava (proximal to the right renal vein) were ligated to ensure unidirectional flow. The bile duct was also tied off because cannulation was not required for a drug with minimal biliary excretion. The rat was then transferred to a perfusion system where the liver was perfused *in situ*.

The perfusion apparatus was modified from that described previously (Semple and Xia, 1994). The perfusion system, maintained in a temperature-controlled (37°C) cabinet, included two reservoir units, a peristaltic pump (Ismatec SA, Vario-pumpsystem, Cole-Parmer, Niles, IL, USA), a silastic tubing oxygenator, a bubble trap/filter device and two three-way stopcocks which were used to facilitate the switch between antegrade and retrograde flow to the liver during perfusion (Pang and Terrell, 1981). A pH meter (PHM84 Research pH meter, Radiometer A/S Copenhagen, Denmark) and a biological oxygen monitor (YSI model 5300, YSI, Inc., Yellow Springs, OH, USA) were interconnected with the flow-line before and after the liver, respectively, to monitor the pH of inlet perfusate and the oxygen content of outlet perfusate. A syringe pump (Model 975, Harvard Apparatus, South Natick, MA, USA) was attached to the perfusion line approximately 1 cm from the entry of the liver to add Aminosyn II 10% amino acids injection. The concentrations of amino acids in the perfusate were controlled by the infusion rate set in the syringe pump.

The liver was perfused in a single-pass mode at a constant rate of about 15 ml/min/liver. The perfusion medium consisted of 20% (v/v) washed outdated human red

blood cells, 1% bovine serum albumin and 0.1% dextrose in a Krebs-Henseleit bicarbonate buffer. Human red cells were washed with equal volumes of physiological saline three times, followed by equal volumes of Krebs-Henseleit bicarbonate buffer three times. The washed red blood cells were used immediately. The perfusate was buffered to pH 7.2 - 7.4 and oxygenated by equilibration with 95% O₂/5%CO₂ in the silastic tubing oxygenator. To achieve a reduced level of oxygen delivery, the perfusate was equilibrated with a mixture of 95% O₂/5%CO₂ and 95% N₂/5%CO₂. The proportion of oxygen to nitrogen determined the oxygen level in the perfusate.

The viability of the liver was assessed by: 1) rate of oxygen consumption; 2) maintenance of metoprolol steady state; and 3) physical appearance of the liver.

8.2.3 Experimental Design

The experiment was designed to examine the effect of amino acids on the metabolism of metoprolol during antegrade and retrograde perfusion in the liver under different perfusion and coinfusion conditions. Twenty-four rat livers were randomly divided into 3 groups (N = 8 each): 1) Hypo-O₂/High AA group. The livers in this group were perfused with hypo-oxygenated perfusion medium equilibrated with the mixture of nitrogen and oxygen, and coinfused with high levels of amino acids. 2) Normal O₂/High AA group. The livers were perfused with the perfusate normally oxygenated with 95% O₂/5%CO₂, and coinfused with high levels of amino acids. 3) Normal O₂/Low AA group. The livers were perfused under the normal oxygenation condition, and coinfused with low levels of amino acids.

The perfusion of each liver preparation was conducted in antegrade direction following retrograde direction, or *vice versa*. During both antegrade and retrograde perfusion, amino acids were introduced into the liver. Thus, the total perfusion duration consisted of four phases: antegrade (Ante), antegrade with amino acids (Ante-AA), retrograde (Retro), and retrograde with amino acids (Retro-AA). This enabled each liver to act as its own control for both direction of flow and effect of amino acids. The order of flow direction was randomized. In each group, antegrade perfusion preceded retrograde perfusion for four liver preparations, and retrograde perfusion preceded antegrade perfusion for the other four.

Following a 20 min stabilization period of perfusion with blank oxygenated perfusion medium, the liver was perfused with the medium containing 5.84 μM (2 $\mu\text{g/ml}$) metoprolol. The initiation of metoprolol infusion was defined as time 0. After 30 min, when metoprolol and metabolite concentrations and oxygen consumption rates had been at steady state for 15 - 20 min, a balanced mixture of amino acids (Aminosyn® II 10% amino acids injection) was introduced into the inlet perfusate at a rate of either 0.42 ml/min (High AA group) or 0.21 ml/min (Low AA group). The final concentrations of each constituent amino acid in the perfusate are given in Table 8.1 for the High AA group, in which the total concentration of amino acids was 21.5 mM, whereas the final concentrations for the Low AA group were half the values of those for the High AA group. The coinfusion of amino acids was continued for 30 min. At 75 min, the direction of flow was reversed from antegrade to retrograde (or *vice versa*). After a 15 min period of stabilization, the mixture of amino acids was introduced to the liver from 90 to 120 min. The perfusion was allowed to run a further 10 min with the perfusion medium

containing metoprolol. A blank perfusate sample was collected from the vena cava at time 0; after time 0, effluent samples were collected every 5 min over the entire perfusion period of 130 min. Each sample consisted of a 30 sec collection of effluent. After centrifugation at $1000 \times g$, the plasma of perfusate samples was separated, and immediately stored at -20°C until analysis.

8.2.4 Analysis of Metoprolol and Metabolites

The concentrations of metoprolol and its metabolites, α -hydroxymetoprolol and O-demethylmetoprolol in liver perfusate samples were determined simultaneously using a revalidated HPLC method as described previously (Wang and Semple, 1997) with some modifications. In brief, to 1 ml of liver perfusate plasma was added 50 μl of nadolol (5 $\mu\text{g}/\text{ml}$, internal standard) and 2 ml of 1 M sodium carbonate buffer (pH 10.5). The drug, metabolites and internal standard were extracted into 5 ml of a mixture of diethyl ether-dichloromethane (4:1, v/v) after vortex mixing for 10 min and centrifugation at $2500 \times g$ for 10 min. The organic layer was transferred to a clean glass tube and evaporated to dryness under nitrogen at room temperature. The residues were reconstituted in 300 μl of mobile phase, and 100 μl of which was injected into the HPLC system.

The HPLC system consisted of a Waters 510 pump, Waters 710B WISP automatic injector, Waters Baseline data system (Millipore-Waters, Mississauga, ON), and a Spectroflow 980 fluorescence detector (Applied Biosystems, Ransey, NJ, USA) set at an excitation wavelength of 224 nm. The separation of the individual components

was achieved on a 250 × 4 mm I.D., 5 µm particle size Lichrospher 60 RP-select B column (E. Merck, Darmstadt, Germany). The mobile phase was water-acetonitrile (87:13, v/v), containing 0.3% (v/v) triethylamine and phosphoric acid to adjust the apparent pH to 3.0. The flow rate was 1 ml/min.

Calibration curves were linear over the concentration range studied, i.e. 50 - 1000 ng/ml for metoprolol and α-hydroxymetoprolol and 20 - 500 ng/ml for O-demethylmetoprolol. The intra- and inter- run coefficients of variation were less than 10% for all analytes. Quality control (QC) samples duplicated at low, medium and high concentrations over the range of calibration curves of the drug and metabolites were incorporated into each run, and all those concentrations were within 11% error of the nominal concentrations.

8.2.5 Oxygen Delivery and Consumption Determinations

The oxygen tension in the outflow perfusate was monitored throughout the liver perfusion experiment by the online YSI5300 biological oxygen monitor. The partial pressures of oxygen (pO₂) in the effluent were recorded every 5 min when perfusate samples were collected. The hemoglobin contents (Hb) and pO₂ in the inflow perfusate were determined using a blood gas analyzer (Model 288, CIBA-Corning, Medfield, MA, USA). The oxygen content (O₂Ct) in either the inflow or outflow perfusate was calculated from the following equation (Staub, 1992):

$$O_2Ct(ml / dl) = 1.34 \times Hb \times \%O_2Sat + 0.003 \times pO_2 \quad (8.1)$$

where Hb and pO₂ are expressed in g/dl and mmHg, respectively, and %O₂Sat is the

percentage of oxygen saturation of hemoglobin, which could be estimated from the pO₂ according to the Manual of the CIBA-Corning 288 Blood Gas System. Thus, the oxygen delivery rate (O₂Del) in the influent was determined by Eq 8.2 (Brouwer and Thurman, 1991):

$$O_2Del(\mu\text{mol}/\text{min}/\text{g liver}) = \frac{O_2Ct(\text{Inflow}) \times Q \times 39.3}{\text{LiverWt} \times 100} \quad (8.2)$$

where Q is the perfusion flow rate (ml/min) and LiverWt is the weight of the liver (g).

The oxygen consumption rate (O₂Csm) was calculated by

$$O_2Csm(\mu\text{mol}/\text{min}/\text{g liver}) = \frac{[O_2Ct(\text{Inflow}) - O_2Ct(\text{Outflow})] \times Q \times 39.3}{\text{LiverWt} \times 100} \quad (8.3)$$

The oxygen extraction (O₂Ext) was calculated from

$$O_2Ext(\%) = \frac{O_2Csm}{O_2Del} \times 100 \quad (8.4)$$

8.2.6 Pharmacokinetic Analysis

The effluent concentrations of metoprolol and the metabolites at steady state for the four periods (Ante, Ante-AA, Retro, and Retro-AA) were calculated by averaging the last three measured data points in each period, which were determined to be at steady state according to the method described previously (Semple and Xia, 1994). The efficiency of hepatic metabolism was described by the hepatic extraction ratio (E) and hepatic clearance (CL) at steady state. In each period of liver perfusion, E and CL of metoprolol were calculated by the following equations:

$$E = \frac{C_{in} - C_{out}}{C_{in}} \quad (8.5)$$

$$CL = Q \times E \quad (8.6)$$

where C_{in} is the inflow concentration and C_{out} is the steady state outflow concentration of metoprolol. Metabolite formation clearance (CL_f) for α -hydroxymetoprolol and O-demethylmetoprolol was calculated as

$$CL_f = \frac{Q \cdot C_M}{C_{in}} \quad (8.7)$$

where C_M is the effluent metabolite concentration at steady state.

8.2.7 Statistical Analysis

Data are expressed as the mean \pm standard deviation. The difference between groups was evaluated by a one-way ANOVA, and the difference within groups was evaluated by a nested randomized complete block ANOVA (the model includes the rat nested within the order of perfusion directions, the order of perfusion directions, and the perfusion phase). Comparisons between antegrade and retrograde perfusions were made by paired t-test. A value of $P < 0.05$ was considered statistically significant.

Table 8.1. Concentrations of individual amino acids in the influent perfusate to the liver for the High AA group ^a

Amino acids	mM
Essential amino acids	
<i>L</i> -Isoleucine	1.39
<i>L</i> -Leucine	2.11
<i>L</i> -Lysine	1.41
<i>L</i> -Methionine	0.32
<i>L</i> -Phenylalanine	0.50
<i>L</i> -Threonine	0.93
<i>L</i> -Tryptophan	0.27
<i>L</i> -Valine	1.18
Nonessential amino acids	
<i>L</i> -Alanine	3.09
<i>L</i> -Arginine	1.62
<i>L</i> -Aspartic acid	1.46
<i>L</i> -Glutamic acid	1.39
Glycine	1.84
<i>L</i> -Histidine	0.54
<i>L</i> -Proline	1.74
<i>L</i> -Serine	1.40
N-Acetyl- <i>L</i> -tyrosine	0.34

^a The concentrations of the individual amino acids for the Low AA group were half the above values.

8.3 Results

8.3.1 Hypo-O₂/High AA Group

8.3.1.1 Viability of Isolated Perfused Liver

All of the livers were well perfused as evidenced by all lobes showing an equal and even color, the absence of leakage, adequate hepatic oxygen consumption and maintenance of metoprolol steady state during the whole period of perfusion. The mean physiological parameters of the perfused livers are shown in Table 8.2. The oxygen consumption rate and effluent concentrations of metoprolol, α -hydroxymetoprolol and O-demethylmetoprolol quickly reached steady state (< 15 min) for both antegrade and retrograde perfusions (Figure 8.1). The order of flow direction in the group had no effect on the time to reach the steady state, oxygen consumption, metoprolol clearance and metabolite formation clearance of liver ($P > 0.05$, nested ANOVA).

8.3.1.2 Oxygen Consumption

In the Hypo-O₂/High AA group, livers were perfused with the hypo-oxygenated perfusion medium with an average O₂ delivery rate of 3.40 ± 0.52 $\mu\text{mol/min/g}$ liver, approximately half of that in the normally oxygenated perfusion medium. The mean oxygen consumption was 2.49 ± 0.62 $\mu\text{mol/min/g}$ liver during the Ante phase and 2.41 ± 0.56 $\mu\text{mol/min/g}$ liver during the Retro phase, respectively (Table 8.3). The change in direction of flow thus did not alter the oxygen consumption of the liver ($P > 0.05$). The

mean oxygen extraction during the Ante and Retro phases were 72.6 ± 8.8 and 70.5 ± 9.2 %, respectively.

Coinfusion of high levels of amino acids caused an increase in oxygen consumption during both antegrade and retrograde perfusion. The mean oxygen extraction increased to 82.2 ± 7.0 % during the Ante-AA phase and 79.4 ± 5.4 % during the Retro-AA phase (Table 8.3). Also the magnitude of change in oxygen consumption caused by amino acids during antegrade perfusion (13.8 ± 7.4 %) was approximate to that during retrograde perfusion (13.5 ± 9.8 %, $P > 0.05$).

8.3.1.3 Metoprolol Metabolism

The hepatic extraction ratios and clearances of metoprolol, and the metabolite formation clearances during the four phases, Ante, Ante-AA, Retro, and Retro-AA, are shown in Table 8.3. During the Ante phase, the mean hepatic clearance of metoprolol was 1.56 ± 0.15 ml/min/g liver, and its extraction ratio was 87.6 ± 6.2 %. The mean formation clearances of α -hydroxymetoprolol and O-demethylmetoprolol were 0.460 ± 0.079 and 0.102 ± 0.069 ml/min/g liver, respectively, indicating that about 29.5% of the metoprolol clearance was mediated via the α -hydroxylation metabolism pathway, whereas only 6.5% of clearance was mediated via O-demethylation. During the Retro phase, the mean hepatic clearance of metoprolol was 1.51 ± 0.09 ml/min/g liver, and its extraction ratio was 85.3 ± 4.6 %. The mean formation clearances of α -hydroxymetoprolol and O-demethylmetoprolol were 0.449 ± 0.064 and 0.114 ± 0.071 ml/min/g liver, respectively, accounting for 29.8% and 7.5% of total clearance,

respectively. Hence, the metabolism of metoprolol was not altered during retrograde perfusion, as evidenced by no significant difference in metoprolol clearances and metabolite formation clearances between the Ante and Retro phases.

Coinfusion of high levels of amino acids caused a reduction in metoprolol metabolism during both antegrade and retrograde perfusion. However, the magnitude of the change was smaller during retrograde perfusion than during antegrade perfusion (Figure 8.2). Metoprolol clearance was significantly decreased by coinfusion of amino acids from 1.56 ± 0.15 to 1.32 ± 0.19 ml/min/g liver during antegrade perfusion, and from 1.51 ± 0.09 to 1.35 ± 0.14 ml/min/g liver during retrograde perfusion. The percentage decrease during antegrade perfusion was 15.6 ± 5.0 %, which was significantly larger than 11.0 ± 4.8 % decrease during retrograde perfusion. Consistently, the formation clearance of α -hydroxymetoprolol was reduced by 9.6 ± 4.6 % when coinfused with amino acids during antegrade perfusion. The reduction by amino acids during retrograde perfusion was only 2.0 ± 5.8 %, which showed significant difference from that of antegrade perfusion. The percentage of α -hydroxylation accounting for the total metoprolol clearance seemed to slightly increase after coinfusion of amino acids from 29.5% to 31.8% during antegrade perfusion and from 29.8% to 32.8% during retrograde perfusion. Unexpectedly, coinfusion of amino acids caused significant increases in the formation clearance of O-demethylmetoprolol, 35.3 ± 18.3 % for antegrade perfusion and 26.2 ± 12.1 % for retrograde perfusion. The percentage of O-demethylation accounting for the total clearance of metoprolol increased from 6.6% during the Ante phase to 10.1% during the Ante-AA phase, and from 7.5% for the Retro phase to 10.2% for the Retro-AA Phase.

