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INTERPATIENT VARIABILITY WITH THE DISPOSITION OF VERAPAMIL

by

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Abstract

The pharmacokinetics of verapamil were evaluated in the young and elderly after steady state administration of a sustained release formulation. The objective was to determine whether the interindividual metabolic variability of verapamil and its main metabolites could be correlated with the enzymatic activity of CYP3A4 by using dextromethorphan (DM) as an *in vivo* probe of CYP3A4 activity.

Pharmacokinetic analysis showed a significant age related increase in the clearance of verapamil. Verapamil interindividual variability could not be correlated with formation of 3-methoxy-morphinan. Our results suggest that verapamil metabolism may be significantly mediated by other cytochromes.

Résumé

La pharmacocinétique de verapamil à été evaluée chez les jeunes et les personne âgées après administration d'une preparation orale pendant 7 jours. L'objectif consitait à determiner si la variabilité métabolique, du verapamil et metabolite, entre individus était due à l'activité enzymatiquedu CYP3A4 en utilisant le dextromethorphan comme sonde *in vivo* de l'activité CYP3A4.

L'analyse pharmacocinétique a montré que les personnes âgées présentent statistiquement une réduction significative dans la capacité à la formation de 3-methoxy-morphinan. Nos résultats suggèrent l'implication de d'autre cytochromes dans le métabolisme du verapamil.

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Chapter 1

INTRODUCTION

There are significant inter-patient variations in the pharmacokinetic disposition and clinical effects of verapamil. These differences are due to a variety of parameters including the formulation of the drug and the characteristics of the patient. The route of administration plays a key role in stereoselective first pass clearance which favors the active S-verapamil relative to R-verapamil. Thus, the formulation itself, *i.e.* i.v. *vs* oral, immediate *vs* sustained release, will lead to differences in total and relative systemic exposures of S- and R-verapamil.

A key patient determinant is age. The results of a recent study demonstrated that the systemic clearances of S- and R-verapamil are significantly reduced in older healthy males relative to younger male subjects (1). In addition, older hypertensive patients have a greater hypotensive response, but less cardiac A-V nodal conduction delay after single i.v. doses. Gender may also be an important factor as the clearance of S-verapamil is significantly greater in elderly women than in elderly men after i.v. administration (2).

An additional fundamental source of inter-patient differences in verapamil clearance and effect is the ability of the individual to metabolize drug. The majority of the N-demethylation of verapamil is mediated by the microsomal isoform CYP3A4 (3). This enzyme is located in the liver and upper gut wall (4). Thus, a source of formulation and patient dependent differences in verapamil clearance may be where the absorption of the drug takes place {stomach *vs* gut} and the concentration and activity of CYP3A4 enzymes at that site {as well as in the liver}(4).

The activities of metabolizing enzymes can be assessed using probe-drug phenotyping. This approach uses innocuous probe drugs which are metabolized by well characterized enzymes. The activities of the target enzymes are reflected in the metabolite to parent ratio of the probe drug. The antitussive dextromethorphan (DM), for example, is metabolized in two steps by two isozymes of the cytochrome P450 (CYP) family of drug-metabolizing enzymes (5). Thus, the ratio of the O-demethylated metabolite to its parent (DM) is an index of CYP2D6 activity while the N-demethylated DM/DM ratio is indicative of CYP3A4 activity.

Objective

The objectives of the study are to determine the effect of age and CYP3A4 metabolic activity on the steady-state systemic exposure of verapamil and metabolites after 7 day administration of sustained release verapamil. In order to achieve these goals, the proposed project has been designed to achieve the following **experimental objectives**:

Objective 1: Determine the steady-state plasma concentrations of verapamil and the respective metabolites;

Objective 2: Measure the activity of CYP3A4 in each patient by using DM as an *in vivo* probe of CYP3A4;

Objective 3: Correlate the data obtained in Objectives 1 and 2 with age of the patients.

It is anticipated that the results of this study will help address the following questions: What are the sources of inter-patient variations in verapamil exposure and can they be predicted by simple pretreatment probe drug phenotyping? Is DM a suitable probe of CYP3A4 activity?

1.1 Verapamil Description

Verapamil is a calcium ion influx inhibitor (slow-channel blocker or calcium ion antagonist). Chemically it is benzeneacetonitrile, alpha- (3-((2 -(3, 4- dimethoxyphenyl) ethyl) methylamino) propyl)- 3, 4 - dimethoxy- alpha - (1 - methylethyl) hydrochloride. Verapamil HCl is an almost white, crystalline powder, practically free of odor, with a bitter taste. It is soluble in water, chloroform, and methanol (6).

Verapamil is not chemically related to other cardioactive drugs. It is a calcium ion influx inhibitor that exerts its pharmacologic effects by modulating the influx of ionic calcium across the cell membrane of the arterial smooth muscle as well as in conductile and contractile myocardial cells (7).

1.2 Clinical Uses and Therapeutic Effects

Verapamil is used in hypertension, angina and supraventricular tachyhrythmias. Vascular smooth muscle appears to be most sensitive but relaxation can be shown for bronchiolar, gastrointestinal and uterine smooth muscle (8). In the vascular system, arterioles appear to be more sensitive than veins. The reduction in peripheral vascular resistance is one of the mechanisms by which verapamil may benefit patients with angina and hypertension. Verapamil also blocks alpha adrenoreceptors, which may contribute to peripheral vasodilation (8).

Verapamil also dilates the main coronary arteries and coronary arterioles, both in normal and ischemic regions. This property increases myocardial oxygen delivery in patients with coronary artery spasm and is responsible for the effectiveness of verapamil in unstable angina (9). Verapamil regularly reduces the total systemic resistance (afterload) against which the heart works both at rest and at a given level of exercise by dilating peripheral arterioles (7).

Verapamil prolongs the effective refractory period within the AV node and slows AV conduction in a age related manner (10,11). The young have a greater electrocardiographic PR prolongation when compared to the elderly after verapamil administration. Swartz *et al.* (11) demonstrated that when heart rate was fixed PR prolongation was no longer observed suggesting that this is an indirect effect on the AV node. Instead it may be associated with impaired baroreflex response, altered sympathetic tone and perhaps altered parasympathetic tone associated with aging (10).

Verapamil may interfere with sinus-node impulse generation and may induce sinus arrest or sinoatrial block (7). Verapamil does not alter normal atrial action potential or intraventricular conduction time, but depresses amplitude, velocity of depolarisation, and conduction in depressed atrial fibers (7). Verapamil may also shorten the antegrade effective refractory period of the accessory bypass tract (7).

1.3 Cytochrome P450s

The cytochrome P450 (CYP450) gene superfamily is made up of diverse collection of haem containing monooxygenases that have an incredible range of substrate specificities. P450s represent a superfamily of enzymes and are found in animals, plants, yeast and bacteria (12) . Some P450s are involved in pathways of steroid biosynthesis and do not metabolise foreign compounds. The vast majority of P450s appear to exist solely to oxidise chemicals that are not normal constituents of the body. P450s are also involved in the metabolism of carcinogens and can activate a procarcinogen into a carcinogenic compound (13). P450 are therefore important in both drug clearance and carcinogen activation.

The CYP450s are known as phase 1 drug metabolism enzymes. They catalyse the introduction of an atom of molecular oxygen into a substrate. This oxidation reaction facilitates increased clearance and metabolism by phase II enzymes. Phase II enzymes involve conjugation reactions to molecules such as glucuronic acid. (14). Drugs are eliminated after phase I or phase II metabolism or a combination of both. The ultimate objective of metabolism is to make the substrate less lipophilic and more hydrophilic resulting in a more polar moiety to facilitate excretion.

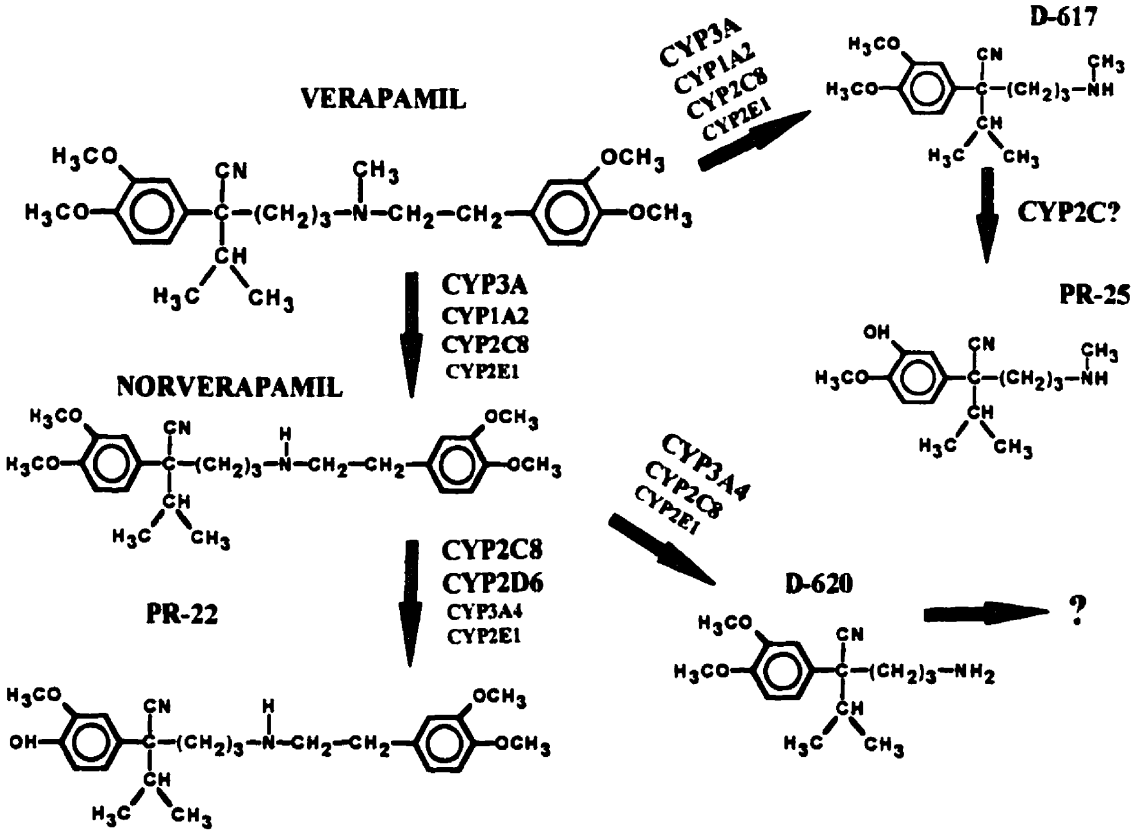
CYP450 enzymes may be regulated by many different factors, both endogenous and exogenous (15). Smoking, alcohol consumption and diet have all been identified as factors and may influence enzyme levels. Phenobarbital and food substances such as cruciferous vegetables (brussel sprouts, cabbage, broccoli, cauliflower and spinach), charcoal broiled food , and cigarette smoking have all been implicated as enzyme inducers (12).

The amounts and types of isoenzymes synthesized is important in determining how an individual will respond to drugs. Therefore, understanding metabolic patterns are important in determining how individuals may respond not only to drugs but to carcinogens and chemical toxins. An extensive database is accumulating on the substrate specificities of the major CYP450 enzymes expressed in the human liver (CYP1A2, 2A6,

2B6, 2D6, 2E1, 3A4/5, 4A9/11). In vitro assays have been used to determine which enzyme or enzymes are involved in a specific drugs metabolism.

1.4 Metabolism of Verapamil

Figure 1



CYP3A subfamily includes the most abundantly expressed cytochromes P450s in the liver. The major forms expressed in the liver are CYP3A4 and CYP3A5 (12). CYP3A5 is expressed in only 10-20% of human livers (17). No genetic polymorphisms have been detected except for an apparent genetic association of low activity phenotypes in certain families having a cystic fibrosis patient (18). CYP3A4 is associated with a wide interindividual variability (19) which is likely attributed to environmental induction/inhibition and/or physiologic factors as opposed to genetic diversity.

Verapamil is metabolised predominantly by CYP3A4 (3). There is also evidence suggesting a significant involvement of CYP1A2 (3,20) and CYP2C8 (21,22). Verapamil undergoes extensive first pass clearance in man with the major metabolic steps comprising formation of norverapamil (NV) and D617 (figure) (23).

Cashman (1989) described the formation of D617 but not NV to be mediated via the flavin containing monooxygenases. However, Ayesh (25) investigated the oxidative N-dealkylation of verapamil in a family with 2 proposti with an inherited deficiency of trimethylamine-N-oxidation and found no functional segregation. There have been no additional reports identifying flavin containing monoxygenases in the metabolism of verapamil.

Kroemer *et al.* (3) characterised the enzymes involved in the formation of D617 and NV. By using an antibody directed against CYP3A4/3A5 he inhibited formation of D617 and NV by an average of 60%. These data clearly indicate that a substantial portion of NV and D617 is formed via CYP3A4/3A5. It also suggests that there are other enzymes involved in these pathways.

Formation of D617 was correlated with the expression of CYP3A ($r=0.85$ $p<0.001$) and CYP1A2 ($r=0.57$ $p<0.05$) with racemic verapamil and formation of NV with the expression of CYP3A ($r=0.58$ $p<0.01$) and CYP1A2 ($r=0.5$ $p<0.05$). Sesardic *et al.* (65) also identified CYP1A2 in the formation of NV.

Recently, the role of CYP1A2 in verapamils metabolim has been questioned. Tracy (21) identified CYP3A4 and CYP2C8 as primarily mediating formation of NV and D617 with minor contributions from CYP2E1. Interestingly, no metabolism was noted with CYP1A2.

