IN VITRO STUDIES SUGGEST INVOLVEMENT OF CYP2D6 IN THE METABOLISM OF SIMVASTATIN AND ACTIVE METABOLITES

Thesis submitted to Department of Pharmacology,
School of Pharmacy, Faculty of Mathematics and Natural Sciences,
University of Oslo
for the degree Candidata pharmaciae

Olaug Sveinsgjerd Fenne

November 2003

IN VITRO STUDIES SUGGEST INVOLVEMENT OF CYP2D6 IN THE METABOLISM OF SIMVASTATIN AND ACTIVE METABOLITES

Thesis submitted to Department of Pharmacology, School of Pharmacy,
Faculty of Mathematics and Natural Sciences, University of Oslo
for the degree Candidata pharmaciae

Olaug Sveinsgjerd Fenne

Supervisors:
PhD student Monica Hermann
PhD student Espen Molden
Associate professor Hege Christensen

November 2003

ACKNOWLEDGEMENTS

First, I want to express my great appreciation to Monica Hermann and Espen Molden for their invaluable contributions, support and incredible engagement through this year. Thanks to Hege Christensen for her suggestions during the completion of this thesis and for providing excellent pc equipment.

I am grateful to Leon Reubsaet at Department of Pharmaceutical Analysis for his important contributions during the HPLC-MS method development.

Siri Johannesen and Petter Arnesen are acknowledged for the preparation of recombinant CYP microsomes.

Thanks to everyone at Department of Pharmacology for providing a friendly and stimulating environment and Hanne for the many enjoyable lunch breaks and good conversations during the year.

Finally, thanks to Tore, family and friends for their support and interest.

Blindern, 15. november 2003

Olaug Sveinsgjerd Fenne

TABLE OF CONTENTS

ABSTRACT

Aim: The HMG-CoA reductase inhibitor, simvastatin (SV), is known to be extensively metabolised by CYP3A4 in humans. However, clinical studies have suggested a possible involvement of the polymorphic enzyme CYP2D6 in the metabolism of one or more of the active metabolites of the SV. The present *in vitro* study was conducted to investigate the role of CYP2D6 in SV metabolism, and in particular clarify whether any of the SV metabolites are possible substrates for CYP2D6.

Methods: Two different strategies were used. First, SV was incubated with human liver microsomes (HLM) in absence and presence of potent CYP3A4 or CYP2D6 inhibitors. Then, incubation of SV was performed with microsomes of recombinant CYP3A4 and CYP2D6 (T5-3A4 and T5-2D6). SV was first incubated with T5-3A4 microsomes, then, the remaining SV and metabolites produced by T5-3A4 were subjected to metabolism with T5-2D6 microsomes. SV and metabolites were analysed by a high-performance liquid chromatography-mass spectrometry (HPLC-MS) method, developed in the present work.

Results: Upon incubation of SV with HLM, five possible SV related metabolites were detected. All of the deduced metabolites were found to increase with incubation time. The metabolites together represented four different phase I metabolic modifications of SV, giving rise to the metabolites 6′-CH_2OH-SV (I), 3′-OH-SV (II), 6′-exomethylene-SV (III), 3′-OH-6′-CH_2OH-SV (IV) and 6′-COOH-SV (V). Inhibition studies and studies with recombinant CYP microsomes showed that CYP3A4 was involved in all these reactions. CYP2D6 was apparently not catalysing any of the reactions, but a clear trend of unspecific accumulation of SV and all measured metabolites in presence of the CYP2D6 inhibitor quinidine indicated that CYP2D6 might be responsible for a metabolic reaction not detected in this work. This was supported by an unspecific decline of SV and the metabolites produced by T5-3A4 following T5-2D6 incubation, which could not be reflected as new chromatographic peaks after T5-D6 incubations.

Conclusion: The present *in vitro* study suggests that CYP2D6 is involved in metabolism of SV and several phase I metabolites with pharmacological activity (in their acid form). However, the potential *in vivo* importance of CYP2D6 involvement needs to be evaluated in a

study where pharmacokinetic measurements of SV and metabolites are investigated in relation to CYP2D6 phenotype. Attempts should also be made to identify the metabolites apparently produced via CYP2D6.

ABBREVIATIONS

API	Atmospheric pressure ionisation
CHD	Coronary heart disease
CI	Chemical ionisation
CYP	Cytochrome P450
EM	Extensive metabolisers
ES	Electrospray
ESI	Electrospray ionisation
HEM	Heterozygous extensive metabolisers
HLM	Humane liver microsomes
HMG-CoA	3-Hydroxy-3-methylglutaryl coenzyme A
HPLC	High performance liquid chromatography
I.S.	Internal standard
LDL	Low-density lipoprotein
LV	Lovastatin
M1	Desacetyldiltiazem
M2	*N*-demethyldiltiazem
M4	*O*-demethyldiltiazem
M6	*N, O*-didemethyldiltiazem
MS	Mass spectrometry
m/z	Mass-to-charge ratio
NADPH	Nicotinamid adenine dinucleotide phosphate
PM	Poor metabolisers
SD	Standard deviation
SV	Simvastatin
SVA	Simvastatin acid
T5-3A4	Recombinant human liver cells transfected with CYP3A4
T5-2D6	Recombinant human liver cells transfected with CYP2D6
UM	Ultra rapid metabolisers
V/V	Volume/volume
Q.C	Quality control
I	6´-CH_2OH-simvastatin

II	3´-OH-simvastatin
III	6´-exomethylene simvastatin
IV	3´-OH-6´-CH_2OH-simvastatin
V	6´-COOH-simvastatin

1. INTRODUCTION

1.1 AIM OF STUDY

The HMG-CoA reductase inhibitor, simvastatin (SV), is known to be extensively metabolised by CYP3A4 in humans [1]. It has been speculated that the polymorphic enzyme CYP2D6 could be involved in the metabolism of active SV metabolites based on an apparent association of CYP2D6 phenotype and clinical effect [2, 3]. Thus, the aim of this study was to investigate the possible involvement of CYP2D6 in SV metabolism by performing *in vitro* experiments using human liver microsomes (HLM) and recombinant CYP microsomes.

1.2 DRUG METABOLISM

Drug metabolism refers to the process by which drugs are biochemically modified to facilitate their excretion from the body. Lipophilic drugs are converted into more hydrophilic, usually less active or inactive metabolites. However, this biotransformation can also generate metabolites with potent biological activity or even toxic properties. The enzyme systems involved in metabolism of drugs are primarily localised in the liver, but other tissues and organs like the mucosa of the intestine, kidneys, lungs, white blood cells and skin have metabolic capacity as well [4]. Drug metabolic reactions may be divided into two categories; phase I and phase II. Phase I involves modifications such as oxidation, dealkylation, hydrolysis and reduction, while phase II metabolism involves conjugation of glucuronide, glycin or sulphate to the parent drug or its phase I metabolites [5].

1.2.1 CYTOCHROME P450 MONOOXYGENASE SYSTEM

The most important enzyme system involved in drug metabolism is the cytochrome P450 (CYP) enzyme system of microsomal mixed function oxidases. This system is involved in the metabolism of 50-60 % of all clinically used drugs as well as being important in the metabolism of many xenobiotic chemicals [6]. The CYP enzymes are primarily located in the endoplasmatic reticulum of liver cells (Fig. 1-1), but some are also found in the intestinal mucosa [7].

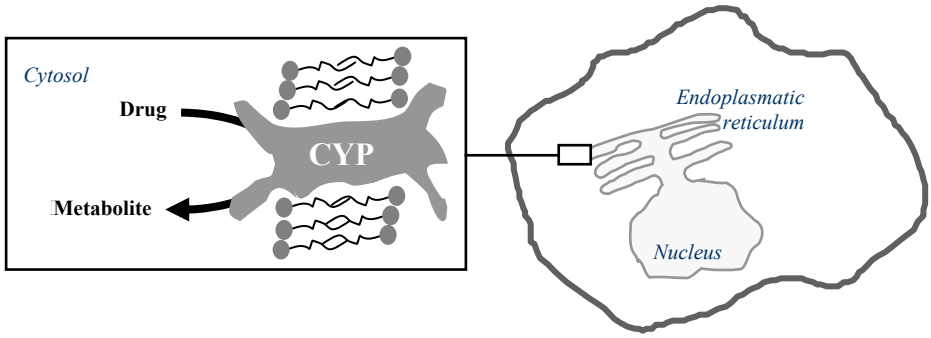


Fig. 1-1: Cellular localization of the cytochrome P450 enzymes [8].

The CYP system comprises a superfamily of enzymes. A nomenclature system was introduced in 1987 [9], which classifies enzymes into families and subfamilies according to their overlaps in amino acid sequence. Sequence homology greater than 40 % assembles enzymes into families (indicated by an Arabic number, e.g. CYP3), while enzymes within subfamilies share greater than 55% homology (indicated by the capital letter following the family designation, e.g. CYP3A). The final Arabic number following the subfamily designation indicates the individual isoforms within each subfamily (e.g. CYP3A4) [10]. In humans, about 60 different CYP enzymes have been described [11], but only a few are important in drug metabolism. The CYP1, CYP2, and CYP3-families are importantly involved in drug metabolism and their representatives in humans are CYP2C9, CYP2C19, CYP2D6, CYP1A2, and CYP3A4. CYP3A4 is by far the most important by being involved in approximately 50 % of all CYP mediated drug metabolism, whereas 20 % are substrates for CYP2D6 [4, 6, 12].

1.2.2 CYP ENZYMES AND INTERINDIVIDUAL VARIABILITY

Despite the expansive knowledge on the field genetics and drug metabolism the past decade, it is still difficult to predict the outcome of drug therapy for a given individual. The drug level in plasma can vary 1000-fold between two persons having the same weight and drug dosage [13]. Such variation may arise from a range of environmental factors affecting drug metabolising capacity (dietary habits, age, sex, cigarette smoking, drug interactions and disease states). More important is probably the genetic diversity amongst the drug metabolising enzymes. Approximately 40 % of all human CYP dependent metabolism is carried out by genetic polymorphic enzymes [6]. CYP2C9, CYP2C19 and CYP2D6 are the most important polymorphic enzymes, while no important genetically different forms have been found in CYP3A4 [6]. CYP2D6 is one of the best characterised and studied polymorphic

drug metabolising enzymes. As many as 70 allelic variants have so far been found for CYP2D6 [14]. Of these, more than 15 encode an inactive enzyme or no enzyme at all, while others encode enzyme with reduced, normal or increased activity. This genetic diversity divides people into 4 CYP2D6 phenotypic subpopulations of metabolisers; i.e. poor (PM), intermediate (IM), extensive (EM) and ultra rapid (UM). The frequencies of these phenotypes show a marked interethnic variability. In Caucasians, about 7 % are poor metabolisers, compared with only ~1 % of Orientals [4, 14].

CYP2D6 catalyses the metabolism of a quite large group of drugs, including antidepressants, antipsychotics, analgetics and cardiovascular drugs. For implicated drugs, individual differences in CYP2D6 phenotype can indeed lead to clinically significant variability in drug response. In general, CYP2D6 PMs are particularly susceptible to drug accumulation and adverse reactions, while ineffective therapy could be the outcome for CYP2D6 UMs. However, several aspects need to be taken into account, including pharmacological activity of metabolites and degree of metabolism via the polymorphic enzyme compared to other routes of elimination. Moreover, it has been shown that active metabolites could be substrates for polymorphic enzymes in contrast to the parent compound [15]. This implies that the parent drug might be pharmacologically unaffected by variability in metabolic phenotypes, but not its therapeutic response. The drug's therapeutic index must also be considered with respect to the severity of genetic variability. Drugs with steep dose-response curves are more likely to cause adverse effects and drug toxicity. The complexity of this issue shows that the clinical impact of genetic polymorphism needs to be closely studied for each separate drug.

1.3 STRATEGIES TO INVESTIGATE THE ROLE OF CYP ENZYMES IN DRUG METABOLISM

Drug metabolism is one of the major determinants for the systemic drug exposure of many drugs. Thus, pharmaceutical industry has not surprisingly showed a growing awareness of the critical role drug metabolism constitutes in drug therapy. During drug development, the pharmaceutical companies now seek to obtain early information on human metabolic processes of new drug candidates [16]. This includes determination of metabolic pathways and identification of drug metabolising enzymes to predict the importance of polymorphic enzymes as well as potential drug interactions [12, 16]. Several *in vitro* enzyme systems have

emerged as a suitable tool at this stage of the process. Preclinical *in vitro* information on drug metabolism in humans can contribute to the design of the important pharmacokinetic and toxicological studies on animals in the early phase of drug development [16]. On a later stage, when the drug moves into phases of high-cost clinical studies, the *in vitro* data can give valuable contributions to the design of these studies (e.g. genetic and drug interaction studies).

