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Research Paper

Interconversion Pharmacokinetics of Simvastatin and its Hydroxy Acid in Dogs: Effects of Gemfibrozil

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Purpose. To characterize the pharmacokinetics of simvastatin (SV) and simvastatin acid (SVA), a lactone–acid pair known to undergo reversible metabolism, and to better understand mechanisms underlying pharmacokinetic interactions observed between SV and gemfibrozil.
Methods. Pharmacokinetic studies were conducted after intravenous administration of SV and SVA to dogs pretreated with a vehicle or gemfibrozil. *In vitro* metabolism of SVA in dog hepatocytes as well as *in vitro* hepatic and plasma conversion of SV/SVA were investigated in the absence and presence of gemfibrozil.
Results. In control animals, the irreversible elimination clearances of SV (CL_{10}) and SVA (CL_{20}) were 10.5 and 18.6 ml min^{-1} kg^{-1}, respectively. The formation clearance of SVA from SV (CL_{12} = 4.8 ml min^{-1} kg^{-1}) was 8-fold greater than that of SV from SVA (CL_{21} = 0.6 ml min^{-1} kg^{-1}), and the recycled fraction was relatively minor (0.009). In gemfibrozil-treated animals, CL_{10} was essentially unchanged, whereas CL_{12}, CL_{20}, CL_{21}, and recycled fraction were significantly decreased to 2.9, 9, 0.14 ml min^{-1} kg^{-1}, and 0.003, respectively. In control dogs, values for real volume of distribution at steady state ($V_{ss,real}$) of SV (2.3 L kg^{-1}) were much larger than the corresponding values of SVA (0.3 L kg^{-1}). Gemfibrozil treatment did not affect $V_{ss,real}$ of either SV or SVA. In dog hepatocytes, gemfibrozil modestly affected the formation of CYP3A-mediated oxidative metabolites (IC_{50} > 200 μM) and β-oxidative products (IC_{50} ~100 μM), but markedly inhibited the glucuronidation-mediated lactonization of SVA and the glucuronidation of an SVA β-oxidation product (IC_{50} = 18 μM). In *in vitro* dog and human liver S9 and plasma, hydrolysis of SV to SVA was much faster than that of SVA to SV. Gemfibrozil (250 μM) had a minimal inhibitory effect on the hydrolysis of either SV to SVA or SVA to SV in dog and human liver S9, but had a significant (~60%) inhibitory effect on the SV to SVA hydrolysis in both dog and human plasma.
Conclusions. In dogs, the interconversion process favored the formation of SVA and was less efficient than the irreversible elimination processes of SV and SVA. Treatment with gemfibrozil did not affect the distribution of SV/SVA, but rather affected the elimination of SVA and the SV/SVA interconversion processes. Gemfibrozil decreased CL_{20} and CL_{21} likely via its inhibitory effect on the glucuronidation of SVA, and not on the CYP3A-mediated oxidative metabolism of SV or SVA, the β-oxidation of SVA, nor the SVA to SV hydrolysis. The decrease in CL_{12} might be due in part to the inhibitory effect of gemfibrozil on SV to SVA hydrolysis in plasma. Similar rationales may also be applicable to studies in humans and/or other statin lactone–acid pairs.

KEY WORDS: gemfibrozil; hepatocytes; interconversion; metabolism; pharmacokinetics; simvastatin; simvastatin acid; statins.

INTRODUCTION

3-Hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, or "statins," which target the rate-limiting enzyme in cholesterol biosynthesis, are used widely for the treatment of hypercholesterolemia and hypertriglyceridemia (1). Of the statins available on the market, simvastatin (SV) and lovastatin are pharmacologically inactive lactones. Upon conversion to their corresponding hydroxy acid form, simvastatin hydroxy acid (SVA) and lovastatin hydroxy acid, respectively, they serve as potent competitive inhibitors of HMG-CoA reductase (2). All statins undergo varying degrees of metabolism in both animals and humans (3–8), and their metabolism is known

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ABBREVIATIONS: CL_{app}, apparent clearance; CL_{real}, real clearance; CL_{10}, irreversible elimination clearance of SV; CL_{12}, formation clearance of SVA from SV; CL_{20}, irreversible elimination clearance of SVA; CL_{21}, formation clearance of SV from SVA; SV, simvastatin; SVA, simvastatin acid; RF, recycled fraction; $V_{ss,app}$, apparent volume of distribution at steady state; $V_{ss,real}$, real volume of distribution at steady state.

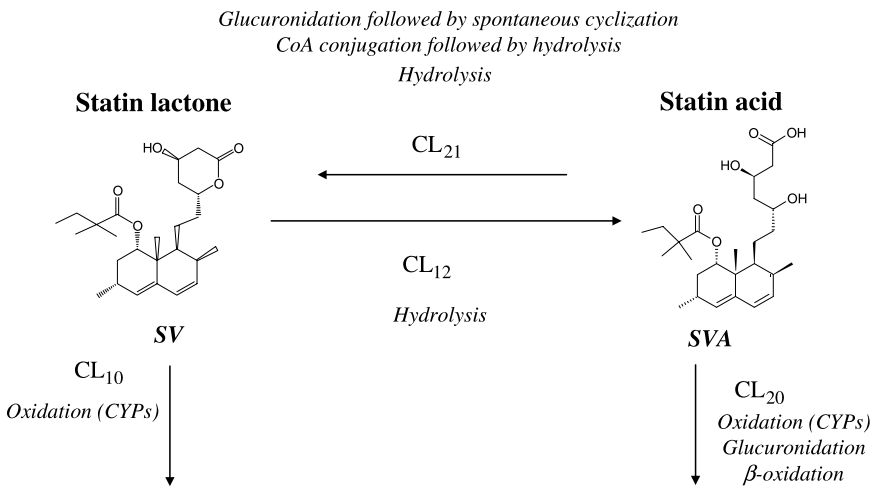

Fig. 1. Metabolic scheme of SV and SVA interconversion.