In 1991, Kroemer *et al.* observed using prepared microsomes from 10 human livers the formation of D617 by N-dealkylation from verapamil with subsequent O-demethylation to PR-25. Kroemer (26) also observed formation of NV (N-demethylation from verapamil) and subsequent N-dealkylation to D620 or O-demethylation to PR-22. (figure). Verapamil was also O-demethylated to form D703 or D702 (not included in figure). The enzymes responsible for O-demethylation of verapamil have been suggested to be mediated by CYP2C (22). Formation of PR22 has been recently identified to be

mediated primarily by CYP2C8 and CYP2D6 with minor contributions from CYP2E1 and CYP3A4 (21). There is currently no information suggesting further metabolism of D620.

1.5 First Pass

The interindividual variation in the pharmacokinetics (PK) of verapamil is typical of drugs which undergo extensive first pass metabolism. When discussing first pass we typically think of the liver, but there are also significant contributions from other organs such as the gut and the lung. (27).

The liver remains the major source of biotransformation of drugs. The rate of drug elimination by the liver is dependent on the hepatic blood flow , its ability to metabolise, bind and excrete (through the bilary tract) drugs, and the free fraction of drug in blood or plasma (28). In humans, the liver to intestine CYP450 ratio has been reported as about 20, suggesting that the contribution of intestinal phase 1 biotransformation to the overall metabolism of the drug is unlikely to be important (28). More recently Kolars (29) and Fromm (30) have provided evidence suggesting that the gut is also a major source of biotransformation for phase I reactions.

Rometi *et al.* (31) reported that phase II enzymes are comparable in the gut wall and liver. There role in the metabolism of orally administeredethinylestradiol has been extensively documented and are known to be conjugated in the gut wall.

CYP3A4 is expressed in gastric and intestinal tissues (29,32). Cyclosporine metabolism is mediated primarily by CYP3A4 (33). A consider part of cyclosporine is metabolised in the gut before reaching the liver (33). Kolars (34) demonstrated that CYP3A4 in human bowel enterocytes is inducible by rifampin, and Morel (35) demonstrated no induction in human hepatocytes. The gut may be an important source of interindividual variation associated with metabolism of CYP3A4 substrates.

There has been direct evidence indicating the importance of the gut in verapamil presystemic metabolism. Using stable isotope technology Fromm (30) designed a study to assess the contribution of prehepatic and hepatic enzymes (induced by rifampin) to total clearance of verapamil and demonstrated the importance of the gut wall in metabolism of

verapamil. Stable isotope technology provides a tool for investigation of systemic and oral clearance during chronic drug administration without stopping oral dosing. By using the deuterium labelled i.v. verapamil formulation, the investigator was able to distinguish both routes of administration by using mass spectrometry. His results quite elegantly demonstrated that the increase in first pass of verapamil occurs in the mucosa of the gastrointestinal tract before reaching the liver. Therefore, current scientific data identify the presence of CYP3A4 enzyme in the gut wall, and importantly, that it is inducible. These gut wall enzymes represent an important site of drug metabolism for verapamil as well as other CYP3A4 substrates.

Metabolism can also occur in the gut lumen (28). Metabolic enzymes in the gut lumen originate from exocrine glands, cells shed from mucosal lining and the gut flora. There are also intestinal microorganisms (aerobic and anaerobic) located in the colon and the lower portion of the small intestine (28) which inactivate host enzymes. Therefore, enzyme activity generally is higher in the mucosal epithelial cells of the duodenum and jejunum (28).

In the duodenum there have been very small amounts of CYP2C detected (200 fold lower than liver) but no CYP1A2 (35). CYP2C has been identified in verapamil metabolism, however, the contribution of the duodenum in verapamil presystemic metabolism is unknown.

1.6 Calcium Channel

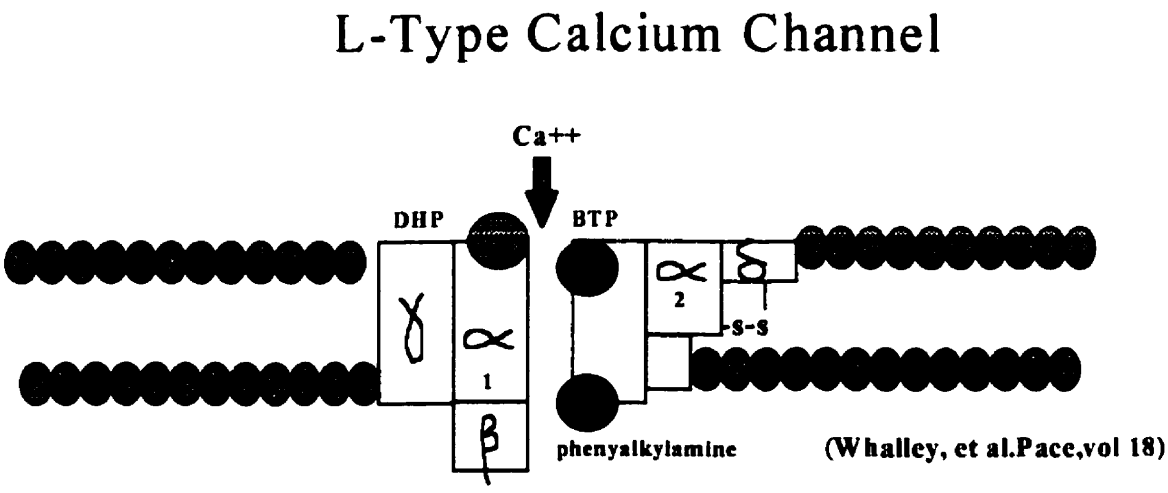


Figure 2: Diagramatic representation of an L-Type calcium channel. Dihydropyridine (DHP), Benzothiazepine (BTP)

Verapamil interacts with L-type Ca^{2+} channels which are transmembrane proteins with multiple subunits ($\alpha 1$, β, γ and $\alpha 2$, γ subunits) whose conductance depends on transmembrane voltage (37). The $\alpha 1$ subunit is sufficient to function as a voltage gated ion channel (37). Calcium channels can be inhibited by 2 other classes of organic blocking drugs, the dihydropyridines and benzothiazepines. Each class appears to occupy a distinct site on the $\alpha 1$ subunit.

Verapamil has a high pKa and at physiologic pH a large fraction of the drug is in charged form and the solubility is low. The verapamil binding site is located at the cytosolic (intracellular) surface of the Ca^{2+} channel. Because of their low lipid solubility, these drugs access their binding site predominately via the open channel (38). As the transmembrane voltage changes the channel passes through 3 distinct states. At a voltage below threshold (-40mV), the channel is in the resting state (closed). At a voltage above threshold, the channel opens. After depolarization, the channel assumes an inactive state (38).

T and N type Ca^{2+} channels are less sensitive to Ca^{2+} channel blockers. Therefore, tissues in which these channels play a major role (neurons and most secretory glands) are much less affected than cardiac and smooth muscle (39).

1.7 Chirality of Verapamil

Verapamil has a single stereogenic chiral centre which results in two stereoisomers Verapamil is used as a racemic mixture of (-) S and (+) R- verapamil. Stereochemical aspects of verapamil are important because the enantiomers differ in terms of pharmacologic activity. The S enantiomer has been shown to be preferentially metabolised, especially after oral dosing (40).

Animal and human studies have demonstrated that the S-verapamil is the more potent enantiomer and is responsible for the negative dromotrophic activity on atrioventricular conduction. During initial studies with verapamil it was found that when verapamil was administered i.v., it appeared to be 2-3 times more potent than oral formulations in causing changes in conductivity in the heart (40,41). We know that this effect is a result of unequal bioavailabilities of R and S-verapamil and that the route of administration will have a significant effect on the bioavailabilities of these enantiomers.

1.8 Pharmacokinetics

Verapamil is a prototypic chiral drug with a high first pass clearance. Verapamil metabolism has been studied after i.v. and oral administration of C^{14} R and S verapamil in healthy subjects. (23,42). Extensive first pass elimination (extraction fraction=0.79) from portal circulation results in low systemic bioavailability (10-20%) (23) with less than 5% of the drug eliminated unchanged. A total of 12 metabolites, accounting for 50-55 % of the dose administered, were isolated from urine and identified (23). Most of the metabolites identified in the urine are in trace amounts with the exception of NV, D617, D620, PR22, PR23 and PR25.

Following oral administration the drug is almost completely absorbed (43). The volume of distribution in humans ranges from 162-380L (44). The elimination half life ranges from 2- 10 hrs (44). During long term treatment, oral clearance decreases (10) and bioavailabilty increases (45) due to saturation of first pass metabolism. NV plasma concentrations approximate those of verapamil following single or multiple oral doses of the parent drug. Pronounced intra and interindiviidual variation in clearance and bioavailability have been observed among the young and elderly (1).

Pharmacokinetic differences for enantiomers of verapamil have been reported. After oral dosing the R enantiomer is much more prevalent. In studies comparing i.v. to oral therapy, the i.v. dose resulted in equal amounts of the 2 enantiomers (46) where as oral dosing depended on the formulation used. Sustained release (SR) dosage form of racemic verapamil yields an R:S ratio of 4:1 wheras the immediate release (IR) yields an R:S ratio of 3:1. The SR dosage form with its intentionally lower release rate of 30 mg/hr has a lower absorption rate, than IR with a release rate 40 mg/hr. Therfore, different formulations will have a profound influence on the pharmacodynamic response.

The actual extent of binding of verapamil to plasma proteins is dependent on the route of administration. The free fraction of S-verapamil is 12% after i.v. dosing and 23% after oral IR dosing and is always twice that of R verapamil (6% i.v. and 13% oral) (46). These differences in free fraction contribute to the volume of distribution of S-verapamil being about twice that of R-verapamil (6.4L/kg vs 2.7 l/kg) and plasma clearance of S being about twice that of R (18ml/min/kg vs 10 ml/min/kg) (46).

In patients with liver cirrhosis, pharmacokinetric parameters are grossly altered. Clearance decreases, elimination half life is prolonged and bioavailabilty more than doubles. In addition the volume of distribution increases (45). Renal disease does not appear to affect substantially the PK of verapamil.

Because of the complex PK associated with multiple dose administration and the variation in individual patient responsiveness to the drug, standard dosing recommendations are difficult to determine. Use of verapamil must be titrated to a clinical end point (45).

1.9 Sources of Pharmacokinetic (PK) Variability

Current PK data are difficult to interpret. There are wide variations in parameters reported and these are often contradictory. Factors affecting variation include age, diet, disease, environmental pollutants, ethnic background, genetic polymorphism, gender and formulation. Experimental approaches may also contribute, reflecting variation among subject size, subject variability (age, gender, disease state), study design, analytical assays and data analysis techniques. These factors can influence our interpretations and may affect our conclusions.

Age related metabolic differences for verapamil and other drugs have been documented. There are no standards defining how young is young and how old is old. Human and animal studies describing the effects of age on the metabolism of drugs have been reported. Different studies will have different definitions of young and elderly and their conclusions define age related attributes. Interpreting results is difficult and I will focus on trends associated with increasing age.

Physiologic changes need to be considered as a possible source of pharmacological variability. Body composition in the elderly is characterised by a decrease of lean body mass and an increase in total body fat. Such age related physiological changes, along with changes in hepatic function may be expected to result in PK changes. As body fat increases this leads to an increase in compartmentalisation resulting in an increased volume of distribution.

Changes in the gut associated with age include increased gastric pH and a decrease in gastrointestinal motility. Not much is known about drug metabolising enzymes in the gut with increased age (47).

Autopsy reports have shown a decline in weight of livers between the ages of 20-80 years (47). This may have important consequences on overall metabolic function and may contribute to impaired drug metabolism.

Pharmacodynamic responses may be altered among the very young (newborns) and the very elderly. Verapamil is administered in children for the treatment of supraventricular arrhythmias (SVT) and in the elderly for angina, hypertension and SVT.

The immature heart is less sensitive than the adult heart, this may be due to a number of factors including: 1) immaturity of the autonomic nervous system and autonomic receptors 2) PK factors affecting drug metabolism and distribution 3) ultrastructural properties related to drug and ionic permeation and 4) the influence of immature cellular metabolism on transmembrane permeability and ionic gradients (48). The elderly have less PR prolongation when given verapamil and it has been suuggested that this difference is attributed to impaired baroreflex response, altered sympathetic tone and perhaps altered parasympathetic tone associated with aging (10). These physiologic differences contribute to the difficulty observed in correlating therapeutic drug levels and pharmacodynamic (PD) response.

Albumin and alpha 1 acid glycoprotein are major plasma proteins that bind drugs. An increase in binding would result in a decrease in clearance causing an increase in bioavailability. Diseases such as arthritis have been associated with increased alpha 1 glycoprotein levels in plasma (49). Age associated changes in the levels of albumin and glycoprotein (49) have not been conclusive, however protein binding studies with verapamil consistently have reported changes in protein binding (1, 10).

Abernethy *et al.*(50) looked at a hypertensive population of young (23-36 years) and elderly (61-102 years) and found that the elderly had decreased clearance and a prolongation of half life when compared with the young. His data suggested that impaired drug clearance continues to increase with advancing age into the 80's and 90's. Swartz *et al.* (51) looked at stereoselective verapamil clearance and found that aging decreases both S and R verapamil clearance and was also related to an increase in elimination half life.

These studies suggest that the changes in clearance associated with aging are attributed to changes in metabolism and not volume of distribution. Since verapamil is used extensively among geriatric hypertensives, these studies provide important valuable information which may help improve current treatments.