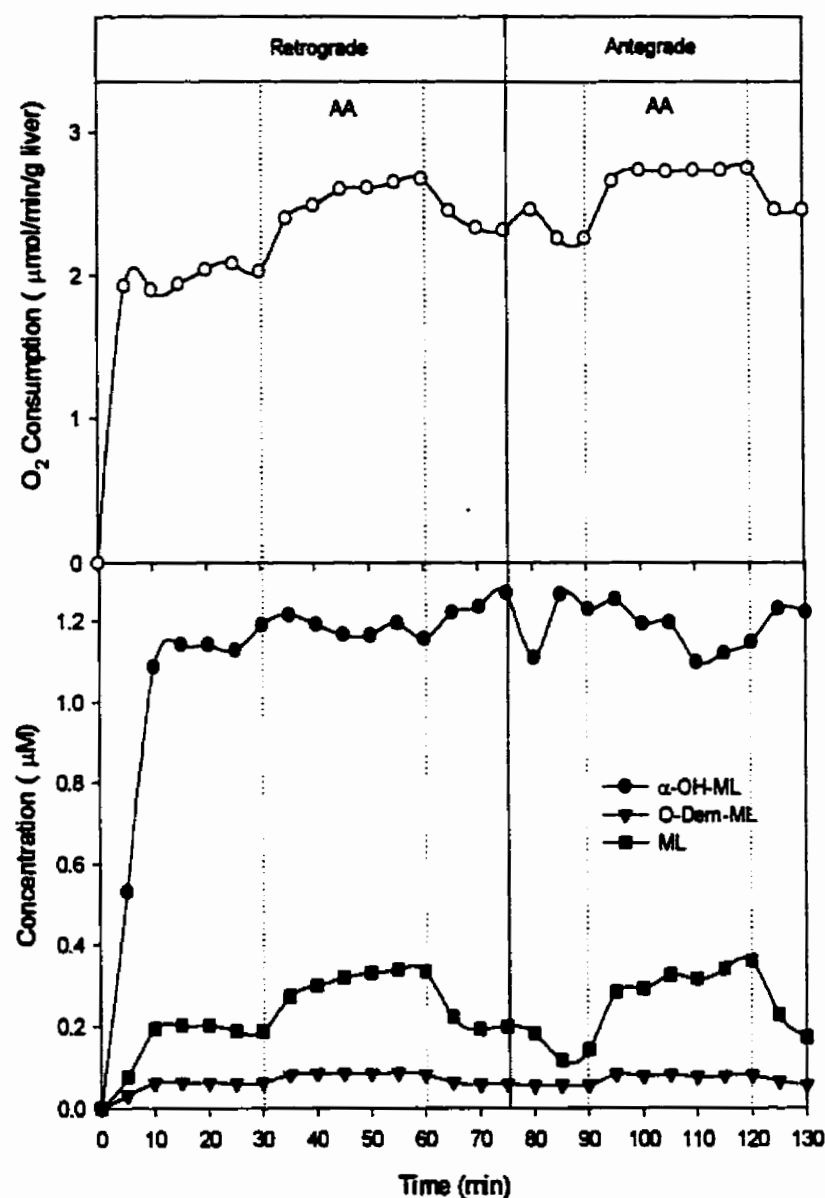


Figure 8.1. Time courses of hepatic oxygen consumption and effluent concentrations of metoprolol (ML), α -hydroxymetoprolol (α -OH-ML) and O-demethylmetoprolol (O-Dem-ML) in a perfused liver from the Hypo- O_2 /High AA group.

The liver was perfused with $5.84 \mu\text{M}$ metoprolol in a hypo-oxygenated (O_2 delivery rate: $3.35 \mu\text{mol/min/g liver}$) erythrocyte-enriched perfusion medium from 0 to the end of the experiment, and high levels of amino acids were coinfused from 30 to 60 min and 90 to 120 min. The direction of perfusion was reversed after 75 min.

Table 8.2. Physiological parameters (mean \pm SD) of isolated, perfused rat livers

Group	N	Liver weight (g)	Perfusion flow rate (ml/min/g liver)	pH	O₂ delivery rate (μmol/min/g liver)
Hypo-O ₂ /High AA	8	7.19 \pm 0.99	1.78 \pm 0.13	7.26 \pm 0.07	3.40 \pm 0.52
Normal O ₂ /High AA	8	7.76 \pm 0.89	1.80 \pm 0.20	7.26 \pm 0.13	5.66 \pm 0.85
Normal O ₂ /Low AA	8	8.70 \pm 0.82	1.74 \pm 0.20	7.24 \pm 0.04	5.21 \pm 0.72

Table 8.3. Effect of high levels of amino acids on oxygenation and pharmacokinetic parameters (mean \pm SD, N = 8) of metoprolol and its metabolites during antegrade and retrograde perfusion in the isolated rat liver perfused with a hypo-oxygenated medium (Hypo-O₂/High AA group)

	Antegrade perfusion			Retrograde perfusion		
	No AA	AA	%Change *	No AA	AA	%Change *
O ₂ consumption (μ mol/min/g liver)	2.49 \pm 0.62	2.81 \pm 0.60 ^a	13.8 \pm 7.4	2.41 \pm 0.56	2.71 \pm 0.53 ^b	13.5 \pm 9.8
O ₂ extraction (%)	72.6 \pm 8.9	82.2 \pm 7.0 ^a	13.8 \pm 7.4	70.5 \pm 9.2	79.4 \pm 5.4 ^b	13.5 \pm 9.8
Metoprolol E (%)	87.6 \pm 6.2	74.1 \pm 8.8 ^a	-15.6 \pm 5.0	85.3 \pm 4.6	76.0 \pm 7.2 ^b	-11.0 \pm 4.8 ^c
Metoprolol CL (ml/min/g liver)	1.56 \pm 0.15	1.32 \pm 0.19 ^a	-15.6 \pm 5.0	1.51 \pm 0.09	1.35 \pm 0.14 ^b	-11.0 \pm 4.8 ^c
α -Hydroxymetoprolol CL _r (ml/min/g liver)	0.460 \pm 0.079	0.418 \pm 0.085 ^a	-9.6 \pm 4.6	0.449 \pm 0.064	0.439 \pm 0.063	-2.0 \pm 5.8 ^c
O-Demethylmetoprolol CL _r (ml/min/g liver)	0.102 \pm 0.069	0.132 \pm 0.081 ^a	35.3 \pm 18.3	0.114 \pm 0.071	0.139 \pm 0.078 ^b	26.2 \pm 12.1

^a Significant difference (P < 0.05) between No AA and AA during antegrade perfusion by paired t-test.

^b Significant difference (P < 0.05) between No AA and AA during retrograde perfusion by paired t-test.

^c Significant difference (P < 0.05) between antegrade and retrograde perfusion by paired t-test

* Percentage change upon coinfusion of AAs during antegrade or retrograde perfusion, calculated as $\frac{([AA] - [No AA]) \times 100}{[No AA]}$.

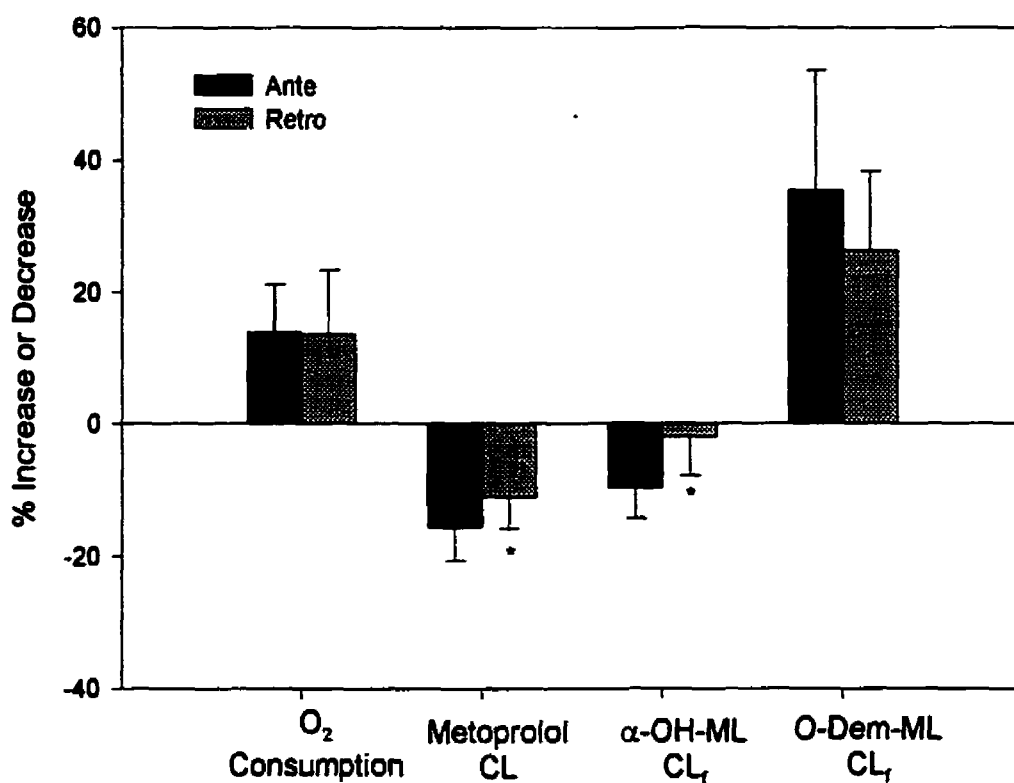


Figure 8.2. The percentage change (mean \pm SD, N = 8) in oxygen consumption, metoprolol clearance (CL), α -hydroxymetoprolol formation clearance (α -OH-ML CL_f) and O-demethylmetoprolol formation clearance (O-Dem-ML CL_f) after coinfusion of high levels of amino acids in the isolated rat liver perfused with a hypo-oxygenated medium during antegrade (Ante) and retrograde (Retro) perfusion (Hypo-O₂/High AA group).

* indicates significant difference between antegrade and retrograde perfusion by paired t-test (P < 0.05).

8.3.2 Normal O₂/High AA group

8.3.2.1 Viability of Isolated Perfused Liver

The physiological parameters of the perfused liver are shown in Table 8.2. All of the livers were well perfused as described in the Hypo-O₂/High AA group. The time to reach steady state was less than 15 min for metoprolol and its metabolites during both antegrade and retrograde perfusion. The time courses were similar to those depicted in Figure 8.1. The order of perfusion direction had no effect on the time courses and levels of oxygen consumption, and metoprolol metabolism in the perfused livers ($P > 0.05$, nested ANOVA).

8.3.2.2 Oxygen Consumption

In this group, livers were perfused with a normally oxygenated perfusion medium. The mean oxygen delivery rate to the liver was 5.66 ± 0.85 $\mu\text{mol/min/g}$ liver. The mean oxygen consumption was 3.62 ± 0.56 $\mu\text{mol/min/g}$ liver, and the mean oxygen extraction ratio was 63.4% during the Ante phase (Table 8.4). During the Retro phase, the mean oxygen consumption was 3.63 ± 0.46 $\mu\text{mol/min/g}$ liver, and the mean oxygen extraction ratio was 63.8%. There was no significant difference between antegrade and retrograde in hepatic oxygen consumption ($P > 0.05$, paired \pm test).

Coinfusion of high levels of amino acids caused $16.1 \pm 12.8\%$ and $14.4 \pm 7.8\%$ increase in oxygen consumption during antegrade and retrograde perfusion, respectively. The difference in the magnitude of amino acid –induced increase was not significant between antegrade and retrograde perfusion phases ($P > 0.05$, paired t-test).

8.3.2.3 Metoprolol Metabolism

The pharmacokinetic parameters of metoprolol and its metabolites during the Ante, Ante-AA, Retro, Retro-AA phases are shown in Table 8.4. During antegrade and retrograde perfusion without coinfusion of amino acids, the hepatic extraction ratio of metoprolol was 86.9 ± 5.7 and $86.3 \pm 5.3\%$, respectively, and the hepatic clearance of metoprolol was 1.56 ± 0.24 and 1.56 ± 0.22 ml/min/g liver, respectively. The mean formation clearance of α -hydroxymetoprolol was 0.449 ± 0.095 ml/min/g liver during the Ante phase and 0.452 ± 0.087 ml/min/g liver during the Retro phase, accounting for about 29.0 and 29.1% of the total clearance of metoprolol, respectively. The mean formation clearance of O-demethylmetoprolol was 0.073 ± 0.018 ml/min/g liver during antegrade perfusion and 0.082 ± 0.021 ml/min/g liver during retrograde perfusion, accounting for 4.8 and 5.5% of the total clearance, respectively. No significant difference in metoprolol metabolism was found between the antegrade and retrograde perfusion phases.

Coinfusion of high levels of amino acids decreased the hepatic clearance of metoprolol by $8.7 \pm 3.3\%$ during antegrade perfusion and by $6.3 \pm 1.9\%$ during retrograde perfusion (Figure 8.3). The magnitude of decrease for the retrograde perfusion was less than that for the antegrade perfusion; however, the difference was statistically insignificant ($P > 0.05$, paired t-test). During both antegrade and retrograde perfusion, the alteration in α -hydroxymetoprolol formation clearance caused by amino acids exhibited large variation among the eight liver preparations in the group. In five

liver preparations, amino acids decreased the formation clearance of α -hydroxymetoprolol by 1.5 to 25.9%, but in the other three preparations, the formation clearance was increased by 1.3 to 12.2%. The formation clearance of α -hydroxymetoprolol in the Ante-AA and Retro-AA phases accounted for 30.5 and 29.6% of the total clearances, respectively. Furthermore, the formation clearance of O-demethylmetoprolol was increased by 32.0 ± 18.8 and $21.9 \pm 15.6\%$ after coinfusion of amino acids during antegrade and retrograde perfusion, respectively. The percentage of O-demethylmetoprolol accounting for metoprolol metabolism increased from 4.8 to 6.8% and from 5.5 to 6.9% during the Ante-AA and Retro-AA phase, respectively.

Table 8.4. Effect of high levels of amino acids on oxygenation and pharmacokinetic parameters (mean \pm SD, N = 8) of metoprolol and its metabolites during antegrade and retrograde perfusion in the isolated rat liver perfused with a normally oxygenated medium (Normal O₂/High AA group)

	Antegrade perfusion			Retrograde perfusion		
	No AA	AA	%Change *	No AA	AA	%Change *
O ₂ consumption (μ mol/min/g liver)	3.62 \pm 0.56	4.14 \pm 0.45 ^a	16.1 \pm 12.8	3.63 \pm 0.46	4.13 \pm 0.41 ^b	14.4 \pm 7.8
O ₂ extraction (%)	63.4 \pm 10.9	72.7 \pm 8.6 ^a	16.1 \pm 12.8	63.8 \pm 9.7	72.5 \pm 7.9 ^b	14.4 \pm 7.8
Metoprolol E (%)	86.9 \pm 5.7	79.4 \pm 6.7 ^a	-8.7 \pm 3.3	86.3 \pm 5.3	80.8 \pm 5.4 ^b	-6.3 \pm 1.9
Metoprolol CL (ml/min/g liver)	1.56 \pm 0.24	1.42 \pm 0.22 ^a	-8.7 \pm 3.3	1.56 \pm 0.22	1.46 \pm 0.24 ^b	-6.3 \pm 1.9
α -Hydroxymetoprolol CL _r (ml/min/g liver)	0.449 \pm 0.095	0.432 \pm 0.091	-3.1 \pm 3.2	0.452 \pm 0.087	0.433 \pm 0.102	-4.5 \pm 10.4
O-Demethylmetoprolol CL _r (ml/min/g liver)	0.073 \pm 0.018	0.095 \pm 0.017 ^a	32.0 \pm 18.8	0.082 \pm 0.021	0.098 \pm 0.019 ^b	21.9 \pm 15.6

^a Significant difference (P < 0.05) between No AA and AA during antegrade perfusion by paired t-test.

^b Significant difference (P < 0.05) between No AA and AA during retrograde perfusion by paired t-test.

* Percentage change upon coinfusion of AA during antegrade or retrograde perfusion, calculated as $[(AA) - (No\ AA)] \times 100 \div [No\ AA]$.

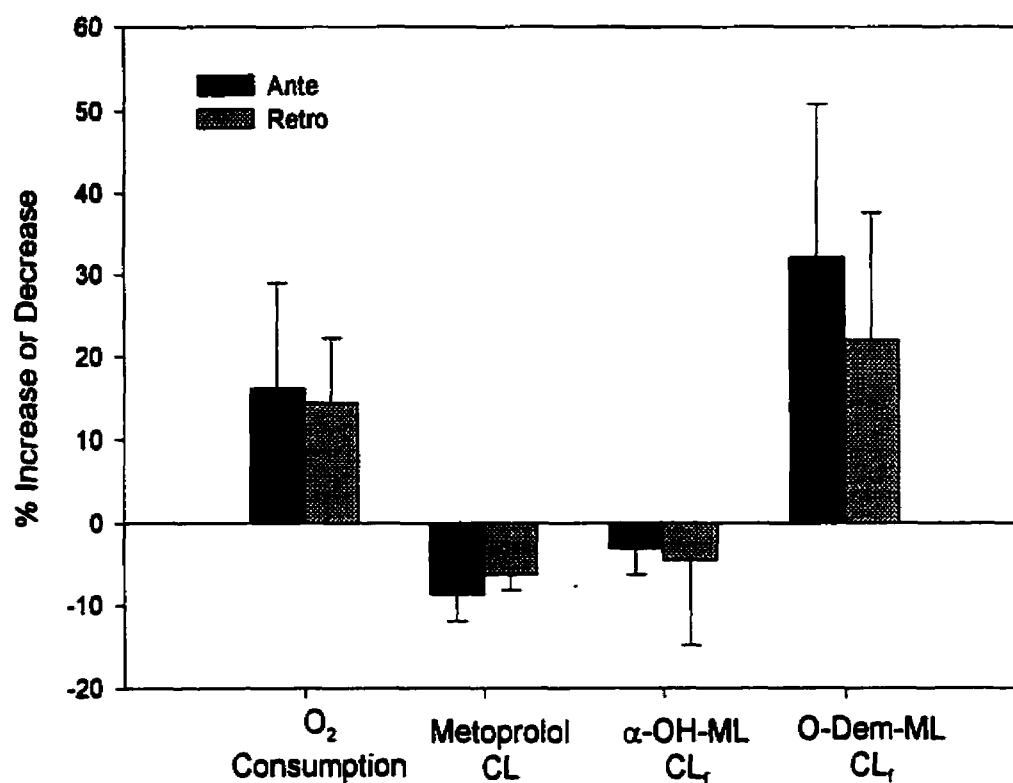


Figure 8.3. The percentage change (mean \pm SD, N = 8) in oxygen consumption, metoprolol clearance (CL), α -hydroxymetoprolol formation clearance (α -OH-ML CL_f) and O-demethylmetoprolol formation clearance (O-Dem-ML CL_f) after coinfusion of high levels of amino acids in the isolated rat liver perfused with a normally oxygenated medium during antegrade (Ante) and retrograde (Retro) perfusion (Normal O₂/High AA group).

8.3.3 Normal O₂/Low AA group

8.3.3.1 Viability of Isolated Perfused Liver

The physiological parameters of the perfused liver are shown in Table 8.2. All of the livers were well perfused as described in the Hypo-O₂/High AA group. The time courses of oxygen consumption and concentrations of metoprolol and its metabolites were similar to those depicted in Figure 8.1. The order of antegrade and retrograde direction had no effect on the time to reach steady state, and the rate of oxygen consumption, metoprolol clearance and metabolite formation clearance at steady state ($P > 0.05$, nested ANOVA).