Two major approaches can be used to study the *in vitro* metabolic profile of a drug:

- Incubate the drug with differentiated cellular models, such as isolated and cultured hepatocytes or liver tissue slices.
- Incubate the drug with subcellular fractions of endoplasmic reticulum (microsomes).

Hepatocytes are unique in that they provide a cellular integrity with intact enzyme architecture, membrane and phase II metabolism. They are thereby the closest model for reflecting the *in vivo* situation of many xenobiotics. However, this model involves transport of the substrate across the cell membrane, which may affect the exposure of the substrate or the inhibitor to the metabolising enzymes. A major drawback with primary hepatocyte cultures is their lack of ability to grow *in vitro.* As a consequence, these must be prepared from suitable liver tissue (biopsies) each time they are used [12, 17, 18].

Microsomes are produced by differential centrifugation of liver cells. Human liver microsomes (HLM) are also commercially available. Studies with HLM are primarily performed with a pool of microsomes from several donors. This eliminates the important issue of inter-individual variability. Microsomes perform rapid metabolism, they are easy to use and can be stored at -80° C for a longer period without lack of activity. Although microsomes have become the most frequently used model to determine *in vitro* drug metabolism, there is still a need for standardisation of the procedures used. Differences in incubation conditions or substrate concentrations make it difficult to compare and validate studies that investigate metabolism of similar compounds [19].

When the metabolic profile of a drug has been described, the following strategies are frequently applied to identify the CYP enzyme(s) responsible for a particular metabolic reaction:

- Co-incubate the drug with HLM in absence and presence of selective and potent CYP inhibitors.
- Incubate the drug with recombinant enzyme systems, which have been genetically manipulated to express only one single CYP enzyme (either cultured cells or microsomes isolated from such cells).

Often, these two approaches are combined to confirm the importance of a particular enzyme in a certain metabolic reaction of a drug.

1.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY- AN IMPORTANT TOOL IN METABOLITE IDENTIFICATION

Drug metabolism studies require highly sensitive analytical methods. High performance liquid chromatography coupled to mass spectrometry, HPLC-MS, has become the most suitable procedure for such studies [20]. HPLC-MS is widely used in both qualitative and quantitative analysis in research and drug development [21]. MS is a highly sensitive detector, but the major advantage is that it enables both mass-specific (single-MS) as well as structural (tandem-MS) information. Thus, metabolites for which no reference compounds exist can be identified with a high degree of certainty. Continuous improvements in this technology have made it applicable to almost every analyte. Low detection limits (pico-level), short run time, high specificity, precision and accuracy are some of its many advantages [22].

1.4.1 INSTRUMENTATION

A mass spectrometer separates charged atoms or molecules according to their mass-to-charge ratio (*m/z*). The instrument consists of an inlet system (here: chromatographic), ion source, capillary, skimmers and lenses, mass analyser and an ion detector (Fig. 1-2). The most important step in mass spectrometry is the ionisation procedure, as it is the charge that makes it possible to select individual ions and measure their mass. A sample can be introduced into the ion source via several different ionisation techniques. Atmospheric pressure ionisation (API) techniques, such as chemical ionisation (CI) and electrospray (ESI), are ideal to combine with liquid chromatography.

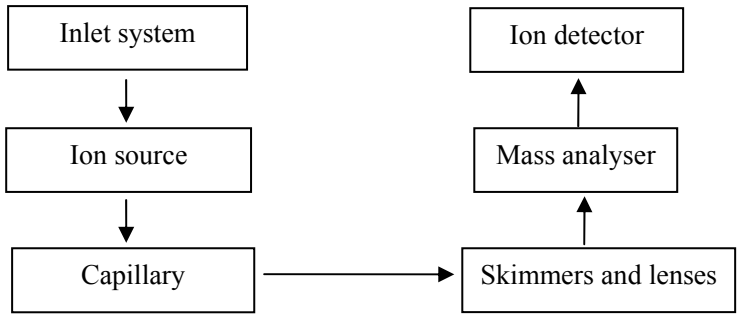


Fig. 1-2: Schematic presentation of the instrumentation in MS [22].

In ESI, the HPLC eluent is pumped through a capillary, which carries a high potential. The potential together with a nebulising gas cause the solvent to break into charged droplets of one polarity. A hot drying gas makes the droplets evaporate and a vacuum system accelerates the ions towards the analyser region. In APCI, ionisation is achieved by the use of an ionised, heated reagent gas like methane or ammonia that forces charges onto the molecules. This latter technique tends to have an advantage for less polar, thermally stabile compounds. For both techniques, it is of great importance that mobile phases and buffers used are volatile. Salt formation in the ion source and clotting of the capillary can occur if unsuitable solvents are used. A potential problem in HPLC-MS analysis is the formation of adduct ions like $[M+NH_4]^+$ or $[M+Na]^+$ in addition to the preferred molecule ion $[M+H]^+$. Adduct ions could be formed even if the mobile phase does not contain the ions. Simply placing analytes in glass bottles or glass auto-sampler vials extract trace quantities of sodium ions and can lead to formation of undesirable adducts. Multiple ionic forms of the analytes are undesirable due to loss of sensitivity and reproducibility. This problem can be overcome by adding a HPLC mobile phase additive that will favour the formation of a single, major molecular ion [22, 23].

1.5 CHARACTERISTICS OF SIMVASTATIN

1.5.1 PHYSICAL/CHEMICAL DATA

- Formula: $C_{25}H_{28}O_5$
- Chemical name: Butanoic acid, 2,2-dimethyl-1S,3R,7S,8S,8aR-1,2,3,7,8a-hexahydro- 3,7-dimethyl-8-[2-[2R,4R)tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl] ethyl]-1-naphtalenyl ester
- White, crystalline, non-hygroscopic solid

- Solubility: insoluble in water (0,03mg/ml), soluble in polar, organic solvents

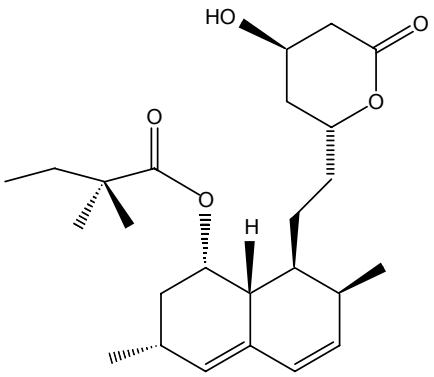


Fig.1-3: Chemical structure of simvastatin

1.5.2 CLINICAL USE

Simvastatin (Fig. 1-3) is a HMG-CoA reductase inhibitor (statin), widely used in the treatment of hypercholesterolemia. The consumption of statins in Norway have increased steeply since 1994, and the sales increased threefold from 1997 to 2002, measured in number of sold DDDs (defined daily dose) (Fig. 1-4). Simvastatin and atorvastatin were number one and two on the list of top-selling prescription only medicines in Norway in 2002, and statins as a group constituted 8 % of the total sales [24].

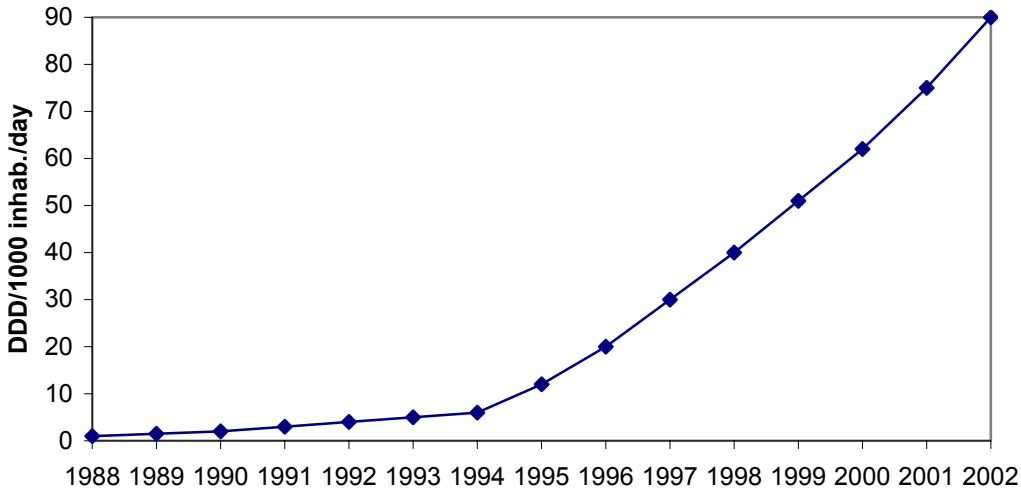


Fig. 1-4: Sales of serum lipid reducing agents in Norway in the period 1988 – 2002 [24].

This explosive increase in consumption must be seen in connection with several major placebo controlled clinical trials that have been performed with statins since 1994 [25-29]. Together, these have demonstrated the efficacy of statin treatment in reducing the risk of coronary heart disease (CHD) events, the leading cause of deaths in industrialized countries. Statins have shown a reduction in the relative risk of myocardial infarction and CHD events by 23-37 % in primary as well as secondary prevention therapy [30]. Recent evidence suggests that more aggressive LDL-cholesterol lowering may provide greater clinical benefits, even for individuals with moderately elevated or average lipid level [31].

1.5.3 MODE OF ACTION AND TOLERABILITY

Simvastatin lowers cholesterol by inhibition of the enzyme 3-hydroxy-3-methylglutaryl coenzyme (HMG-CoA) reductase, which catalyses the rate limiting step in cholesterol biosynthesis in the body. This effectively reduces the cholesterol synthesis and leads to an up-regulation of cell surface low density lipoprotein receptors and subsequent decreased level of LDL-cholesterol [32]. Currently, there are 5 statins on the norwegian market; lovastatin (Mevacor®), simvastatin (Zocor®), pravastatin (Pravachol®), fluvastatin (Lescol®) and atorvastatin (Lipitor®) [33]. As a group, the statins are generally well tolerated. However, muscle abnormalities may occur .These can range from benign myalgia to myopathy (ten fold elevation in creatin kinase level) which can further progress to the life threatening condition rhabdomyolysis [32, 34]. The statin cerivastatin (Lipobay®) was withdrawn from the market in 2001 because of high frequency of serious myopathy and rhabdomyolysis [35]. When using statins in monotherapy the incidence of myopathy and rhabdomyolysis are approximately 0,1-0,5 % and 0,04-0,25 %, respectively. The incidence of these side effects show dose dependence and is increased when SV is concomitantly administered with other agents that can cause muscle toxicity (e.g. fibric acid derivates) or agents that are potential inhibitors of statin metabolism [31, 34, 36]. Statins are in most cases prescribed on a long-term basis, and many patients will typically receive multi-drug treatment (polypharmacy) during this periode. Thus, possible interactions with other drugs deserve high attention.

1.5.4 METABOLISM

With the exception of simvastatin (SV) and lovastatin (LV), all current statins are administrated as the active β-hydroxy acid form. SV and LV are administered as inactive lactone prodrugs and must be hydrolysed *in vivo* to their corresponding, pharmacologically active β-hydroxy acid forms [1, 37]. The absorption of simvastatin is approximately 60-80%, while bioavailability is only 5 % [38]. This low systemic bioavaliability is due to the high degree of presystemic metabolism. Several *in vivo* and *in vitro* studies [39-41] have shown that SV undergoes extensive metabolism to several hydroxylated metabolites, as well as hydrolysis of the lactone ring to yield the active open acid form. Among these, simvastatin acid (SVA) is probably the most potent inhibitor of HMG-CoA reductase, but some of the oxidative metabolites are also active in their acidic forms [32, 40, 41].

A metabolic pattern of SV was proposed in 1990 by Vickers *et al.* through studies with rat/mice liver microsomes and human bile extract [41]. Four major metabolites were formed upon incubation with rat and mice microsomes (6`-OH-SV which was rearranged to 3´-OH-SV upon acidic conditions, 3``-OH-SV, 6-exomethylen-SV and SVA). In addition, two other metabolites, 6´-CH_2OH-SV and 6´-COOH-SV were found in human bile (Fig. 1-5). The two biliary metabolites, were found to have approximately 90 and 40 % HMG-CoA reductase inhibitory activity, respectively (compared to SVA) [41]. Later, Prueksaritanont *et al.* described another metabolite, 3´,5´-dihydrodiol-SV, in studies with SV and HLM [1]. In this latter study, four major oxidative metabolites were observed (Fig. 1-6).