1.10 Pharmacodymamics (PD)

Verapamil causes suppression of atrioventricular conduction, suppression of sinoatrial node automaticity, impairment in myocardial contractility and peripheral vascular relaxation (52). However, the clinical effects seen after verapamil administration are the summation of these direct drug effects and the responses of cardiovascular reflexes. One important reflex contributer is the baroreceptor mediated increase in beta adrenergic tone in response to peripheral vasodilation (50). S-Verapamil delays atrioventricular nodal conduction much more potently and is extensively metabolised during first pass. The pharmacologic potency of S-verapamil is 10-20 times greater than that of R-verapamil in terms of negative dromotrophic affect on AV conduction. (53). Echizen *et al.* (40) demonstrated that 3-4 times higher total plasma concentrations are required to elicit the same negative dromotrophic effect after oral as after i.v. administration. This difference is explained by stereoselective first pass metabolism which leads to preferential biotransformation of the more potent S-enantiomer, thus leading to the predominannce of R-verapamil in plasma.

Dog studies have demonstrated that NV has about 20% of the vasodilating activity of verapamil, wheras the other N-dealkylated metabolites have no vasodilatory activity (54). The O-demethylated metabolite of verapamil exhibits the same potency as the parent drug. However, their contribution is negligible to the overall pharmacological effect since this metabolite is present in the plasma as a glucuronide which has no pharmacological activity (23).

The effects of verapamil administration on AV node conductivity have been controversial. Neugebauer *et al.*(54) reported that none of the metabolites possess any dromotrophic effects on atrioventricular conduction. However, more recent evidence in the isolated rabbit heart by Johnson *et al.* (55) suggests that NV and D620 may posses significant effects on AV nodal conduction. In humans, Swartz *et al.* (51) demonstrated that during transesophageal pacing there is no change in AV nodal conduction and that changes in AV nodal conduction are a result of an indirect effect.

D617 and D620 have also been evaluated but their PD contributions appear to be minimal. Neugebauer (54) evaluated coronory blood flow in a canine model and found that NV had 22% the homodynamic potency of verapamil, while D617 had 3% and D620 had 4% of the potency of verapamil on the surface ECG. AV nodal conduction and refractoriness was not assessed for D617 and D620. The clinical significance is not known because the protein binding of these metabolites is not known. The greater the protein binding of these metabolites the less significant the effect will be.

Arnold *et al.* (48) looked at age related differences in the PD of verapamil in adult dogs and puppies. The ability of verapamil to prolong PR interval was not observed in puppies even though they had blood levels of verapamil equal to or greater than those seen in adult dogs. He postulated that this difference may be due to a reduced or immature slow inward current (I_{si}) and may account for the lack of effect on the PR interval in the puppies. Reflex tachycardia was not observed in the puppies even though they had a significant hypotensive response.

Human studies have demonstrated that the elderly have a greater hypotensive response compared to the young (1, 10). The young exhibit reflex tachycardia, compared with decrease in HR among the elderly. Less PR prolongation in the elderly after R and S verapamil has also been observed. However, these age related differences in AV conduction were not noted during transesophageal atrial pacing for R and S (51). No age related difference in AV conduction to either enantiomer was seen when pacing was used to eliminate frequency dependent effects caused by differences HR response. PR interval was therefore not an accurate reflection of AV conduction. So aging may be associated with differences in sensitivity.

The PD responses observed indicate an interaction between direct drug effect and age and disease related changes in the hemodynamic and autonomic nervous system function (10). Stereoselective PD are preserved with aging but BP, HR and PR interval show age dependent responses.

Table 1

Pharmacodymamics Summary	
PD effects	greater potency of S vs R
negative dromotrophic	10-20x
negative ionotropic	15x
negative chronotropic	5x
vasodilation	2.5x

Longethreth (46)

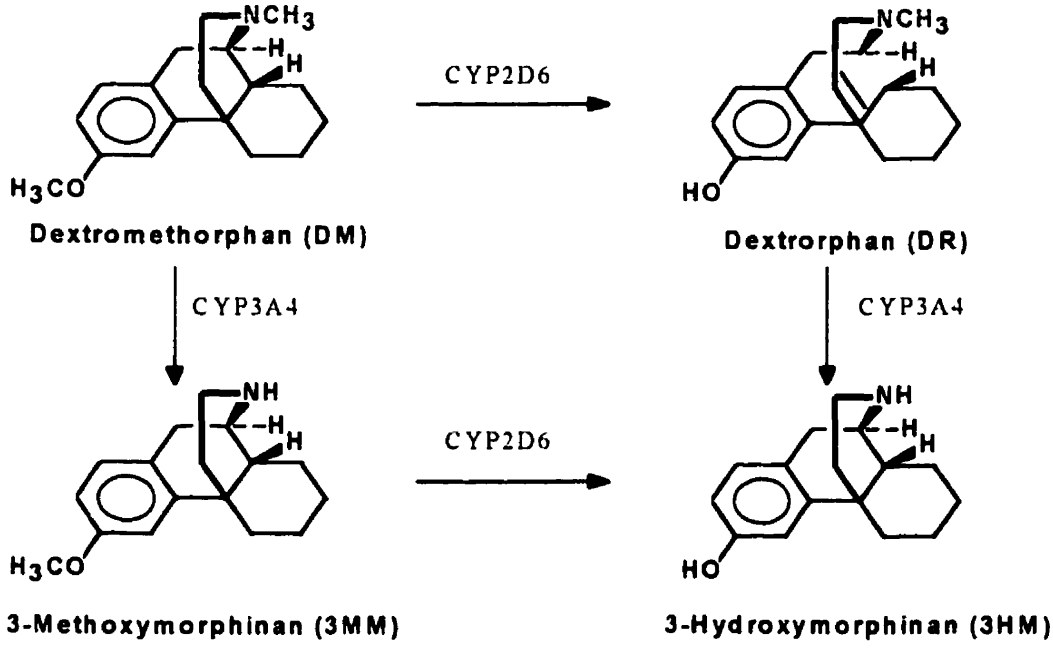
1.11 Protein Binding

Protein binding can be estimated by *in vitro* experiments. In vitro studies have shown that verapamil is bound to albumin (60%) and to alpha 1-acid glycoprotein (56). Verapmil has been shown to be enantiooselective in protein binding with the free fraction of S-verapamil being twice that of the R-enantiomer (43), thereby contributing to the stereoselective disposition observed *in vivo*.

In man, verapamil is highly bound (90%) to plasma proteins (57). No age related differences have been observed in protein binding (1).

1.12

DEXTROMETHORPHAN



Dextromethorphan is a non-narcotic, non-prescription drug commonly used for coughing. Robitussin DM is a cough syrup that is available over the counter. Side effects associated with a single dose of Robitussin DM may include nausea and dizziness, however these occur rarely and disappear rapidly if they occur. DM undergoes parallel O-dethylation to dextrophan (Dex) via CYP2D6 and N-demethylaytion to 3-methoxymorphinan (3MM) via CYP3A4. 3-hydroxymorphinan , a didemethylated metabolite is formed secondarily. (58).

The metabolism of DM as a probe drug has been used to probe genetically determined CYP2D6 activity and has been investigated *in vivo* and *in vitro* (59). The

relationship between genotyping and phenotyping of CYP2D6 is over 90% (60) and its use as a suitable probe of CYP2D6 remains uncontroversial.

There have been no reports identifying a genetic polymorphism for CYP3A4. However, there is considerable interindividual variation associated with its expression. DM is currently being investigated as a probe of CYP3A4 activity and its use as a suitable probe of CYP3A4 is controversial.

Gorski *et al.* (61) characterised DM N-demethylation in human liver microsomes. His findings indicated that there was no significant correlation between 3MM formation and association between CY1A2, CYP2D6, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP2E1. He concluded that 3MM formation appeared to be catalysed primarily by CYP3A4 and to a lesser extent by CYP3A5 *in vitro* in humans. Recently, through inhibition studies, Schmider *et al.* (58) investigated in an *in vitro* system the formation of Dex and 3 MM. His results demonstrated that that formation of 3MM is not solely mediated by CYP3A3/4 and may not constitute a suitable index reaction .

In vivo data regarding the use of DM as a probe for CYP3A4 are promising. Jones *et al.* (62) investigated the use of DM as an *in vivo* marker of CYP3A4 and found that DM was a suitable CYTP3A4 probe which was independent of CYP2D6 activity. Ducharme *et al.* (5) found similar results and were able to correlate the AUC of NV with DM/3MM. P450 CYP3A concentration can be estimated by an erythromycin breath test which can be used to calculate the initial cyclosporin dose requires to obtain cyclosporin blood trough concentrations in the therapeutic window (63). Phenotyping of individuals with erythomycin has revealed a large degree of interindividual variability, but no evidence of polymorphism (64). However, its usefulness in predicting the fate of orally administered drugs remains unclear (5).

There is considerable interindividual variation in both microsomal CYP3A4 activity and CYP3A4 substrate disposition (65). CYP3A4 activity has been identified in the gut (4) and has significant effects on the presystemic metabolism of orally administered drugs (30). Since there is considerable interindividual variation associated with CYP3A4 activity, identifying a suitable orally administered probe drug would represent an important advance in clinical practice.

Chapter 2

METHODS

2.1 Subjects

Nineteen non smoking healthy males who were taking no medication and had normal physical examination and screening laboratory parameters (complete blood count, electrolytes, liver function tests, urinalysis, and electrocardiogram) participated after giving written informed consent to the protocol which had been approved by the Roger Williams Hospital human research review committee. Eight younger subjects (mean ± SEM age 22 ± 1 year, all < 30) were compared to 11 older subjects (69 ± 1 year, all > 65 years). All subjects were within 25% of their ideal body weight and had no acute illness (e.g. viral syndrome) for at least 2 weeks prior to study.

2.2 Study Design

An achiral method was developed to measure verapamil and metabolites in the sustained release samples. Subjects were recruited at Roger Williams Hospital Clinic Research Unit in Providence, R.I.. The subjects took a SR formulation of verapamil hydrochloride (Calan SR, G.D. Searle and Co.) by mouth every 24 hours. This was continued for 6 doses at which time the subject was admitted to the Clinical Research Unit. The 7th dose was administered at 0800 hours and blood samples (10 ml) were taken at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4, 5, 6, 7, 10, 12, 16, and 24 hours from an indwelling 20 gauge cannula which was placed in the arm prior to the last dose. Plasma was separated by centrifugation and stored at -80 C° until time of analysis.

In order to determine individual patterns of metabolism, the volunteers took two teaspoons (10 ml) of cough syrup containing the test drug dextromethorphan (DM) (Robitussin-DM) prior to verapamil treatment. The volunteers collected two (2) urine samples

(of about 20 ml, or four teaspoons, each): the first one before ingesting DM (pre-dose urine sample) and the second one 4 hours after DM ingestion (4-hr urine sample). During those 4 hrs, the volunteers did not need to collect their urine or to abstain from urinating. However, during those 4 hrs, the volunteers abstained from coffee or any caffeinated beverage (colas, hot chocolate, tea).

All urine and plasma samples were analyzed in the Pharmacokinetics Laboratory at the Montreal General Hospital laboratory using a previously developed and validated analytical method (5).

2.4 Chemicals

Verapamil HCL (alpha-(3-((2-(3,4-dimethoxyphenyl) ethyl) methylamino) propyl) -3,4 - dimethoxy - alpha- (1-methylethyl) hydrochloride, norverapamil HCL, D-617 HCL, D-620-HCL, PR-22 HCL, PR-25 HCL and gallapmil HCL (internal standard) were all provided by Searle Research and Development (Chicago, IL, USA)(Figure 1).

Acetonitrile (CH3CN), acetic acid and methanol, all of analytical grade were purchased from J.T. Baker. Diethylammonium acetate was supplied by A&C (American Chemical Ltd.). Hydrochloric acid (HCL) was supplied by BDH (Ville St.Laurent, Canada). Distilled water was obtained from a Millipore, Milli-Q water delivery system.

2.5 Apparatus

The modular high performance liquid chromatography (HPLC) system consisted of a Spectra Physics P-2000 modular pump, a Spectra Physics AS-3000 auto sampler, a Spectra Physics FL-2000 fluorescence detector, a Spectra Physics analytical data jet integrator. The excitation wavelength and the emission wavelength of the fluorescence detector were set at 236 and 310 nm respectively. The column configuration consisted of a Regis C18 guard column and a 15 cm reversible ODSII Regis column coated on a 5 micron silica gel support .

2.6 Chromatoghraphic conditions

The chromatographic separation of verapamil and metabolites was achieved with a mobile phase (MP) consisting of distilled water (0.3%DEA) pH 4 - acetonitrile (69:31, v,v). The pH was adjusted to 4 by using acetic acid before diluting to the appropriate volume. The MP was prepared in 4 litre batches and degassed under vacuum conditions through a 0.45 micron membrane supplied from Millipore. The flow rate was 1.0 ml /min and ambient temperature was used throughout the study.

2.7 Aqueous Stock Solutions

Verapamil and the metabolites were weighed and dissolved in methanol at the following concentrations: Verapamil (10000 ng/ml), NV (10000 ng/ml), PR22 (2000 ng/ml) PR25 (2000 ng/ml), D620 (2000 ng/ml), and D617 (2000 ng/ml). These solutions were stored in the dark in a -80 C° freezer . Appropriate volumes were taken from each solution and placed in a 50 ml polypropylene tube and evaporated to dryness under a stream of air to prepare a stock solution containing the parent compound and its metabolites. Appropriate amounts of the stock solution were combined and diluted with the MP to give solutions of the following concentrations: Verapamil and norverapamil at 10 micrograms/ml and the other metabolites, D617, D620, PR22, PR25, at 2 μg /ml (stock solution A) and stored in the dark at -20 C°. Serial dilutions of the stock gave rise to working solutions containing parent compound and metabolites of 2.8, 2.4, 1.9, 1.4, 0.96, 0.48 and 0.24 micrograms/ml.