to be complex, involving acid–lactone interconversion via various pathways (9). As summarized in Fig. 1, statin lactones are hydrolyzed to their open acids chemically or enzymatically by esterases or paraoxonases (PONs). Statin acids are converted to the corresponding lactones via the acyl glucuronide intermediate and via the CoASH-dependent pathway. Both acyl glucuronide and acyl CoA derivatives may revert to statin acids by hydrolysis. In addition, statin lactones are irreversibly and exclusively cleared by the well-known P450-mediated oxidation, whereas statin acids are irreversibly cleared by the P450-mediated oxidation, β-oxidation, and the glucuronidation processes. For SV, SVA, atorvastatin, and its lactone form, CYP3A is primarily involved (10–12), whereas for cerivastatin, both CYP2C8 and CYP3A play important roles in mediating the oxidative reaction (13).

Gemfibrozil, a fibrate derivative commonly used in combination with statins to treat patients with mixed hyperlipidemia (14,15), has been shown to affect the pharmacokinetics of several statins to various degrees in humans (16–19). Among statins studied, cerivastatin showed the highest magnitude of interactions. Through *in vivo* (dog) and *in vitro* liver microsomal (dog and human) studies, the pharmacokinetic interactions between gemfibrozil and SV were demonstrated not to be mediated by the inhibitory effect of gemfibrozil on the CYP3A-mediated oxidative metabolism of SV or SVA nor by induction of plasma hydrolysis of SV to SVA, but rather at least in part by its inhibitory effect primarily on the glucuronidation of SVA (20). In the case of cerivastatin, it was proposed to be due partly to dual inhibitory effects of gemfibrozil on the glucuronidation and the CYP2C8-mediated oxidation of cerivastatin (20). It is noteworthy that in our earlier dog studies, effects of gemfibrozil on β-oxidation of statin hydroxyl acids have not been investigated. Theoretically, the observed pharmacokinetic interactions in this species could also be attributable to inhibitory effects of gemfibrozil on the β-oxidation pathway. In addition, although inhibition of statin glucuronidation was observed in both the *in vivo* and *in vitro* experiments, the IC_{50} value of gemfibrozil (~200 μM for SVA) obtained in the previous liver microsomal study was closer to the total (~350 μM) and not the unbound (~3 μM) plasma concentrations of gemfibrozil observed *in vivo* (20). This appeared to contradict the general belief that protein binding is an important factor when considering drug interactions. We attributed this apparent contradiction in part to effects of the detergent used in the *in vitro* liver microsomal model (20). Furthermore, to date, the interconversion pharmacokinetics of all statins, including SV/SVA, have not been characterized in animals or humans. Significant reversible metabolism is a known confounding factor for pharmacokinetic parameters obtained using a classical method, and fundamental clearances characterizing interconversion and irreversible processes separately are needed to adequately describe the pharmacokinetics of compounds undergoing appreciable interconversion (21–23).

Thus, we set out to characterize the interconversion pharmacokinetics of a statin lactone and statin hydroxy acid pair, using SV/SVA as model compounds in dogs. Effects of gemfibrozil on each of the fundamental clearances of SV/SVA also were investigated. In addition, *in vitro* metabolism studies were conducted using dog hepatocytes and dog and human liver subcellular fractions and plasma in the absence and presence of gemfibrozil.

MATERIALS AND METHODS

Materials

SV, SVA, [$^{13}CD_3$]SV, and [$^{13}CD_3$]SVA were synthesized at Merck Research Laboratories (Rahway, NJ, USA). Gemfibrozil, 2-bromooctanoic acid, and ketoconazole were obtained from Sigma (St. Louis, MO, USA). Solvents used for analysis were of analytical or high-performance liquid chromatography (HPLC) grade. Pooled dog (n = 10) and human (n = 10) liver S9 preparations were purchased from Xenotech LLC (Kansas City, KS, USA). Dog hepatocytes from four different donors were prepared in-house after collagenase digestion. The cells were resuspended in 10 mM HEPES buffer for a final concentration of 2×10^6 cells mL^{-1}, and cell viability (>85%) was determined by trypan blue exclusion before use.

In Vivo Studies

All studies were reviewed and approved by the Merck Research Laboratories Institutional Animal Care and Use

Committee. The *in vivo* studies were carried out in a crossover fashion, with at least a 10-day washout period. Beagle dogs (n = 4, 9–11 kg) were pretreated with either vehicle (0.5% methyl cellulose suspension) or gemfibrozil (75 mg kg^{-1} p.o., in 0.5% methyl cellulose suspension) twice daily for 5 days. The dose of gemfibrozil used in this study has been shown to significantly affect the pharmacokinetics of SV and SVA after oral administration of SV in this species (20). The animals were fasted overnight before SV or SVA administration on day 5. On the morning of day 5, SV or SVA was infused at 0.4 or 1.2 mg kg^{-1}, respectively, via a femoral vein for 20 min, to dogs; blood samples were collected at 0, 10, 20 (end of iv infusion), 30, 50, 70, 90, 120, 180, 240, 360, 480, 600, and 1,440 min after SV or SVA administration. Plasma samples were separated immediately at 10°C and kept frozen at −20°C.

In Vitro Studies

Experiments were conducted using dog or human liver S9 (2 mg mL^{-1}) or freshly obtained plasma (0.2 mL) in 50 mM Tris buffer (pH 7.4) incubated with SV or SVA (10 μM). At various times during a 3-h incubation period, the reaction was stopped by the addition of 0.3 mL ice-cold 0.1 M ammonium acetate buffer (pH 4.5). The samples were then extracted immediately and analyzed for SV and SVA by liquid chromatography–tandem mass spectrometry (LC-MS/MS) as described below. Control incubations were performed using 50 mM Tris buffer pH 7.4. Effects of gemfibrozil on the hydrolysis processes also were examined by coincubating gemfibrozil (250 μM) with liver S9 or plasma before the reaction was initiated with SV or SVA.