8.3.3.2 Oxygen Consumption

Eight livers were perfused with normally oxygenated medium with a mean oxygen delivery rate of 5.21 ± 0.72 $\mu\text{mol}/\text{min}/\text{g}$ liver. The mean oxygen consumption of the liver was 3.47 ± 0.59 $\mu\text{mol}/\text{min}/\text{g}$ liver during the Ante phase, and 3.38 ± 0.63 during the Retro phase, where no significant difference existed (Table 8.5). The mean oxygen extraction ratio was 67.3 ± 13.1 % during the Ante phase and 65.4 ± 11.8 % during the Retro phase.

Coinfusion of low levels of amino acids increased hepatic oxygen consumption by 0.76 to 39.6 % with an average of 10.8% during antegrade perfusion, and by 1.4 to 23.4% with an average of 7.9% during retrograde perfusion. The difference in the increase between antegrade and retrograde perfusion was not significant.

8.3.3.3 Metoprolol Metabolism

The pharmacokinetic parameters of metoprolol metabolism during the Ante, Ante-AA, Retro, and Retro-AA phases of liver perfusion are summarized in Table 8.5. The mean metoprolol clearance was 1.49 ± 0.15 ml/min/g liver in the Ante phase and 1.47 ± 0.20 ml/min/g /liver in the Retro phase. The formation clearance of α -hydroxymetoprolol and O-demethylmetoprolol was 0.384 ± 0.026 (25.8% of total clearance) and 0.064 ± 0.024 ml/min/g liver (4.3% of total clearance) in the Ante phase, respectively; and 0.360 ± 0.062 (24.6% of total clearance) and 0.068 ± 0.024 ml/min/g liver (4.6% of total clearance) in the Retro phase, respectively. There was no significant difference in the hepatic extraction ratio and clearance of metoprolol, the formation clearance of α -hydroxymetoprolol, and the formation clearance of O-demethylmetoprolol during antegrade and retrograde perfusion.

Coinfusion of low levels of amino acids decreased metoprolol metabolism, as evidenced by decreasing the metoprolol extraction ratio and metoprolol clearance by an average of 3.8% (unchanged to 10.9%) during antegrade perfusion, and by an average of 3.4% (unchanged to 9.5%) during retrograde perfusion of all eight livers in this group (Figure 8.4). However, α -hydroxymetoprolol formation clearance was not significantly altered by low levels of amino acids during either antegrade or retrograde perfusion. The formation clearance of O-demethylmetoprolol was increased after coinfusion of amino acids by $21.6 \pm 11.5\%$ during antegrade perfusion and by $11.8 \pm 6.6\%$ during retrograde perfusion.

Table 8.5. Effect of low levels of amino acids on oxygenation and pharmacokinetic parameters (mean \pm SD, N = 8) of metoprolol and its metabolites during antegrade and retrograde perfusion in the isolated rat liver perfused with a normally oxygenated medium (Normal O₂/Low AA group)

	Antegrade perfusion			Retrograde perfusion		
	No AA	AA	%Change *	No AA	AA	%Change *
O ₂ consumption (μ mol/min/g liver)	3.47 \pm 0.59	3.79 \pm 0.45 ^a	10.8 \pm 13.1	3.38 \pm 0.63	3.63 \pm 0.59 ^b	7.9 \pm 6.9
O ₂ extraction (%)	67.3 \pm 13.1	73.7 \pm 11.1 ^a	10.8 \pm 13.1	65.4 \pm 11.8	70.1 \pm 10.3 ^b	7.9 \pm 6.9
Metoprolol E (%)	89.4 \pm 5.0	86.0 \pm 6.0 ^a	-3.8 \pm 3.7	88.8 \pm 4.8	85.9 \pm 7.3 ^b	-3.4 \pm 3.7
Metoprolol CL (ml/min/g liver)	1.49 \pm 0.15	1.43 \pm 0.18 ^a	-3.8 \pm 3.8	1.47 \pm 0.20	1.41 \pm 0.18 ^b	-3.4 \pm 3.9
α -Hydroxymetoprolol CL _r (ml/min/g liver)	0.384 \pm 0.026	0.392 \pm 0.038	2.0 \pm 4.6	0.360 \pm 0.062	0.373 \pm 0.060	4.3 \pm 9.6
O-Demethylmetoprolol CL _r (ml/min/g liver)	0.064 \pm 0.024	0.077 \pm 0.025 ^a	21.6 \pm 11.5	0.068 \pm 0.024	0.076 \pm 0.029 ^b	11.8 \pm 6.6

^a Significant difference (P < 0.05) between No AA and AA during antegrade perfusion by paired t-test.

^b Significant difference (P < 0.05) between No AA and AA during retrograde perfusion by paired t-test.

* Percentage change upon coinfusion of AA during antegrade or retrograde perfusion, calculated as $\frac{([AA] - [No\ AA]) \times 100}{[No\ AA]}$.

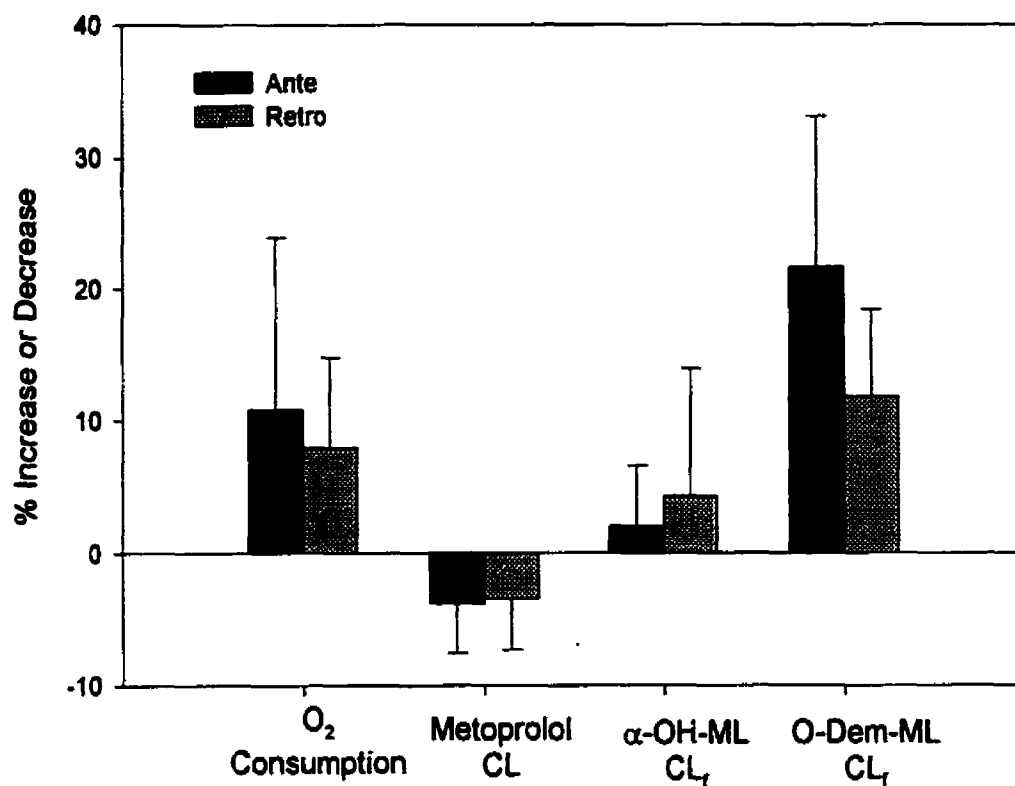


Figure 8.4. The percentage change (mean \pm SD, N = 8) in oxygen consumption, metoprolol clearance (CL), α -hydroxymetoprolol formation clearance (α -OH-ML CL_f) and O-demethylmetoprolol formation clearance (O-Dem-ML CL_f) after coinfusion of low levels of amino acids in the isolated rat liver perfused with a normally oxygenated medium during antegrade (Ante) and retrograde (Retro) perfusion (Normal O₂/Low AA group).

8.3.4 Comparison between groups

In the absence of amino acids, the metoprolol extraction ratio was found comparable (85 – 89%) between the three groups in both the antegrade and retrograde perfusion phases ($P > 0.05$, one-way ANOVA), although the oxygen extraction was higher in the Hypo-O₂/High AA group than in the other two groups ($P < 0.05$, one-way ANOVA). The effect of amino acids on metoprolol metabolism differed between groups. The order of decreases in metoprolol clearance by amino acids was Hypo-O₂/High AA group > Normal O₂/High AA group > Normal O₂/Low AA group in either the antegrade or retrograde perfusion mode ($P < 0.05$, one-way ANOVA). Amino acids also increased the oxygen extraction to a higher level in the Hypo-O₂/High AA group as compared with the other two groups.

8.4 Discussion

8.4.1 Experimental design

Our previous study showed that a balanced mixture of amino acids reversibly reduced the V_{\max} values of metoprolol and the two metabolites, α -hydroxymetoprolol and O-demethylmetoprolol in the isolated rat liver perfused with Krebs bicarbonate buffer (Wang and Semple, 1997). Amino acids also increased hepatic oxygen consumption until oxygen in the perfusion buffer was almost completely depleted. Possible mechanisms influencing the V_{\max} have been discussed, including direct inhibition of metabolic enzymes by amino acids and/or cosubstrate (NADPH or oxygen) limitation. In our later study of rat liver microsomes (Alcorn, 1997), however, no direct inhibition of metoprolol metabolism was observed at physiological levels of amino acids. We have therefore hypothesized that the amino acid-mediated pericentral oxygen limitation is the most plausible mechanism involving in the amino acid-metoprolol interaction.

The present study was designed to illustrate the effect of oxygen depletion by amino acids on metoprolol metabolism and its relevance to the food effect. Unlike the previous work, a single-pass *in situ* liver perfusion system with erythrocyte-enriched Krebs-Henseleit bicarbonate buffer was used, whereby hepatic oxygen supply and perfusion directions were adjustable during the experiment. The hepatic oxygen delivery rate and oxygen consumption rate were precisely monitored, and the concentrations of metoprolol and its two metabolites, α -hydroxymetoprolol and O-demethylmetoprolol, were also measured throughout the perfusion. This study, therefore, allow us to inspect

the relationship between the oxygen level and metoprolol metabolism in the presence of amino acids in the liver.

8.4.2 Metoprolol metabolism

Metoprolol is extensively metabolized in the liver of humans and other animals (Ablad et al., 1975). In the present study for all three groups, metoprolol was rapidly eliminated in the liver perfused in either antegrade or retrograde mode, with a mean clearance of ~ 1.5 ml/min/g liver, which corresponds to a $\sim 13\%$ hepatic availability. This availability was higher than the $\sim 4\%$ of bioavailability reported in rat *in vivo* studies (Borg et al., 1975a). The difference is not surprising because the metabolism of metoprolol is saturable, although the inlet concentration of metoprolol employed was relatively low ($5.84 \mu\text{M}$). The lower *in vivo* availability of metoprolol could be also caused by 1) additional metabolic sites other than the liver; and /or 2) impaired function of the isolated perfused liver. However, there is no evidence of these two possibilities. There are three major metabolites found in rat urine and rat liver microsomes, which are α -hydroxymetoprolol, O-demethylmetoprolol, and metoprolol acid, respectively accounting for about 25%, 3.5% and 62% of the total metabolites produced (Borg et al., 1975b; Arfwidsson et al., 1976). Two metabolites, α -hydroxymetoprolol and O-demethylmetoprolol, were detected in the liver perfusion system, and their relative amounts accounted for about 28% and 5%, respectively, consistent with the reported values for the rat urine and rat liver microsomes.

Coinfusion of amino acids in the perfused rat liver caused a decrease in metoprolol clearance in all the three groups, but to a different extent. This suggests that

amino acids inhibit metoprolol metabolism as discussed in the previous study (Wang and Semple, 1997). The effluent concentrations of metoprolol rose in the presence of amino acids, and recovered to the pre-amino acid levels after termination of amino acid infusion, indicating that the effect of amino acids was temporary and reversible. It is hence unlikely that amino acids caused permanent damage to the hepatic metabolic enzyme system. Along with the decrease in metoprolol clearance, a reduction in α -hydroxymetoprolol formation clearance occurred during infusion with amino acids. The magnitude of reduction became more obvious when a larger decrease in parent drug clearance was present. The reduction in α -hydroxymetoprolol formation did not account for the change in total clearance of metoprolol since O-demethylation comprised larger portion of metoprolol metabolism. Unexpectedly, the formation clearance of O-demethylmetoprolol was considerably increased by amino acids. O-Demethylmetoprolol is further oxidized to a secondary metabolite, metoprolol acid, which we did not measure in this study. The increase in O-demethylmetoprolol probably could result from inhibition of the secondary metabolic step (from O-demethylmetoprolol to metoprolol acid) to a larger extent than that of the first step (from metoprolol to O-demethylmetoprolol). The formation of O-demethylmetoprolol is mediated by CYP2D6, whereas the formation of metoprolol acid could be mediated by aldehyde dehydrogenase. Very likely the sensitivity of these two enzymatic systems to oxygen are different. It is difficult to tell what really happened with O-demethylation during infusion of amino acids unless metoprolol acid is measured.

8.4.3 Hepatic oxygen level

In addition to the decrease in metoprolol clearance, coadministration of amino acids caused an increase in hepatic oxygen consumption, suggesting that oxygen limitation could contribute to the mechanism of inhibition. It has been long recognized from *in vitro* and whole organ studies that the hepatic metabolism of many drugs can be affected by the oxygen level in the liver (Woodroffe et al., 1995). Especially for the drugs which undergo extensive oxidation *via* hepatic cytochrome P-450 enzymes, an acute decrease in oxygen results in a marked reduction of drug elimination in the liver (Jones et al., 1984; Angus et al., 1989; Elliott et al., 1993a). In the isolated perfused liver, an hypoxic threshold of oxidatively metabolized drugs has been identified to describe the critical rate of oxygen delivery below which the rate of drug metabolism begins to decrease, as oxygen becomes rate-limiting. The oxygen delivery thresholds vary from 2.5 to 6 $\mu\text{mol}/\text{min}/\text{g}$ liver depending upon the substrate and the experiment (Angus et al., 1989; Elliott et al., 1993; Hickey et al., 1996). However, no such oxygen threshold has been determined for metoprolol. One may assume that the oxygen threshold of metoprolol is similar to that of propranolol, which was reported to be 2.5 (Hickey et al., 1996) or 5 - 6 $\mu\text{mol}/\text{min}/\text{g}$ liver (Elliott et al., 1993a; 1993b), since both of these drugs are mainly oxidized by the cytochrome P-450 enzymes in the rat liver. In our present study, two oxygen delivery rates were applied to the perfused rat liver. In the Normal O_2 /High AA group, the rate of oxygen delivery to the liver was 5.66 ± 0.85 $\mu\text{mol}/\text{min}/\text{g}$ liver, matching the normal physiological range of 5 - 7 $\mu\text{mol}/\text{min}/\text{g}$ liver in the rat *in vivo* (Bredfeldt et al., 1985; Carmichael et al., 1993). In contrast, the oxygen delivery in the Hypo O_2 /High AA group was 3.40 ± 0.52 $\mu\text{mol}/\text{min}/\text{g}$ liver, comparable

to that in the previous study where the liver was perfused with Krebs bicarbonate buffer only (Wang and Semple, 1997). The clearance of metoprolol, however, was similar in both groups, suggesting that the oxygen threshold of metoprolol may be lower than 3.4 $\mu\text{mol}/\text{min}/\text{g}$ liver. Oxygen thus should not be the rate-limiting factor for metoprolol metabolism in the liver perfused with either the normally oxygenated or hypo-oxygenated medium.

However, the oxygen condition would change when an extra oxidative load is placed in the liver. Because the metabolism of amino acids in the liver, including membrane transport and gluconeogenesis, is an oxygen consuming process (Seifter and Englard, 1994), livers will increase oxygen extraction when amino acids are present. In the Hypo O_2 /High AA group, coadministration of amino acids caused a 13.8% increase in oxygen consumption along with a 15.6% decrease in metoprolol clearance (Table 8.3). The increased oxygen consumption brought about a hepatic oxygen extraction increase from 72.6% up to 82.2%. The oxygen supply in the liver thus could become limiting so that metoprolol metabolism was inhibited. The magnitude of the amino acid-induced decrease in metoprolol metabolism was smaller when the oxygen delivery rate was increased. In the Normal O_2 /High AA group, the same concentrations of amino acids led to an oxygen extraction of 72.7% and only a decrease of 8.7% in metoprolol clearance (Table 8.4). It is apparent that the higher oxygen delivery somewhat compensated for the consumption of oxygen caused by the metabolism of amino acids. The metabolism of a drug by the intact liver depends on several factors, including uptake of the substrate by the hepatocyte, enzyme activity, transport of the metabolite across the canalicular or sinusoidal membrane, and availability of essential co-factors,

such as oxygen and NADPH. Since the steady-states of metoprolol and its metabolites were reached immediately upon coinfusion of amino acids, it is unlikely that amino acids inhibit metoprolol uptake and metabolite transport across the membrane. Furthermore, no direct inhibition of drug-metabolizing enzymes was observed (Alcorn, 1997). It appears most likely that the limitation of oxygen is a major contributing factor to the inhibition of metoprolol metabolism caused by amino acids, while NADPH depletion, which was not tested in our experiments, can not be completely excluded.