2.8 Standard Curve

A 7 point standard curve was prepared by serial dilution. 2.4 ml of stock solution A was adjusted to a volume of 40 ml in a 50 ml polypropylene tube resulting in a concentration of 600 ng/ml for verapamil and NV and 120 ng/ml for the remaining metabolites. By serial dilution the following samples were prepared for verapamil and NV at the following concentrations : 500, 400, 300, 200, 100 and 50 ng/ml. The remaining metabolites contained

120, 100, 80, 60, 40, 20 and 10 ng/ml. Triplicate samples were prepared for each concentration. Calibration curves plotting analyte plasma concentration as a function of the analyte/IS peak area ratios were derived for verapamil and each of the metabolites. The recoveries were determined by using spiked plasma concentrations for verapamil and NV at 600, 300 and 50 ng/ml whereas the other analytes, D-617, D-620, PR22- and PR-25, were at 120, 60, and 10 ng /ml. The peak area ratios of the 3 extracted samples of the analytes were compared to 2 aqueous unextracted samples to derive percent recovery.

2.9 Accuracy and Precision

For intra day and inter day validation studies, control samples were prepared from drug free plasma with verapamil and metabolites added at 3 different levels. The low control sample contained 75 ng of verapamil and NV as well as 15 ng/ml for the other metabolites(D-617, D-620, PR-22, PR-25), medium control contained 333.3 ng/ml of verapamil and NV as well as 66 ng/ml of the remaining metabolites, the high control contained 540 ng/ml of verapamil and norverapmil as well as 100 ng/ml of the remaining metabolites. A dilution assay was also assessed to verify if dilution of samples off the curve could be reliable read. This became an issue during the quantification of patient samples. Occasionally, a patient would have a value for D617 or D620 which was not on the curve. By diluting a series of known spiked plasma samples, we could accurately predict the concentration with a coefficient variation of 6%.

2.10 Assay of Plasma samples

The chromatograms resulting from the analysis of blank plasma and plasma spiked with internal standard are presented in figure 4. Standard curves were derived for verapamil and metabolites with the following regression equations and correlation coefficients (table 2).

Table 2	Regression Equations derived from standard curves
Analyte	**Equation**
Verapamil	y = 0.0065x - 0.081
Norverapamil	y = 0.0063x - 0.091
PR25	y = 0.0028x-0.0089
PR22	y = 0.0052x -0.0048
D617	y = 0.004x - 0.00058
D620	y= 0.0047x - 0.0025

Table 3	Chromatographic parameters
Analyte	k'
Pr-25	6.0
D-620	8.0
D-617	10.0
PR-25	30.0
Verapamil	60
Norverapamil	70
Gallapamil	80

2.11 Sample Preparation

After thawing, the plasma samples were vortexed vigorously for 1 minute and 600 ul was transferred to a 1.5 ml amber polypropylene microcentrifuge tube followed by an equal volume (600 ul) of the internal standard gallapamil (0.5ug/ml). The sample was centrifuged for 30 minutes at 13000 RPM. A 1 ml C18 solid phase extraction column (Wennick Scientific)

was conditioned with 2 ml of 100% methanol followed by 2 ml of distilled water. One ml of the resulting supernatant was added to the column and under vacuum conditions the eluent was collected in a plastic tube and discarded. In order to avoid sample contamination the columns were briefly removed from the speed mate vacuum system and replaced after the unit was thoroughly washed with methanol and a sterile glass centrifuge tube was used to collect the sample. The analytes were eluted with 2 ml of methanol (0.3% DEA) and evaporated to dryness under a stream of air. Residues were reconstituted in 250 ul of 0.01 NHCl and centrifuged for 10 minutes at 10C in a Sorval RT60000B refrigerated centrifuge from Dupont. The sample was then placed in a 250 ul autosampler vial and 50 ul injected into the chromatographic system.

Table 4
Recovery of Verapamil and its metabolites from Serum (%Recovery)

Compounds	120 ng/ml	60 ng/ml	10 ng/ml
D617	81.9 ± 8.6	95.3 ± 6.0	81.6 ± 5.3
D620	81.6 ± 11.1	88.1 ± 12.5	87.8 ± 1.4
PR22	82.5 ± 0.9	75.8 ± 8.5	64.8 ± 7.8
PR25	76.4 ± 8.8	81.1 ± 9.7	66.1 ± - 5.6
Compounds	600 ng/ml	300 ng/ml	50 ng/ml
Norverapamil	87.7 ± 2.2	87.5 ± 5.1	74.27 ± 11.6
Verapamil	87.6 ± 2.9	89.2 ± 4.4	76.9 ± 3.3

Figure 4: Actual Chromatogram from an elderly patient at 7 hours

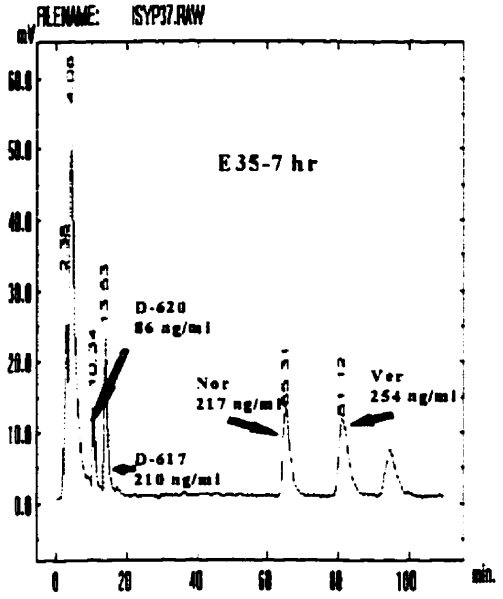


Figure 5: Actual chromatograms representing spiked and blank plasma samples with internal standard (gallapamil).

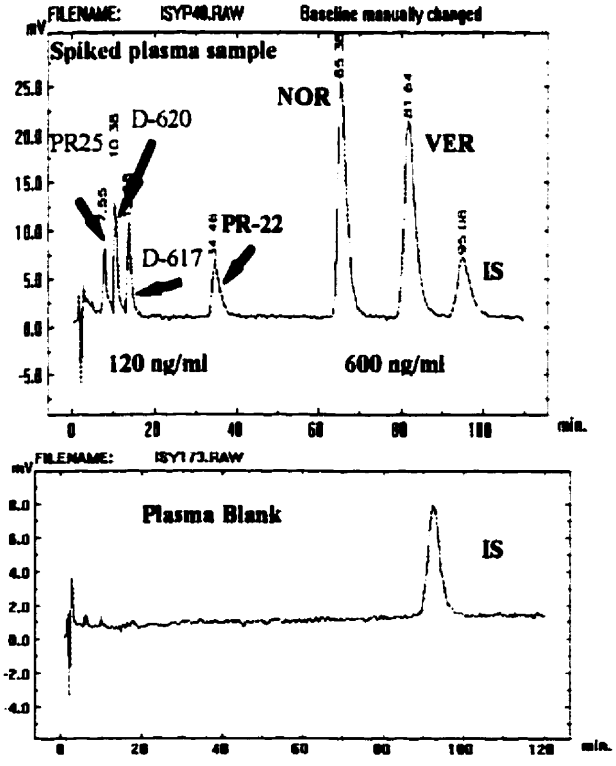


Figure 6

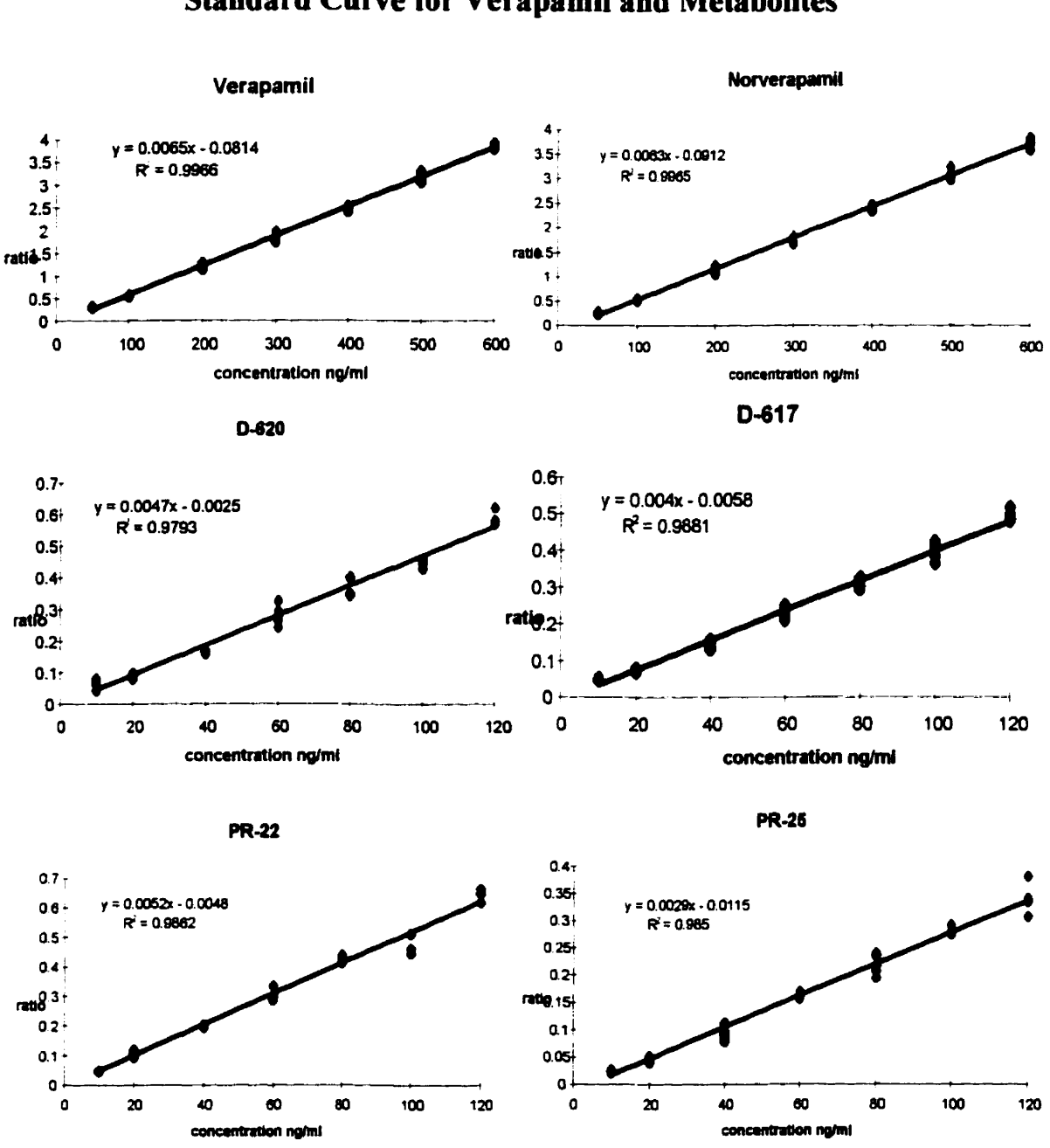


Figure 5 :Y axis represents ratio of analyte/ internal standard (gallapamil). X axis represents prepared plasma sample concentrations (ng/ml) of the analyte. Patient samples were analysed using regression equations derived from these standard curves.

2.12 Pharmacokinetic Data Analysis

Noncompartmental pharmacokinetic parameters were derived from the plasma concentration time curve. The areas under the concentration time curves (AUC) were computed by the trapezoidal rule. Oral clearance was derived from the following :

$$\text{Cl oral} = \frac{Cl}{F} = \frac{D}{AUC\ (0\text{-}24)}$$

The dose (D) was 240 mg for the racemic SR formulation. In order to calculate bioavailability (F) samples were analysed after *I.V.* constant infusion at steady state and was computed by the following:

$$I.V.\ Cl = \frac{Ko}{Css}$$

Ko is the steady state infusion constant (mg/hr) and Css is the concentration (mg/L).

Oral Bioavailability was calculated by $F = \frac{\text{Clearance oral}}{\text{Clearance } I.V.}$

Half life could not be accurately assessed due to sampling schedule and formulation.

2.13 Statistical Analysis

Data are presented as mean values +/- standard deviation (SD). Data were compared for age differences using the Mann-Whitney rank sum test since the data are not normally distributed. The level of statistical significance was set at 0.05.

Spearman rank correlation coefficient was used to investigate relationships between DM/3MM vs Molar AUC's of verapamil and metabolites and molar ratios of verapamil to metabolites. The Spearman test was chosen because the data do not appear to obey assumptions of parametric statistics.

Chapter 3

Results

3.1 Non Compartmental Pharamacokinetic Parameters

Mean plasma concentration time curves of total verapamil and metabolites were measured non stereospecifically and are shown in Table 5. PR22 and PR25 were not detected in any subject samples. These analytes undergo glucuronidation *in vivo* and are rapidly excreted as conjugates (66).

The data are presented as mean values ± SD (Table 5). Mann-Whitney test was used to compare the young and elderly . CL_{oral} in the young was 20 ± 6 and 35 ± 7 ml/min/kg in the elderly. Statistical analysis found significant differences for oral clearance between the 2 groups ($p< 0.001$).

Racemic verapamil was administered by zero order at an infusion rate calculated to maintained 200 ng/ml . Total clearance was calculated by the following equation.

$$\text{Total clearance} = \frac{\text{infusion rate (mg/hr)}}{\text{concentration at steady state (mg /L)}}$$

Oral bioavailability (F) was determined by the following relationship: $F = \frac{CL\ total}{CL_{oral}}$

F after SR steady state administration in the young was 36% ± 17 and in the elderly 33% ± 7. Non significant differences were detected between the 2 groups in the 2 populations.