CYP3A4 is importantly involved in the oxidatations of SV indicated in Fig. 1-6 [1], but there is limited data on the enzymes further catalysing the active metabolites or alternative metabolic pathways. In a recent publication, Prueksaritanont *et al.* reported that metabolism of SVA is also mediated by CYP3A4 [42]. However, it was observed that SVA was a much poorer substrate for CYP3A4 enzymes than SV lactone [42].

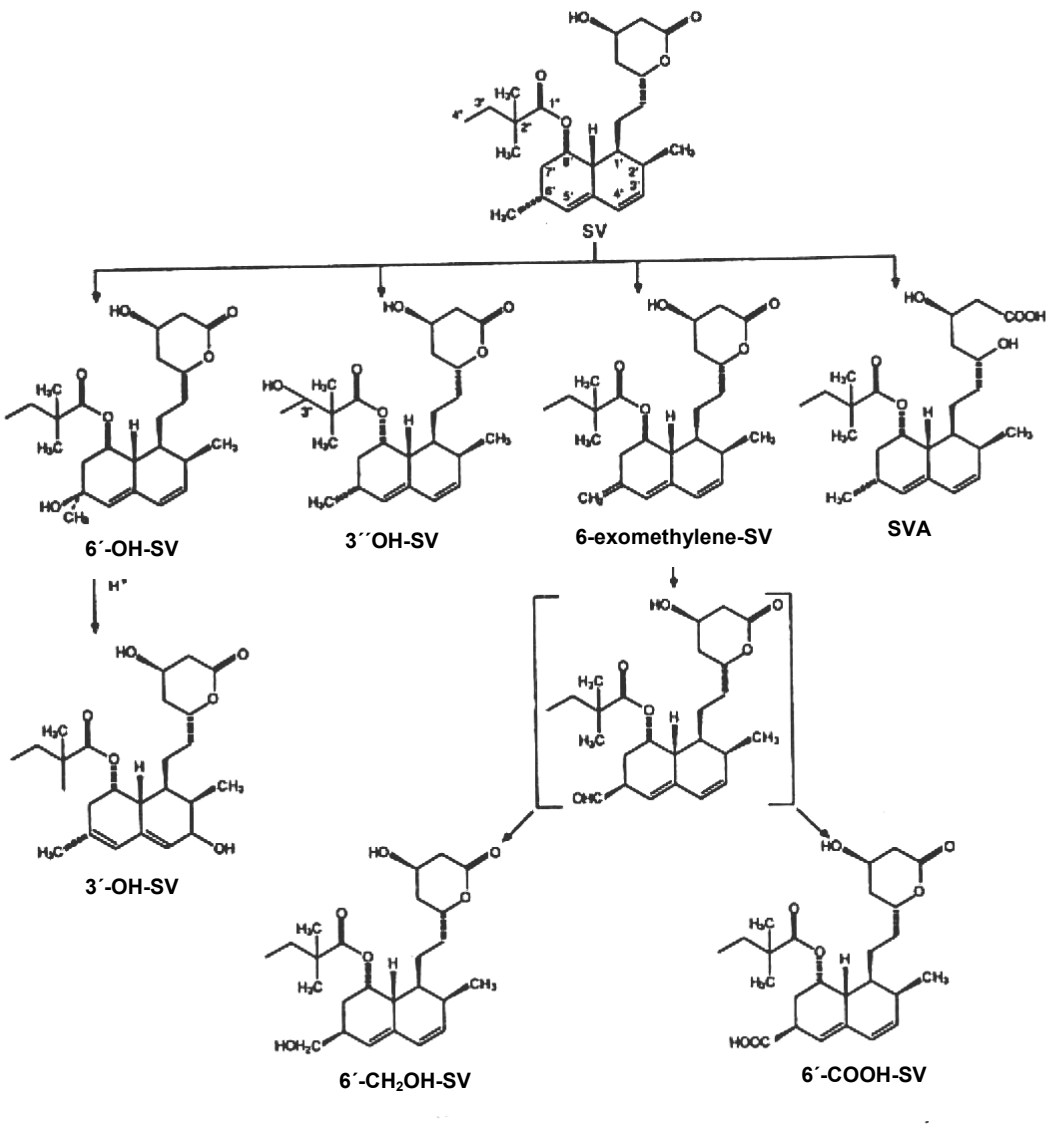


Fig. 1-5: Structures of in vitro and in vivo metabolites of SV.Major *in vitro* metabolites were 6`-OH-SV (rearranged to 3`-OH-SV upon acidic conditions), 3``-OH-SV, 6-exomethylen-SV and SVA. These were obtained by incubations with liver microsomes from rats and mice. Biliary metabolites (in human) also included 6`$CH_2$OH-SV and 6`COOH-SV [41].

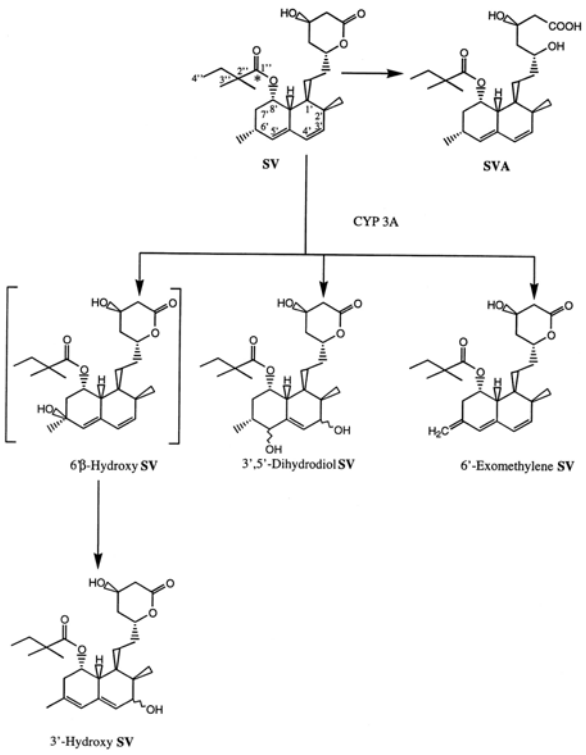


Fig. 1-6: Major metabolites observed in incubation of SV with HLM by Prueksaritanont *et al* [1].

Although CYP2D6 has been shown not to be involved in the reactions described in Fig. 1-6, clinical studies have recently suggested a possible involvement of CYP2D6 in the metabolism of SV. Nordin *et al.* [3] found that cholesterol in serum during treatment with 40 mg SV correlated negatively with debrisoquine ratio, which is an *in vivo* probe for CYP 2D6 activity (Fig. 1-7). However, none of the 8 healthy volunteers in the study were CYP2D6 PMs. Similar observations were reported by Mulder *et al*. [2, 43, 44], who found a significantly higher cholesterol reduction per mg administered dose in CYP2D6 PMs compared to EMs (Table 1-1). In addition, 80 % of the PMs had to discontinue the therapy because of adverse events, whereas one patient with UM phenotype showed a very low cholesterol reduction even at a daily dose of 40 mg SV.

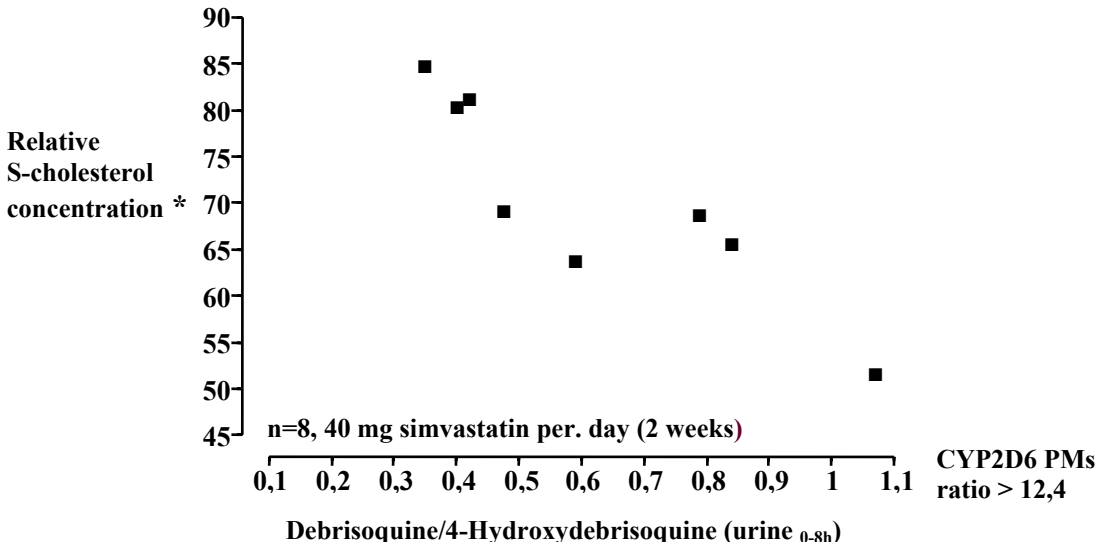


Fig.1-7: Cholesterol in serum after simvastatin treatment [3]. * Fasting serum cholesterol concentration during SV treatment, expressed as % of pre-treatment concentration.

Table 1-1: Association of CYP2D6 phenotype and efficacy per dose SV administered and intolerability of SV treatment in 88 patients with hypercholesterolemia [2].

CYP2D6 phenotype	Cholesterol reduction (mmol/L) pr. mg simvastatin	Number of intolerants (%)
PM (n=5)	0,23	80
HEM (n=28)	0,20	46
EM (n=54)	0,10	17
UM (n=1)	0,01	0

PM = poor metabolisers
HEM = heterozygous extensive metabolisers
EM = homozygous extensive metabolisers
UM = ultra rapid metabolisers

Based on these clinical results, the authors speculated that one or more of the active metabolites of SV is metabolised by the polymorphic CYP2D6 enzyme. Lack of pharmacokinetic measurements in the referred studies prohibited a verifiation of this assumption. The fact that another group, Geisel *et al*., claim that they did not find an assosiation between SV effect and CYP2D6 phenotype in a third, unpublished study [43], implies that there is great uncertainity about the importance of CYP2D6 phenotype for the total pharmacological response of SV. In view of this uncertainity and the widespread use of this drug, the aim of present study was to investigate the possible role of CYP2D6 in SV metabolism, and in particular to clarify whether any of the SV metabolites are possible substrates for CYP2D6.

2. MATERIALS AND METHODS

CHEMICALS

Acetonitrile, HPLC grade	Chemi-Teknik A/S, Oslo, Norway
Acetic acid, glacial, analytical grade	Chemi-Teknik A/S, Oslo, Norway
Acetone, HPLC grade	Chemi-Teknik A/S, Oslo, Norway
B-nicotinamid adenine dinucleotide-phosphate reduced tetrasodium salt (NADPH)	SIGMA, Steinheld, Germany
Cis (+)-desacetyldiltiazem HCl	Tanabe Seyaku, Osaka, Japan
Deionised water (19,3 MΩ-cm)	Easypure UV, Barnstead, Iowa, USA
Formic acid, analytical grade	MERCK, Darmstadt, Germany
Erythromycin	SIGMA, Steinheld, Germany
Lovastatin	MERCK, Rahway NJ, USA
Magnesium sulphate, analytical grade	MERCK, Darmstadt, Germany
Methanol, HPLC grade	Chemi-Teknik A/S, Oslo, Norway
Methylamine 40%, analytical grade	Chemi-Teknik A/S, Oslo, Norway
Nitrogen gas	AGA Progas A/S, Oslo, Norway
Simvastatin	MERCK, Rahway NJ, USA
Sulphuric acid, analytical grade	MERCK, Darmstadt, Germany
Tris (hydroxymethyl) aminomethane	Chemi-Teknik A/S, Oslo, Norway
Quinidine hydrochloride monohydrate	SIGMA, Steinheld, Germany

2.1 *IN VITRO* METABOLISM OF SIMVASTATIN

2.1.1 STUDIES WITH HUMAN LIVER MICROSOMES

HLM, (20 mg protein/ml) pooled from 50 subjects were purchased from In Vitro Technologies, Baltimore, USA. These were stored in liquid nitrogen (-196° C) and carefully thawed on ice before use. The incubation reaction was carried out in 2 ml eppendorf vials with a safe sealing, and incubation conditions were chosen on the basis of a previous master thesis by Eili T. Kase [18]. A typical incubation mixture in a final volume of 500 µl contained 389 µl 200 mM Tris-H_2SO_4 buffer (pH 7,5), 78 µl 10 mM NADPH (dissolved in 200 mM Tris-H_2SO_4 buffer), 15 µl HLM and 13 µl 20 mM $MgSO_4$ (dissolved in sterile water). SV was pre-dissolved in acetone [41] and added to the incubation mixture to a final concentration of 100 µM. Acetone concentration in the final incubate did not exceed 1%. The reaction mixtures were incubated for 10 and 60 minutes at 37° C in a Fermaks incubator with a Vari-mix Thermolyne (Dan Meszansky A/S, Oslo, Norway).