8.4.4 Retrograde perfusion

The differences in zonal distribution of enzymes in the acinus may contribute to the inhibition of metoprolol metabolism by amino acids because periportally located metabolism of amino acids has the priority to utilize the co-substrate, oxygen, in comparison to metoprolol metabolism for which the enzymes are located in the pericentral zone. This hypothesis was tested by using retrograde liver perfusion. Although it is a non-physiological liver model, retrograde perfusion becomes a useful technique for examining acinar heterogeneity. It is believed that short-term retrograde perfusion has no deleterious effect on the liver in term of K^+ , SGOT, SGPT, and LDH, and ultrastructure determined by light and electron microscopy (Pang, 1984). Retrograde perfusion, however, causes an increase in sinusoidal volume and surface area (Bass et al., 1989; St.Pierre et al, 1989). An increase in sinusoidal surface area might increase the hepatic clearance of a drug whose clearance is rate-limited by uptake into the hepatocyte. This case, however, should not apply to metoprolol, which is a flow-dependent drug and is considered to rapidly enter into the cell. Indeed, our experiments

revealed that no difference existed between antegrade and retrograde perfusion in both metoprolol clearance and oxygen consumption, indicating that the hepatic ability of metoprolol metabolism was not altered by changing the perfusion direction under either hypo-oxygenation or normal oxygenation conditions.

In the Hypo O₂/High AA group, the decrease of metoprolol clearance by amino acids during antegrade perfusion was significantly greater than during retrograde perfusion. Consistently, the decrease of α -hydroxymetoprolol formation clearance was also greater with antegrade than with retrograde perfusion (Table 8.3, Figure 8.2). The results suggest that inhibition of metoprolol metabolism caused by amino acids is partially reversed by retrograde perfusion. These observations strongly support the hypothesis we proposed above that the metabolism of amino acids in the liver produces a pericentral oxygen deprivation which leads to the impairment of metoprolol metabolism. The retrograde perfusion, however, only offset about 30% of the decrease in metoprolol clearance of the antegrade perfusion. The relatively small impact of retrograde perfusion implies that the oxygen available for metoprolol metabolism is still deprived by the metabolism of amino acids during retrograde perfusion, although to a lesser extent. Hence the observations could be explained by: 1) A change in activities of amino acid metabolizing enzymes due to retrograde perfusion. Although the enzymes involved in the metabolism of amino acids are preferentially located in the periportal zone of the acinus, the enzyme activity depends upon the direction of flow when the substrate supply is not limiting (Häussinger and Gerok, 1986). 2) Periportal distribution of some cytochrome P-450 isoenzymes. Although most cytochrome P-450 isoenzymes are indeed pericentrally located, a few are more evenly distributed within the acinus

(Thurman et al., 1986; Butler et al., 1992). Metoprolol is mainly oxidized in the rat liver via more than one cytochrome P-450 isoforms, which have not been clearly identified yet (Barham et al., 1994). The exact zonal distribution of metabolic enzymes for metoprolol is not clear. 3) Higher oxygen affinities for the amino acid metabolizing enzyme systems than for the metoprolol metabolizing enzyme systems. In contrast to the Hypo O₂/High AA group, the amino acid-mediated decrease of metoprolol clearance during retrograde perfusion was slightly but not significantly different from antegrade perfusion in the Normal O₂/High AA group, suggesting that the effect of zonal heterogeneity was trivial when the liver was normally oxygenated. The result is consistent with a previously reported study of propranolol, in which the difference in clearance between antegrade and retrograde perfusion was only demonstrated during hypoxia, not under conditions of normal oxygenation (Elliot, et al. 1993b).

It should be noted that the results from our previous study (Wang and Semple, 1997) differed markedly with respect to various observations for Hypo O₂/High AA group in our present study (Table 8.3), even though the oxygen delivery rate was comparable. In terms of the same inlet concentration of metoprolol (5.84 μ M) as the present study, the differences in the previous study are as follows: 1) a smaller extraction ratio of metoprolol (62% compared to 87.6%); 2) a larger percentage of α -hydroxymetoprolol (60% compared to 29.5%) and O-demethylmetoprolol (12% compared to 6.6%) formation clearance accounting for the total metoprolol clearance; 3) a larger decrease (37.2% compared to 15.6%) in metoprolol clearance by amino acids; and 4) a decrease in O-demethylmetoprolol formation clearance by amino acids (compared to an increase). Because the liver was perfused with hemoglobin-free Krebs-

Henseleit bicarbonate buffer saturated with oxygen in our previous work, the oxygen capacity was much lower than the erythrocyte-enriched buffer of the present study. Even though the oxygen delivery rate was comparable by increasing the flow rate up to ~3 ml/min/g liver, oxygen was less supplied with the result that the liver almost completely depleted oxygen in the perfusate with an extraction of 95%. Based upon our present findings regarding the relationship between oxygen and metoprolol metabolism, it can be concluded that the differences between the two studies arose because of the poor oxygen availability in the previous liver perfusion system.

8.4.5 Portal concentrations of amino acids

Comparing the results from the Normal O₂/High AA group with the Normal O₂/Low AA group, the lower level of amino acids caused a smaller decrease in metoprolol clearance and smaller changes in the formation clearance of metabolites (Tables 8.4 and 8.5). This is in agreement with our previous finding that the degree of metabolic inhibition of propranolol was linearly correlated with the amino acid concentration in the influent buffer (Semple and Xia, 1995). It is questionable as to the level of amino acids infused to the liver required to simulate the plasma concentration of amino acids in the portal vein after a high protein meal. No doubt, during the postprandial period, there is an abrupt rise of free amino acids in the portal circulation (Rerat et al., 1976). In the dog, 1 hr after ingestion of a protein meal, the portal concentration of dietary amino acids reaches a plateau, 2 to 3 times that in the preabsorptive period, which is maintained for 8 - 12 hr (Elwyn, 1965; 1970). Unfortunately, there is no such measurement available in man. In the present study, we

applied two levels of amino acids delivered to the perfused liver: the Low AA group, in which livers were infused with the amino acid mixture at about 3 times the normal plasma concentration in the rat portal vein (Sax et al, 1988a; 1988b), and the High AA group, in which the amino acid concentration was double that of the Low AA group. The results revealed that the higher level of amino acids produced a larger effect on metoprolol metabolism without damage to the liver.

8.4.6 Relevance to the food effect

At this point, we wish to answer the question: how the inhibition of metoprolol metabolism caused by amino acid-induced hepatic oxygen limitation in the perfused rat liver model would be relevant to the food effect observed in human studies. Although there is no direct evidence that ingestion of amino acids or protein can cause hepatic deprivation of oxygen, human studies have demonstrated that after a protein meal the splanchnic oxygen consumption increases considerably by 35 - 50%, but the splanchnic blood flow only increases by 13 - 35% (Brandt et al., 1955; Brundin, 1993; Brundin and Wahren, 1994), that is, the postprandial increase in oxygen extraction from the splanchnic blood happens to meet the oxygen demand without increasing the blood flow (oxygen supply) to the same extent. The splanchnic oxygen consumption mainly includes the intestinal and hepatic oxygen required for digestion, absorption, and processing of the protein ingested. A rise in intestinal oxygen consumption may result in a reduction in oxygen content in the portal vein, which is simply the sum of outflows of the extrahepatic splanchnic organs. Meanwhile, the hepatic arterial oxygen delivery is reduced as hepatic arterial flow, normally a major oxygen supply to the liver, does not

change or even decreases after a meal, whereas portal venous flow increases to account for the increase in total hepatic blood flow (Chou, 1983; Lautt, 1983; Dauzat et al, 1994). This is the so-called 'hepatic artery buffer response'. As a consequence, deprivation of oxygen may occur in the liver after a protein meal due to increased hepatic oxygen consumption with less or unchanged hepatic oxygen delivery. On the other hand, oral ingestion of glucose or fat does not change splanchnic oxygen consumption but increases splanchnic blood flow (Brundin and Wahren, 1993; Brundin et al., 1996; Brundin, 1998). Because protein (amino acids) seems to be an important component of a meal that causes the 'food effect' with high hepatic extraction ratio drugs (McLean, et al., 1981; Walle, et al., 1981) rather than carbohydrates (glucose) and fat (Chow and Lalka, 1993; Ogiso, 1994), we postulate that amino acid-mediated hepatic oxygen deprivation may be involved in the mechanism of the 'food effect'. Nevertheless, *in vivo* studies are needed to demonstrate this hypothesis.

8.5 Conclusion

The present study in the perfused rat liver has revealed that amino acids cause a reversible decrease in metoprolol metabolism. The magnitude of amino acid effect is proportional to the hepatic oxygen delivery rate and the concentration of amino acids. Retrograde perfusion partially reverses the inhibition of metoprolol metabolism by amino acids, indicating that pericentral oxygen depletion may be a mechanism contributing to the inhibition. This amino acid-metoprolol interaction could be a key to understanding the 'food effect' on high hepatic extraction ratio drugs. We also realize that the liver perfusion model has to be used with recognition of its limitations involved and hence its results should be carefully interpreted since the model *per se* is functioning under an oxygen-limited condition.

CHAPTER 9

EFFECT OF AMINO ACID INGESTION ON THE PHARMACOKINETICS OF METOPROLOL IN THE RAT ¹

9.1. Introduction

In man, food intake has been reported to enhance the oral bioavailability of certain high hepatic first-pass drugs, such as propranolol (Melander et al., 1977), metoprolol (Melander et al., 1977), propafenone (Axelson et al., 1987) and diprafenone (Koytchev et al., 1996). The average area under the plasma concentration-time curve after oral doses of these drugs (AUC_{oral}) has been shown to increase by >40% when they were given with a meal. This phenomenon is called the 'food effect'. The mechanism of the food effect may involve changes in drug absorption from the gut and/or presystemic metabolism. Most drugs which exhibit the food effect are known to be completely absorbed from the gastrointestinal tract under the fasting condition and almost solely metabolized in the liver, thus a reduction in hepatic extraction, rather than an increase in the extent of absorption from the gastrointestinal tract, most likely contributes to the increase in AUC after an oral dose. It was proposed that a food-induced transient increase in hepatic blood flow could result in the observed increase in AUC_{oral} (McLean et al., 1978; Elvin et al., 1981; Walle et al., 1981; Heinzow et al., 1984; Olanoff et al., 1986). However, the observed time course of the change in hepatic blood flow caused

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by food consumption is not sufficient to explain the magnitude of the increase in AUC_{oral} (Svensson et al., 1983; Semple et al., 1990). Additionally, a posture-induced increase in hepatic blood flow, approximating that after food ingestion, was shown not to cause an increase in AUC_{oral} (Modi et al., 1988). Therefore, there may be other more important mechanisms involved in the 'food effect', such as acute inhibition of hepatic metabolism, i.e., a decrease in hepatic intrinsic clearance.

Most human diets are complex mixtures of macro-nutrients such as carbohydrates, fats and proteins, which are digested and transported into the portal vein as glucose, fructose, free fatty acids and amino acids, etc. These macro-nutrients are widely recognized to be modulating factors in the metabolism of drugs (Anderson, 1988; Yang and Yoo, 1988). Of these components in the diet of humans, however, protein appears to be the most responsible for the 'food effect'. Human studies have demonstrated that protein-rich meals unquestionably enhance the AUC_{oral} of propranolol (Melander et al., 1977; Mclean et al., 1981; Walle et al., 1981; Liedholm and Melander, 1986; Olanoff et al., 1986; Liedholm et al., 1990), metoprolol (Melander et al., 1977) and propafenone (Axelson et al., 1987), whereas there is no or less effect of carbohydrate-rich meals (McLean et al., 1981; Walle et al., 1981; Jackman et al., 1981; Liedholm et al., 1990). In rats, a high protein diet can cause a small increase in the AUC_{oral} of propranolol (Ogiso et al., 1994), while ingestion of fatty acids and glucose decreases the AUC_{oral} (Chow and Lalka, 1993; Ogiso et al., 1994). Furthermore, studies in the isolated, perfused liver have shown that amino acids, digestive products of protein, reversibly inhibit the metabolism of propranolol and metoprolol via regulation of the oxygen supply in the liver (Semple and Xia, 1995; Wang and Semple, 1997; Chapter 8).

It is very likely that metabolic inhibition by amino acids may contribute to the 'food effect', but no propranolol- or metoprolol- amino acid interaction *in vivo* has been reported.

Metoprolol is a high hepatic extraction drug exhibiting an average AUC increase of about 40% when given with a protein-rich meal in man (Melander et al., 1977). Data from studies in the isolated perfused rat liver have shown that the major pathways of metoprolol metabolism can be inhibited by coinfusion with amino acids (Wang and Semple, 1997; Chapter 8). To examine if amino acids can interact with metoprolol *in vivo* and how this interaction is related to the 'food effect' observed in man, we have specifically investigated the effect of a mixture of amino acids on the kinetics of metoprolol and its metabolites after an oral dose in rats. Metoprolol is rapidly and completely absorbed from the gastrointestinal tract in the rat, and is eliminated mainly via extensive hepatic oxidation into α -hydroxymetoprolol, O-demethylmetoprolol and metoprolol acid (Arfwidsson et al, 1976; Borg et al., 1975a). If there is an interaction between metoprolol and amino acids similar to that observed in the perfused rat liver, one could expect changes in the kinetics of both parent drug and metabolites after an oral dose administered with amino acids.

9.2 Materials and Methods

9.2.1 Chemicals

R, S-Metoprolol tartrate and nadolol (internal standard) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). α -Hydroxymetoprolol (H119/66) *p*-OH benzoate and O-demethylmetoprolol (H105/22) *p*-OH benzoate were gifts from Astra (Hässle, Sweden). Aminosyn® II 10% Amino Acids Injection came from Abbott Laboratories Ltd. (Montreal, Quebec, Canada). All solvents were HPLC grade obtained from BDH Inc. (Toronto, Ontario, Canada). All other chemicals used were analytical grade from BDH Inc.

9.2.2 Animal experiments

Animals. Male Sprague-Dawley rats (Charles River, St. Constant, Quebec, Canada), weighing 250-310 g, were used. The animals were allowed to acclimatize for 3-4 days before commencement of the experiment. They were maintained on standard laboratory chow and water *ad libitum* in accordance with the guidelines of the Canadian Council on Animal Care.

Surgical procedure. One day before drug administration, each animal was anesthetized with inhaled halothane (MTC Pharmaceuticals, Cambridge, Ontario, Canada), and a right external jugular vein cannula was implanted according to the procedures described previously (Waynforth and Flecknell, 1994). The jugular cannula consisted of 7 cm of polyethylene tubing (PE 50, 0.58 cm i.d. \times 0.965 cm o.d.) tipped

with 3.2 cm of bevel-ended silastic tubing (Silastic®, 0.51 cm i.d. × 0.94 cm o.d., Dow Corning, Midland, MI, USA). The cannula was gas sterilized with 8.6% ethylene oxide and 91.4% chlorotetrafluorethane before use. The distal end of the polyethylene tubing was passed subcutaneously to emerge at the dorsal aspect of the neck. Heparin (100 U/ml) in 0.9% saline was flushed into the catheter after implantation. All animals regained consciousness within 10 min after the operation, and were allowed free access to water and food until 12 hr prior to dosing.

Dosing and sampling. On the study day, the fasted animals were randomly divided into two groups, each consisting of 8 or 10 rats, to receive either 12 ml/kg of a balanced mixture of dissolved amino acids (10% Aminosyn® II, Table 9.1) or an equivalent volume of distilled water. The total amount of amino acids approximated the content of an animal protein meal (Deferrari et al., 1988). Metoprolol at a dose of 10 mg/kg was given via gastric intubation with amino acids or water. At 0, 5, 10, 15, 20, 30, 40, 50, 60, 75, 90, 120, 180, 240, and 360 min after drug administration, blood samples of approximately 250 µl were drawn through the jugular vein cannula with heparinized syringes. An equal volume of saline was administered through the jugular vein cannula immediately after blood sampling to replace the lost blood. The samples were centrifuged immediately, and the plasma was separated and stored at -20°C until the next day for assay.

9.2.3 Analytical procedure

The concentrations of metoprolol and its metabolites, α -hydroxymetoprolol and O-demethylmetoprolol in liver perfusate samples were determined simultaneously using

a revalidated HPLC-fluorescence method as described previously (Wang and Semple, 1997) with some modifications. In brief, to 100 μ l of plasma were added 50 μ l of nadolol (1 μ g/ml, internal standard) and 2 ml of 1 M sodium carbonate buffer (pH 10.5). The drug, metabolites and internal standard were extracted into 5 ml of a mixture of diethyl ether-dichloromethane (4:1, v/v) after vortex mixing for 8 min and centrifugation at $2500 \times g$ for 10 min. The organic layer was transferred to a clean glass tube and evaporated to dryness under nitrogen at room temperature. The residues were reconstituted in 120 μ l of mobile phase, and 100 μ l was injected into the HPLC system.

Calibration curves were linear ($r \geq 0.99$) over the range of 15-200 ng/ml for metoprolol, 10-1000 ng/ml for α -hydroxymetoprolol and 10-100 ng/ml for O-demethylmetoprolol, respectively. The intra- and inter- run coefficients of variation (CV) were less than 10% for all compounds, except at their limit of quantitation (LOQ) where the intra- and inter-run CV were less than 15%. Quality control (QC) samples in duplicate at three concentrations (one near the lowest, one in the middle, and one close to the highest end of the calibration curve) were incorporated into each run. To accept the run, at least four of the six QC samples must be within $\pm 20\%$ of their respective nominal values, and any two that may lie outside the $\pm 20\%$ must not be at the same concentration.