Area under the curve (AUC) was calculated by the trapezoidal rule at 0-24 hours and is presented in Table 5 and Figure 7. Verapamil AUC in the young was 1583 ± 457 and in the elderly was 2570 ± 589 ng·hr/ml ($p< 0.005$). The AUC for NV in the young was 2060± 457 and in the elderly 2670 ± 596 ng·hr/ml ($p<0.05$). Non significant AUC differences were detected for D617 and D620. These data suggest that aging decreases verapamil clearance

leading to significant increases in pharmacologically active parent and norverapmil (20% activity) levels in the systemic circulation.

The elimination half life could not be accurately estimated due to the sustained release characteristics of the drug and the sampling schedule. In order to assess the half life all processes of absorption, distribution and clearance of the drug should be "first order" (67). Single dose is therefore preferred to multiple dose administration particularly since verapamil exhibits multicompartmental PK during multidose administration (68). After the distribution phase is complete, the serum concentration curve enters a slower release phase of disappearance, due mainly to irreversible drug elimination or clearance. Elimination half life is calculated based on the pattern of decline during the slower elimination phase, after the equilibrium of drug distribution is complete (67).

Plasma samples were obtained at steady state following oral administration of 240 mg of a SR preparation. The formulation has been designed to release the active drug at a slower rate in order to maintain therapeutic drug levels following once daily administration. Since plasma samples were taken for 24 hours it is difficult to properly assess the elimination phase of this formulation. Figure 18 presents the concentration time curves for 6 subjects. E31 has an elimination phase which may allow an accurate assessment of half life, however, subjects E34 and Y08 demonstrate the difficulty in assessing elimination half life after steady state administration.

The volume of distribution (Vd) is a hypothetical volume term relating the total amount of drug in the body to its concentration in serum or plasma. The relationship between half life , Vd, and clearance can be expressed as follows:

$$\text{Half life} = \frac{0.693 \times Vd}{\text{Clearance}} \quad (8)$$

Since we are unable to assess half life we can not calculate the Vd. These non compartmental PK parameters have been calculated extensively in the literature. During multidose administration the half life has been reported to range from 4.5 to 12 hours (product monograph). The Vd ranges from 103 to 597 litres (44) following oral administration of verapamil.

Table 5 Pharmacokinetic parameters for SR verapamil at steady state in the Young and Elderly

Phamacokinetic parameters		Young n=8	Elderly n=11
Age(years)		22 ± 6	69 ± 1
Weight (kg)		79 ± 3	81 ± 3
CL oral (ml/min/kg)		20 ± 6	35 ± 7 *
Biavailability (%) F		36 ± 17	33 ± 7
AUC (0-24) ng· hr/ml)			
	Ver	1583 ± 457	2570 ± 589 **
	NV	2060 ± 457	2670 ± 596 ***
	D-617	1826 ± 442	2152 ± 616
	D-620	729 ± 93	802 ± 235

Data are mean values ± SD.
Mann-Whitney analysis obtained the following significant results. *p <0.001,
** p < 0.005, *** p < 0.05

Figure 7

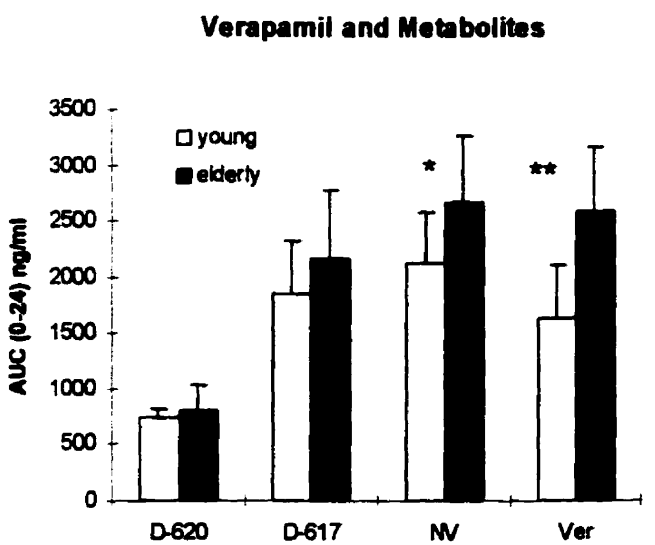


Figure 7:Data presented as mean ± SD. Mann Whitney analysis showed significance for AUC(0-24) concentrations of NV* and Verapamil** * p< 0.05 ** p< 0.005

3.2 Molar Parent Compound to Metabolite Ratio for Verapamil

The molar ratios of parent compound to metabolite were evaluated as an index of metabolic activity. The following molecular weights were used to transform our data to molar values: verapamil 491 g/mol, NV 477 g/mol, D617 327 g/mol, and D620 313 g/mol. The main enzyme involved in the N demethylation of verapamil to NV is CYP3A4, but other enzymes, including CYP2C and CYP1A2, have also been implicated in this reaction. (21, 22, 26) Verapamil N-dealkylation to D-617 is mainly mediated by CYP3A4 but there are also suggestions that CYP1A2 and CYP2C play an role in this pathway. The conversion of NV to D620 is mediated mainly by CYP3A4, and there is also evidence suggesting a minor role of CYP2C and CYP1A2.

The data show that the elderly have greater mean ratios of ver/NV, Ver/D-617 and NV/D-620 (Figure 8). These ratios were compared using a Mann-Whitney test and significance was found for Ver/NV and NV/D620. This data suggest that the elderly are less effective in these metabolic pathways.

Figure 8

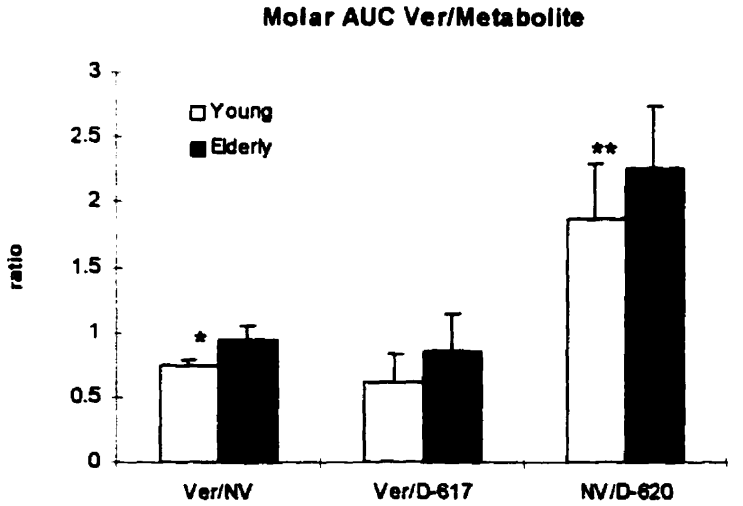


Figure 8 Data are presented as mean ± SD. Mann Whitney showed significant difference in the Ver/NV ratio * $p < 0.001$, NV/D620 **$p < 0.05$ among the young and elderly.

3.3 Probit Analysis of Verapamil/Metabolite Molar ratios

In order to determine whether we are looking at two distinct populations (fast and slow) of metabolizers, a probit analysis of the data was done (Figures 9, 10). This will allow us to determine whether the differences in metabolism are part of a normally distributed population or whether we are dealing with a skewed population.

Ver/D-617: Probit analysis suggests a better fit with a bimodal distribution (Figure 11). The antimode was calculated by the following regression equations:

Extensives metabolizers $y = 4.04x + 2.40$
Poor metabolizers $y = 9.04x + 2.90$

The antimode was calculated by solving the equation for x which is the point at which the two populations cross. The antimode is equal to log -0.0996 which relates to a Ver/NV ratio of 0.8 The frequency distribution suggests a trend with the young being skewed to the left (fast) and the elderly being skewed to the right (slow).

Ver/NV: We have previously shown that there is a statistical significance between the young and elderly when mean ver/nv ratios were looked at. Our probit analysis suggest that our data fit better with a one population model. However, if we look at the frequency distribution in figure 11 there appears to be a trend suggesting that the young are skewed to the left (fast metabolizers) and the elderly are skewed to the right (slower metabolizers).

NV/D-620: The probit analysis suggest a better fit with a one population model and the frequency distribution appears normally distributed without any obvious age related trends.

Probit Analysis of Verapamil/Metabolite Molar AUC ratios among the Young and Elderly

Figure 9

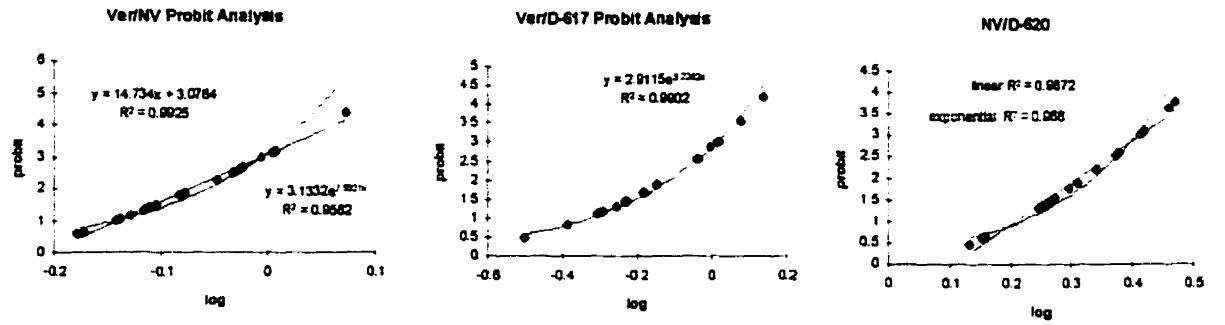


Figure 9 Probit analysis of Ver/NV, Ver/D-617 and NV/D-620. Ver/NV and NV/D-620 are fitted better with linear regression suggesting a unimodal population. Ver/D-617 is fitted better with by exponential regression suggesting a bimodal population.

Figure 10

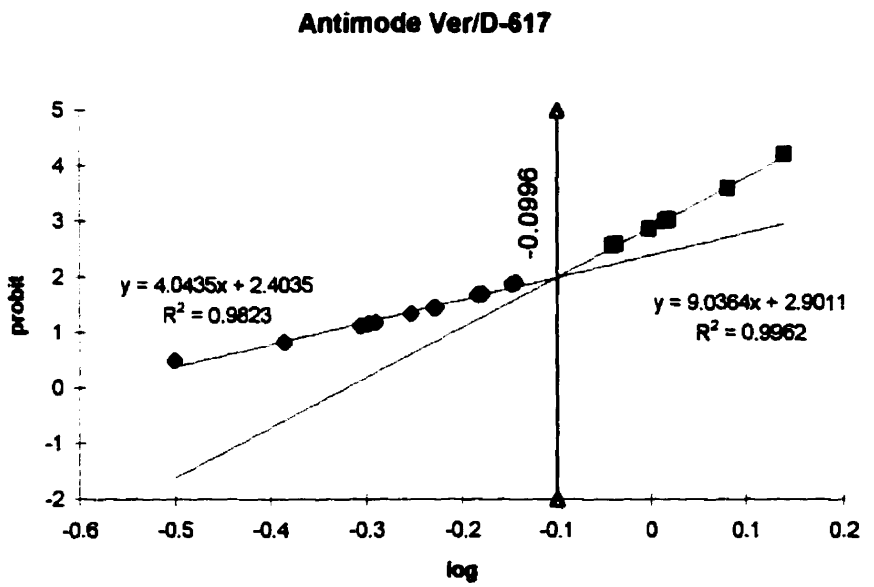


Figure 10: Probit analysis reveals an antimode occurring at a verapamil/D617 ratio of 0.79 and represents the theoretical division among extensive and poor metabolisers.

Frequency Distributions of verapamil / metabolite Molar AUC ratios among the young and elderly

Figure 11

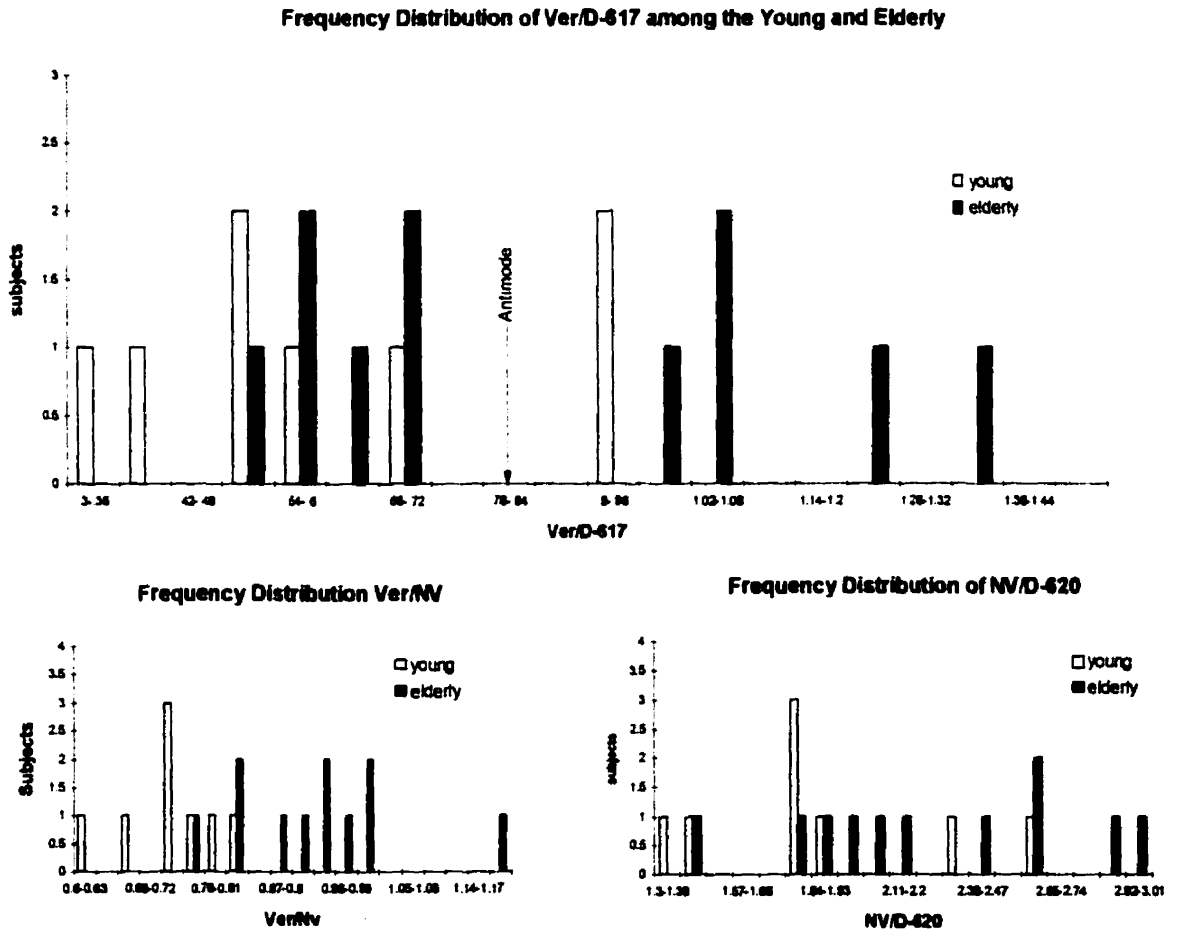


Figure 11: Frequency distribution of the young and elderly for Ver/NV, Ver/D-617 and NV/D-620 Molar ratios.