Effects of gemfibrozil on the metabolism of SVA also were examined in dog hepatocytes, using a protocol similar to that published earlier (24). In brief, a typical incubation mixture, in a final volume of 0.5 mL, contained 2×10^6 dog hepatocytes, and metabolic inhibitors (gemfibrozil, 10–200 μM; ketoconazole, 1 μM; 2-bromooctanoic acid, 200 μM) or vehicle used to prepare the inhibitors (50% acetonitrile in water). For all experiments and for each hepatocyte preparation, incubations were done in triplicate. The rection was started by the addition of SVA (20-μM final concentration) after a 3-min preincubation at 37°C and terminated by the addition of acetonitrile after incubation for 40 min.

Analytical Procedures

Quantification of levels of SV and SVA in dog plasma and dog and human liver S9 was accomplished using LC-MS/MS, as described previously (20). In brief, SV and SVA were extracted at 4°C from dog plasma using a solid-phase extraction method, and detected by turbo ions pray on a PE Sciex API 300 tandem mass spectrometer with within-run polarity switching between negative ion (for SVA) and positive ion (for SV) monitoring. Stable isotope labeled analogs of the compounds of interest ($[^{13}CD_3]$SV and $[^{13}CD_3]$SVA) were used as internal standards. The precursor–product ion transitions monitored were m/z 439.2 $(M - H)^- \rightarrow$ 319.1 (for $[^{13}CD_3]$SVA), m/z 435.2 $(M - H)^- \rightarrow$ 319.1 (for SVA), m/z 423.1 $(M + H)^+ \rightarrow$ 199.1 (for $[^{13}CD_3]$SV), and m/z 419.1 $(M+H)^+ \rightarrow$ 199.1 (for SV). Interday and intraday precision (%RSD) and accuracy were < 10% RSD and 100–106% for both SV and SVA. The lower limit of quantitation was 1 nM for both compounds. The interconversion between SV and SVA during sample preparation was ≤0.2% for SVA → SV and ≤0.3% for SV → SVA.

Levels of SVA metabolites (3′-hydroxy SVA, β-oxidation products B1 and B2, and B2 glucuronide) in dog hepatocyte incubations were quantified using either HPLC with UV detection or an on-line IN/US β-RAM radioactivity detector (IN/US Systems, Tampa, FL, USA) or both and LC-MS/MS, as described previously (24).

Data Analysis

The area under the plasma concentration–time profile (AUC) was calculated from time zero to the last detectable sampling time using the linear trapezoidal rule. The peak plasma concentration (C_{max}) and the time at which this peak occurred (T_{max}) were determined by observation. Apparent clearance (CL_{app}) values for SV or SVA were calculated as SV or SVA i.v. dose divided by their respective AUC from time zero to infinity (AUC_{0-inf}). Apparent volume of distribution at steady-state ($V_{ss,app}$) values were estimated by conventional moment analysis as i.v. dose multiplied by the first moment of the plasma concentration–time profile (AUMC) and divided by $(AUC_{0-inf})^2$.

The interconversion model for SV and SVA is shown in Fig. 1, and determinations of their pharmacokinetic parameters were similar to those described earlier for prednisone and prednisolone (23). This model assumes that both SV and SVA have linear and stationary disposition and that elimination and interconversion of both compounds occur via their central compartments. Note that based on apparent terminal $t_{1/2}$ determination of SV and SVA (see Results), a possibility for some nonlinearity could not be ruled out. Consequently, the SV/SVA parameters estimated based on this simple interconversion model may be considered as approximates.

The four fundamental clearances, namely, irreversible elimination clearance of SV (CL_{10}) and SVA (CL_{20}), and the interconversion clearance of SV to SVA (CL_{12}) and SVA to SV (CL_{21}) were estimated based on the following equations:

$$CL_{10} = \frac{\left[\left(\text{Dose}^{SV} \times AUC^{SVA}_{SVA}\right) - \left(\text{Dose}^{SVA} \times AUC^{SV}_{SVA}\right)\right]}{\left[\left(AUC^{SV}_{SV} \times AUC^{SVA}_{SVA}\right) - \left(AUC^{SV}_{SVA} \times AUC^{SVA}_{SV}\right)\right]}$$

$$CL_{20} = \frac{\left[\left(\text{Dose}^{SVA} \times AUC^{SV}_{SV}\right) - \left(\text{Dose}^{SV} \times AUC^{SVA}_{SV}\right)\right]}{\left[\left(AUC^{SV}_{SV} \times AUC^{SVA}_{SVA}\right) - \left(AUC^{SV}_{SVA} \times AUC^{SVA}_{SV}\right)\right]}$$

$$CL_{12} = \frac{\left[\left(\text{Dose}^{SVA} \times AUC^{SV}_{SVA}\right)\right]}{\left[\left(AUC^{SV}_{SV} \times AUC^{SVA}_{SVA}\right) - \left(AUC^{SV}_{SVA} \times AUC^{SVA}_{SV}\right)\right]}$$

$$CL_{21} = \frac{\left[\left(\text{Dose}^{SV} \times AUC^{SVA}_{SV}\right)\right]}{\left[\left(AUC^{SV}_{SV} \times AUC^{SVA}_{SVA}\right) - \left(AUC^{SV}_{SVA} \times AUC^{SVA}_{SV}\right)\right]}$$

where the superscripts refer to the dosed compound, and the subscripts refer to the measured compound. In addition, the real clearance (CL_{real}) and recycled fraction (RF) were also determined as follows:

$$CL_{real}^{SV} = CL_{10} + CL_{12}$$

$$CL_{real}^{SVA} = CL_{20} + CL_{21}$$

$$RF = [CL_{12}/(CL_{10} + CL_{12})] \times [CL_{21}/(CL_{20} + CL_{21})]$$

In addition, values for real volume of distribution at steady state ($V_{ss,real}$) were also calculated as described by Ebling and Jusko (23) as follows:

$$V_{ss,real}^{SV} = \frac{V_{ss,app}^{SV} - \left(Kd_{SVA}^{SV} \times V_{ss,app}^{SVA}\right)}{1 - \left(Kd_{SVA}^{SV} \times Kd_{SV}^{SVA}\right)}$$

$$V_{ss,real}^{SVA} = \frac{V_{ss,app}^{SVA} - \left(Kd_{SV}^{SVA} \times V_{ss,app}^{SV}\right)}{1 - \left(Kd_{SVA}^{SV} \times Kd_{SV}^{SVA}\right)}$$

$$Kd_{SVA}^{SV} = \frac{Dose^{SV} \times AUC_{SVA}^{SV} \times AUC_{SV}^{SVA}}{Dose^{SVA} \times AUC_{SV}^{SV} \times AUC_{SV}^{SV}}$$

$$Kd_{SV}^{SVA} = \frac{Dose^{SVA} \times AUC_{SV}^{SVA} \times AUC_{SVA}^{SV}}{Dose^{SV} \times AUC_{SVA}^{SVA} \times AUC_{SVA}^{SVA}}$$

Statistical Analysis

Statistical analysis was performed using a two-tailed paired *t* test. A p value of <0.05 was considered statistically significant.

RESULTS

Interconversion Pharmacokinetics in Control Dogs

The plasma concentration–time profiles of SV and SVA after intravenous administration of SV and SVA are shown in Fig. 2A and B, respectively. In each case, the concentrations of the administered drug were higher than those of the corresponding metabolites. The AUC of SVA accounted for 25% of that of SV after SV administration, whereas the AUC of SV was 4% of that of SVA after SVA administration (Table I). After either SV or SVA administration, the plasma profiles of SV appeared to decline more or less in parallel to each other (Fig. 2A and B). However, the calculated terminal $t_{1/2}$ of SV was slightly longer (~30%) than that of SVA (Table I), a feature suggestive of a possible nonlinearity in the pharmacokinetic model. Due to assay sensitivity limitation, the terminal $t_{1/2}$ of SV could not be accurately determined after SVA administration.

Values for clearances of SV and SVA are shown in Table II. In vehicle-treated dogs, CL_{12}, which reflects the formation clearance of SVA from SV, was approximately 8-fold more rapid than CL_{21}, the formation clearance of SV from SVA. The irreversible clearance of SVA (CL_{20}) was almost two times faster than that of SV (CL_{10}). Both the irreversible processes were much more rapid than the interconversion clearances CL_{12} (~2- to 4-fold) and CL_{21} (>15-fold). The RF was estimated to be small (0.009, Table II), and values for the CL_{real} for SV and SVA were close to the corresponding values for CL_{app} (Table II). In control dogs, the CL_{real} or CL_{app} value of SV (~15 mL min^{-1} kg^{-1}) was slightly lower than the respective value of SVA (~19 mL min^{-1} kg^{-1}).

In vehicle-treated animals, values for $V_{ss,real}$ of SV were much larger (~7-fold) than those of SVA (Table II), consistent with the fact that SV is more lipophilic in nature than SVA. Also, for both SV and SVA, values for $V_{ss,real}$ were comparable to their corresponding values for $V_{ss,app}$ (Table II).

Interconversion Pharmacokinetics in Gemfibrozil-Treated Dogs

As was the case in control animals, the concentrations of the administered drug also were higher than those of the corresponding metabolites in gemfibrozil-treated dogs (Fig. 3A and B). The AUC of SVA accounted for about 30%

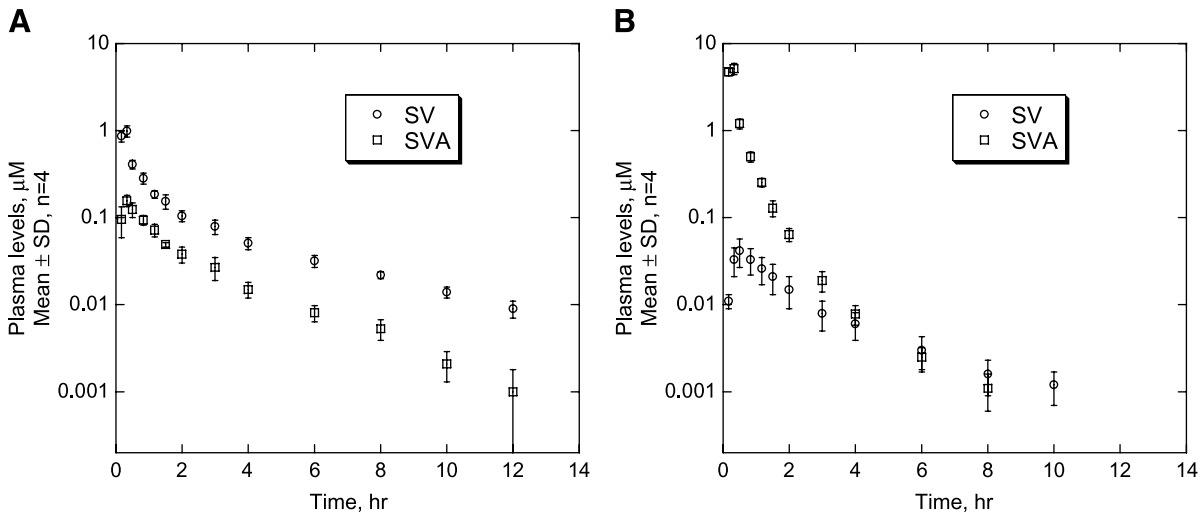

Fig. 2. SV and SVA plasma profiles after intravenous administration of SV (0.4 mg kg^{-1}, A) or SVA (1.2 mg kg^{-1}, B) to vehicle-treated dogs.