Incubations were performed in absence (i.e. control) and presence of potent inhibitors of CYP3A4 (erythromycin 10 and 100 µM) and CYP2D6 (quinidine 5 and 25 µM). All experiments were carried out with 4 parallels at each experimental condition. In the experiments with erythromycin, the inhibitor was pre-incubated with HLM and NADPH for 30 minutes at 37° C (to obtain metabolite complexation) before adding SV in Tris-buffer and $MgSO_4$. The non-competitive inhibitor, quinidine, was co-incubated with the substrate. Incubations were terminated after 10 or 60 minutes by the addition of 1 ml acetonitrile (4° C). After 10 minutes on ice, the vials were centrifuged on a Universal 32R centrifuge (Hettich zentrifugen, Tuttlingen, Germany) for 15 minutes at 14000 rpm. To exclude separation of organic and aqueous phases, the whole supernatant was transferred to a new eppendorf vial and mixed for 10 seconds on a MS 1 Minishaker (Heigar laboratorieutstyr, Oslo, Norway). Then, 500 µl of the supernatant was mixed with 50 µl of internal standard solution (lovastatin (LV), 100 µg/ml, dissolved in acetonitrile) and subsequently evaporated to dryness by nitrogen gas. The residue was redissolved in 120 µl mobile phase A (20 % acetonitrile, 80% methylamine buffer, see section 2.3). This solution was centrifuged once more for 10 minutes at 14000 rpm, and 100 µl of the supernatant was finally injected onto the HPLC-MS system.

2.1.2 STUDIES WITH RECOMBINANT CYP ENZYMES

Recombinant human liver epithelial cell lines with specific expression of CYP3A4 (T5-3A4) and CYP2D6 (T5-2D6) were provided from the Nestlé Research Centre, Lausanne, Switzerland. Microsomal fractions from these cells were prepared according to the method described in the previous master thesis by Eili T. Kase [18]. The microsomes from these recombinant enzyme systems were used in an experimental procedure where the purpose was to study a possible CYP2D6 mediated, subsequent metabolism of SV metabolites following production via CYP3A4 microsomes. Incubations were performed with the same conditions as described in section 2.1.1, with minor modifications (see below). A schematic presentation of the incubation procedure used is illustrated in figure 2-1. First, 100 µM SV was incubated with T5-3A4 microsomes for 60 minutes. After termination of the reaction and removal of the proteins, one third of the supernatant was evaporated and reconstituted for analysis by HPLC-MS (see section 2.2), whereas another third part was re-incubated with T5-2D6 microsomes for 60 minutes. The supernatant from this latter incubation was also analysed by HPLC-MS, and the resulting metabolite pattern was compared with that found after incubation with T5-3A4.

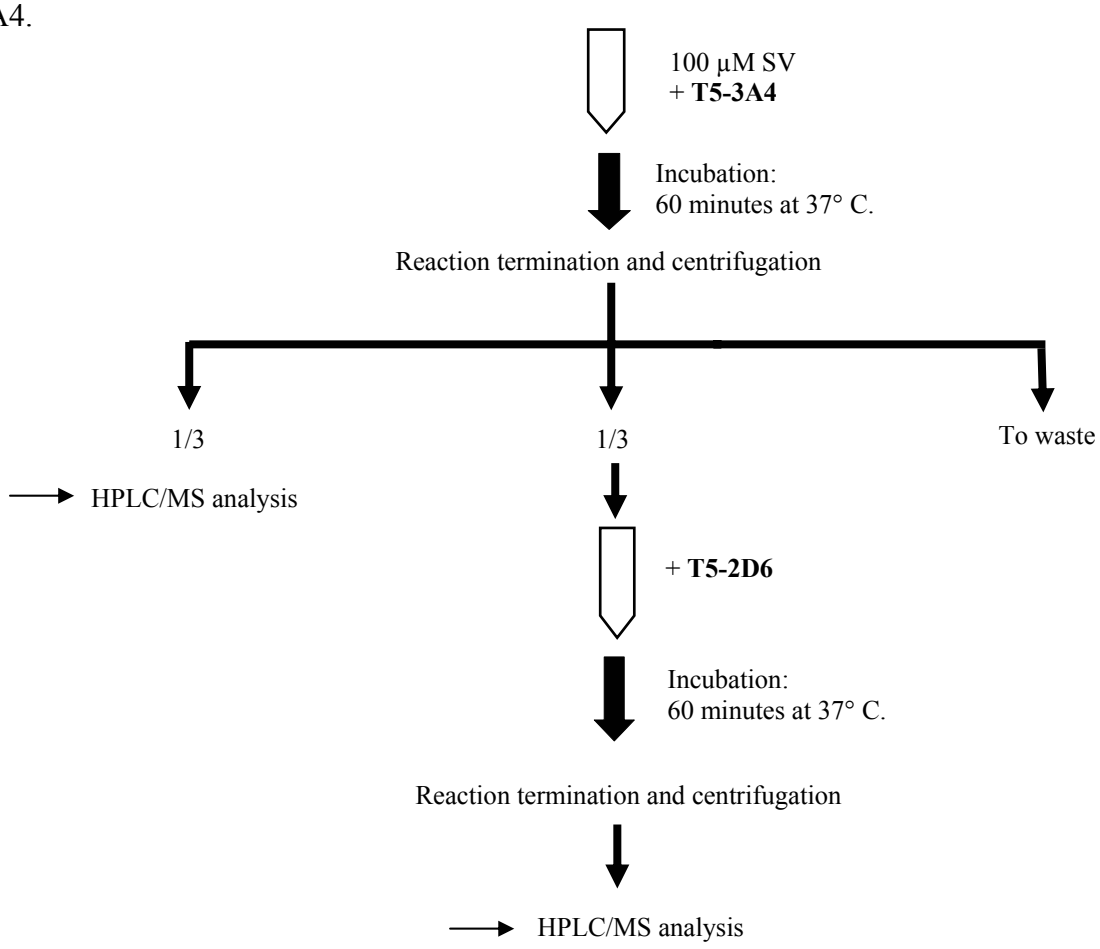


Fig. 2-1: Schematic presentation of the procedure used to study the sequential metabolism via CYP3A4 and CYP2D6 in microsomes from T5-3A4 and T5-2D6 recombinant cells.

The incubation mixture contained 112 µl T5-3A4 microsome suspension in a total volume of 500 µl (=0,67-0,87 mg protein/ml). The amount of Tris-H_2SO_4 buffer was accordingly reduced to 292 µl. The reaction was started by the addition of 112 µl T5-3A4 microsomes following a 3 minutes pre-incubation at 37° C of the substrate, NADPH and $MgSO_4$. After termination of the reaction and removal of proteins (section 2.1.1), 500 µl of the supernatant was added 50 µl I.S, evaporated to dryness, resolved in mobile phase A and analysed by HPLC-MS. Another 500 µl part of the supernatant was also evaporated to dryness. To the residue 39 µl NADPH and 155 µl sterile water was added. This solution was pre-incubated for 3 minutes at 37° C before addition of 56 µl T5-2D6 microsome suspension to a final volume of 250 µl (=0,67-0,87 mg protein/ml). The reaction was terminated after 60 minutes by the addition of 500 ml acetonitrile (4° C). I.S. (50 µl) was added after 10 minutes on ice, and proteins were removed before the whole supernatant (~750 µl) was evaporated to dryness. The final residue was redissolved in 120 µl mobile phase A and injected onto the HPLC-MS system.

In order to confirm the functionality of this procedure, a known substrate for CYP3A4 and CYP2D6, the diltiazem metabolite desacetyldiltiazem (M1), was incubated following the same procedure as for SV. It has earlier been shown that CYP2D6 and CYP3A4 are mediating the *O*-and *N*-demethylation of M1, respectively (Fig. 2-2) [45]. The substrate concentration was 100 µM, and the samples were analysed as described by Molden *et al.* [46].

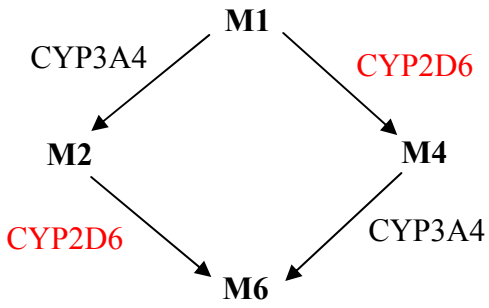


Fig. 2-2: Metabolic pathways of desacetyldiltiazem, M1[45]. Abbreviations used; M1=desacetyldiltiazem, M2=*N*-desmethyldesacetyldiltiazem, M4=*O*-desmethyldesacetyldiltiazem, M6= *N*, *O*-di-desmethyldesacetyldiltiazem.

2.2 DEVELOPMENT AND APPLICATION OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY ANALYSIS OF SIMVASTATIN AND ITS *IN VITRO* METABOLITES

2.2.1 THE CHROMATOGRAPHIC SEPARATION

The HPLC equipment (ThermoFinnigan, Austin, Texas, USA) consisted of an AS3000 autosampler and a P4000 quaternary pump. Aliquots (100 µl) of the samples were placed in the chilled autosampler tray (4° C). A reversed phase C18 column (Omnisphere C18, 30 x 2 mm, 3 µm, Varian, Harbor City, CA, USA) was used for chromatographic separation of SV and its metabolites. The analytical column was preceded by a guard column (Chromguard, 10 x 2 mm, 5 µm, Varian, Harbor City, CA, USA). A linear gradient mobile phase system eluted the analytes, as illustrated in Fig. 2-3. Solution A consisted of a 20/80 (v/v) mixture of acetonitrile and 2 mM methylamine buffer (adjusted to pH 4.5 with glacial acetic acid), whereas solution B had the same components in a relationship 80/20.

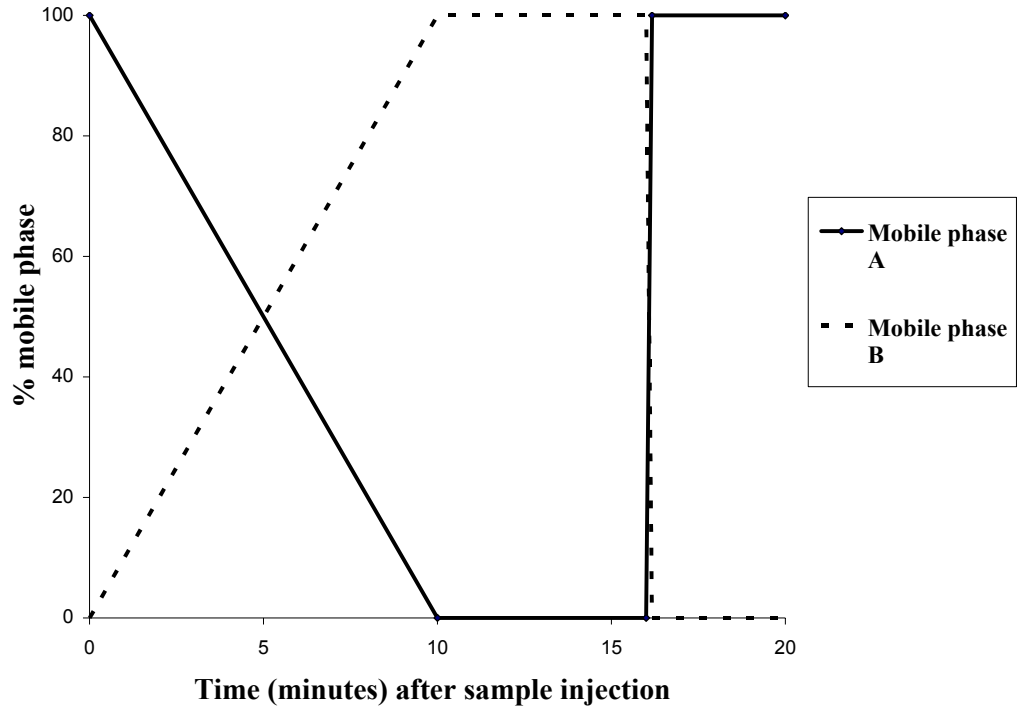


Fig. 2-3: The linear gradient system used for the chromatographic separation of SV and its metabolites.

The mobile phase changed from 100 % A to 100% B in 10 minutes. From 10 to 16 minutes of the run the mobile phase was 100 % B, then reversed to 100 % A over 17 seconds. The flow-rate was set at 0.2 ml/min during the first 16.1 minutes. During the last 3 minutes of the run, the column was re-equilibrated with mobile phase A (with a flow-rate set at 0.4 ml/min) before injection of another sample. The total run time was 19 minutes per sample. During the first 2 and last 3 minutes of the run the flow was sent to waste.