3.4 Urine Molar ratios of DM/3MM

After collecting a pre dose morning urine, the subjects (11 elderly and 8 young received a single oral dose of 10 ml of Robitussin DM containing 30 mg of DM hydrobromide or 23 mg of DM base. At 4 hours after DM administration a 4 hour spot urine was collected.. The major pathway involved in DM metabolism is mediated by CYP2D6 and has been successfully used as an index of 2D6 activity. In vitro studies have shown CYP3A4 as a minor pathway in DM's metabolism. To illustrate the predominance of this pathway the urinary levels of DM, 3MM and Dextrophan are shown (Figure 12). Recently, Ducharme et al (5) described analytical and clinical data for the simultaneous determination of CYP2D6 and CYP3A4 activity. Current scientific data suggest that the gut wall is an important source of prehepatic metabolism and DM appears to be a promising probe to evaluate the first pass effect.

CYP3A4 phenotype was determined in the young and the elderly by using the urinary molar ratio of DM over 3MM as an index of CYP3A4 activity (Figure 13). The molar ratios of DM/Dex are used to assess the CYP2D6 activity and have been included only for illustration. These data were not used in our analysis since there have been no reports of CYP2D6 activity in the metabolism of verapamil. Molar ratios must be to evaluate data since we are not collecting total urine and monitoring fluid intake. Molar ratio therefore allow us to assess individual CYP3A4 activities.

DM/3MM ratio in the young ranged from 0.5 - 1.6 and from 0.63 - 4.1 in the elderly. No statistically significant difference was found between the two groups. Two elderly subjects were clearly poor metabolizers and allowed us to calculate an antimode. Since we expected a small proportion of the population to be poor metabilizers of CYP3A4, these results were not surprising. The regression equations used to calculate the antimode were:

Poor metabolizers $y=9.25x - 0.734$

Extensive metabolizers $y=2.65x + 1.62$

The point at which the two lines cross represents the antimode and can be calculated by solving for x. The antimode is equal to 0.357 log which relates to a DM/3MM 2.27.

Figure 12

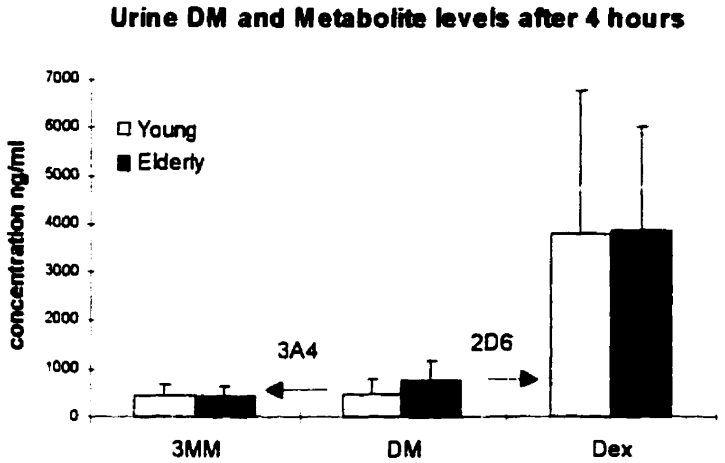


Figure 12: This representation has been provided to illustrate the predominant pathway in the metabolism of DM which is mediated by CYP2D6.
Abbreviations: Dextromethorphan (DM), 3 methoxymorphinan (3MM), dextrophan (Dex). Data are presented as mean ± SD. Mann Whitney analysis showed non significant differences among urine concentrations of DM, 3MM and Dex among the young and elderly.

Figure 13

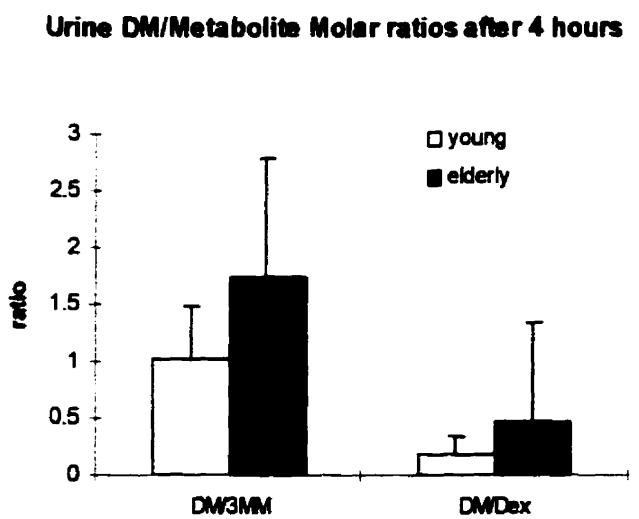


Figure 13 Data presented as mean ± SD. Mann Whitney showed non significant differences between the young and elderly and their DM/metabolite ratios.

Figure 14 .

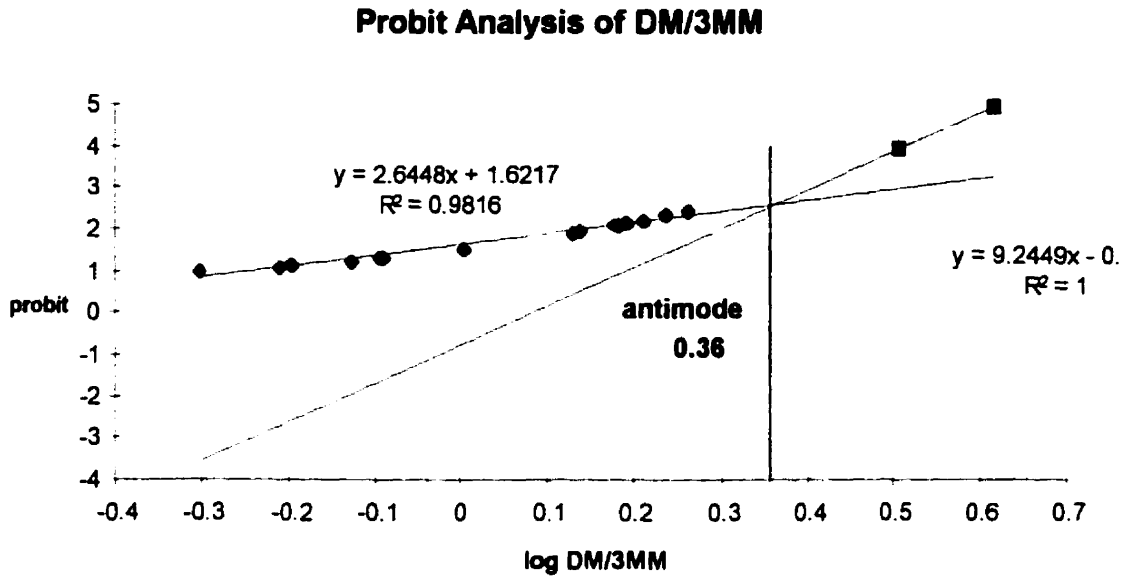


Figure 14: Probit analysis and frequency distribution for DM/3MM

Figure 15

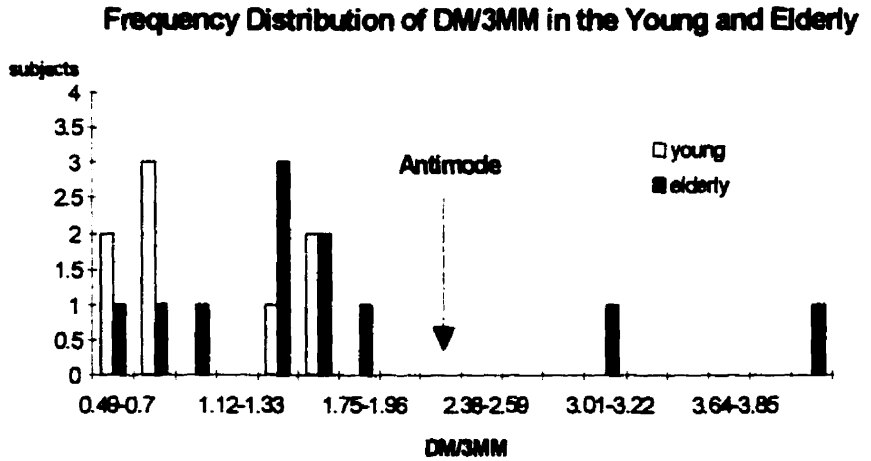


Figure 15: Frequency distribution of molar dextromethorphan (DM) over molar 3 methoxy morphinan (3MM) among the young and elderly. Extensive and poor metabolisers are divided by an antimode of 2.2

Probit Analysis of Molar AUC Verapamil and metabolite levels among the young and elderly

Figure 16

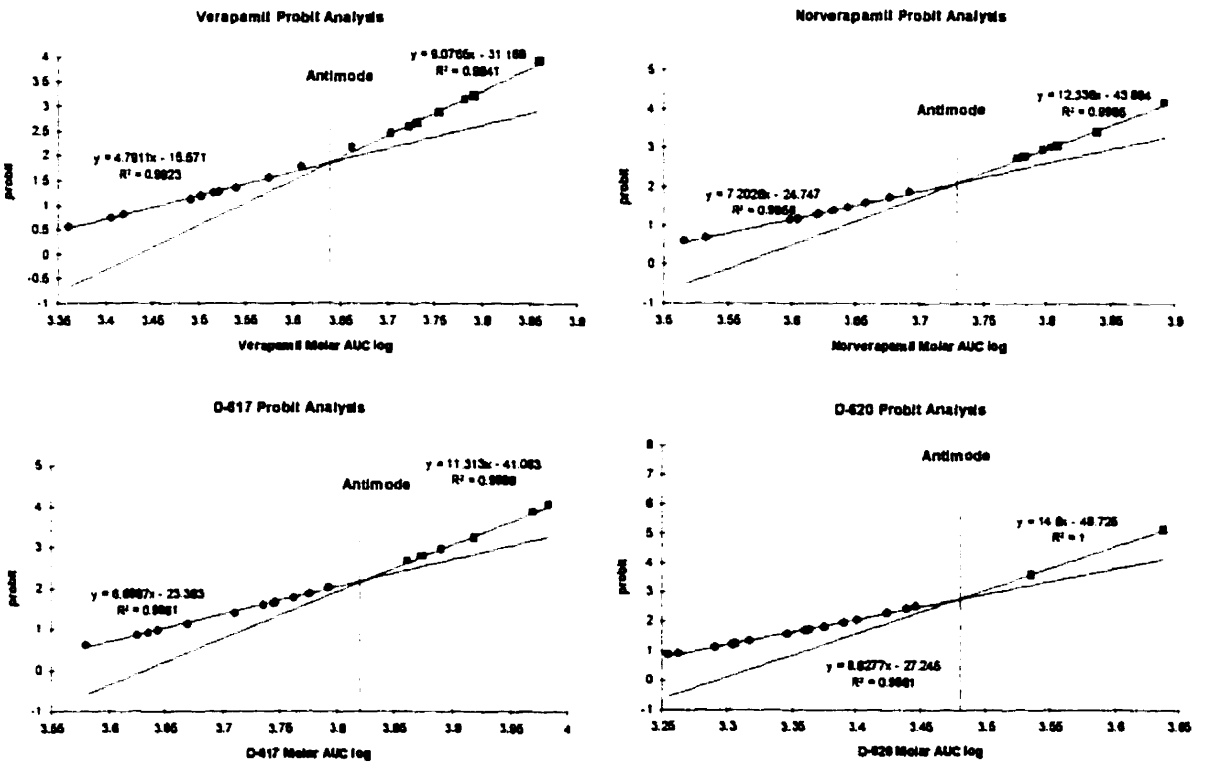


Figure 16: Probit analysis of verapamil and metabolite molar AUC levels among the young and elderly

Frequency Distributions of Verapamil and Metabolite Molar AUC's among the Young and Elderly