Table I. Pharmacokinetic Parameters of SV and SVA After Intravenous Administration of SV or SVA to Vehicle- or Gemfibrozil-Treated Dogs

Compound administered	Dose (mg kg^{-1})	Analytes	Pharmacokinetic parameters			
			AUC (μM h)	C_{max} (μM)	T_{max} (h)	Terminal $t_{1/2}$ (h)
Vehicle-treated dog						
SV	0.4	SV	1.06 ± 0.13	N/A	N/A	3.1 ± 0.4
		SVA	0.26 ± 0.05	2.19 ± 0.34	3.0 ± 0.0	2.3 ± 0.6
		SVA/SV	0.25 ± 0.06			
SVA	1.2	SV	0.09 ± 0.03	0.04 ± 0.01	0.4 ± 0.1	N/A
		SVA	2.34 ± 0.25	N/A	N/A	1.3 ± 0.3
		SV/SVA	0.04 ± 0.01			
Gemfibrozil-treated dog						
SV	0.4	SV	1.10 ± 0.18	N/A	N/A	3.0 ± 0.3
		SVA	0.34 ± 0.07	3.14 ± 0.97	3.0 ± 0.0	2.2 ± 0.3
		SVA/SV	0.31 ± 0.09			
SVA	1.2	SV	0.05 ± 0.02	0.04 ± 0.01	0.5 ± 0.1	N/A
		SVA	5.05 ± 1.34*	N/A	N/A	1.1 ± 0.3
		SV/SVA	0.01 ± 0.00			

Results are mean ± SD, $n = 4$.
N/A = not available.
*$p < 0.05$, statistically significant difference from vehicle-treated dogs.

of that of SV after SV administration, and the AUC of SV was only 1% of that of SVA after SVA administration (Table I). Whereas gemfibrozil treatment significantly decreased the AUC of SVA after SVA administration, it did not significantly affect the AUC values of either SV or SVA after SV administration or of SV after SVA administration (Table I). The terminal $t_{1/2}$ of SV after SV administration or of SVA after SVA administration also was not affected by gemfibrozil treatment (Table I). Interestingly, as was the case in control dogs, the calculated terminal $t_{1/2}$ of SV was slightly longer than that of SVA after SV administration to gemfibrozil-treated dogs (Table I), raising again a slight possibility for nonlinear pharmacokinetics.

In gemfibrozil-treated dogs, except for the irreversible clearance of SV (CL_{10}), all the three fundamental clearances (CL_{12}, CL_{21} and CL_{20}) were significantly reduced as compared to those in control animals (Table II). The magnitude of decrease was more pronounced for CL_{21} (~4-fold), than that for CL_{20} and CL_{12} (~2-fold). Gemfibrozil treatment also significantly and markedly (~3-fold) decreased the RF value (Table II). In addition, the CL_{real} and CL_{app} of SVA, but not SV, also were significantly decreased (~2-fold) in gemfibrozil-treated animals (Table II). However, treatment with gemfibrozil did not affect $V_{ss,real}$ of either SV or SVA (Table II). Note that attempts have also been made to estimate volume of distribution at central compartment (V_c), using a two-compartment model, and the results showed that gemfibrozil had a minimal effect on V_c of both SV (0.33 ± 0.10 and 0.36 ± 0.16 L kg^{-1} in control and gemfibrozil-treated dogs, respectively) and SVA (0.12 ± 0.03 and 0.12 ± 0.01 L kg^{-1} in control and gemfibrozil-treated animals, respectively).

Hydrolysis of SV and SVA in Liver S9 and Plasma

In dog and human liver S9, the hydrolysis rate of SV to SVA was about 5% per hour (Table III). Control experi-

Table II. Interconversion Pharmacokinetic Parameters of SV and SVA After Intravenous Administration of SV or SVA to Vehicle- or Gemfibrozil-Treated Dogs

Parameters	Unit	Vehicle-treated	Gemfibrozil-treated
CL_{10}	mL min^{-1} kg^{-1}	10.5 ± 1.0	11.7 ± 1.0
CL_{20}	mL min^{-1} kg^{-1}	18.6 ± 1.8	9.1 ± 2.7*
CL_{12}	mL min^{-1} kg^{-1}	4.8 ± 1.2	2.9 ± 1.2*
CL_{21}	mL min^{-1} kg^{-1}	0.57 ± 0.16	0.14 ± 0.02*
CL_{real}^{SV}	mL min^{-1} kg^{-1}	15.3 ± 1.5	14.6 ± 1.2
CL_{real}^{SVA}	mL min^{-1} kg^{-1}	19.1 ± 1.8	9.3* ± 2.6
CL_{app}^{SV}	mL min^{-1} kg^{-1}	15.2 ± 1.5	14.5 ± 1.2
CL_{app}^{SVA}	mL min^{-1} kg^{-1}	19.0 ± 1.8	9.3* ± 2.6
RF		0.009 ± 0.001	0.003 ± 0.001*
$V_{ss,real}^{SV}$	L kg^{-1}	2.38 ± 0.24	2.35 ± 0.15
$V_{ss,real}^{SVA}$	L kg^{-1}	0.34 ± 0.04	0.34 ± 0.05
$V_{ss,app}^{SV}$	L kg^{-1}	2.39 ± 0.34	2.36 ± 0.15
$V_{ss,app}^{SVA}$	L kg^{-1}	0.37 ± 0.04	2.35 ± 0.15

Values are mean ± SD, $n = 4$.
*$p < 0.05$, statistically significant difference from vehicle-treated dogs.