2.2.2 MASS SPECTROMETRIC DETECTION

The HPLC equipment was coupled to a ThermoQuest LCQ^{DUO} ion trap MS detector (ThermoFinnigan, Austin, Texas, USA), and the HPLC-MS interface was operated with an electrospray (ESI) inlet. The analysis was performed in positive mode (ESI^+). Tuning of the detector and optimisation of the ESI conditions were performed by injection of SV in mobile phase B (1000 ng/ml). A summary of the MS conditions used in the analysis of SV and metabolites is shown in appendix 1.

The detector was operated in single-MS, full scan mode. In the initial phase of the analytical method development, 1 mM formic acid was added to the mobile phase to improve sensitivity in the positive-ion mode. However, formation of various adduct ions of SV and metabolites, such as $[M+Na]^+$ and $[M+NH_4]^+$ was not optimal. Zhao *et al*. have investigated the effects of ammonium and various different alkylammonium acetate as mobile phase additives on the ionisation of SV and SVA [23] and observed that methylammonium acetate significantly improved the ion signal intensity of SV. By adding this buffer to the mobile phase system the methylammonium adduct of SV, $[SV+CH_3NH_3]^+$, was observed as the only major molecule ion, while the formation of other adduct ions were successfully suppressed. Thus, it was decided to add methylamine buffer (2mM, pH 4.5) to the mobile phase in the further development.

With methylamine as a mobile phase additive methylammonium adduct ions, $[M+CH_3NH_3]^+$ of both SV, I.S. and possible *in vitro* metabolites were expected to be the major ions in the full scan spectra. Due to lack of reference compounds, SV metabolites were deduced from MS peaks that showed a clear increase in intensity by incubation time. Their structural nature was interpreted from the characteristic mass-to-charge ratios of the detected peaks and their chromatographic retention times.

2.2.3 EVALUATION OF METHOD PRECISION

A large batch of quality control (q.c) samples, containing 5 µg/ml SV and 5 µg/ml I.S dissolved in mobile phase A, was prepared to evaluate inter- and intra assay variability in analytical precision. These samples were stored at -20° C, and two parallels were included in each analytical run (first and final analysis of each experiment).

3. RESULTS AND DISCUSSION

3.1 *IN VITRO* METABOLISM OF SIMVASTATIN

3.1.1 STUDIES WITH HLM IN ABSENCE OF INHIBITORS.

Fig. 3-1 illustrates a typical ESI–single-MS chromatogram derived from incubates of HLM with SV in absence of CYP inhibitors. SV lactone was eluted ~12.1 minutes after sample injection, whereas its corresponding hydroxy acid (SVA) was eluted at ~9.6 minutes. The retention time of the I.S (lovastatin) was ~11.3 and ~8.9 minutes for the lactone and acid form, respectively.

Reference compounds of SV metabolites were not available. However, based on mass-specific detection and chromatographic features, five MS peaks were interpreted to be lactone metabolites of SV (denoted I-V in Fig. 3-1). The *m/z* values present in the five MS peaks and the corresponding *m/z* values of the hydroxy acid forms (formed by non-enzymatic hydrolysis), are shown in Table 3-1. Except for metabolite 6′-COOH-SV (V), the corresponding hydroxy acid form of these lactone metabolites was apparent in the spectra (denoted IA-IVA in Fig. 3-1). In the further presentation, denominations and values of the metabolites I-V include both the lactone and acid forms.

Table 3-1: SV, I.S. and SV metabolites (I-V) present in HLM incubates, with corresponding masses (*m/z*) of their methylamine adduct, $[M+CH_3NH_3]^+$. Incubations were carried out as described in 2.1.1, in absence of inhibitors.

COMPOUND	LACTONE (*m/z*)	ACID (*m/z*)
SV	450	468
I.S.	436	454
6′-CH_2OH-SV (I)	466	484
3′-OH-SV (II)	466	484
6′-exomethylene-SV (III)	448	466
3′-OH-6′-CH_2OH-SV (IV)	482	500
6′-COOH-SV (V)	480	498

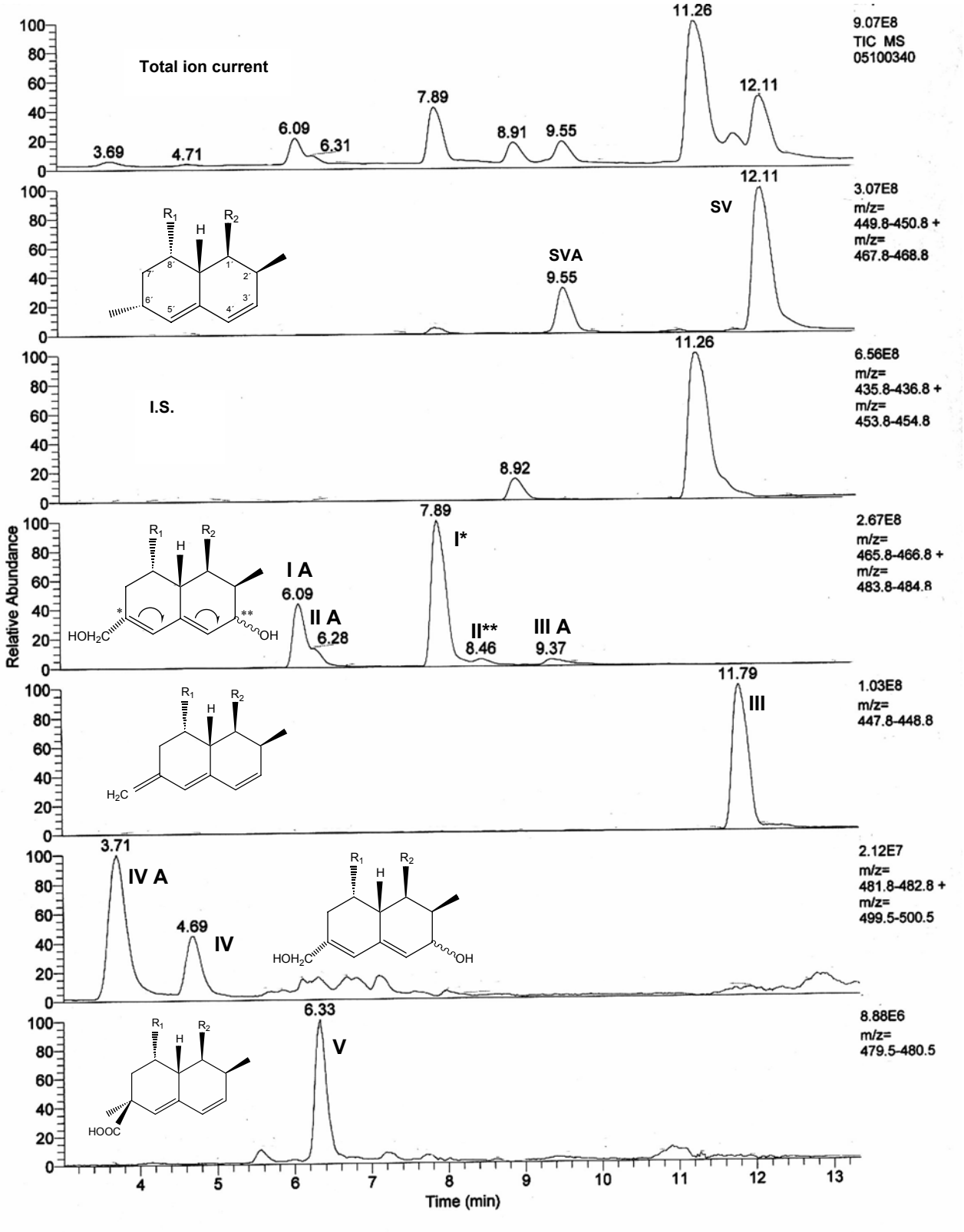
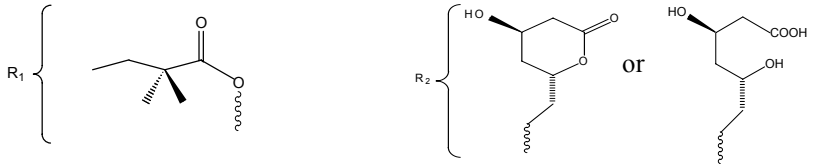


Fig. 3-1: A representative ESI-single MS chromatogram of SV and metabolites in a HLM incubate, presented as total ion current (sum of all compounds detected) and *m/z*-selected, individual chromatographic spectra of SV, I.S. and SV metabolites Structures of SV and the proposed metabolites I-V are presented in the figure. Incubations were carried out as described in 2.1.1, in absence of inhibitors.



(*) I hydroxylated in 6´-position, (**) II hydroxylated in 3´-position.

The increased signal intensities of all the deduced metabolites (I-V) with time in HLM incubations provided further evidence that these compounds were metabolites of SV (Fig. 3-2). Correspondingly, the level of SV declined by 44 % from 10 to 60 minutes of incubation.

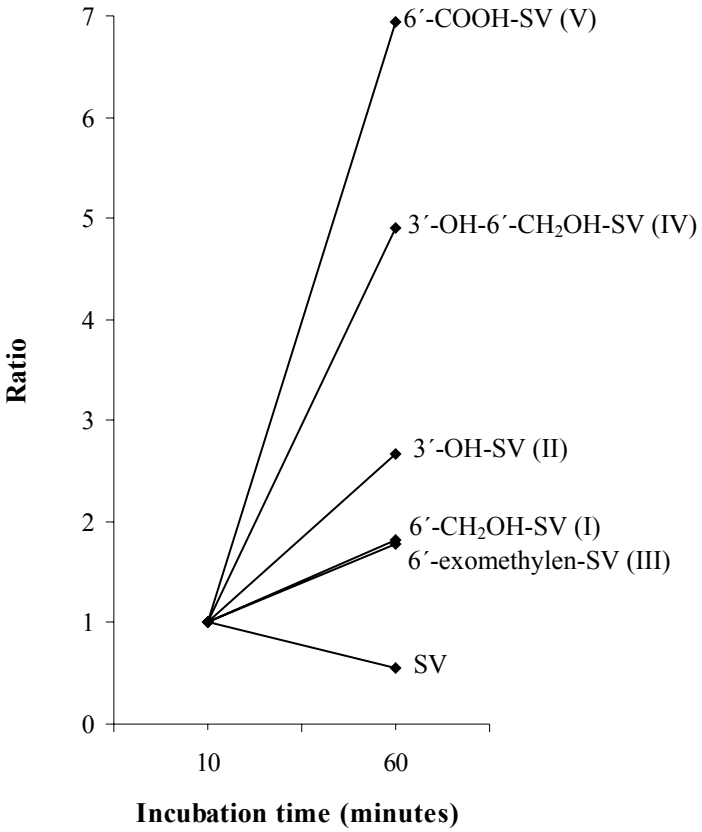


Fig. 3-2: Effect of incubation time on the relative levels of SV and deduced metabolites (I-V) in the HLM incubates. Values are presented as mean (n=4) ratios of the MS-signal intensities after 10 and 60 minutes incubation (ratio=MS $peaks_{60min}$ / $peaks_{10min}$, i.e. ratio at 10 minutes=1 for all agents). Incubations were carried out as described in 2.1.1, in absence of inhibitors.

The presence of two closely eluted peaks both with *m/z*=466 (one major peak at ~7.9 min and one minor peak at ~8.5 min, Fig. 3-1) was interpreted as two distinct mono-hydroxylated lactone metabolites. As the metabolite 6´-COOH-SV (V) also was formed in HLM experiments, it is reasonable to believe that its reduced precursor, 6´-CH_2OH-SV (I) represented one of the mono-hydroxylated metabolites. The other mono-hydroxylated metabolite was interpreted to be 3´-OH-SV (II), based on its presence in former HLM experiments with SV [1]. This latter metabolite will have a slightly longer retention time (i.e. less chromatographic retention on a reversed phase system) compared to 6´-CH_2OH-SV (I), as seen in Fig. 3-1. The corresponding hydroxy acids of 6´-CH_2OH-SV (I) and 3´-OH-SV were also seen as two peaks in the chromatographic spectra (~6.1 and ~6.3 min).

Previous *in* vitro studies with SV have not reported the metabolites 6´-CH_2OH-SV (I) and 6´-COOH-SV (V). However, together with 3´-OH-SV (II), these metabolites are described to be the most abundant SV metabolites in human bile [41]. The HMG-CoA reductase inhibitory activity of the hydroxy acid form of these metabolites were examined by Vickers *et al.* [41]. Compared to SVA, metabolite 6´-CH_2OH-SV (I) and 6´-COOH-SV (V) showed approximately 90 % and 40 % inhibiting activity, respectively, whereas metabolite 3´-OH-SV (II) completely lacked inhibitory activity [41].