Figure: 17

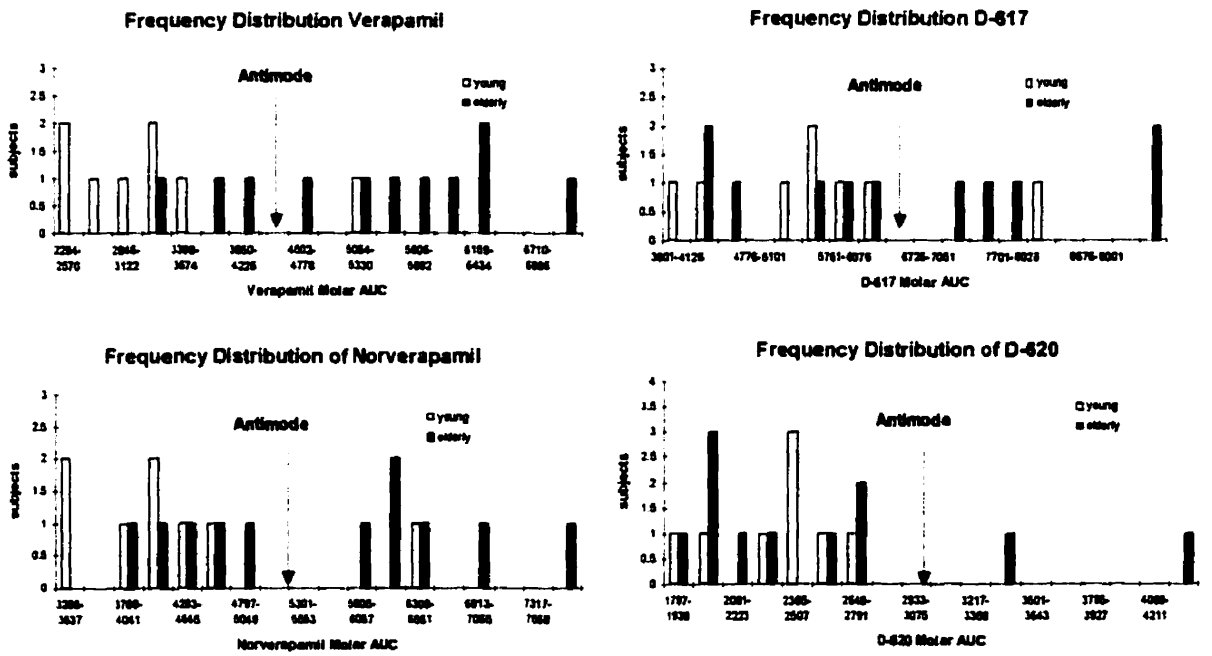


Figure 17: The antimode was calculated following probit analysis for Verapamil, NV, D620 and D617.

3.5 Correlation of DM/3MM with Verapamil's Metabolism

DM/3MM ratios were poorly correlated with Ver/NV (r_s 0.27), Ver/ D-617 (r_s -0.11) and NV/D-620 (r_s 0.05) with the young and the elderly (figure 8). Correlations were also done separately between the young and elderly, with r_s values equally as low. However, when correlations were made with molar AUC levels of verapamil, NV, D617 and D620, the results were much more promising and are presented in Table 6 and Figure 7.

Among the young there appears to be a correlation with D617 AUC Molar levels with an r_s value of 0.38 and a decreased correlation with D620 (r_s 0.24) which is a secondary metabolite formed after the transformation of NV (figure 1). It is not surprising that this is a less specific indicator of enzyme activity. However in the elderly the correlation of D617 (r_s 0.48) and D620 (r_s 0..46) are similar. The correlation with NV (r_s 0.52) in the elderly is the most significant pathway.

Table 6

Spearman Rank Correlation

	Young and Elderly	**Elderly**	**Young**
AUC	DM3MM	DM3MM	DM3MM
D620	0.32	0.46	0.24
D617	0.51	0.48	0.38
NV	0.28	0.52	-0.38
VER	0.34	0.26	-0.21
VER/NV	0.27	-0.22	0.02
VER/D617	-0.11	-0.12	-0.48
NV/D620	0.05	-0.10	-0.43
WT	-0.16	-0.37	-0.31

Table 6: Values are presented as r_s. Negative values indicate a negative correlation between verapamil and metabolite AUC's and ratios. AUC are in Molar/hr.

Figure 18

Inter Individual Variation in the Metabolism of SR

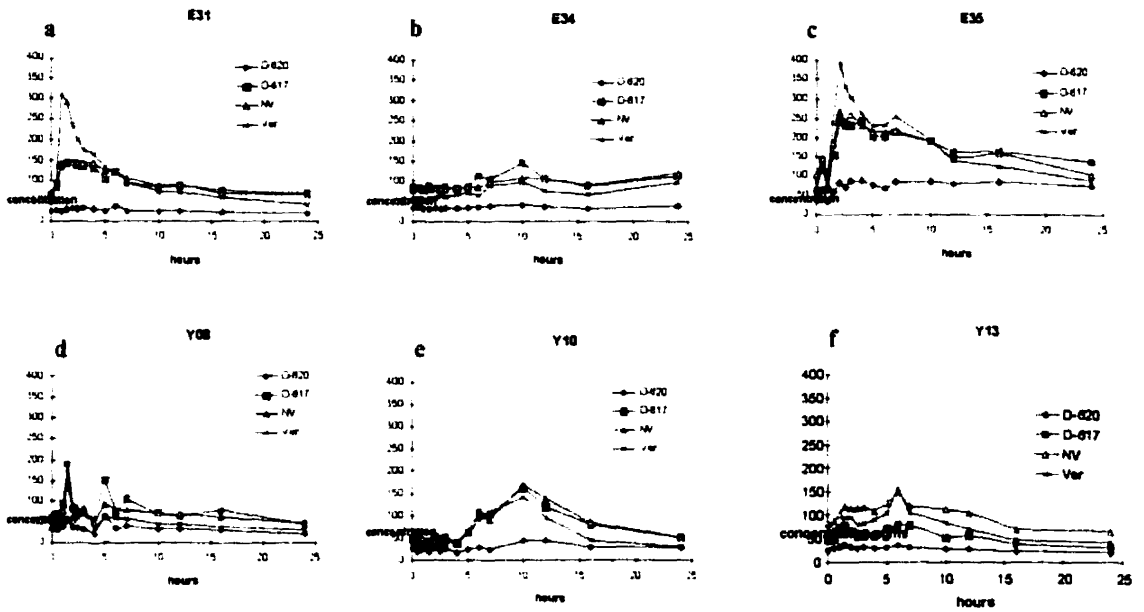


Figure 18: Plasma samples were measured by HPLC. Blood samples were taken during a 24 hour period. Verapamil and metabolite concentrations are in ng/ml and time is in hours.

Chapter 4

Discussion

There are 3 types of formulations commercially available for verapamil, immediate release, sustained release (SR) and intravenous. The route of administration (oral vs *I.V.*) and formulation (IR vs SR) will have profound influences on verapamil pharmacodynamics (PDs) and pharmacokinetics (PKs) . There are age related differences with the metabolism of verapamil (1,10) and there is some evidence suggesting that gender may also be a factor (2). The greatest contribution to diversity is likely a reflection of the diversity observed among cytochrome P450's.

P450s make up a large percentage of the enzymes produced in liver and there is evidence demonstrating both inducibility and inhibition of these enzymes (69). In clinical practice therapeutic dosing is generally based on body weight and occasionally on age, disease state and clinical response. Rarely are genetics considered or how much grapefruit juice (inhibitor) or cigarettes (inducer) patients have per day. Identifying appropriate probe drugs to reliably assess enzymatic activity may be useful to physicians by providing a means of optimizing treatments and reducing drug related toxicities. It may be argued that this is too unrealistic and costly to be a part of clinical practice. This may be true in the treatment of hypertension where dosing can be adjusted to a clinical endpoint (reduction of BP) without significant consequences to the life of the patient. However, in HIV infection and cancer, knowing a patient's enzymatic profile may be a life saving tool.

Maintaining therapeutic levels of drug in HIV therapy is critical in suppressing viral growth. This situation may also be applicable in cancer therapy except the target is malignant cells. Our laboratory is currently trying to identify suitable probes to provide individual enzymatic profiles for CYP2D6, CYP1A2, CYP3A4, N-acetyl transferase (NAT2) ,glucuronidation and sulfation phenotypes in HIV and cancer patients. The physiologic changes associated with disease progression, as well as exposure to multiple drug therapies may significantly influence metabolic activity. By monitoring individual

metabolic activity physicians can modify therapies to ensure optimal therapeutic responses throughout the course of the disease.

CYP3A is one of the most important cytochrome P450 isoforms responsible for drug metabolism by humans because it is the major such enzyme in critical tissues such as the gastrointestinal tract and liver (28). This study has been designed to evaluate the use of dextromethorphan (DM) as an *in vivo* probe of CYP3A4 activity. The erythromycin breath test has been used to measure CYP3A activity (70) and has been applied in the treatment of cyclosporin A (69). However, this test requires the use of *i.v.* administration of radioactive erythromycin and is doubtful that that it can be used to predict the fate of an orally administered drug.

Verapamil is mainly metabolised by CYP3A4 and *in vitro* evidence has also suggested a minor role of CYP1A2 (3) and CYP2C8 (21,22) in verapamil metabolism. CYP3A activity can be readily modulated by inducers like rifampicin and several anticonvulsant agents, and many important inhibitors exist such as azole antifungal agents and macrolide antibiotics.(71). Metabolism involving CYP3A is also likely affected by liver disease as well as aging (71) with modest differences associated with gender (2). CYP3A is capable of activating aflatoxins and benzo[a]pyrene 7,8-diol (69) and is therefore important in both drug clearance and carcinogen activation.

CYP3A proteins are abundantly expressed in extrahepatic tissues especially the gut (4). This has extremely important implications for the metabolism of orally administered drugs that serve as substrates for this enzyme. Recently, DM has been proposed as a promising new probe drug to assess the first pass effect (5) of orally administered CYP3A4 substrates.

Our objective is to assess CYP3A4 activity among the young and elderly by using DM as an *in vivo* probe and to correlate these findings with the interindividual variability observed in verapamil metabolism. We evaluated the PK of a SR formulation of verapmil because the characteristics of this formulation provide optimal levels of metabolite formation by presystemic metabolism.

Pharmacokinetic data in this report were analyzed by noncompartmental methods. The analysis of SR verapamil indicates that there is a significant age related decrease in

the clearance of verapamil. These data are consistent with previously reported observations (1,10) among the young and elderly. Due to sampling schedule and formulation characteristics it was not possible to accurately assess elimination half life. Since clearance is directly proportional to half life and inversely proportional to Vd we can not assess the source of variation.

Previous PK studies have been controversial regarding the source of age related changes in clearance. Most data indicate non significant differences in elimination half life suggesting that the observations are related to the Vd. Abernethy's (10) study found significant changes in elimination half life suggesting that there is an age related decrease in enzymatic activity. Our data suggest that Vd and elimination half life are likely playing an important role in the observed age related decrease in clearance.

Bioavailability represents the amount of drug available after first pass metabolism. Percent bioavailability in the young is 36 ± 17 vs 33 ± 7 (mean ± SD) in the elderly. Our results indicate that there are no age related differences in the bioavailabilty of verapamil. There is however an interesting difference in the variability observed within each group. The young have a large variability (17%) which may suggest that they have a greater variability in presystemic metabolism. Among the elderly, the variability is only 7% and may suggest that the liver is a greater source of variability as opposed to the gut. However, these observations are likely influenced by other factors. Our small unequal populations make it difficult to justify these suggestions. Further, changes in drug absorption unrelated to metabolism, such as gastric pH and intestinal motility, will also have profound effects on bioavailability (39). More appropriate studies designed to specifically evaluate presystemic metabolism are needed to identify the extent of the gut as a source of interindividual variability with verapamil metabolism.

Molar AUCs were calculated as moles/L/hr using the trapezoidal rule. There are significant differences observed in the AUCs (0-24) for verapamil and NV among the young and the elderly (figure 7). No significant differences were observed between the AUC of D620 and D617 although there is a trend indicating greater mean plasma levels among the elderly. The source of this variation reflects changes in the clearance of the drug and may have significant effects on pharmacodynamic responses. These results

indicate that the elderly are exposed to greater levels of pharmacologically active compounds verapamil and NV(20% activity of parent)(50) . The clinical implications of these findings have been previously reported and physicians have been cautioned to be more careful when prescribing drugs among their geriatric patients (51).

The AUC ratios of verapamil over metabolite were evaluated as an index of metabolic activity. It is assumed that the ratios reflect the individual rate of conversion of verapamil to NV, NV to D620 and verapamil to D617 and that these reactions are mediated primarily by CYP3A4 (72). However, recent evidence suggests the involvement of CYP2C8 and CYP1A2 may also be playing an important function in these reactions (21). The extent of the involvement of CYP2C8 and CYP1A2 has been assessed by *in vitro* experiments and the results are controversial. Kroemer (3) report that the formation of NV was mediated primarily by CYP3A4 (r=0.85) and CYP1A2 (r=0.5). Tracy (21) did not identify CYP1A2 in the formation of NV but found in addition to CYP3A4(r=0.70) , involvement of CYP2C8 (r=0.47) and minor contributions from CYP2E1(0.10). Tracy (21) also identified the formation of D-620 from NV being similarly mediated by CYP3A4 with minor contributions from CYP2C8 and CYP2E1.

Our data indicate significant age related differences in the transformation of verapamil to NV and NV to D617 (figure 8) and suggest decreased metabolism among the elderly. The comparison between verapamil/D-617 in the young and elderly did not reach statistical significance but did indicate a trend towards a reduced metabolism among the elderly. These results indicate that with increasing age there is a decrease in metabolism resulting in increased levels of parent compound and metabolites in the systemic circulation.

In order to identify whether these age related changes are a result of two distinct populations separated by age, a probit analysis was done. The analysis of verapamil to metabolite ratios suggests that we may be dealing with a skewed population in the formation of D-617 and D-620 (figure 9). The formation of norverapmil appears to be most consistent with a normally distributed population. The antimode represents the theoretical division between extensive (fast) and poor metabolisers. A clear antimode was

found for Verapamil/D-617 suggesting that the population may be divided among fast and poor metabolisers. No antimode was found for NV/D-620.