9.2.4 Pharmacokinetic Analysis

Pharmacokinetic parameters were determined by WINNONLIN Standard Edition Version 1.1 (Scientific Consulting Inc., Lexington, KY, U.S.A). Non-

compartmental methods were used to describe the disposition of metoprolol, in which no assumption for a specific compartment model is required. The linear trapezoidal method was used to calculate area under the concentration-time curve from 0 to 60 min ($AUC_{0-60min}$) and from 0 to t^* (AUC_{0-t^*}), and area under the first moment concentration-time curve from 0 to t^* ($AUMC_{0-t^*}$). The t^* is the time of the last measurable concentration (C^*). The area under the concentration-time curve ($AUC_{0-\infty}$) and area under the first moment concentration-time curve from time 0 to infinity ($AUMC_{0-\infty}$) were calculated from

$$AUC_{0-\infty} = AUC_{0-t^*} + \frac{C^*}{\lambda_z} \quad (9.1)$$

and

$$AUMC_{0-\infty} = AUMC_{0-t^*} + \frac{t^* C^*}{\lambda_z} + \frac{C^*}{\lambda_z^2} \quad (9.2)$$

, respectively, where the λ_z is 2.303 times the slope of the terminal exponential phase of a plot of log concentration-time. The λ_z was estimated by linear regression using the last 3-6 concentration data points. The half-life of the terminal phase in the plasma concentration-time curve ($t_{1/2(z)}$) was calculated from

$$t_{1/2(z)} = \frac{0.693}{\lambda_z} \quad (9.3)$$

Oral clearance (CL_{oral}) was calculated as

$$CL_{oral} = \frac{Dose}{AUC_{0-\infty}} \quad (9.4)$$

The mean residence time (MRT) was calculated as

$$\text{MRT} = \frac{\text{AUMC}}{\text{AUC}} \quad (9.5)$$

9.2.5 Statistical Analysis

Since a parallel study design was applied, an unpaired Student's t-test was used to evaluate the significance of differences between amino acid and water (control) groups. All pharmacokinetic parameters, except t_{max} , were log transformed prior to statistical analysis. A value of $P < 0.05$ was considered statistically significant. Data are expressed as the arithmetic mean \pm standard deviation unless otherwise indicated.

Table 9.1. Composition of Aminosyn® II 10% Amino Acid Injection

Amino acids	mg/100ml	mM
Essential amino acids		
<i>L</i> -Isoleucine	660	50.4
<i>L</i> -Leucine	1000	76.3
<i>L</i> -Lysine	1050	71.9
<i>L</i> -Methionine	172	11.5
<i>L</i> -Phenylalanine	298	18.1
<i>L</i> -Threonine	400	33.6
<i>L</i> -Tryptophan	200	9.8
<i>L</i> -Valine	500	42.7
Nonessential amino acids		
<i>L</i> -Alanine	993	111.6
<i>L</i> -Arginine	1018	58.5
<i>L</i> -Aspartic acid	700	52.6
<i>L</i> -Glutamic acid	738	50.2
Glycine	500	66.7
<i>L</i> -Histidine	300	19.4
<i>L</i> -Proline	722	62.8
<i>L</i> -Serine	530	50.5
N-Acetyl- <i>L</i> -tyrosine	270	14.9

9.3 Results

An individual plasma concentration-time curve of metoprolol and its metabolites for each group is shown in Figure 9.1. The mean plasma concentration-time curves of metoprolol, α -hydroxymetoprolol and O-demethylmetoprolol are shown in Figure 9.2, and the corresponding pharmacokinetic parameters are given in Tables 9. 2, 9.3 and 9.4. The mean $AUC_{0-\infty}$ of metoprolol showed an 18% increase after ingestion of amino acids. There was a larger increase (33%) in the mean $AUC_{0-60min}$ of metoprolol, which represents the drug levels during the early entry phase. But the differences were not significant. The mean peak plasma concentration (C_{max}) was numerically but not significantly higher in the amino acids-treated animals than that in the control animals. The failure to show significance between treatments may be due to the large variation between individual animals, which is a typical feature of high hepatic first-pass drugs. The differences in the time to reach the peak concentration (t_{max}), half-life of the terminal phase ($t_{1/2(z)}$), MRT of metoprolol between the amino acid group and control group were small and not statistically significant. Since metoprolol is almost completely absorbed and solely metabolized by the liver, its CL_{oral} is equivalent to the total apparent intrinsic hepatic clearance (CL_i). Compared with the hepatic blood flow rate in the rat (about 10 ml/min/liver), the mean CL_{oral} (887 ml/min in the amino acid group and 917 ml/min in the control group) was much higher, confirming that metoprolol is a high hepatic extraction ratio drug.

In contrast, the mean C_{\max} of α -hydroxymetoprolol in the amino acid group was significantly lower than that in the control group, and the t_{\max} showed a significant prolongation in the amino acids-treated animals (45 ± 12 min vs. 18 ± 6 min). However, the mean $AUC_{0-\infty}$ and $AUC_{0-60\text{min}}$ of α -hydroxymetoprolol were not significantly different between the two groups, although the mean $AUC_{0-60\text{min}}$ was smaller following administration of amino acids. Similarly, the mean t_{\max} of O-demethylmetoprolol was significantly prolonged by 25 min in the animals administered with amino acids, but there was no significant difference in the mean C_{\max} , $AUC_{0-\infty}$, and $AUC_{0-60\text{min}}$. The mean half-lives of the terminal phase ($t_{1/2(z)}$) of both α -hydroxymetoprolol and O-demethylmetoprolol were not different in the amino acid group from the control group. The results indicated that amino acids caused a delayed appearance of the measured metabolites.

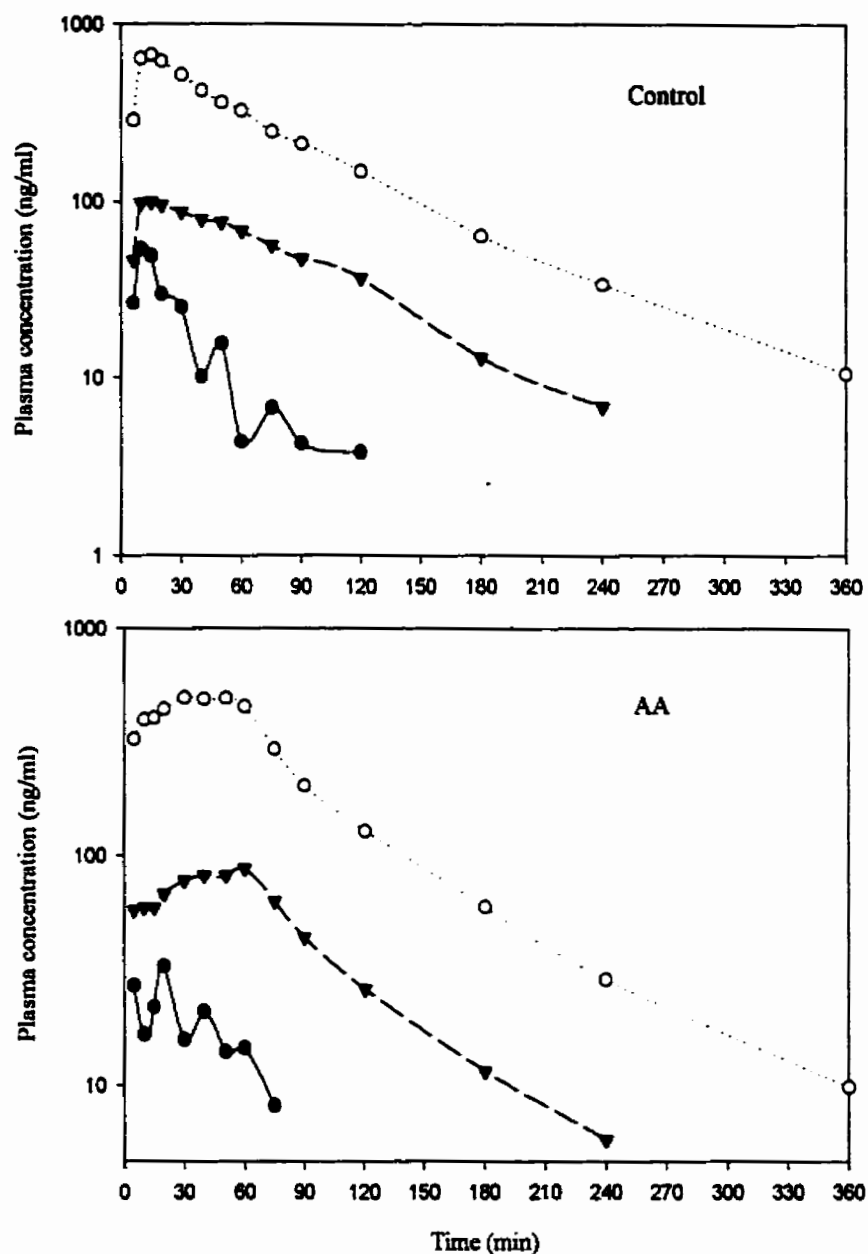


Figure 9.1. Examples of plasma concentrations of metoprolol (●), α -hydroxymetoprolol (▽) and O-demethylmetoprolol (○) after a single oral dose of metoprolol (10 mg/kg) administered with water (Control) and amino acids (AA).

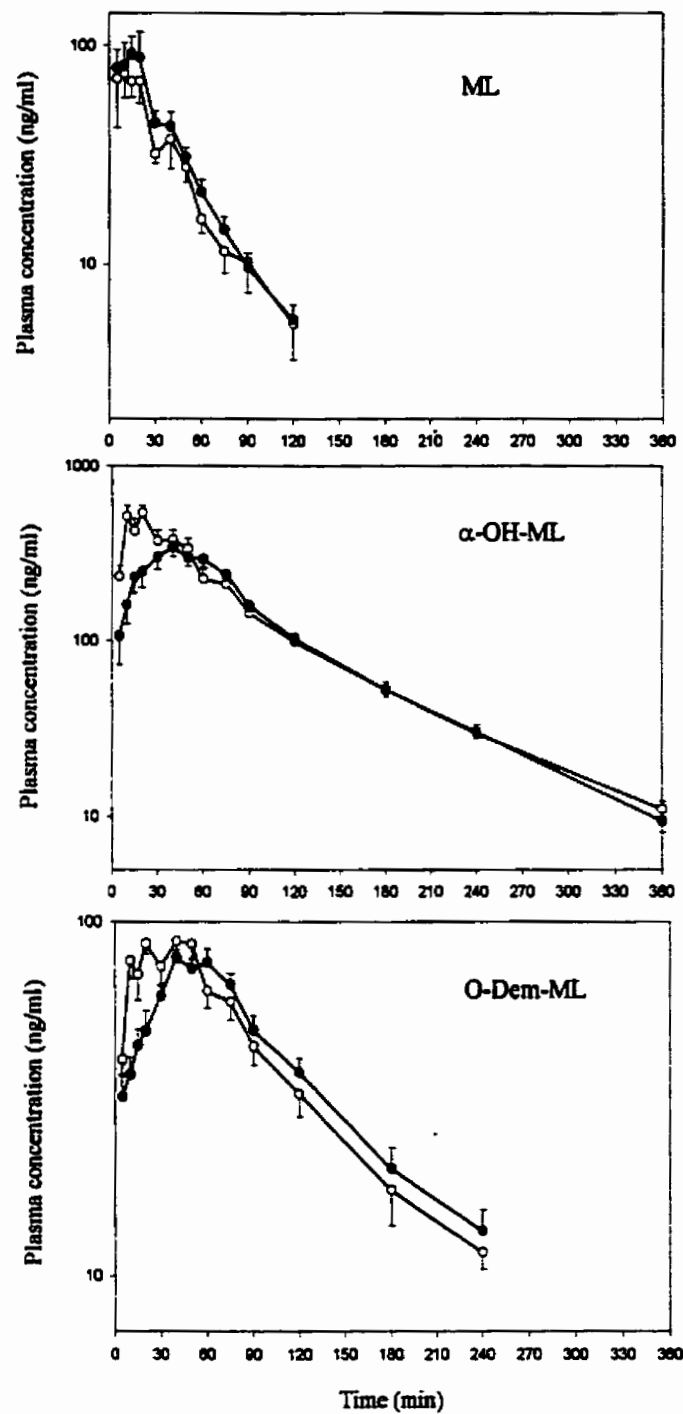


Figure 9.2 Mean plasma concentrations (mean, SEM) of metoprolol (ML), α -hydroxymetoprolol (α -OH-ML) and O-demethylmetoprolol (O-Dem-ML) after a single oral dose of metoprolol (10 mg/kg) administered with water (μ) and amino acids (λ).

Table 9.2. Mean pharmacokinetic parameters (mean \pm SD) of metoprolol in rats after a single oral dose of metoprolol (10 mg/kg) administered with water and amino acids

	Water (control) (n = 8)	Amino acids (n = 10)
C_{max} (ng ml ⁻¹)	90 \pm 48	120 \pm 86
t_{max} (min)	15 \pm 5	14 \pm 6
AUC _{0-∞} (ng min ml ⁻¹)	3210 \pm 1133	3760 \pm 1798
AUC _{0-60 min} (ng min ml ⁻¹)	1387 \pm 466	1852 \pm 1321
λ_z (min ⁻¹)	0.026 \pm 0.015	0.031 \pm 0.015
$t_{1/2(z)}$ (min)	32 \pm 12	27 \pm 11
MRT (min)	49 \pm 17	45 \pm 13
CL _{oral} (ml min ⁻¹)	917 \pm 263	887 \pm 437

C_{max} , the peak concentration.

t_{max} , the time of peak concentration.

Table 9.3. Mean pharmacokinetic parameters (mean \pm SD) of α -hydroxymetoprolol in rats after a single oral dose of metoprolol (10 mg/kg) administered with water and amino acids

	Water (control) (n = 8)	Amino acids (n = 10)
C_{max} (ng ml ⁻¹)	488 \pm 236	333 \pm 121*
t_{max} (min)	18 \pm 6	45 \pm 12*
AUC _{0-∞} (ng min ml ⁻¹)	37981 \pm 13697	35111 \pm 7814
AUC _{0-60 min} (ng min ml ⁻¹)	18011 \pm 9204	14619 \pm 6027
λ_z (min ⁻¹)	0.010 \pm 0.002	0.011 \pm 0.003
$t_{1/2(z)}$ (min)	68 \pm 13	70 \pm 18
MRT (min)	94 \pm 22	103 \pm 22

C_{max} , the peak concentration.

t_{max} , the time of peak concentration.

* Significantly different from control group ($P < 0.05$).

Table 9.4. Mean pharmacokinetic parameters (mean \pm SD) of O-demethylmetoprolol in rats after a single oral dose of metoprolol (10 mg/kg) administered with water and amino acids

	Water (control) (n = 8)	Amino acids (n = 10)
C_{max} (ng ml ⁻¹)	84 \pm 23	82 \pm 17
t_{max} (min)	29 \pm 16	54 \pm 11*
AUC _{0-∞} (ng min ml ⁻¹)	10283 \pm 3554	10170 \pm 2995
AUC _{0-60 min} (ng min ml ⁻¹)	3857 \pm 1262	3397 \pm 810
λ_z (min ⁻¹)	0.011 \pm 0.004	0.018 \pm 0.021
$t_{1/2(z)}$ (min)	73 \pm 28	67 \pm 19
MRT (min)	116 \pm 40	112 \pm 30

C_{max} , the peak concentration.

t_{max} , the time of peak concentration.

* Significantly different from control group (P < 0.05).

9.4 Discussion

The present *in vivo* study demonstrates the effect of ingestion of an amino acid mixture, simulating the amino acid content of an animal protein meal, on the disposition of a high hepatic first-pass drug exhibiting the 'food effect', metoprolol. The results show a trend of change in metoprolol disposition similar to that in the 'food effect' observed in humans, suggesting that amino acids could be one of the dietary components responsible for the 'food effect'.

The mean AUC_{oral} of metoprolol was increased by 18% after orally administered with a mixture solution of amino acids. A larger increase (33%) was observed in the mean $AUC_{0-60 \text{ min}}$, indicating that the increase in bioavailability occurred in the early phase of drug disposition of the body, most likely during the first-pass metabolism period. The increase was not significant, however, and there was a large variation observed between individual animals. This may be due to the extremely low bioavailability of metoprolol caused by high hepatic first-pass extraction. An enhancement in the administration dose of this drug may reduce the variation, but may also reduce the effect of amino acids. In this case, a crossover experimental design would be ideal to demonstrate the effect of amino acids instead of the present parallel study. However, we had previously experienced technical difficulties in performing such a crossover experiment *in vivo* in rats (Alcorn, 1997). A pilot study of propranolol demonstrated that the stresses of blood sampling and volume depletion, along with the pharmacological effect of propranolol caused the death of all experimental rats in the second crossover period.

The effect of amino acids on the parent drug mean AUC_{oral} was not associated with a decreased mean AUC of metabolites. The mean AUC values of α -hydroxymetoprolol and O-demethylmetoprolol had no or little change with coadministration of amino acids. This means that amino acids seemed not to cause long-term inhibition of hepatic metabolizing enzymes. However, it is noteworthy that the t_{max} values of both metabolites were significantly prolonged, and the mean C_{max} of α -hydroxymetoprolol was significantly reduced after amino acid treatment. The observed delayed appearance of metabolites could be caused by: (1) a decreased absorption rate of parent drug and/or (2) a decreased formation rate of metabolites. The former reason is unlikely because no prolongation of the t_{max} of metoprolol was observed. Moreover, the decreased absorption rate should result in a decrease instead of an increase in the mean AUC_{oral} of parent drug since the degree of saturation of hepatic metabolism is lessened during the absorption phase. A decreased formation rate of metabolites could have occurred if the metabolism of parent drug was temporarily inhibited during the early phase, and returned to the normal level afterwards. Our data from studies in the perfused rat liver have shown that the apparent intrinsic clearance can be temporarily reduced by coinfusion of amino acids into the liver due to an amino acid-mediated limitation of oxygen supply to the drug metabolizing enzyme system (Chapter 8). So it seems that when given with amino acids in the *in vivo* rat, metoprolol can be absorbed into the portal vein along with a bolus influx of amino acids, which leads to a transient impairment of the drug metabolizing enzyme activity during the absorption phase of metoprolol. More metoprolol thus escapes from the hepatic first-pass metabolism into the circulation, resulting in an increased AUC of parent drug. On the other hand, the

escaped metoprolol will eventually be eliminated through metabolism by the liver. The temporarily inhibited hepatic metabolism would only delay the appearance of metabolites, but not change the extent of hepatic metabolism over the whole course of drug disposition. Therefore, the increased AUC_{oral} of parent drug (albeit not of statistical significance) along with the delayed appearance of metabolites may be attributed to the short-term inhibition of hepatic metabolism by amino acids during the early phase of drug disposition.