6´-Exomethylen-SV (III) eluted closely to SV lactone as expected from their structural similarities. The acid form, was interpreted to be the third peak with m/z=466 (IIIA, eluted at ~9.4 min, Fig. 3-1). The di-hydroxylated SV metabolite, IV, is likely the combined metabolite of the two mono-hydroxylated metabolites, 6´-CH_2OH-SV (I) and 3´-OH-SV (II), namely 3´-OH-6´-CH_2OH-SV (IV). Previous metabolism studies with SV have not described this metabolite, but it might be similar to one of the unidentified *in vivo* metabolites reported in human bile [41]. The sequential HPLC retention times of the suggested metabolites correlated well with the chromatographic features of Vickers *et al.*[41], who characterised their *in vitro* and *in vivo* metabolites by comparing retention times with reference compounds.

The dissimilarities in metabolite profile between the present HLM experiment and the findings of Prueksaritanont *et al.* (section 1.5.4) [1] are likely due to differences in assay conditions. A range of factors, including pH and ionic strength, influences CYP activities [17]. It has been reported that pH significantly affected the metabolite formation of cyclosporine via CYP3A4 [18]. While Prueksaritanont *et al.* performed their experiments with a sodium phosphate buffer at pH 7.4 [1], the present study was carried out with a Tris buffer at pH 7.5. In addition, it is unclear whether magnesium should be added (and in which amount) to microsomal incubations mixtures as this cofactor could create unwanted kinetic artefacts [17]. Prueksaritanont *et al.* used a 20 fold-higher magnesium concentration compared to present study. Thus, although similar SV concentration (100 µM) was applied in the HLM studies, there are differences in the incubation conditions that may explain different metabolite profiles. In contrast to Prueksaritanont *et al*, who only described one of the three major human biliary metabolites (3´-OH-SV), all three human bile metabolites were found in the present work. It is therefore likely that the results provided here better reflect the *in vivo* situation than those of the previous study.

6′-CH_2OH-SV (I) was the most abundant metabolite in the experiments (Table 3-2). The level of this metabolite was >3-fold higher than the other oxidative metabolites. It was observed that the metabolites hydroxylated in 3′-position (3′-OH-SV (II) and 3′-OH-6′-CH_2OH-SV (IV)) appeared dominantly as hydroxy acid forms, in contrast to SV and the metabolites 6′-CH_2OH-SV (I) and 6′-exomethylene-SV (III), where the lactone forms were the predominant form (Table 3-2 and Fig. 3-1). A possible explanation is that hydroxylation in 3′-position could involve hydrogen binding between the 3′-OH-group and a hydroxyl group in the open acid form, a situation that would favour the hydroxy acid form over the closed lactone form.

Table 3-2: Relative MS-peak intensities (sum of lactone and acid form) and lactone/acid ratios of the deduced SV metabolites in HLM incubate. The values for relative MS-peak intensities (mean ± SD, n=4) are presented as ratios of the most abundant metabolite, 6′-CH_2OH-SV (I). Incubations were carried out as described in 2.1.1, in absence of inhibitors.

SV metabolites	Relative intensities	Lactone/acid ratio
6′-CH_2OH-SV (I)	1	2,3 ± 0,4
3′-OH-SV (II)	0,15 ± 0,004	0,53 ± 0,2
6′-exomethylen-SV (III)	0,32 ± 0,02	7,0 ± 0,4
3′-OH-6′-CH_2OH-SV (IV)	0,11 ± 0,03	0,56 ± 0,07
6′-COOH-SV (V)	0,06 ± 0,03	only detected as lactone

Altogether, four distinct oxidative modifications of SV were detected in the current HLM experiments. Based on these reactions, an oxidative metabolite pattern is proposed in Fig. 3-3, where 3′-OH-6′-exomethylene-SV (VI) and 3′-OH-6′-COOH-SV (VII) are outlined as end-stage metabolites of the oxidative reactions. Trace amounts of 3′-OH-6′-exomethylene-SV (VI) appeared as a peak at ~8.2 min (not indicated in Fig. 3-1), but its peak intensity was too low to be detected with sufficient accuracy. The metabolite 3′-OH-6′-COOH-SV (VII) was not present in the MS chromatogram after incubations of SV with HLM. The absence of this metabolite probably reflected that it is too hydrophilic to be detected in the present mobile phase system (eluted in the front).

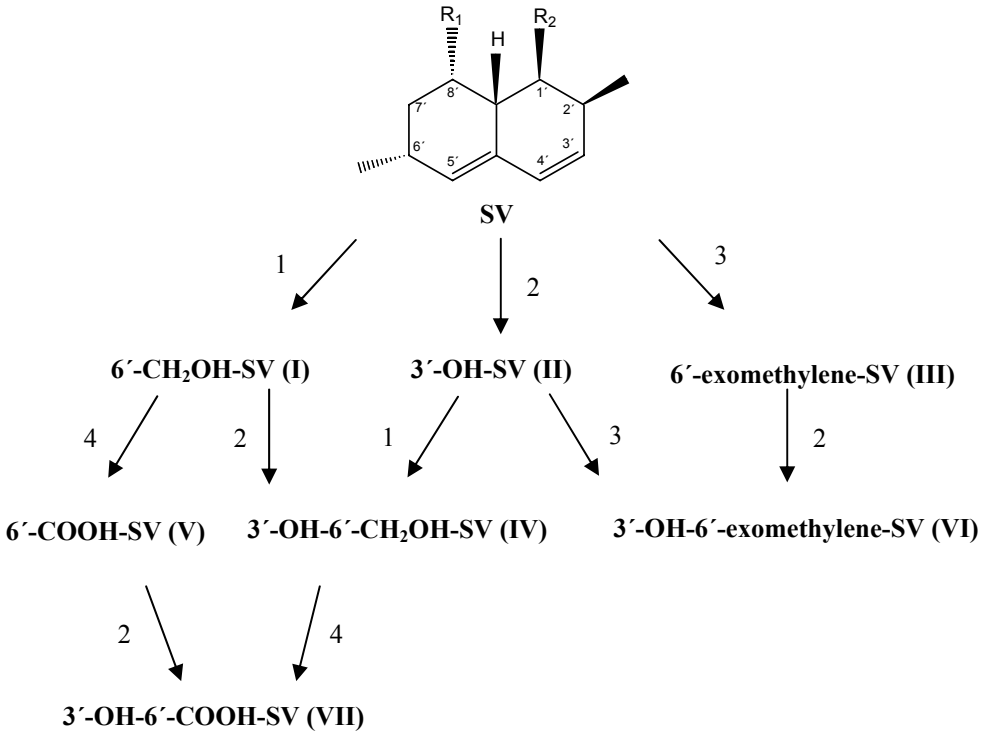


Fig. 3-3: Proposed metabolite pattern of SV. The numbers 1-4 represent the four distinct oxidative modifications detected in the HLM experiments. R_1 and R_2, see Fig. 3-1.

3.1.2 STUDIES WITH HLM IN PRESENCE OF INHIBITORS

The CYP3A4 inhibitor erythromycin inhibited SV metabolism, in accordance with previous experiments [1]. SV level increased by 20-70 % (ratio 1,2-1,7) in presence of erythromycin, and this inhibition was dose-dependent (Fig. 3-4). Additionally, the inhibition of SV metabolism increased with time (Fig. 3-4). This agrees with the fact that erythromycin performs inhibition through metabolite complexation [47], which is a time dependent event (i.e. inhibitory effect increased with time).

The level of the metabolites 3´-OH-SV (II) and 6´-exomethylen-SV (III) showed a small decline in presence of erythromycin (Fig. 3-4), also in agreement with previous *in vitro* study [1]. Likewise, the previously not reported metabolite 3´-OH-6´-CH_2OH-SV (IV) followed the same pattern as its precursor 3´-OH-SV (II) in presence of erythromycin. The decline observed for 3´-OH-SV (II), 6´-exomethylene-SV (III) and 3´-OH-6´-CH_2OH-SV (IV) was dose-dependent within both incubation times applied (10 and 60 min, Fig. 3-4). Based on the increased inhibition of SV metabolism with time, it was expected that the level of SV

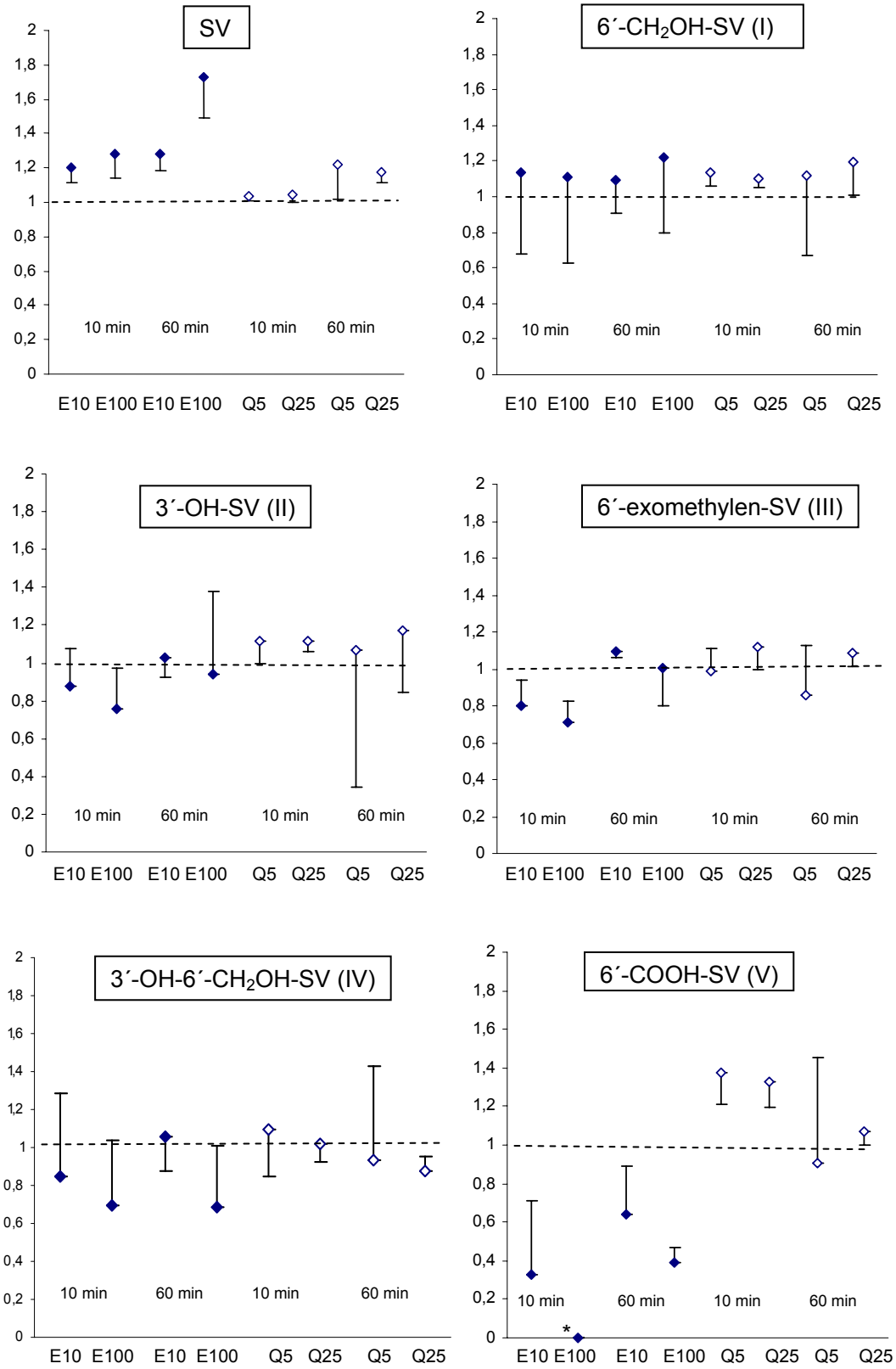


Fig. 3-4: Effect of CYP inhibitors on the levels of SV and metabolites in HLM incubations at different time events and inhibitor concentrations. SV metabolites are expressed as ratio of control activities (i.e. in absence of inhibitors). The dashed line at y=1 represents the control activities. E= erythromycin (10/100µM), filled symbols, Q= quinidine (5/25 µM), open symbols. Results are presented as mean (n=4). SD is shown as + SD for values <1 and – SD for values >1. All incubations were carried out as described in section 2.1.1.
(*) not detected.

metabolites would decrease with time during co-incubations of erythromycin. It was observed that the level of the metabolites 3´-OH-SV (II), 6´-exomethylene-SV (III) and 3´-OH-6´-CH_2OH-SV (IV) actually was relatively higher at 60 minutes compared to 10 minutes incubation. The reason(s) for this observation is unclear, but it might perhaps indicate that the further metabolism of SV metabolites is catalysed via CYP3A4 to a higher degree than SV itself.