The frequency distribution among the young and elderly provides an interesting look and appreciation of the difficulty in predicting metabolism based on age (figure 11). Probit analysis of Ver/NV certainly suggests that we are dealing with a single population. However, if we look at the frequency distribution, there appear to be two normally distributed populations, consisting of the young and elderly. The frequency distribution of NV/D-620 is more complicated ; however there does appear to be a suggestion that with increasing age there is a decrease in metabolism associated with increased age.

Analysis for Ver/D-617 resulted in a clear antimode suggesting that population is divided among fast and poor metabolisers. However, since this division is not completely age related it may prompt one to conclude that these observations are a result of polymorphic enzyme expression attributed to exogenous, endogenous or genetic factors.

Another interpretation may be related to physiologic aging. As we get older there may be a decrease in enzymatic activity resulting in impaired drug metabolism. The elderly, who are part of a normally distributed EM population may not yet have made the transition from EM to PM. This hypothesis is consistent with Abernethy's (50) finding which demonstrated that among the elderly and very elderly there was a continued decrease in metabolism which continued into their 90s. These observations are not surprising since we expect physiologic aging to be associated with a decrease in hepatic function. A recent study with 226 subjects looked at age and cytochrome P450 linked drug metabolism. Antipyrine clearance rate began declining after 40 years of age with a 30% reduction in metabolism after 70 years of age (73). It is evident from the frequency distributions that although there may be a trend suggesting an age related decrease in metabolism, age alone is not an accurate predictor of metabolic activity. These observations reinforce our need to find suitable probes to help predict metabolism among the elderly in order to optimize treatments and reduce toxicity.

Dextromethorphan is O-demethylayted to form dextrophan (dex) and N-demethylated to form 3 methoxymorphinon (3MM). The molar ratio of DM/Dex has been successfully used as an index of CYP2D6 activity. The molar ratio of DM/3MM has been

identified as an index of CYP3A4 activity and its use as an effective probe of CYP3A4 is controversial. Schimer *et al.* (58) investigated the use of DM as a probe of CYP2D6 and CYP3A3/4. His findings suggested that the formation of 3 methoxy morphinan (3MM) is mediated only partly by CYP3A3/4 and therefore is not a reliable index of CYP3A4 *in vitro* or *in vivo*.

A recent analytical method using HPLC (5) has been developed to simultaneously assess CYP2D6 and CYP3A4 after administration of a single dose of Robitussin DM (30 mg). The formation of dextrophan is the major pathway mediated by CYP2D6 and 3MM a minor pathway mediated by CYP3A4 (figure 12). Figure 12 shows mean concentrations of analytes measured in the urine and demonstrates the predominance of CYP2D6 in the metabolism of DM. Spot urine samples were obtained and therefore molar ratios of parent compound to metabolite were used as an index of enzymatic activity.

Analysis of DM/3MM indicates that we have a skewed distribution. 2 elderly subjects are located far from the normal distribution and are considered to be poor metabolisers (PM) of CYP3A4 activity. These two individuals (E44 and E30) are the most important subjects in this study. If we consider that the ratio of DM/3MM is a reliable index of CYP3A4 and that verapamil is metabolised primarily by CYP3A4 then these subjects should correlate with verapamil metabolism as well as other substrates of CYP3A4.

E44 has elevated AUCs for verapamil, NV, D617 and D620. Analysis of AUC levels indicates that he is a poor metaboliser for each of these pathways. Therefore, his ability to metabolise verapamil, NV, D617 and D620 is impaired. He has also been identified as a poor CYP2D6 metaboliser which may or may not be related to a genetic polymorphism. A likely interpretation is that E44 has a decrease in hepatic function resulting in a general impairment of metabolic activity.

E30 has elevated levels of NV and verapamil but only moderate levels of D620 and D617. Contrary to E44, E30 has normal CYP2D6 activity. E30 may represent someone with a low CYP3A4 activity and normal levels of other cytochromes. He may also represent an elderly individual who is in a transitional period of reduced hepatic function.

The reason for his reduced CYP3A4 activity is not the most important point. What we have found is an interesting association between reduced formation of 3MM and impaired metabolism of verapamil.

Spearman correlation analysis of DM/3MM vs verapamil/metabolite ratios and AUCs for verapamil and metabolite are presented in table 6. There does not appear to be a direct association between verapamil metabolism and the formation of 3MM. Perhaps establishing absolute correlations are impractical and it may be more informative to focus these types of studies on the far outliers. There are however some interesting trends which may or may not be related to the activity of CYP3A4. Among the elderly there appears to be an association between systemic levels of D620 (r_s 0.46), D617 (r_s 0.48) and NV (r_s 0.52) but less for verapmil (r_s 0.26). This would suggest that in the elderly when CYP3A4 activity is impaired we still have metabolism of verapamil. CYP2C8 and CYP1A2 have been identified *in vitro* to mediate the formation of NV, D617 and D620. Although the elderly may have impaired CYP3A4 activity, there may be sufficient amounts of enzymes (CYP3A4, CYP2C8 and CYP1A2) present with high affinity for verapamil preventing its accumulation even when CYP3A4 activity is reduced.

If we consider that the metabolism of verapamil is mediated by several enzymes it would be difficult to establish a strong correlation between DM/ 3MM and verapamil AUC ratios. The assumption that DM/3MM is also produced by as single enzyme has been questioned and may also be mediated by more than one enzyme. (58) It is therefore not surprising that in the elderly we are unable to establish a correlation between DM./3MM and verapamil/NV, verapamil/D617 and NV/D620.

AUCs are a measure of systemic exposure of a drug. The amount of drug available will depend not only on metabolic activity but is also influenced by absorption, elimination, volume of distribution and kidney function. Since verapamil and perhaps DM metabolism may be mediated through multiple enzymes, AUC may be a reasonable alternative. Although we cannot use it as an absolute measure it may be useful in identifying trends

The associations between DM/3MM and AUCs of D620, D617 and NV in the elderly are certainly not straightforward but do suggest the involvement of other

cytochromes. When there is a decrease in the formation of 3MM (mediated primarily by CYP3A4) we see an accumulation of systemic D620, D617 and NV but no increase in systemic verapamil. These data are consistent with the involvement of other enzymes in verapamil metabolism. A role of CYP1A2 and CYP2C8 has been identified in verapamil metabolism (3,21,22). When CYP3A4 is deficient there may be enough enzymes available which prevent the accumulation of verapamil and therefore even when CYP3A4 function is impaired there are alternative enzymes (or sufficient CYP3A4) available to prevent verapamil accumulation.

Among the elderly the AUC levels of D617, NV and D620 are elevated when CYP3A4 levels are decreased. Since these enzymes are accumulating it would suggest that their metabolism is being impaired. We know that D617 and NV are further metabolised by CYP2C8 (21,22). The association between poor CYP3A4 metabolisers and an increase in D620, D617 and NV among the elderly may suggest a decrease in CYP2C8 activity resulting in an accumulation of these metabolites.

CYP3A4 is a major metabolic enzyme produced by the liver. Among the elderly, when CYP3A4 activity is impaired, it may be due to a decrease in overall metabolic function. Since CYP3A4 is the most abundantly produced enzyme there may be enough present to metabolise verapamil, but insufficient amounts of CYP2C8 to remove D617 and NV, leading to their accumulation.

These observations in the elderly are likely attributed to decreases in overall hepatic function. However, these correlations may also be attributed to other factors such as the volume of distribution. When we look at total systemic (AUC) levels of metabolites we are assessing clearance and not metabolic activity. When we look at ratios we are compensating somewhat for individual changes in the volume of distribution. Among the elderly and young, 15% and 10% of the variability associated in DM/3MM could be explained by weight. Therefore in the elderly a causal link between DM/3MM and AUCs of D620, D617 and NV may be attributed to differences in clearance related to the volume of distribution.

Among the young there is a decrease in the clearance of D617 associated with impaired CYP3A4 activity. As in the elderly, this may be attributed to a decrease in CYP2C8 activity resulting from impaired hepatic function.

Interestingly, among the young the clearance of NV appears to be negatively associated with DM/3MM. This would imply that when CYP3A4 activity is decreased there is an increase in the clearance of NV. This may suggest that the young are able to compensate for their CYP3A4 deficiency either by an intrinsic mechanism or through exposures to exogenous inducers.

Previously we indicated that in young a decrease in CYP3A4 activity may be associated with a decrease in hepatic function resulting in simultaneous decreases in the levels of CYP2C8. Therefore, if we are dealing with impaired liver function in the young how do we explain that their metabolism of NV is improved. *In vitro* data has shown that NV is metabolised by CYP3A4, CYP2C8 and CYP2E1 to form D620, or alternatively by CYP2C8 and CYP2D6 to form PR22 (26). There is no evidence suggesting the inducibility of CYP2D6 and CYP2C8 in the literature; however, there is evidence demonstrating the inducibility of CYP2E1.

In rats, administration of ethanol causes an increase in CYP2E1 protein without affecting its mRNA; it may be posttranscriptionally regulated through substrate induced protein stabilization both in rats and humans (69). During reduced hepatic function the young may have sufficient levels of CYP3A4 with the consequences that there are only minor affects on its substrates. However, CYP2C8 may be less abundant, resulting in more dramatic differences in the metabolism of its substrates. A possible hypothesis may be that the young who have a reduced hepatic function may have increased expression of CYP2E1 which is rapidly transforming NV to D620, resulting in a decrease in the AUC levels of NV. It is currently not known which enzymes are involved in the metabolism of D620 and therefore no explanation can be provided regarding the poor correlation with D620.

Indirectly, our data has demonstrated, both in the young and elderly, the importance of cytochromes other than CYP3A4 in the metabolism of verapamil. We were unable to find a correlation between DM/3MM and the ratio of verapamil to metabolite

among the elderly. However, in the young there appears to be a negative correlation between CYP3A4 activity and transformation of verapamil/D617 and NV/D620. The ratio of verapamil to D617 and NV to D620 may reflect the enzymatic activity of CYP3A4, CYP2C8 and CYP1A2. There have been no studies demonstrating the involvement of CYP1A2 in the formation of D620, however the structural similarities between these two pathways suggest that they are mediated by the same enzymes.

No genetic based polymorphism has been identified for CYP3A4 and it is reasonable to assume that a decrease in CYP3A4 activity could be related to decreased hepatic activity. In the young when the activity of CYP3A4 is decreased there is an increase in formation of D617 and D620. The increased metabolism of verapamil to D617 and NV to D620 when CYP3A4 levels are decreased indicates that there are likely other enzymes involved in these pathways. Since we do not observe this in the elderly it suggests that the young have a greater metabolic capacity.

When the enzymatic activity in the young was compared to the elderly there were statistically higher levels of enzymatic activity as reflected by the verapamil/NV and NV/D620 ratio. Although the difference between verapamil/D617 ratio in the young and elderly did not reach statistical significance, the data indicate that D617 is produced to a greater extent in the young. These observations suggest that when there is a decrease in CYP3A4 activity in the young, there are sufficient amounts of alternative enzymes capable of mediating these reactions. CYP1A2 has been identified in the formation of D617 and may also be involved in the formation of D620. CYP1A2 is an inducible enzyme (69) and it may be present in greater amounts among the young which may explain the increase in metabolite formation when CYP3A4 activity is reduced. Approximately 40% of an American population showed rapid metabolism of caffeine (74) which is mediated by CYP1A2.

These results indicate that although DM is an interesting probe for CYP3A4 activity it does not appear to be informative in predicting the metabolism of verapamil. Although CYP3A4 has been suggested as the primary enzyme for verapamil, there are several other enzymes which have been implicated in the metabolism of verapamil. Thus, the involvement of other cytochromes in verapamil metabolism makes it difficult to use

DM as a probe. It was initially thought that verapamil metabolism was mediated primarily by CYP3A4. Its use may be limited in identifying individuals who are far removed from a normal distribution who are more likely to exhibit impaired drug metabolism.

Among the elderly, there is a causal link between DM/3MM and the clearance of D620, D617 and NV. This association is unlikely to be related to CYP3A4 activity and may be a result of impaired hepatic activity. Among the young there appears to be a link between CYP3A4 activity and increases and decreases in metabolite formation which may be associated with impaired CYP2C8 activity resulting from a decrease in hepatic function and/or increased CYP1A2 activity. These hypothesis have been suggested as a possible interpretation of our results but further studies are needed to establish conclusive evidence of the *in vivo* involvement of CYP2C8 and CYP1A2 in verapamil metabolism.

Our data suggest that CYP3A4 may not be the only source of interindividual variation observed with the metabolism of verapamil. The use of DM as an *in vivo* probe of CYP3A4 activity can not be evaluated by using verapamil as a model substrate of CYP3A4. Although DM/3MM ratio cannot provide an absolute measure of verapamil metabolism it may be useful in identifying trends. DM is an orally administered probe which undergoes both gut and liver metabolism. Its use as a suitable probe of presystemic metabolism should continue to be explored.

Our results indicate that there are significant age-related differences in the metabolism of verapamil which can be attributed to changes in metabolic activity associated with increasing age. This is important because in developed countries 20% of the population is over 65 yrs of age. (14). The elderly represent the fastest growing segment of the population and are the heaviest consumers of drugs. In addition it has been demonstarting that the elderly are particularly sensitive to the adverse effects of drugs and may have a greater sensitivity to environmental toxins (14). As the population of elderly patients increases these types of studies on age related effects and drug responses become increasingly more important. The simultaneous involvement of both physiologic changes and various clinical factors and disease states, together with issues involving lifestyle and diet, makes identifying appropriate dosing strategies solely on the basis of age difficult.

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