The effect of amino acids observed in this study resembles the results from a human study of the food effect on an important model drug, propranolol (Liedholm et al, 1990), in which a protein-rich meal was shown to increase the AUC_{oral} of propranolol and delay the appearance of naphthoxylactic acid, a major oxidative metabolite of propranolol, without alteration in its AUC value. The authors argued that the increase in parent drug AUC_{oral} but no change in metabolite AUC could be consequent to a transient increase in the absorption rate caused by the alternative absorption of the drug from a food-filled stomach, rather than enzyme inhibition by food. However, the argument can hardly explain the prolonged t_{max} of parent drug observed in the reported study, which should be shortened if the absorption rate is increased. According to the above discussion, the metabolite AUC may not be decreased even if the hepatic drug metabolism is inhibited, as long as the inhibition is short-lived. This is why most studies on the 'food effect' failed to show a decrease in metabolite AUC, which had been considered to accompany the increased parent drug AUC_{oral} (Walle et al., 1981; Liedholm et al., 1990). One exception is the reported reduction in the primary

glucuronic acid conjugation of propranolol after food intake, but its magnitude can not account for the enhancement in propranolol AUC_{oral} (Liedholm and Melander, 1986).

Although the increased bioavailability after a high protein meal has been reproduced in many human studies, a similar 'food effect' has not been successfully demonstrated in animal models, such as rats (Chow and Lalka, 1993; Ogiso et al., 1994; Alcorn, 1997) and dogs (Bai et al., 1985; Power et al., 1995). The reason may be due to species differences in the process of digestion and absorption of food components, and the resultant timing of hepatic hyperemic response and oxygen consumption. For example, protein can be digested and absorbed rapidly in both humans and dogs, resulting in an abrupt rise of dietary amino acids in the portal circulation (Rerat et al., 1976; Elwyn, 1970) and an enhancement in hepatic blood flow lasting 1-3 hr for humans and 3-7 hr for dogs (Bai et al., 1985). In contrast, rats exhibit a fluctuating pattern of protein digestion/amino acid absorption such that an oral protein load causes a small and discontinuous elevation (double peaks) in portal concentrations of amino acids and hepatic blood flow (Fernández-López et al., 1992). This may explain why a protein meal (egg white) did not cause an increase in the AUC_{oral} of propranolol but double peaks in the plasma concentration-time curve in the rat (Alcorn, 1997). In another rat study (Ogiso et al., 1994), ingestion of skimmed milk increased the mean AUC_{oral} of propranolol by 39% (not significantly), in agreement with the results of the present study. The appearance of amino acids in the portal blood is more dramatic and rapid when amino acid mixtures (probably as well as protein solution, such as skimmed milk) are given orally than after a solid protein meal because the complex hydrolytic process of protein is avoided in the gastrointestinal tract (Deferrari et al., 1988). By ingestion of

amino acid mixtures instead of a protein meal, therefore, we may reduce the species difference and thereby produce the 'food effect' in rats.

In summary, our present study has demonstrated an increasing trend in AUC_{oral} of metoprolol after orally administered with an amino acid mixture in rats. The result resembles the observations related to the effect of high protein food on propranolol disposition in humans, and is consistent with the observations in the perfused rat liver that amino acids temporally inhibit the hepatic metabolism of metoprolol due to amino acid-mediated oxygen deprivation. Hence, amino acids may be one of the key components causing a decrease in hepatic intrinsic clearance of the drug and thereby an increase in the AUC_{oral} (the phenomenon of the 'food effect'). However, the cause of the 'food effect' is complex, involving more than one mechanism. The transient increase in hepatic blood flow by food can never be neglected, even though its role in the 'food effect' must be minor (McLean et al., 1978; Olanoff et al., 1986; Svensson et al., 1983; Modi et al., 1988). Moreover, neurohormonal responses to food may be involved in the increased bioavailability of the drug (Power et al., 1995). Also food-induced alteration in dissociation of the drug-protein complex and diffusion of drug from blood into the hepatocytes can not be simply excluded in mechanisms of the 'food effect', although no hard evidence has been reported to reject or accept this possibility. Above all, a high absorption rate of drug seems to be an essential element to display the 'food effect' phenomenon. Any factors that cause a reduction in drug absorption rate, such as use of a slow-release formulation (Byrne et al., 1984; Liedholm and Melander, 1986) and ingestion of high carbohydrate meals or glucose (Liedholm et al., 1990; Chow and

Lalka, 1993), could enhance hepatic extraction of the drug and thus prevent the increased bioavailability after oral doses with food.

CHAPTER 10

CONCLUSIONS

10.1 Overall discussion

The main purpose of this work was to understand the mechanism of the food effect on the drugs which undergo extensive hepatic first pass metabolism. We hypothesized that inhibition of hepatic metabolism of these drugs by dietary amino acids could be the cause of the increased bioavailability after an oral dose with high protein food. To test the hypothesis, we have investigated the drug-amino acid interaction by selecting the isolated perfused rat liver and the intact rat as the animal model systems, and propafenone and metoprolol as the model drugs. This work has therefore been accomplished in three parts:

I. Establishment of the isolated, perfused rat liver preparations

The isolated, perfused rat liver preparation has many advantages for studying drugs which are eliminated mainly via hepatic metabolism. Two rat liver perfusion systems have been established. One is the isolated rat liver perfused with oxygenated Krebs buffer in a single pass mode. The other is a more complex liver perfusion system equipped to the exchange of antegrade and retrograde perfusion direction, in which erythrocyte-enriched medium is used. These two perfusion systems have been successfully applied for different purposes in the various metabolic studies conducted.

II. Evaluation of propafenone as a model drug

As its oral bioavailability was observed to be dramatically influenced by food ingestion in a human study, propafenone was initially chosen as a model drug for studying the mechanism of drug-food interactions. However, the kinetics and metabolism of this drug in the rat were unknown. We have therefore studied the disposition of propafenone in the isolated, perfused rat liver. With the information that we obtained, we are able to evaluate the usefulness of propafenone as a model drug in the rat.

First, a sensitive and convenient HPLC method for the analysis of propafenone enantiomers in rat liver perfusate and human plasma was developed using GTC as a chiral derivatizing reagent. The method was completely validated and was shown to be a reliable analytical method for rat liver perfusate samples. It also has potential to be a useful assay in human studies. Application of this method in a pilot study of propafenone kinetics in the isolated, perfused rat liver indicates that propafenone is highly bound to the hepatic tissue and its disposition is stereoselective.

By using HPLC/MS and MS/MS spectrometry, the phase I and II metabolism of propafenone in the isolated, perfused rat liver has been profiled. Hydroxylation in the terminal phenyl ring, which is not found in humans, is the major metabolic pathway in rat liver, whereas N-dealkylation and hydroxylation in the middle phenyl ring are relatively of minor importance. In addition to the oxidation, a considerable proportion of the parent drug and the hydroxylated metabolites are subject to glucuronidation. Four glucuronide conjugates have been found and structurally characterized, including two

newly identified glucuronides of hydroxylated propafenone derivatives. These studies represent the first report on the metabolism of propafenone in the rat.

We conclude that propafenone is not an appropriate model drug for studying the drug-food interaction in the isolated, perfused rat liver and the *in vivo* rat. The reasons are: 1) Propafenone is highly bound to hepatic tissue. Its approach to steady state can be greatly prolonged at non-saturating inlet concentrations in perfused rat livers, therefore, saturating inlet concentrations have to be employed. The resultant high inlet concentrations would reduce the effect of amino acids and may limit its usefulness in mechanistic studies in the perfused rat liver. 2) Propafenone undergoes different hepatic metabolic pathways in the rat from that in humans. The differences may be due to different metabolizing enzymes in the liver between these species. Therefore the rat should be used cautiously as an animal model in metabolic studies of propafenone. Nevertheless, propafenone could be a good model drug for human studies.

Unlike propafenone, metoprolol has much less hepatic tissue binding. Therefore, a steady state is achievable within a short time even at non-saturating inlet concentrations. Also, it undergoes hepatic metabolism in rats, which resembles that in humans. More importantly, previous studies in our laboratory have demonstrated an interaction between metoprolol and amino acids in the isolated, perfused rat liver. These evidences warrant the continuity to use metoprolol as a model drug in the proposed perfused rat liver study and the *in vivo* rat study.

III. Interactions between metoprolol and amino acids

A balanced mixture of amino acids has been found to inhibit the metabolism of propranolol and metoprolol in the isolated perfused rat liver. Previous studies in our laboratory have revealed that the inhibition might be due to oxygen limitation caused by the concurrent metabolism of amino acids in the liver, rather than direct interaction between amino acids and the drug metabolizing enzymes. In order to understand the mechanism of the inhibition and thereby its relevance to the food effect observed in humans, the interactions between metoprolol and amino acids have been studied in the isolated perfused rat liver with different levels of oxygen delivery in either antegrade or retrograde perfusions. The studies have shown that amino acids cause a temporary and reversible decrease in metoprolol metabolism under both hypo- and normal hepatic oxygenation. The magnitude of inhibition is proportional to the hepatic oxygen delivery rate. Moreover, retrograde perfusion partially reversed the inhibition of metoprolol metabolism by amino acids, suggesting that oxygen deprivation in the pericentral zone of the liver, resulting from preferential oxygen utilization by the metabolism of amino acids, is a contributing mechanism to the inhibition. The magnitude of inhibition is also dependent on the inlet concentration of coinfused amino acids. These findings are relevant to the phenomena of the food effect observed in humans. Firstly, the temporary inhibition of metoprolol metabolism by amino acids is consistent with what is required to explain the food-induced increase in the AUC_{0-t} observed in humans. Secondly, ingestion of proteins (amino acids), rather than carbohydrates or fat, results in oxygen depletion from the hepatic effluent in human studies, even though a large increase in hepatic blood flow occurs. Thirdly, the magnitude of the food effect in human subjects

is also found to correlate with the protein content of the meal. The relevance to the food effect observed in humans supports the conclusion that amino acid-mediated hepatic oxygen deprivation is involved in the mechanism of the 'food effect'. This study gave the first solid evidence linking meal-related oxygen utilization to drug metabolism that has ever been uncovered since Wang and Semple (1997) postulated the possibility.

To validate the findings in the isolated perfused rat liver, the interactions between metoprolol and amino acids have been studied in the *in vivo* rat. The results are in agreement with those observations in the isolated, perfused rat liver. The mean AUC_{oral} of metoprolol shows an increasing trend after been given with a mixture of amino acids in the rat. The mean t_{max} of α -hydroxymetoprolol, a major metabolite in rats, is significantly prolonged, while the mean C_{max} of this metabolite is reduced. The changes in these pharmacokinetic parameters appear very likely to be due to a temporary inhibition of hepatic metoprolol metabolism by amino acids. The effect of amino acids on the kinetics of these drugs in rats also resembles the food effect in humans, indicating that amino acids are the key component in the food effect. These findings are of great significance to gain understanding of the mechanism of the 'food effect'. This is the first time that a dietary component-related effect on the pharmacokinetics of high first-pass drugs, resembling to the food effect observed in humans, has been demonstrated in the *in vivo* rat. It not only provides evidence of the effect of amino acids on drug metabolism, but also demonstrates that the *in vivo* rat is a useful animal model for studying the food effect under certain conditions.

10.2 Future research

The metabolic interaction of amino acids with metoprolol should be further studied in a large animal species, such as the dog. The larger species of animal model can provide larger blood samples and, therefore, a crossover experiment design can be carried out. The crossover design is very important to demonstrate a significant difference between control and amino acid treatment groups, because a large interindividual variation of high first-pass drugs exists. The drawback of the dog as an animal model is that the food effect has not yet been demonstrated in this species. By minimizing the physiological processes in the digestion of a protein food, i.e. by giving amino acids instead of a protein food, the dog could be a useful animal model to demonstrate the effect of food components. Propafenone, as well as metoprolol, could be used as the model drug to test the interaction with amino acids in the dog. Meanwhile, oxygen probes can be placed into the hepatic vessels to measure the oxygen level before and after ingestion of amino acids. This would be of help to understand the role of oxygen in the regulation of drug metabolism.

If the interaction of amino acids with metoprolol or propafenone is observed in the dog study and its pattern is similar to the 'food effect' in humans, it will be necessary to conduct a human study to eventually illustrate the relevance of the amino acid effect to the food effect on the pharmacokinetics and metabolism of metoprolol or propafenone. The study can be carried out by a crossover design including three treatment groups, that is, fasting, amino acid treatment and protein meal groups. The difference among the treatment groups can be analyzed by ANOVA.

If positive results are observed in the human study, it will be worth expanding the drug-amino acid interaction studies to related clinical situations, such as chronic dosing, the effect of dietary habits, hypertensive patients, etc.

10.3 Conclusions

The *ex vivo* and *in vivo* interaction between metoprolol and amino acids strongly support our hypothesis that inhibition of drug metabolism by dietary components (amino acids) is a significant mechanism contributing to the increased bioavailability of high hepatic first pass drugs by intake of food. The inhibition is probably caused by elevated consumption of oxygen in the splanchnic tissues during the absorptive and digestive phases of dietary protein and metabolism of absorbed amino acids. These findings provide insights to the mechanism of the 'food effect' observed in humans, and also are of importance to basic science. This understanding may lead to evolving rational scheduling of high first-pass drugs in relation to meals.

REFERENCES

- Aaes-Jorgensen, T., Liedholm, H. and Melander, A. (1987) Influence of food intake on the bioavailability of zuclopenthixol. *Drug Nutr. Interact.* 5: 157-160.
- Ablad, B., Borg, K. O., Carlsson, E., Ek, L., Johnson, G., Malmfors, T. and Regårdh, C. G. (1975) A survey of the pharmacological properties of metoprolol in animals and man. *Acta. Pharmacol. Toxicol.* 36 (Suppl. V): 7-23.
- Alcorn, M. J. (1997) The food effect: investigations into possible animal models and mechanisms. Master's thesis, Department of Veterinary Physiological Sciences, Western College of Veterinary Medicine, University of Saskatchewan.
- Ameer, B. and Weintraub, R. A. (1997) Drug interactions with grapefruit juice. *Clin. Pharmacokinet.* 33: 103-121.
- Anderson, K. E. (1988) Influences of diet and nutrition on clinical pharmacokinetics. *Clin. Pharmacokinet.* 14: 325-346.
- Angus, P. W., Mihaly, G. W., Morgan, D. J. and Smallwood, R. A. (1989) Oxygen dependence of omeprazole clearance and sulfone and sulfide metabolite formation in the isolated perfused rat liver. *J. Pharmacol. Exp. Ther.* 250: 1043-1047.
- Angus, P. W., Morgan, D. J. and Smallwood, R. A. (1990) Review article: hypoxia and hepatic drug metabolism-clinical implications. *Aliment. Pharmacol. Ther.* 4: 213-225.
- Arfvidsson, A., Borg, K. O., Hoffmann, K.-J. and Skanberg, I. (1976) Metabolism of metoprolol in the rat in vitro and in vivo. *Xenobiotica* 6: 691-711.
- Axelson, J. E., Chan, G. L.-Y., Kirsten, E. B., Mason, W. D., Lanman, R. C. and Kerr, C. R. (1987) Food increases the bioavailability of propafenone. *Br. J. Clin. Pharmacol.* 23: 735-741.
- Bai, S. A., Walle, U. K., and Walle, T. (1985) Influence of food on the intravenous and oral dose kinetics of propranolol in the dog. *J. Pharmacokinet. Biopharm.* 13: 229-241.
- Barham, H. M., Lennard, M. S. and Tucker, G. T. (1994) An evaluation of cytochrome P450 isoform activities in the female dark agouti (DA) rat: relevance to its use as a model of the CYP2D6 poor metaboliser phenotype. *Biochem. Pharmacol.* 47: 1295-1307.
- Barnwell, S. G., Laudanski, T., Dwyer, M., Story, M. J., Guard, P., Cole, S. and

Attwood, D. (1993) Reduced bioavailability of atenolol in man: the role of bile acids. *Int. J. Pharm.* 89: 245-250.

Bass, N. M., Manning, J. A. and Weisiger, R. A. (1989) Increased sinusoidal volume and solute extraction during retrograde liver perfusion. *Am. J. Physiol.* 256 (6 pt 1): G1041-G1048.

Belpaire, F. M., Wijnant, P., Temmerman, A., Rasmussen, B. B. and Brøsen, K. (1998) The oxidative metabolism of metoprolol in human liver microsomes: inhibition by the selective serotonin reuptake inhibitors. *Eur. J. Clin. Pharmacol.* 54: 261-264.

Benfield, P., Clissold, S. P. and Brogden, R. N. (1986) Metoprolol. An updated review of its pharmacodynamic and pharmacokinetic properties and therapeutic efficacy in hypertension, ischaemic heart disease and related cardiovascular disorders. *Drugs* 31: 376-429.

Bogertoft, C., Carlsson, I., Ekenved, G. and Magnusson, A. (1978) Influence of food on the absorption of acetylsalicylic acid from enteric coated dosage forms. *Eur. J. Clin. Pharmacol.* 14: 351-355.

Benowitz, N. L. (1998) Antihypertensive agents. In: *Basic and Clinical Pharmacology*, pp 153-178, Katzung, B. G. (ed.) Appleton & Lange, Stamford, Connecticut.