The level of the most abundant metabolite, 6´-CH_2OH-SV (I) increased with 10-20 % (ratio 1,1-1,2) in presence of erythromycin (Fig. 3-4). This observation might indicate that 6´-CH_2OH-SV (I) is a better CYP3A4-substrate than its precursor SV or alternatively, that other enzymes than CYP3A4 are important in catalysing the formation of 6´-CH_2OH-SV (I). The metabolite 6´-COOH-SV (V), the likely subsequent metabolite of 6´-CH_2OH-SV (I), showed the relatively largest decline amongst the measured compounds in presence of erythromycin (Fig. 3-4). Incubation with 100 μM erythromycin for 10 minutes resulted in undetectable amounts of this metabolite, whereas the other conditions showed a decline of 40-70 % (ratio 0,6-0,3) compared to control. As for 3´-OH-SV (II), 6´-exomethylene-SV (III) and 3´-OH-6´-CH_2OH-SV (IV), the relative level of 6´-COOH-SV (V) was higher after 60 minutes compared to 10 minutes of incubation.

Co-incubation of quinidine, known as a potent inhibitor of CYP2D6, generally increased the level of all measured compounds compared to control (increase in 19 of 24 experimental conditions, Fig. 3-4). In contrast to erythromycin, quinidine did not demonstrate a dose dependent effect on SV and metabolites. A possible explanation is that quinidine performs 100 % inhibition of CYP2D6 even at 5 μM, hence, adding more quinidine will not affect the degree of inhibition. The time dependent effect of inhibition seen with erythromycin was not observed for quinidine (Fig. 3-4). This is likely due to the fact that erythromycin and quinidines mediates different type of inhibition mechanisms. Quinidine is a non-competitive inhibitor [48] where inhibition is independent of time.

Unlike the studies with erythromycin, none of the measured metabolites decreased in presence of quinidine. Based on these observations, it could be speculated that CYP2D6 catalyses a common, unexplored, metabolic pathway of both SV and metabolites, not detected

in present work. It is possible that this presumed oxidation by CYP2D6 leads to a structural alteration, where the resulting products would not be detected under the applied MS conditions. Alternatively, the reaction could imply an increase in hydrophilicity, which leads to elution of the CYP2D6 mediated metabolites before the flow is directed to the MS detector.

3.1.3 STUDIES WITH RECOMBINANT CYP ENZYMES

In view of the speculations that one or more of the active metabolites of SV is metabolised by CYP2D6 [2, 3], an experiment was performed where SV first was subjected to CYP3A4 metabolism (T5-3A4), and then the remaining SV and metabolites produced by T5-3A4 were subjected to further metabolism with T5-2D6 microsomes.

Incubation of SV with T5-3A4 resulted in formation of 6′-CH_2OH-SV (I), 3′-OH-SV (II) and 6′-exomethylen-SV (III), but the two secondary metabolites, 3′-OH-6′-CH_2OH-SV (IV) and 6′-COOH-SV (V) were not detected. This was probably due to the relatively low formation of the precursors 6′-CH_2OH-SV (I) and 3-OH-SV (II) in T5-3A4 incubations (10 fold lower than in HLM). Ratios of the three metabolites formed after incubation with T5-3A4 microsomes were compared with corresponding ratios of metabolites formed after incubation with HLM (Table 3-3). This was performed to evaluate the relative importance of CYP3A4 activity for the levels of the primary metabolites formed in the HLM experiments described in section 3.1.1. The higher relative levels of 6′-CH_2OH-SV (I) and 6′-exomethylen-SV (III) compared to 3′-OH-SV (II) in HLM versus T5-3A4 experiments might indicate that these former metabolites are produced to a greater extent through non-CYP3A4 metabolism than 3′-OH-SV (II). The analogous proportions of metabolite 6′-CH_2OH-SV (I) and 6′-exomethylen-SV (III) in both the HLM and the T5-3A4 incubate, could reflect that these two metabolites follow identical enzymekinetic profiles.

Table 3-3: Comparison of SV metabolite ratios in HLM and T5-3A4 incubates. The values are presented as mean ± SD (n=4). I= 6′-CH_2OH-SV, II= 3′-OH-SV, III= 6′-exomethylen SV.

Metabolite ratio	HLM	T5-3A4
I/II	6,8 ± 0,2	4,8 ± 2,1
I/III	3,2 ± 0,2	3,2 ± 0,4
III/II	2,2 ± 0,2	1,5 ± 0,7

In the combined experiment with T5-3A4 and T5-2D6 microsomes it was observed a slight decrease (~10%) in SV level after incubation with T5-2D6 compared to the level measured after incubation with T5-3A4, while the metabolites 6´-CH_2OH-SV (I), 3´-OH-SV (II) and 6-exomethylen-SV (III) was reduced by about 30, 15 and 30 %, respectively (Fig. 3-5). This decrease in all detected compounds upon incubation with T5-2D6 further supports that CYP2D6 is involved in the metabolism of SV and metabolites. However, the final end-stages of these metabolic reactions have not been determined in this study.

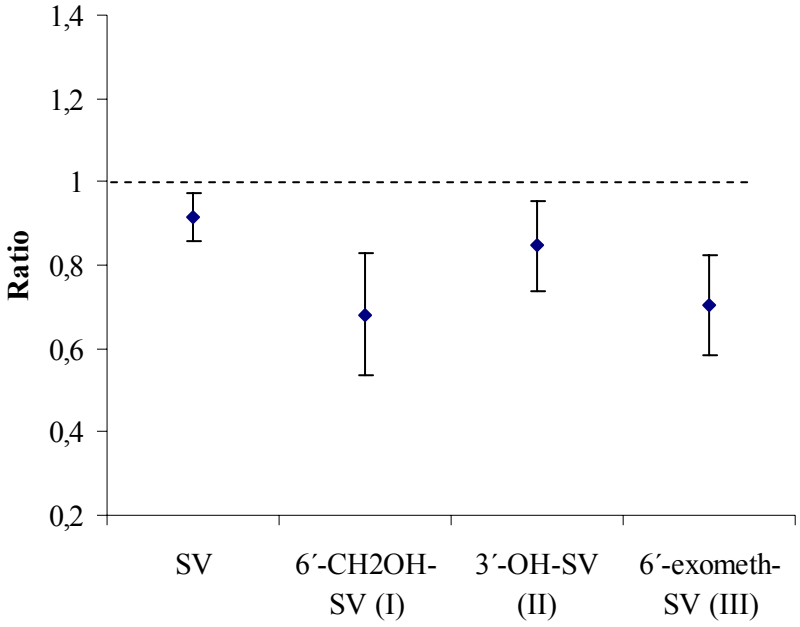


Fig. 3-5: Relative levels (ratios) of SV and metabolites after T5-2D6 incubation compared to level after T5-3A4 incubation. The dashed line at y=1 represents the levels after incubation with T5-3A4. Results are presented as mean ± SD (n=4). Incubations were carried out as described in section 2.1.2.

The functionality of the procedure used to study the sequential SV metabolism by T5-3A4 and T5-2D6 microsomes was verified by the performance of an analogous experiment with the metabolite of the calcium-channel blocker diltiazem, desacetyldiltiazem, M1. After incubation with T5-3A4, the *N*-demethylated metabolite M2 was the only metabolite detected in the resulting MS chromatogram (Fig. 3-6A). During subsequent T5-2D6 experiments, M1 was transformed to the *O*-demethylated metabolite M4, whereas M2 was converted to the *N, O*-didesmethylated metabolite M6 (Fig. 3-6B). This was in agreement with the expected metabolite profile illustrated in Fig. 2-2 (section 2.1.2). The level of the primary substrate, desacetyl-diltiazem (M1) and its CYP3A4 mediated metabolite, *N*-desmethyldesacetyl-diltiazem (M2), decreased by about 35 and 70 %, respectively, after incubation with T5-2D6

(data not shown). These findings indicate that the procedure used in the studies with SV and recombinant CYP enzymes was suitable for its purpose.

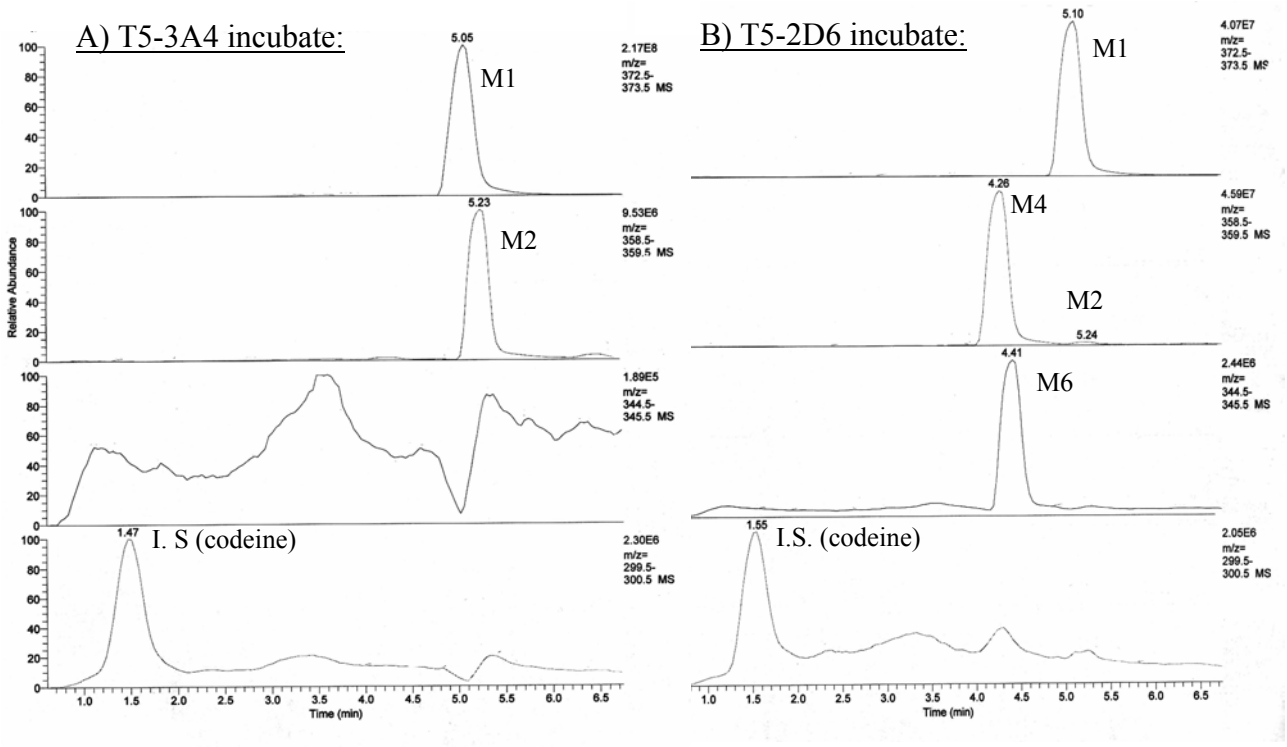


Fig. 3-6: MS chromatogram after incubation of M1 with T5-3A4 microsomes (A) and subsequently with T5-2D6 microsomes (B). Incubations were carried out as described in section 2.1.2. Abbreviations used; M1=desacetyldiltiazem, M2=*N*-desmethyldesacetyldiltiazem,M4=*O*-desmethyldesacetyldiltiazem, M6= *N*,*O*-di-desmethyldesacetyldiltiazem.

3.2 ANALYTICAL CONSIDERATIONS

The initial use of 1 mM formic acid as a mobile phase additive resulted in multiple adduct formation. This unfavourable situation was significantly improved by the addition of 2 mM methylamine to the mobile phase, as 98 % of the total ion signal for SV was observed as methylamine adduct $[SV + CH_3NH_3]^+$ (Table 3-4).

Due to lack of reference compounds of SV metabolites, a complete validation of the present HPLC-MS method could not be accomplished. The injection of a quality control (q.c.) sample of SV and I.S. as the first and final sample of each analytical run was performed to monitor possible variability in the MS detection. Table 3-5 summarises the intra- and inter-run variability of the q.c. samples analysed (6 runs, 12 q.c. samples). The degree of variation was

less than 10 %, except from one run where the intra- and inter-run was 11.9 % and 19.2 %, respectively (Table 3-5). These data were considered as satisfactory, and the analytical method developed in the present work is therefore suitable for *in vitro* metabolic studies.