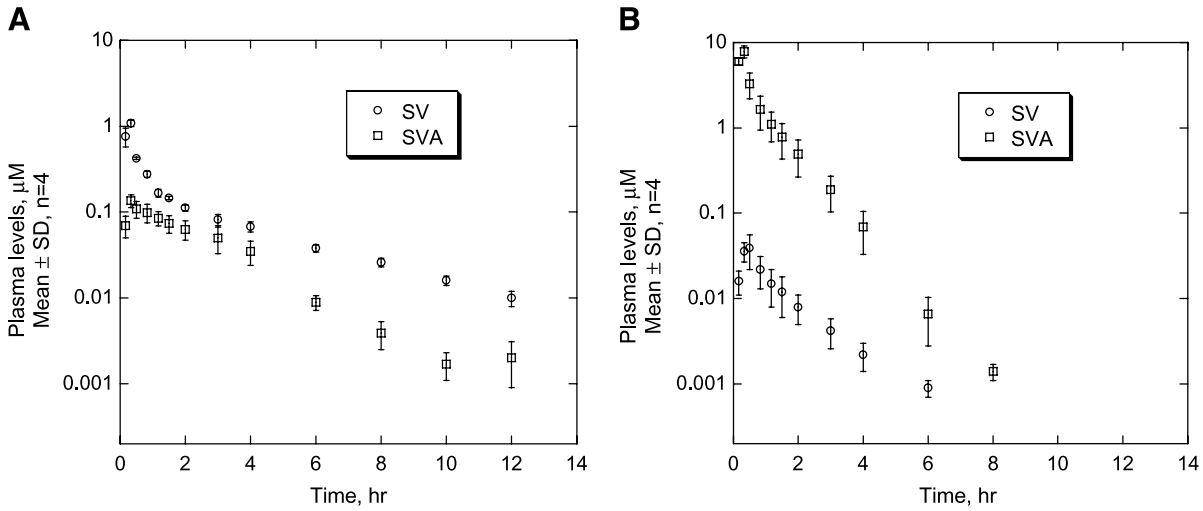

Fig. 3. SV and SVA plasma profiles after intravenous administration of SV (0.4 mg kg^{-1}, A) or SVA (1.2 mg kg^{-1}, B) to gemfibrozil-treated dogs.

ments using pH 7.4 buffer indicated significantly less conversion of SV to SVA (Table III), suggesting that under the studied condition there was an enzymatic component for the hydrolysis of SV in both dog and human liver S9. Similarly, there was also an enzymatic component for the conversion of SVA to SV in liver S9 from both dogs and humans. In both dog and human plasma, the enzymatic hydrolysis of SV to SVA was substantial (~10% per hour), whereas that of SVA to SV was negligible under the present incubation condition (Table III). Overall, the hydrolytic rate of SV to SVA, either chemical or enzymatic, was much higher (>10-fold) than that of SVA to SV (Table III). The rate of either SV to SVA or SVA to SV hydrolysis appeared to be linear for up to 3 h incubation (data not shown). In both species, gemfibrozil (250 μM) had a modest effect on both SV to SVA and SVA to SV hydrolysis in liver S9, whereas it significantly decreased (~60%) the SV to SVA hydrolysis rate in plasma (Table III). Apparently, for both species, the SV/SVA hydrolysis in liver and plasma was mediated by different enzyme systems.

Effect of Gemfibrozil on the Metabolism of SVA in Dog Hepatocytes

As was observed in human hepatocytes (24), major metabolites of SVA observed in dog hepatocytes included those typically associated with oxidation (3′-hydroxy SVA and dihydrodiol), β-oxidation (1′-[5-hydroxy-pentanoic acid] and [1′-propanoic acid] derivatives of SVA; B1 and B2, respectively) and glucuronidation processes (SV and B2 glucuronide). Under the studied conditions, SVA glucuronide ($[M - H]^-$ at m/z 611) was barely detectable, consistent with our earlier finding that the glucuronide conjugate of SVA readily undergoes spontaneous cyclization to form SV at physiological pH (9,24). As was the case in the liver microsomal system (20), gemfibrozil showed modest inhibitory effect (IC_{50} > 200 μM) in dog hepatocytes on the formation of the 3′-hydroxy SVA (Fig. 4) and dihydrodiol SVA (data not shown), both known to be mediated primarily by CYP3A. Formation of the β-oxidation products B1 and B2 was moderately affected by gemfibrozil, with IC_{50} of about 100 μM (Fig. 4). However, gemfibrozil markedly inhibited the lactonization of SVA or the formation of SV, and the glucuronidation of B2, in a concentration-dependent manner (Fig. 4), with IC_{50} values of 18 μM. Control experiments showed that 2-bromooctanoic acid, a known β-oxidation inhibitor (25), inhibited almost completely the β-oxidation products of SVA and the glucuronide conjugate of B2, whereas it minimally inhibited the lactonization and the CYP3A-mediated oxidative metabolites of SVA (data not shown). As expected, ketoconazole, a known inhibitor of CYP3A, inhibited markedly (>60%) the formation of

Table III. Hydrolysis of SV and SVA in Dog and Human Liver S9, Plasma or Buffer in the Absence (Control) or Presence of Gemfibrozil (250 μM)

	SVA formed (% of SV concentration per hour)		SV formed (% of SVA concentration per hour)	
	Control	With gemfibrozil	Control	With gemfibrozil
Dog liver S9	4.6 ± 0.6	4.8 ± 0.4	0.19 ± 0.01	0.20 ± 0.01
Human liver S9	6.4 ± 0.5	6.5 ± 0.3	0.24 ± 0.01	0.21 ± 0.02
Dog plasma	13.4 ± 1.2	8.4 ± 0.6	0.08 ± 0.01	0.07 ± 0.02
Human plasma	11.3 ± 3.0	6.8 ± 0.7	0.06 ± 0.02	0.07 ± 0.01
Buffer	2.6 ± 0.1	2.9 ± 0.1	0.07 ± 0.01	0.13 ± 0.01

Values are mean ± SD of triplicate determinations.