Bodin, N.-O., Borg, K. O., Johansson, R., Ramsay, C.-H. and Skanberg, I. (1975) Tissue distribution of metoprolol-(3H) in the mouse and the rat. *Acta. Pharmacol. Toxicol.* 36 (Suppl. V): 116-124.

Borg, K. O., Carlsson, E., Hoffman, K.-J., Johnsson, T.-E., Thorin, H. and Wallin, B. (1975) Metabolism of metoprolol-(3H) in man, the dog and the rat. *Acta. Pharmacol. Toxicol.* 36 (Suppl. V): 125-135.

Borg, K. O., Fellenius, E., Johansson, R. and Wallborg, M. (1975) Pharmacokinetic studies of metoprolol-(3H) in the rat and the dog. *Acta. Pharmacol. Toxicol.* 36 (Suppl. V): 104-115.

Botsch, S., Gautier, J. C., Beaune, P., Eichelbaum, M. and Kroemer, H. K. (1993) Identification and characterization of the cytochrome P450 enzymes involved in N-dealkylation of propafenone: molecular base for interaction potential and variable disposition of active metabolites. *Mol. Pharmacol.* 43: 120-126.

Brandt, J. L., Castleman, L., Ruskin, H. D., Greenwald, J. and Kelley, J. J. (1955) The effect of oral protein and glucose feeding on splanchnic blood flow and oxygen utilization in normal and cirrhotic subjects. *J. Clin. Invest.* 34: 1017-1025.

Bredfeldt, J. E., Riley, E. M. and Groszmann, R. J. (1983) Hepatic oxygen consumption, in vivo, in the rat. *Experientia* 39: 729-730.

- Bredfeldt, J. E., Riley, E. M. and Groszmann, R. J. (1985) Compensatory mechanisms in response to an elevated hepatic oxygen consumption in chronically ethanol-fed rats. *Am. J. Physiol.* 248 (5 Pt 1): G 507-G511.
- Bressolle, F., Bromet-Petit, M. and Audran, M. (1996) Validation of liquid chromatographic and gas chromatographic methods: Applications to pharmacokinetics. *J. Chromatogr. Biomed. Appl.* 686: 3-10.
- Brode, E., Muller-Peltzer, H. and Hollmann, M. (1988) Comparative pharmacokinetics and clinical pharmacology of propafenone enantiomers after oral administration to man. *Method. Find. Exp. Clin. Pharmacol.* 10: 717-727.
- Brouwer, K. L. R. and Thurman, R. G. (1996) Chapter 10. Isolated perfused liver. In: *Models for Assessing Drug Absorption and Metabolism (Pharmaceutical Biotechnology V 18)*, Borchardt, R. T., Smith, P. L., and Wilson G. (eds) Plenum Press, New York.
- Brundin, T. (1993) Mechanisms of nutrient-induced thermogenesis: total and splanchnic oxygen consumption and blood flow. *Int. J. Obes. Relat. Metab. Disord.* 17 (Suppl. 3): S52-S55.
- Brundin, T. (1998) Whole body and splanchnic metabolic, circulatory, and thermal effects of oral vs. intravenous fat administration. *Am. J. Physiol.* 274 (4 Pt 1): E684-E691.
- Brundin, T. and Wahren, J. (1993) Whole body and splanchnic oxygen consumption and blood flow after oral ingestion of fructose or glucose. *Am. J. Physiol.* 264 (4 Pt 1): E504-E513.
- Brundin, T. and Wahren, J. (1994) Influence of protein ingestion on human splanchnic and whole-body oxygen consumption, blood flow, and blood temperature. *Metabolism* 43: 626-632.
- Brundin, T., Bränström, R. and Wahren, J. (1996) Effects of oral vs. iv glucose administration on splanchnic and extrasplanchnic O₂ uptake and blood flow. *Am. J. Physiol.* 271 (4 Pt 1): E496-E504.
- Brunner, F., Kolmayr, U. and Kukovetz, W. R. (1989) Pharmacokinetics and metabolism of the antiarrhythmic agent [3H]-diprafenone in the rat. *Arzneim.-Forsch. (Drug Res.)* 39: 1430-1435.
- Bryson, H. M., Palmer, K. J., Langtry, H. D. and Fitton, A. (1993) Propafenone - a reappraisal of its pharmacology, pharmacokinetics and therapeutic use in cardiac arrhythmias. *Drugs* 45: 85-130.
- Butler, R., Lindros, K. O., Nordling, A., Johansson, I. and Ingelman-Sundberg, M.

(1992) Zonation of cytochrome P-450 isozyme expression and induction in rat liver. *Eur. J. Biochem.* 240: 407-412.

Byrne, A. J., McNeil, J. J., Harrison, P. M., Louis, W., Tonkin, A. M. and McLean, A.-J. (1984) Stable oral availability of sustained-release propranolol when co-administered with hydralazine or food: evidence implicating substrate delivery rate as a determinant of presystemic drug interaction. *Br. J. Clin. Pharmacol.* 17 (Suppl 1): 45S-50S.

Carmichael, F. J., Crawford, M. W., Khayyam N. and Saldivia, V. (1993) Effect of propofol infusion on splanchnic hemodynamics and liver oxygen consumption in the rat. *Anesthesiology* 79: 1051-1060.

Chou, C. C. (1983) Splanchnic and overall cardiovascular hemodynamics during eating and digestion. *Fed. Proc.* 42: 1658-1661.

Chow, H. H. and Lalka, D. (1993) Pharmacokinetics of D-propranolol following oral, intra-arterial and intraportal administration: contrasting effects of oral glucose pretreatment. *Biopharm. Drug Dispos.* 14: 217-231.

Chow, M. S., Lebsack, C. and Hilleman, D. (1988) Propafenone: a new antiarrhythmic agent. *Clin. Pharm.* 7: 869-877.

Daneshmend, T. K. and Roberts, C. J. C. (1982) The influence of food on the oral and intravenous pharmacokinetics of a high clearance drug: a study with labetalol. *Br. J. Clin. Pharmacol.* 14: 73-78.

Dauzat, M., Lafortune, M., Patriquin, H. and Pomier-Layrargues, G. (1994) Meal induced changes in hepatic and splanchnic circulation: a noninvasive Doppler study in normal humans. *Eur. J. Appl. Physiol.* 68: 373-380.

Deferrari, G., Garibotto, G., Robaudo, C., Sala, M. and Tizianello, A. (1988) Splanchnic exchange of amino acids after amino acid ingestion in patients with chronic renal insufficiency. *Am. J. Clin. Nutr.* 48: 72-83.

Doose, D. R., Minn, F. L., Stellar, S. and Nayak, R. K. (1992) Effects of meals and meal composition on the bioavailability of fenretinide. *J. Clin. Pharmacol.* 32: 1089-1095.

Egginger, G., Lindner W., Vandenbosch, C. and Massart, D. L. (1993) Enantioselective bioanalysis of beta-blocking agents: focus on atenolol, betaxolol, carvedilol, metoprolol, pindolol, propranolol and sotalol. *Biomed. Chromatogr.* 7: 277-295.

Elliott, S. L., Morgan, D. J., Angus, P. W., Ghabrial, H. and Smallwood, R. A. (1993a) Sensitivity of propranolol elimination to hypoxia in the isolated perfused rat liver preparation. *Clin. Exp. Pharmacol. Physiol.* 20: 27-33.

Elliott, S. L., Morgan, D. J., Angus, P. W., Ghabrial, H., Watson, R. G. P. and

Smallwood, R. A. (1993b) The effect of hypoxia on propranolol clearance during antegrade and retrograde flow in the isolated perfused rat liver preparation. 45: 573-578.

Elvin, A. T., Cole, A. F. D., Pieper, J. A., Rolbin, S. H. and Lalka, D. (1981) Effect of food on lidocaine kinetics: mechanism of food-related alterations in high intrinsic clearance drug elimination. *Clin. Pharmacol. Ther.* 30: 455-460.

Elwyn, D. H. (1968) Modification of plasma amino acid patterns by the liver. In: *Protein Nutrition and Free Amino Acid Patterns*, pp 88-106, Brown, H. (ed.) Rutgers University Press, New Brunswick, New Jersey.

Elwyn D. H. (1970) The role of the liver in regulation of amino acid and protein metabolism. In: *Mammalian Protein Metabolism (Volume IV)*, pp 523-557, Munro H. N. (ed.) Academic Press, New York.

Faber, T. S. and Camm, A.J. (1996) The differentiation of propafenone from other class Ic agents, focusing on the effect on ventricular response rate attributable to its beta-blocking action. *Eur. J. Clin. Pharmacol.* 51: 199-208.

Feely, J., Nadeau, J. and Wood, A. J. J. (1983) Effects of feeding on the systemic clearance of indocyanine green and propranolol blood concentrations and plasma binding. *Br. J. Clin. Pharmacol.* 15: 383-385.

Fromm, M. F., Botsch, S., Heinkele, G., Evers, J. and Kroemer, H. K. (1995) Influence of renal function on the steady-state pharmacokinetics of the antiarrhythmic propafenone and its Phase I and Phase II metabolites. *Eur. J. Clin. Pharmacol.* 48: 279-283.

Garg, D. C., Jallad, N. S., Mishriki, A., Kraml, M., Fencik, M. and Weidler, D. J. (1987) Comparative pharmacodynamics and pharmacokinetics of conventional and long-acting propranolol. *J. Clin. Pharmacol.* 27: 390-396.

Gibaldi, M., and Perrier, D. (1982) *Pharmacokinetics*, pp 332. Marcel Dekker, Inc., New York.

Gores, G. J., Kost, L. J. and LaRusso, N. F. (1986) The isolated perfused rat liver: conceptual and practical considerations. *Hepatology* 6: 511-517.

Gut, J., Catin, T., Dayer, P., Kronbach, T., Zanger, U. and Meyer, U. A. (1986) A Debrisoquine/sparteine-type polymorphism of drug oxidation. Purification and characterization of two functionally different human liver cytochrome P-450 isozymes involved in impaired hydroxylation of the prototype substrate bufuralol. *J. Biol. Chem.* 261: 11734-11743.

Hanna, P. E. (1991) Adrenergic agents. In: *Wilson and Gisvold's Textbook of Organic Medicinal and Pharmaceutical Chemistry (9th Ed.)*, pp 413-441, Delgado J. N. and

Remers, W. A. (eds) J. B. Lippincott Company, Philadelphia.

Harron, D. W. G. and Brogden, R. N. (1987) Propafenone. A review of its pharmacodynamic and pharmacokinetic properties, and therapeutic use in the treatment of arrhythmias. *Drugs*, 34: 617-647.

Hartshorn, E. A. (1977) Food and drug interactions. *J. Am. Diet. Assoc.* 70: 15-19.

Hathcock, J. N. (1985) Metabolic mechanism of drug-nutrient interactions. *Fed. Proc.* 44 (1 Pt 1): 124-129.

Häussinger, D. and Gerok, W. (1986) Metabolism of amino acids and ammonia. In: *Regulation of Hepatic Metabolism*, pp 253-291, Thurman R. G., Kauffman F. C. and Jungermann K. (eds) Plenum Press, New York.

Hege, H. G., Lietz, H. and Weymann, J. (1984a) Studies on the metabolism of propafenone, 2nd Comm.: studies on the biotransformation in the dog. *Arzneim.-Forsch. (Drug. Res.)* 34: 972-979.

Hege, H. G., Hollmann, M., Kaumeier, S. and Liets, H. (1984b) The metabolic fate of 2H-labelled propafenone in man. *Eur. J. Drug Metab. Pharmacokinet.* 9: 41-55.

Hege, H. G., Lietz, H. and Weymann, J. (1986) Studies on the metabolism of propafenone, 3rd Comm.: isolation of the conjugated metabolites in the dog and identification using fast atom bombardment mass spectrometry. *Arzneim.-Forsch. (Drug. Res.)* 36: 467-474.

Heinzow, B., Corbett, H., Constantinides, S., Bourne, R. and McLean, A. J. (1984) Interaction between oral hydralazine and propranolol. I. Changes in absorption, presystemic clearance and splanchnic blood flow. *J. Pharmacol. Exp. Ther.* 229: 509-514.

Hickey, P. L., Mclean, A. J., Angus, P. W., Choo, E. F. and Morgan, D. J. (1996) Increased sensitivity of propranolol clearance to reduced oxygen delivery in the isolated perfused cirrhotic rat liver. *Gastroenterology* 111: 1039-1048.

Hii, J. T., Duff, H. J. and Burgess, E. D. (1991) Clinical pharmacokinetics of propafenone. *Clin. Pharmacokinet.* 21: 1-10.

Hollmann, M., Brode, E., Hotz, D., Kaumeier, S. and Kehrhahn, O. H. (1983) Investigations on the pharmacokinetics of propafenone in man. *Arzneim.-Forsch. (Drug Res.)* 33: 763-770.

Jackman, G. P., McLean, A. J., Jennings, G. L. and Bobik, A. (1981) No stereoselective first-pass hepatic extraction of propranolol. *Clin. Pharmacol. Ther.* 30: 291-296.

Jones, D. B., Mihaly, G. W., Smallwood, R. A., Webster, L. K., Morgan, D. J. and Madsen, N. P. (1984) Differential effects of hypoxia on the disposition of propranolol and sodium taurocholate by the isolated perfused rat liver. *Hepatology*, 4: 461-466.

Jusko, W. J. and Levy, G. (1967) Absorption, metabolism and excretion of riboflavin-5'-phosphate in man. *J. Pharm. Sci.* 56: 58-62.

Kates, R. E., Yee, Y. G. and Winkle, R. A. (1985) Metabolite cumulation during chronic propafenone dosing in arrhythmia. *Clin. Pharmacol. Ther.* 37: 610-614.

Kim, M., Shen, D. D., Eddy, A.C. and Nelson, W. L. (1993) Inhibition of the enantioselective oxidative metabolism of metoprolol by verapamil in human liver microsomes. *Drug Metab. Dispos.* 21: 309-317.

Kivisto, K. T., Ojala-Karlsson, P. and Neuvonen, P. J. (1992) Inhibition of norfloxacin absorption by dairy products. *Antimicrob. Agents Chemother.* 36: 489-491.

Knupp, C. A., Milbrath, R. and Barbhuiya, R. H. (1993) Effect of time of food administration on the bioavailability of didanosine from a chewable tablet formulation. *J. Clin. Pharmacol.* 33: 568-573.

Koytchev, R., Alken, R.-G., Mayer, O., Smith, I. and Greenwood, M. (1996) Influence of food in the bioavailability and some pharmacokinetic parameters of diprafenone - a novel antiarrhythmic agent. *Eur. J. Clin. Pharmacol.* 50: 315-319.

Kroemer, H. K., Funck-Brentano, C., Silberstein, D. J., Wood, A. J., Eichelbaum, M., Woosley, R. L. and Roden, D. M. (1989) Stereoselective disposition and pharmacologic activity of propafenone enantiomers. *Circulation*, 79: 1068-1076.

Kroemer, H. K., Mikus, G., Kronbach, T., Meyer, U. A. and Eichelbaum, M. (1989b) In vitro characterization of the human cytochrome P-450 involved in polymorphic oxidation of propafenone. *Clin. Pharmacol. Ther.* 45: 28-33.

Latini, R., Marchi, S., Riva, E., Cavalli, A., Cazzaniga, M. G., Maggioni, A. P. and Volpi, A. (1987) Distribution of propafenone and its active metabolite, 5-hydroxypropafenone, in human tissues. *Am. Heart J.* 113: 843-849.

Lautt, W. W. (1983) Relationship between hepatic blood flow and overall metabolism: the hepatic arterial buffer response. *Fed. Proc.* 42: 1662-1666.

Lennard, M. S. (1985) Quantitative analysis of metoprolol and three of its metabolites in urine and liver microsomes by high-performance liquid chromatography. *J. Chromatogr.* 342: 199-205.

Lennard, M. S., Crewe, H. K., Tucker, G. T. and Woods, H. F. (1986) Metoprolol oxidation by rat liver microsomes. Inhibition by debrisoquine and other drugs. *Biochem.*

Pharmacol. 35: 2757-2761.

Lennard, M. S., Silas, J. H., Freestone, S., Ramsay, L.E., Tucker, G. T. and Woods, H. F. (1982) Oxidative phenotype - a major determinant of metoprolol metabolism and response. *N. Engl. J. Med.* 307: 1558-1560.

Lennard, M. S., Tucker, G. T., Silas, J. H., Freestone, S., Ramsay, L. E. and Woods, H. F. (1983) Differential stereoselective metabolism of metoprolol in extensive and poor debrisoquine metabolizers. *Clin. Pharmacol. Ther.* 34: 732-737.

Lennard, M. S., Tucker, G. T., Woods, H. F., Silas, J. H. and Iyuni, A. O. (1989) Stereoselective metabolism of metoprolol in Caucasians and Nigerians - relationship to debrisoquine oxidation phenotype. *Br. J. Clin. Pharmacol.* 27: 613-616.

Levy, G. and Jusko, W. J. (1966) Factors affecting the absorption of riboflavin in man. *J. Pharm. Sci.* 55: 285-289.

Liedholm, H. and Melander, A. (1986) Concomitant food intake can increase the bioavailability of propranolol by transient inhibition of its presystemic primary conjugation. *Clin. Pharmacol. Ther.* 40: 29-36.

Liedholm, H., Lidén, A., Kroon, L., Melander, A. and Wahlin-Boll, E. (1985) Pharmacokinetics of dicyclanil: low bioavailability improved by food intake. *Drug-Nut. Interact.* 3: 87-92.

Liedholm, H., Wahlin-Boll, E. and Melander, A. (1990) Mechanisms and variations in the food effect on propranolol bioavailability. *Eur. J. Clin. Pharmacol.* 38: 469-475.