Table 3-4: Evaluation of the effect of different mobile phase additives on SV adduct formation. The adducts are expressed as % of the total ion current signal. (*) rearranged from lactone to acid form in the ion source

Adduct	Formic acid additive, 1 mM	Methylamine additive, 2 mM
$[SV+H\ ]^+$	5 %	0,04 %
$[SV+Na]]^+$	10 %	0,08 %
$[SV+NH4]^+$	69 %	0
$[\ SV+\ CH3NH3]^+$	0	98 %
$[SVA+H]^+$ *	15 %	0

Table 3-5: Evaluation of the analytical variability. Data are presented as relative MS-peak intensities of SV in q.c. samples in 6 runs (n=2 in each run).

Intra-run variability= $\frac{p_1-p_2}{p_2}$ * 100 %, Inter-run variability= $\frac{\text{mean}_{tot} - \text{mean}_{run}}{\text{mean}_{tot}}$ * 100 %

Run	Parallel	Rel. peak int.	Intra run variability (%)	Inter run variability (%)
1	1.1	0.887	2.0	5.0
	1.2	0.869		
2	2.1	0.821	2.6	9.9
	2.2	0.843		
3	3.1	0.915	0.51	1.2
	3.2	0.910		
4	4.1	0.902	8.9	6.8
	4.2	0.821		
5	5.1	1.171	11.9	19.2
	5.2	1.032		
6	6.1	0.969	2.2	3.7
	6.2	0.948		
	Mean_{tot}	0.924		

4. CONCLUSION

This is the first *in vitro* study with SV where all three major *in vitro* oxidative metabolites have been produced. The overall results of the conducted experiments indicate that CYP2D6 is involved in the biotransformation of SV. Studies with the CYP2D6 inhibitor quinidine in HLM incubations and experiments with recombinant CYP enzymes suggested that CYP2D6 might be responsible for a unknown metabolic reaction. However, the potential *in vivo* importance of CYP2D6 needs to be evaluated in a study where pharmacokinetic measurements of SV and metabolites are investigated in relation to CYP2D6 phenotype. Further *in vitro* experiments with analytical modifications should also be performed to identify the metabolic reaction possibly mediated by CYP2D6.

REFERENCES

1. Prueksaritanont, T., et al., *In vitro metabolism of simvastatin in humans [SBT]identification of metabolizing enzymes and effect of the drug on hepatic P450s.* Drug Metab Dispos, 1997. **25**(10): p. 1191-9.
2. Mulder, A.B., et al., *Association of polymorphism in the cytochrome CYP2D6 and the efficacy and tolerability of simvastatin.* Clin Pharmacol Ther, 2001. **70**(6): p. 546-51.
3. Nordin, C., et al., *Is the cholesterol-lowering effect of simvastatin influenced by CYP2D6 polymorphism?* Lancet, 1997. **350**(9070): p. 29-30.
4. Hardman, J.G., L.E. Limbird, and A.G. Gilman, *The Pharmacological basis of therapeutics*. 10 ed. 2001: The McGraw Hill Companies.
5. Herfindal, E.T. and D.R. Gourley, *Textbook of therapeutics*. 7 ed. 2000: Lippincott Williams & Wilkins.
6. Ingelman-Sundberg, M., M. Oscarson, and R.A. McLellan, *Polymorphic human cytochrome P450 enzymes: an opportunity for individualized drug treatment.* Trends Pharmacol Sci, 1999. **20**(8): p. 342-9.
7. Lin, J.H. and A.Y. Lu, *Interindividual variability in inhibition and induction of cytochrome P450 enzymes.* Annu Rev Pharmacol Toxicol, 2001. **41**: p. 535-67.
8. Molden, E., *Variability in cytochrome P450 (CYP) mediated metabolism of cardiovascular drugs- clinical implications and practical attempts to avoid potential problems.* Submitted to HeartDrug, 2003.
9. Nebert, D.W., et al., *The P450 gene superfamily: recommended nomenclature.* DNA, 1987. **6**(1): p. 1-11.
10. Rogers, J.F., A.N. Nafziger, and J.S. Bertino, Jr., *Pharmacogenetics affects dosing, efficacy, and toxicity of cytochrome P450-metabolized drugs.* Am J Med, 2002. **113**(9): p. 746-50.
11. *Cytochrome P450 homepage*, http://drnelson.utmem.edu/CytochromeP450.html
12. Donato, M.T. and J.V. Castell, *Strategies and molecular probes to investigate the role of cytochrome P450 in drug metabolism: focus on in vitro studies.* Clin Pharmacokinet, 2003. **42**(2): p. 153-78.
13. Ingelman-Sundberg, M., *Pharmacogenetics: an opportunity for a safer and more efficient pharmacotherapy.* J Intern Med, 2001. **250**(3): p. 186-200.
14. Bertilsson, L., et al., *Molecular genetics of CYP2D6: clinical relevance with focus on psychotropic drugs.* Br J Clin Pharmacol, 2002. **53**(2): p. 111-22.
15. Molden, E., A. Asberg, and H. Christensen, *CYP2D6 is involved in O-demethylation of diltiazem. An in vitro study with transfected human liver cells.* Eur J Clin Pharmacol, 2000. **56**(8): p. 575-9.
16. Lin, J.H. and A.Y. Lu, *Role of pharmacokinetics and metabolism in drug discovery and development.* Pharmacol Rev, 1997. **49**(4): p. 403-49.
17. Tucker, G.T., J.B. Houston, and S.M. Huang, *Optimizing drug development: strategies to assess drug metabolism/transporter interaction potential--towards a consensus.* Br J Clin Pharmacol, 2001. **52**(1): p. 107-17.
18. Kase, E.T., *Metodeutvikling for isolering av mikrosomer fra THLE celler*, in *Department of Pharmacology, School of Pharmacy*. 2002, University of Oslo.
19. Kremers, P. *Liver microsomes: a convenient tool for metabolism studies but..* in *European symposium on the prediction of drug metabolism in man: progress & problems*. 1999. Liege.
20. Clarke, N.J., et al., *Systematic LC/MS metabolite identification in drug discovery.* Anal Chem, 2001. **73**(15): p. 430A-439A.

21. Lee, M.S. and E.H. Kerns, *LC/MS applications in drug development.* Mass Spectrom Rev, 1999. **18**(3-4): p. 187-279.
22. Reubsaet, L., *Use of LC-MS in bio analysis of pharmaceuticals and macromolecules*, in Department of Pharmaceutical Analysis, School of Pharmacy. 2002, University of Oslo. p. 1-55.
23. Zhao, J.J., A.Y. Yang, and J.D. Rogers, *Effects of liquid chromatography mobile phase buffer contents on the ionization and fragmentation of analytes in liquid chromatographic/ionspray tandem mass spectrometric determination.* J Mass Spectrom, 2002. **37**(4): p. 421-33.
24. *Drug consumption in Norway 1998-2002*, http://www.legemiddelforbruk.no, Nasjonalt folkehelseinstitutt.
25. Shepherd, J., et al., *Prevention of coronary heart disease with pravastatin in men with hypercholesterolemia. West of Scotland Coronary Prevention Study Group.* N Engl J Med, 1995. **333**(20): p. 1301-7.
26. Downs, J.R., et al., *Primary prevention of acute coronary events with lovastatin in men and women with average cholesterol levels: results of AFCAPS/TexCAPS. Air Force/Texas Coronary Atherosclerosis Prevention Study.* Jama, 1998. **279**(20): p. 1615-22.
27. *Randomised trial of cholesterol lowering in 4444 patients with coronary heart disease: the Scandinavian Simvastatin Survival Study (4S).* Lancet, 1994. **344**(8934): p. 1383-9.
28. Sacks, F.M., et al., *The effect of pravastatin on coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events Trial investigators.* N Engl J Med, 1996. **335**(14): p. 1001-9.
29. *Prevention of cardiovascular events and death with pravastatin in patients with coronary heart disease and a broad range of initial cholesterol levels. The Long-Term Intervention with Pravastatin in Ischaemic Disease (LIPID) Study Group.* N Engl J Med, 1998. **339**(19): p. 1349-57.
30. Schmitz, G. and W. Drobnik, *Pharmacogenomics and pharmacogenetics of cholesterol-lowering therapy.* Clin Chem Lab Med, 2003. **41**(4): p. 581-9.
31. Jones, P.H., *Comparing HMG-CoA reductase inhibitors.* Clin Cardiol, 2003. **26**(1 Suppl 1): p. I15-20.
32. Williams, D. and J. Feely, *Pharmacokinetic-pharmacodynamic drug interactions with HMG-CoA reductase inhibitors.* Clin Pharmacokinet, 2002. **41**(5): p. 343-70.
33. *Felleskatalogen 2003*.
34. Omar, M.A., J.P. Wilson, and T.S. Cox, *Rhabdomyolysis and HMG-CoA reductase inhibitors.* Ann Pharmacother, 2001. **35**(9): p. 1096-107.
35. Charatan, F., *Bayer decides to withdraw cholesterol lowering drug.* Bmj, 2001. **323**(7309): p. 359.
36. Molden, E. and A. Asberg, *[Metabolic interactions with statins].* Tidsskr Nor Laegeforen, 2001. **121**(2): p. 189-93.
37. Prueksaritanont, T., et al., *beta-Oxidation of simvastatin in mouse liver preparations.* Drug Metab Dispos, 2001. **29**(10): p. 1251-5.
38. Corsini, A., et al., *New insights into the pharmacodynamic and pharmacokinetic properties of statins.* Pharmacol Ther, 1999. **84**(3): p. 413-28.
39. Cheng, H., et al., *Metabolic disposition of simvastatin in patients with T-tube drainage.* Drug Metab Dispos, 1994. **22**(1): p. 139-42.
40. Vickers, S., et al., *Metabolic disposition studies on simvastatin, a cholesterol-lowering prodrug.* Drug Metab Dispos, 1990. **18**(2): p. 138-45.

41. Vickers, S., et al., *In vitro and in vivo biotransformation of simvastatin, an inhibitor of HMG CoA reductase.* Drug Metab Dispos, 1990. **18**(4): p. 476-83.
42. Prueksaritanont, T., B. Ma, and N. Yu, *The human hepatic metabolism of simvastatin hydroxy acid is mediated primarily by CYP3A, and not CYP2D6.* Br J Clin Pharmacol, 2003. **56**(1): p. 120-4.
43. Geisel, J., et al., *The efficacy of simvastatin is not influenced by CYP2D6 polymorphism.* Clin Pharmacol Ther, 2002. **72**(5): p. 595-6.
44. Mulder, A.B., F.A. van den Bergh, and I. Vermes, *Response to "The efficacy of simvastatin is not influenced by CYP2D6 polymorphism" by Geisel et al.* Clin Pharmacol Ther, 2003. **73**(5): p. 475.
45. Molden, E., A. Asberg, and H. Christensen, *Desacetyl-diltiazem displays severalfold higher affinity to CYP2D6 compared with CYP3A4.* Drug Metab Dispos, 2002. **30**(1): p. 1-3.
46. Molden, E., et al., *High-performance liquid chromatography-mass spectrometry analysis of diltiazem and 11 of its phase I metabolites in human plasma.* J Pharm Biomed Anal, 2003. **33**(2): p. 275-85.
47. Lindstrom, T.D., B.R. Hanssen, and S.A. Wrighton, *Cytochrome P-450 complex formation by dirithromycin and other macrolides in rat and human livers.* Antimicrob Agents Chemother, 1993. **37**(2): p. 265-9.
48. Bertelsen, K.M., et al., *Apparent mechanism-based inhibition of human CYP2D6 in vitro by paroxetine: comparison with fluoxetine and quinidine.* Drug Metab Dispos, 2003. **31**(3): p. 289-93.

APPENDIX

MS LCQ ^{Duo} INSTRUMENT STATUS FOR THE ANALYSIS OF SV AND METABOLITES

ESI Source:		
Spray voltage (kV)	:	4,5
Spray current (μA)	:	1,37
Sheat gas flow rate	:	20
Aux gas flow rate	:	10
Capillary voltage (V)	:	4
Capillary temperature °C	:	200
Tube lens (V, sp)	:	-5
Ion optics:		
Multipole 1 offset (V)	:	-1,75
Lens voltage (V)	:	-16
Multipole 2 offset (V)	:	-7,5
Multipole RF Amp (Vp-p, sp)	:	400
Ion detection system:		
Dynode voltage (kV)	:	-14,82
Multiplier voltage (V)	:	-989,13