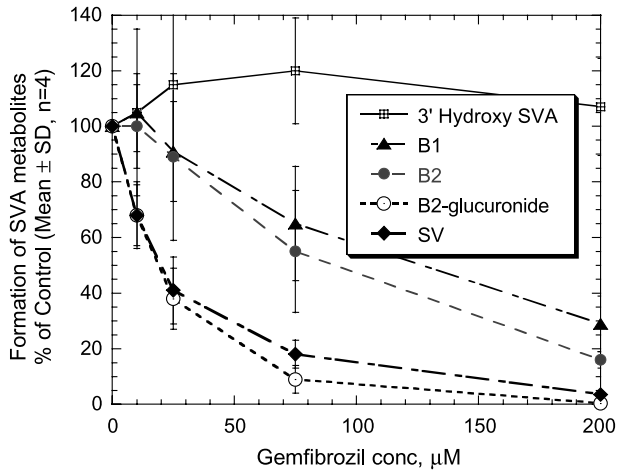

Fig. 4. Effect of gemfibrozil on the metabolism of SVA in dog hepatocytes. Results are expressed as percentage of control values (means ± SD, n = 4 hepatocyte preparations) and were obtained following coincubation of SVA (20 μM) in the presence or absence of gemfibrozil at 37°C for 40 min with dog hepatocytes (2×10^6 cells/mL). Control values ($pmol^{-1}$ min^{-1} per 10^6 cells, mean ± SD, n = 4) for the formation of 3′-hydroxy SVA, B1, B2, B2-glucuronide, and SV were 17.5 ± 4.3, 4.5 ± 2.5, 1.8 ± 1.5, 2.0 ± 0.8, and 5.7 ± 2.7, respectively.

CYP3A-mediated oxidative but not all other metabolites of SVA (data not shown).

DISCUSSION

One of the goals of the present investigation was to characterize pharmacokinetically all processes, including reversible processes, involved in the disposition of SV and SVA. Through this characterization, the underlying mechanisms for each of the parameters observed in the absence or presence of gemfibrozil were examined separately *in vitro*. The results provide several important suggestions, including the following: (1) the interconversion process favored the formation of SVA and was slower than the irreversible process and (2) gemfibrozil primarily affected the metabolism and not the distribution of SV/SVA, as reflected by changes in clearance (CL_{20}, CL_{12}, CL_{21}) and not volume of distribution (V_{ss} and V_c) values. In addition, the reduction in the irreversible clearance of SVA, CL_{20}, was due primarily to the inhibitory effect of gemfibrozil on the glucuronidation pathway, and not CYP3A-mediated oxidation or β-oxidation. Furthermore, the observed decrease in SVA to SV conversion (CL_{21}) could not be attributable to the inhibitory effect of gemfibrozil on the hepatic or plasma SVA to SV hydrolysis, but rather on the glucuronidation-mediated lactonization of SVA. In addition, the decrease in SV to SVA conversion (CL_{12}) was not due to the inhibition of hepatic, but probably plasma, hydrolysis of SV to SVA. The bases for these conclusions and implications of the present study are discussed below.

First, the *in vivo* observation that the SV–SVA interconversion favored the formation of SVA implies that the SV to SVA hydrolysis is much more efficient than the combined rate of the SVA to SV hydrolysis, SVA-CoA hydrolysis, and spontaneous cyclization of SVA glucuronide. Our *in vitro* results, which showed much slower hydrolytic rate of SVA to SV than of SV to SVA in both liver S9 and plasma, are consistent with this view. Based on our previous *in vivo* finding, which revealed that the glucuronidation pathway represents approximately 40% of the i.v. dose of SVA after SVA administration to dogs (20), the irreversible elimination of SVA via the glucuronidation pathway in control dogs (0.4×18.6 mL min^{-1} kg^{-1} = 7 mL min^{-1} kg^{-1}) is approximately 10-fold faster than of the combined SVA to SV conversion processes (CL_{21} = 0.6 mL min^{-1} kg^{-1}). This analysis implies that *in vivo*, SVA glucuronide, once formed, is much more efficiently eliminated irreversibly (via biliary excretion in dogs) than undergoing cyclization to SV. The present *in vivo* results also suggest that the P450-mediated oxidation of SV (CL_{10}) is approximately 2-fold more rapid than the hydrolysis of SV to SVA (CL_{12}) in dogs. The finding that CL_{10} was approximately 2-fold less than CL_{20} suggests that the P450-mediated oxidation of SV is slower than the combination of P450-mediated oxidation, glucuronidation, and β-oxidation of SVA. Based on a preliminary finding that the oxidative metabolism of SV in dog liver microsomes was relatively faster than that of SVA (data not shown), it is conceivable that the glucuronidation and/or β-oxidation processes contribute significantly to the irreversible elimination of SVA in dogs. In addition, the present *in vivo* finding of only ≤1% RF suggest that a relatively minor portion of SV or SVA undergoes interconversion before the irreversible elimination process of SV and SVA takes place. Consequently, the CL_{app} values for SV and SVA were comparable to their respective CL_{real} values (Table II). These results imply that the traditional method could be used to estimate clearances (and volume of distribution) of SV and SVA in dogs, untreated or under conditions that RF values remain low.