Low, L. K. and Castagnoli, N. Jr. (1991) Chapter 3. Metabolic changes of drugs and related organic compounds. In: *Wilson and Gisvold's Textbook of Organic Medicinal and Pharmaceutical Chemistry (9th Ed.)*, pp 45-121, Delgado, J. N. and Remers, W. A. (eds) J. B. Lippincott, Philadelphia.

Luch, J. R. (1983) Metoprolol tartrate In *Analytical Profiles of Drug Substances (Vol. 12)*, pp.325-356, Florey, K. (ed.), American Pharmaceutical Association.

MacKay, J., Mackie A. E., Palmer, J. L., Moulton, A. and Baber, N. S. (1992) Investigation into the mechanism for the improved oral systemic bioavailability of cefuroxime from cefuroxime axetil when taken after food. *Br. J. Clin. Pharmacol.* 33: 326-327.

Masubuchi, Y., Hosolawa, S., Horie, T., Suzuki, T., Ohmori, S., Kitada, M. and Marimatsu, S (1994) Cytochrome P-450 isozymes involved in propranolol metabolism in human liver microsomes. The role of CYP2D6 as ring-hydroxylase and CYP1A2 as N-desisopropylase. *Drug Metab. Dispos.* 22: 909-915.

- Masubuchi, Y., Kagimoto, N., Narimatsu, S., Fujita, S. and Suzuki, T. (1993) Regioselective contribution of the cytochrome P-450 2D subfamily to propranolol metabolism in rat liver microsomes. *Drug Metab. Dispos.* 21: 1012-1016.
- McAinsh, J., Barber, N. S., Holmes, B. F., Young, J. and Ellis, S. H. (1981) Bioavailability of sustained-release propranolol formulations. *Biopharm. Drug Dispos.* 2: 39-48.
- McBride, J. W., Lane, J. R., McCoy, H. G. and Sawchuck, R. J. (1980) Effect on food and circadian variation on the absorption and elimination of propranolol (abstr.) *Clin. Res.* 28: 240.
- McLean, A. J., Isbister, C., Bobik, A. and Dudley, F. J. (1981) Reduction of first-pass hepatic clearance of propranolol by food. *Clin. Pharmacol. Ther.* 30: 31-34.
- McLean, A. J., McNamara, P. J., du Souich, P., Gibaldi, M. and Lalka, D. (1978) Food, splanchnic blood flow and bioavailability of drugs subject to first-pass metabolism. *Clin. Pharmacol. Ther.* 24: 5-10.
- Meijer, D. K. F., Keulemans, K. and Mulder, G. J. (1981) Isolated perfused rat liver technique. In: *Methods in Enzymology (Volume 77)*, pp 81-94, Academic Press, Inc.
- Mehvar, R. (1990) Pharmacokinetics of propafenone enantiomers in rats. *Drug Metab. Dispos.* 18: 987-991.
- Melander, A. (1978) Influence of food on the bioavailability of drugs. *Clin. Pharmacokinet.* 3: 337-351.
- Melander, A. and McLean, A. J. (1983) Influence of food on the presystemic clearance of drugs. *Clin. Pharmacokinet.* 8: 286-296.
- Melander, A., Danielson, B., Schersten, B. and Wahlin, E. (1977) Enhancement of the bioavailability of propranolol and metoprolol by food. *Clin. Pharmacol. Ther.* 22: 108-112.
- Melander, A., Lalka, D. and McLean, A. J. (1988) Influence of food on the presystemic metabolism of drugs. *Pharmacol. Ther.* 38: 253-267.
- Miller, L. L. (1973) Technique of isolated rat liver perfusion. In: *Isolated Liver Perfusion and Its Applications*, pp11-52, Bartosek, I., Guitani, A. and Miller, L. L. (eds) Raven Press, New York.
- Modi, M. W., Hassett, J. M. and Lalka, D. (1988) Influence of posture on hepatic perfusion and presystemic biotransformation of propranolol: simulation of the food effect. *Clin. Pharmacol. Ther.* 44: 268-274.

Murthy, S. S., Shetty, H. U., Nelson, W. L., Jackson, P. R. and Lennard, M. S. (1990) Enantioselective and diastereoselective aspects of the oxidative metabolism of metoprolol. *Biochem. Pharmacol.* 40: 1637-1644.

Narimatsu, S., Kobayash, N., Masubuchi, Y., Horie, T., Kakegawa, T., Kobayashi, H., Hardwick J. P. et al. (2000) Species difference in enantioselectivity for the oxidation of propranolol by cytochrome P450 2D enzymes. *Chem. Biol. Interact.* 127: 73-90.

Naranjo, C. A., Sellers, E. M. and Khouw, V. (1982) Unaltered serum propranolol binding by meal induced variations in fatty acids. *Br. J. Clin. Pharmacol.* 13: 575-576.

Neuvonen, P. J., Kivisto, K. T. and Lehto, P. (1991) Interference of dairy products with the absorption of ciprofloxacin. *Clin. Pharmacol. Ther.* 50: 498-502.

Ogiso, T., Iwaki, M., Tanino, T., Kawafuchi, R. and Hata, S. (1994) Effect of food on propranolol oral clearance and a possible mechanism of this food effect. *Biol. Pharm. Bull.* 17: 112-116.

Ohashi, K., Ebihara, A., Kondo, K. and Usami, M. (1984) Clinical pharmacokinetics and pharmacological actions of a long-acting formulation of propranolol. *Arzneim.-Forsch. (Drug Res.)* 34: 507-512.

Olanoff, L. S., Walle, T., Cowart, T. D., Walle, U. K., Oexmann, M. J. and Conradi, E. C. (1986) Food effects on propranolol systemic and oral clearance: support for a blood flow hypothesis. *Clin. Pharmacol. Ther.* 40: 408-814.

Oti-Amoako, K., Vozeh, S., Ha, H. R. and Follath, F. (1990) The relative potency of major metabolites and enantiomers of propafenone in an experimental reperfusion arrhythmia model. *J. Cardiovasc. Pharmacol.* 15: 75-81.

Otton, S. V., Crewe, H. K., Lennard, M. S., Tucker, G. T. and Woods, H. F. (1998) Use of quinidine inhibition to define the role of the sparteine/debrisoquine cytochrome P-450 in metoprolol oxidation by human liver microsomes. *J. Pharmacol. Exp. Ther.* 247: 242-247.

Pang, K. S. (1984) Liver perfusion studies in drug metabolism and drug toxicity. In: *Drug Metabolism and Drug Toxicity*, pp 331-352, Mitchell J. R. and Horning M. G. (eds) Raven Press, New York.

Pang, K. S. and Terrell, J. A. (1981) Retrograde perfusion to probe the heterogeneous distribution of hepatic drug metabolizing enzymes in rats. *J. Pharmacol. Exp. Ther.* 216: 339-346.

Pang, K. S., and Rowland, M. (1977) Hepatic clearance of drugs. I. Theoretical considerations of a "Well-Stirred" model and a "Parallel Tube" model. Influence of hepatic blood flow, plasma and blood cell binding, and hepatocellular enzymatic activity

on hepatic drug clearance. *J. Pharmacokinet. Biopharm.* 5: 625-653.

Pincus, J. H. and Barry, K. M. (1987) Plasma levels of amino acids correlate with motor fluctuations in Parkinsonism. *Arch. Neurol.* 44: 1006-1009.

Pond, S. M. and Tozer, T. N. (1984) First-pass elimination: basic concepts and clinical consequences. *Clin. Pharmacokinet.* 9: 1-25.

Powell, G. M., Hughes, H. M. and Curtis, C. G. (1989) Isolated perfused liver technology for studying metabolic and toxicological problems. *Drug Metabol. Drug Interact.* 7: 53-86.

Power, J. M., Morgan, D. J. and McLean, A. J. (1995) Effects of sensory (teasing) exposure to food on oral propranolol bioavailability. *Biopharm. Drug Dispos.* 16: 579-589.

Regårdh, C-G. and Johnsson, G. (1980) Clinical pharmacokinetics of metoprolol. *Clin. Pharmacokinet.* 5: 557-569.

Regårdh, C-G., Borg, K. O., Johansson, R., Johnsson, G. and Palmer, L. (1974) Pharmacokinetic studies on the selective β_1 -receptor antagonist metoprolol in man. *J. Pharmacokinet. Biopharm.* 2: 347-364.

Regårdh, C-G., Johnsson, G., Jordö, L. and Sölvell, L. (1975) Comparative bioavailability and effect studies on metoprolol administered as ordinary and slow-release tablets in single and multiple doses. *Acta. Pharmacol. Toxicol.* 36 (Suppl. V): 45-58.

Rerat, A., Corring, T. and Laplace, J. P. (1976) Protein digestion and absorption. In: *Protein Metabolism and Nutrition*, pp 97-138, Cole, D. J. A., Boorman, K. N., Buttery, P. J., Lewis, D., Neale, R. J. and Swan, H. (eds) Butterworths, London.

Roberts, J. and Tumer, N. (1988) Age and diet effects on drug action. *Pharmacol. Ther.* 37: 111-149.

Roos, R. A. C., Tijssen, M. A. J., Van der Velde, E. A. and Breimer, D. D. (1993) The influence of a standard meal on Sinemet CR absorption in patients with Parkinson's disease. *Clin. Neurol. Neurosurg.* 95: 215-219.

Rosenberg, H. A. and Bates, T. R. (1976) Influence of food on nitrofurantoin bioavailability. *Clin. Pharmacol. Ther.* 20: 227-232.

Salmonson, T. (1993) Food effects: regulatory consequences. In: *Bio-International: Bioavailability, Bioequivalence and Pharmacokinetics*, pp 251-255, Midha, K. K. and Blume, H. H. (eds) Medpharm Sci., Stuttgart.

- Sax, H. C., Hasselgren, P.-O., Talamini, M. A., Edwards, L. L. and Fischer, J. E. (1988) Amino acid uptake in isolated, perfused liver: effect of trauma and sepsis. *J. Surg. Res.* 45:50-55.
- Sax, H. C., Talamini, M. A., Hasselgren P.-O., Rosenblum, L., Ogle, C. K. and Fischer, J. E. (1988b) Increased synthesis of secreted hepatic proteins during abdominal sepsis. *J. Surg. Res.* 44: 109-116.
- Schulz-Utermoehl, T., Bennett, A. J., Ellis, S. W., Tucker, G. T., Boobis, A. R. and Edwards, R. J. (1999) Polymorphic debrisoquine 4-hydroxylase activity in the rat is due to differences in CYP2D2 expression. *Pharmacogenetics* 9: 357-366.
- Seifter, S. and Englard, S. (1994) Energy Metabolism. In: *The Liver: Biology and Pathobiology*, pp 323-364, Arias, I. M. (ed.) Raven Press, New York.
- Semple, H. A. and Xia, F. (1994) Propranolol disposition in the single-pass isolated, perfused rat liver in the presence and absence of insulin and glucagon. *Drug Metab. Dispos.* 22: 822-826.
- Semple, H. A. and Xia, F. (1995) Interaction between propranolol and amino acids in the single-pass isolated perfused rat liver. *Drug Metab. Dispos.* 23: 794-798.
- Semple, H. A., Tam, Y. K. and Coutts, R. T. (1990) A computer simulation of the food effect: transient changes in hepatic blood flow and Michaelis-Menten parameters as mediators of hepatic first-pass metabolism and bioavailability of propranolol. *Biopharm. Drug Dispos.* 11: 61-76.
- Shargel, L. and Yu, A. B. C. (1999) Applied biopharmaceutics and pharmacokinetics. pp 99-120, Appleton & Lange, Stamford, Connecticut.
- Shen, G. S., Zhang, Y. D., Li, M. Y., Shen, J. P., Ding, Y. and Huang, D. K. (1993) Effect of dose of metoprolol on its elimination by isolated perfused rat liver in vitro. *Acta. Pharmacol. Sinica.* 14: 539-642.
- Shimada, T., Yamazaki, H., Mimura, M., Inui, Y. and Guengerich, P. (1994) Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. *J. Pharmacol. Exp. Ther.* 270: 414-423.
- Silber, B. M., Holford, N. H. and Riegelman, S. (1983) Dose-dependent elimination of propranolol and its major metabolites in humans. *J. Pharm. Sci.* 72: 725-732.
- Singh, B. N. (1999) Effects of food on clinical pharmacokinetics. *Clin. Pharmacokinetics.* 37: 213-255.
- St.-Pierre, M. V., Schwab, A. J., Goresky, C. A., Lee, W-F. and Pang, K.S. (1989) The

multiple-indicator technique for characterization of normal and retrograde flow in once-through rat liver perfusions. *Hepatology*, 9: 285-296.

Staub, N. C. (1992) Chapter 36. Transport of oxygen and carbon dioxide: tissue oxygenation. In: *Physiology (3rd ed)* pp 590-598, Berne, R. M. and Levy M. N. (eds) Mosby-Year Bood, Inc., Sr. Louis, Missouri.

Svensson, C. K., Edwards, D. J., Mauriello, P. M., Barde, S. H., Foster, A. C., Lanc, R. A., Middleton, E. Jr. and Lalka, D. (1983) Effect of food on hepatic blood flow: implication in the food effect phenomenon. *Clin. Pharmacol. Ther.* 34: 316-323.

Svensson, C. K., Mauriello, P. M., Barde, S. H., Middleton, E. and Lalka, D. (1984) Effects of carbohydrates on estimated hepatic blood flow. *Clin. Pharmacol. Ther.* 35: 660-665.

Tan, W., Li, Q., McKay, G. and Semple, H. A. (1998) Identification and determination of phase I metabolites of propafenone in rat liver perfusate. *J. Pharm. Biomed. Anal.* 16: 991-1003.

Thompson, K. A., Iansmith, D. H. S., Siddoway, L. A., Woosley, R. L. and Roden, D. M. (1988) Potent electrophysiologic effects of the major metabolites of propafenone in canine Purkinje fibers. *J. Pharmacol. Exp. Ther.* 244: 950-955.

Thurman, R. G., Kauffman, F. C. and Baron, J. (1986) Biotransformation and zonal toxicity. In: *Regulation of Hepatic Metabolism*, pp 321-382, Thurman, R. G., Kauffman, F. C. and Jungermann, K. (eds) Plenum Press, New York.

Vermeulen, A. M., Belpaire, F. M., De Smet, F., Vercruysse, I. and Bogaert, M. G. (1993) Aging and the pharmacokinetics and metabolism of metoprolol enantiomers in the rat. *J. Gerontol.* 48: B108-B114.

Wagner, J. G. (1985) Propranolol: Pooled Michaelis-Menten parameters and the effect of input rate on bioavailability. *Clin. Pharmacol. Ther.* 37: 481-487.

Wagner, J. G. (1988) Modeling first-pass metabolism. In: *Pharmacokinetics, Mathematical and Statistical approaches to metabolism and distribution of chemicals and drugs*, pp 129-149, Pecile and Rescigno (eds) Plenum Press, New York.

Wagner, J. G., Antal, E. J., Elvin, A. T., Gillespie, W. R., Pratt, E. A. and Albert, K. S. (1985) Theoretical decrease in systemic availability with decrease in input rate at steady-state for first-pass drugs. *Biopharm. Drug Dispos.* 6: 341-343.

Wahlund, G., Nerme, V., Abrahamsson, T. and Sjöquist, P.-O. (1990) The β_1 and β_2 -adrenoceptor affinity and β_1 -blocking potency of S- and R-metoprolol. *Br. J. Pharmacol.* 99: 592-596.

Walle, T., Fagan, T. C., Walle, U. K., Oexmann, M.-J., Conradi, E. C. and Gaffney, T. E. (1981) Food-induced increase in propranolol bioavailability - relationship to protein and effects on metabolites. *Clin. Pharmacol. Ther.* 30: 790-795.

Walle, T., Oatis, J. E., Walle, U. K. and Knapp, D. R. (1982) New ring-hydroxylated metabolites of propranolol: species differences and stereospecific 7-hydroxylation. *Drug Metab. Dispos.* 10: 122-127.

Walter-Sack, I., and Klotz, U. (1996) Influence of diet and nutritional status on drug metabolism. *Clin. Pharmacokinet.* 31: 47-64.

Wang, B. (1995) Influence of amino acids on the kinetics of metoprolol metabolism using the isolated, perfused rat liver: a study related to the food effect. Master's thesis, College of Pharmacy and Nutrition, University of Saskatchewan.

Wang, B. and Semple, H. A. (1997) Inhibition of metoprolol metabolism by amino acids in perfused rat livers. Insights into the food effects. *Drug Metab. Dispos.* 25: 287-295.

Welling, P. G. (1977) Influence of food and diet on gastrointestinal drug absorption: a review. *J. Pharmacokinet. Biopharm.* 5: 291-334.

Welling, P. G. (1984) Interactions affecting drug absorption. *Clin. Pharmacokinet.* 9: 404-434.

Welling, P. G. (1989) Effects of food on drug absorption. *Pharmacol. Ther.* 43: 425-441.

Welling, P. G. (1996) Effects of food on drug absorption. *Annu. Rev. Nutr.* 16: 383-415.

Wilkinson, G. R. (1987) Clearance approaches in pharmacology. *Pharmacol. Rev.* 39 : 1-47.

Williams, L., Hill, D. P. Jr., Davis, J. A. and Lowenthal, D. T. (1996) Influence of food on the absorption and metabolism of drugs: an update. *Eur. J. Drug Metab. Pharmacokinet.* 21: 201-211.

Wolkoff, A. W., Johansen, K. L. and Goesser, T. (1987) The isolated perfused rat liver: preparation and application. *Anal. Biochem.* 167: 1-14.

Woodroffe, A. J. M., Bayliss, M. K. and Park, G. R. (1995) The effects of hypoxia on drug-metabolizing enzymes. *Drug Metab. Rev.* 27: 471-495.

Yang, C. S. and Yoo, J.-S. H. (1988) Dietary effects on drug metabolism by the mixed-function oxidase system. *Pharmacol. Ther.* 38: 53-72.