Next, the present finding that gemfibrozil had a minimal effect on the irreversible elimination of SV is consistent with our previous observation that gemfibrozil is not a potent inhibitor of CYP3A, the major enzyme responsible for the metabolism of SV in dogs (20) and humans (10). The finding that gemfibrozil decreased the irreversible clearance of SVA implies that gemfibrozil had an inhibitory effect on either one or any combination of the irreversible elimination pathways of SVA. Results from the present dog hepatocyte study substantiated our previous studies (20) that gemfibrozil inhibited the glucuronidation, but not the CYP 3A-mediated oxidation of SVA, and provided additional information regarding its modest inhibitory effect on the β-oxidation process of SVA. β-Oxidation products of SVA, although observed *in vivo* in dogs and humans, are not readily formed in *in vitro* systems other than the hepatocyte model, a more complete system. Our present hepatocyte finding, which showed that the inhibitory effect of gemfibrozil on the β-oxidation pathway was much less than that on the SVA glucuronidation, implies that inhibition of SVA glucuronidation, and not β-oxidation, is a major contributing factor for the reduction in CL_{20} observed in gemfibrozil-pretreated dogs. Both SVA glucuronide formation and β-oxidation are known mechanisms for the conversion of SVA to SV. Taken together with the present *in vitro* results, which showed minimal effect of gemfibrozil on SVA to SV hydrolysis,

inhibition of SVA glucuronidation is also a likely primary cause for the observed reduction in CL_{21} in gemfibrozil-pretreated animals. The present *in vitro* results, which showed significant inhibitory effect (~60%) of gemfibrozil on plasma hydrolysis of SV to SVA, is also consistent with the observed decrease in CL_{12}. However, considering that in our previous study, only a slight (~20%) reduction in the *ex vivo* SV to SVA plasma hydrolysis in dogs pretreated with gemfibrozil as compared to that in control dogs (20), other yet to be identified mechanisms, including possible effects of gemfibrozil on the conversion in different tissues, might also contribute significantly to the decreased CL_{12} in dogs. Other possibilities, which could explain the discrepancy in the magnitude of the inhibition by gemfibrozil obtained in this *in vitro* and previous *ex vivo* result (20), include simultaneous induction and inhibition of plasma enzyme(s) mediating SV to SVA hydrolysis by gemfibrozil, with net inhibitory effect *in vivo* in dogs. Interestingly, gemfibrozil has also recently been shown to act as both an inducer and an inhibitor of CYP2C8 in human hepatocytes (26).

As was observed previously with human liver preparations (24), the IC_{50} values obtained for the inhibitory effect of gemfibrozil on the glucuronidation of SVA in the present dog hepatocyte study (18 μM) was much lower than that obtained in our previous dog liver microsomal study [~200 μM (20)]. Assuming 35% binding to hepatocyte proteins [similar to that obtained with liver microsomes (24)], the unbound IC_{50} value obtained with the hepatocyte model (~6 μM) is close to peak unbound plasma concentrations of gemfibrozil [~3 μM, assuming 99% plasma protein binding (27)] reported in previous pharmacokinetic interaction studies (20). The exact reason for the discrepancy in the IC_{50} values observed between the two *in vitro* systems is presently not known, but our earlier hypothesis related to the effect of detergents used in the liver microsomal system (20,28) or to potential inhibitory activity of oxidative metabolites, but not the glucuronide of gemfibrozil (24), remain viable possibilities. The latter speculation is based on the fact that under the respective *in vitro* inhibition experimental conditions, both oxidation and glucuronidation of gemfibrozil occurred in the hepatocyte system (data not shown), similar to *in vivo* findings (29), whereas only the glucuronide, but not oxidative metabolites of gemfibrozil, would be formed in the liver microsomal study [with uridine diphosphate glucuronic acid (UDPGA) as a cofactor]. In this regard, a major oxidative metabolite of gemfibrozil has recently been tested not to be an inhibitor of CYP2C8 (30), but, unfortunately, there have been no reports regarding its effect on the glucuronidation of statins. Along this view, the glucuronide conjugate of gemfibrozil has recently been shown to inhibit CYP2C8-mediated metabolism of cerivastatin (30). However, this finding appeared inconsistent with our results, which showed almost identical IC_{50} values for the inhibitory effect of gemfibrozil on the CYP2C8-mediated oxidation of cerivastatin obtained in the liver microsomal (20) and hepatocyte systems (24). The reason for this apparent discrepancy remains to be investigated. Recently, a transporter–enzyme interplay has also been postulated as a potential cause for the discrepancy in the magnitude of metabolic drug interactions observed between hepatic microsomal and hepatocyte systems for digoxin (31). In the case of SV and SVA, evidence is lacking to support this view. SV and SVA are known to be passively transported efficiently into hepatocytes due to their high lipophilic nature (32). In addition, SV is not, and SVA is at best a weak substrate of P-glycoprotein (33). Furthermore, gemfibrozil, an inhibitor of the uptake transporters OATP1B1 (30) and OATPC, is not an inhibitor of the efflux transporters P-glycoprotein and MRP2 (34).

Although quantitative differences may exist, there are several qualitative similarities in the pharmacokinetics and metabolism of SV and SVA between dogs and humans. After SV oral administration to humans, the exposure of SVA was higher than that observed in dogs (~40–50% of SV), but nevertheless not more than that of SV (16,35), similar to the observation in dogs. In addition, as was the case in dogs, the AUC of SVA was ≤5% of that of SV after an oral administration of SVA to humans (Merck Research Laboratories, unpublished data). The present *in vitro* finding, which showed that the SV to SVA hydrolysis in human livers and plasma (Table III) is much faster than the corresponding SVA to SV hydrolysis, suggests that the *in vivo* interconversion process in humans possibly also favors the formation of SVA, similar to dogs. In addition, based on our previous *in vitro* metabolism studies that demonstrated that gemfibrozil had inhibitory effects on SVA glucuronidation in human liver microsomes and hepatocytes, it may be anticipated that gemfibrozil would also reduce the CL_{20} and CL_{21} in humans via similar mechanisms to those demonstrated in previous and present dog studies. Likewise, based on the present *in vitro* plasma hydrolysis, a possibility exists that gemfibrozil may also decrease CL_{12} in humans, as was the case in dogs.

In conclusion, the interconversion pharmacokinetic analyses, together with the present findings from *in vitro* metabolism experiments and previous *in vivo* and *in vitro* studies, provide additional mechanistic understanding of the interconversion and irreversible processes of SV/SVA in the absence and presence of gemfibrozil. Considering similarities in the reversible metabolism associated with all statins, this analytical approach may also be conceptually applicable to other statin lactone–acid pairs.

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