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Regional Intestinal Absorption and Biliary Excretion of Fluvastatin in the Rat: Possible Involvement of mrp2

Anders Lindahl,[†] Åsa Sjöberg,[‡] Ulf Bredberg,[‡] Helena Toreson,[‡] Anna-Lena Ungell,[‡] and Hans Lennernäs*[,†]

Department of Pharmacy, Box 580, Biomedical Center, Uppsala University, S-751 23 Uppsala, Sweden, and DMPK and Bioanalytical Chemistry, AstraZeneca, S-431 83 Mölndal, Sweden

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Abstract: The first purpose of this study was to investigate the in vivo absorption, biliary secretion, and first-pass effect of fluvastatin following regional intestinal dosing in the rat. We also examined the membrane transport mechanisms and made in silico predictions of the relative importance of various intestinal regions to the human absorption of fluvastatin. Fluvastatin was administered intravenously (2, 10, and 20 μmol/kg) and into the duodenum (1.46, 2.92, 7.32, and 14.6 μmol/kg), jejunum (14.6 μmol/kg), ileum (1.46 and 14.6 μmol/kg), and colon (1.46 and 14.6 μmol/kg) as a solution to conscious rats. In a separate group of rats, bile was collected after an iv dose of fluvastatin (2 μmol/kg). In the Caco-2 model the bidirectional transport of fluvastatin (16 μM) was investigated with and without various efflux inhibitors (verapamil, vinblastine, probenecid, and indomethacin, 160 μM). The human in vivo absorption of fluvastatin from an oral immediate release tablet and that from an oral extended release tablet (both 40 mg) were simulated in GastroPlus. Neither the dose nor the intestinal region influenced the bioavailability of fluvastatin significantly. The rate of absorption was, however, affected by both the dose and the site of administration; duodenum = jejunum > colon > ileum, and higher following the high dose. Increasing the iv dose from 2 to 20 μmol/kg decreased the clearance (26 $\pm$ 3 to 12 $\pm$ 1 mL/min/kg), the hepatic extraction (66 $\pm$ 8 to 30 $\pm$ 2%), and the volume of distribution (7.3 $\pm$ 0.3 to 2.1 $\pm$ 0.7 L/kg) for fluvastatin ($p < 0.05$). Neither bile cannulation nor bile sampling affected the pharmacokinetics. Fluvastatin was secreted into the bile, probably by active transport. The in vitro permeability for fluvastatin was high ($>10 \times 10^{-6}$ cm/s). Indomethacin, but not the other inhibitors, affected the transport in both directions suggesting mrp2 to be involved. In silico, 93% of the dose was absorbed from the small intestine and 6% from the colon when given as an immediate release formulation. The corresponding values for an extended release formulation were 21% and 74%, respectively. In conclusion, fluvastatin exhibits dose-dependent pharmacokinetics in the rat. The rate of absorption (C_{max}, T_{max}, and C_{max}/AUC_{lqc}) from the intestinal tract is both region and dose-dependent in the rat. This may be due to the involvement of mrp2 in the intestine and/or in the liver. These absorption properties have to be considered in the development of an extended release formulation of fluvastatin.

Keywords: Fluvastatin; HMG-CoA reductase inhibitor; intestinal transport; biliary excretion; pharmacokinetics; rat; Caco-2; in silico simulation

Introduction

Regional differences in physiology and biochemistry along the gastrointestinal tract are major determinants for in vivo drug absorption. Though many aspects of in vivo drug absorption, such as dissolution, transit, permeability, and metabolism, have been the focus for several decades, the importance of regional difference has not been thoroughly investigated. Accordingly, more investigations on regional drug absorption mechanisms should be beneficial for our understanding of in vivo drug absorption and be extremely valuable for the development of new drug formulations.

* Corresponding author: Department of Pharmacy, Box 580, Biomedical Center, Uppsala University, S-751 23 Uppsala, Sweden. Tel: +46 18 471 4317. Fax: +46 18 471 4223. E-mail: hans.lennernas@farmaci.uu.se.

[†] Uppsala University.

[‡] AstraZeneca.

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Fluvastatin is a synthetic inhibitor of hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase, which is the rate-limiting enzyme in the biosynthesis of cholesterol. Accordingly, fluvastatin is used for the treatment of hypercholesterolemia. Oral doses of 20−80 mg of fluvastatin per day for four to six weeks reduced the LDL cholesterol by 20−32% in patients with hypercholesterolemia compared to baseline.[1] As the liver is the major cholesterol-producing organ, it is also the main pharmacological target organ for HMG-CoA reductase inhibitors. The potential side effects of these compounds during long-time treatment depend in part on the degree of extrahepatic exposure.[2] A high liver extraction minimizes the systemic exposure of the parent drug and can thus be considered as a desirable property of HMG-CoA reductase inhibitors.

The hepatic extraction of fluvastatin during the first pass in humans is approximately 70%, which is consistent with the 29% bioavailability reported for a 10 mg oral dose.[3,4] However, a 40 mg oral dose of fluvastatin to healthy volunteers led to a 2−3-fold disproportional increase in the area under the plasma concentration−time curve (AUC), suggesting saturated extraction during the first pass.[4] This phenomenon has also been reported to occur in the mouse, rat, dog, and monkey following higher oral doses of fluvastatin.[5] After intravenous administration of radioactively labeled fluvastatin to these animal species, 84−98% of the radioactivity was recovered in the feces, with 7−22% thereof being attributable to the parent drug.[5] This suggests that fluvastatin and its metabolites are excreted into the bile and/or directly into the intestine, and that fluvastatin might be involved in enterohepatic circulation. Such circulation might be beneficial for the treatment of hypercholesterolemia as the drug is repeatedly presented to the major target organ.

To our knowledge, there is no information available in the literature on the in vivo absorption of fluvastatin, or of any other inhibitor of HMG-CoA reductase, from the lower gastrointestinal tract in humans. We have found that the human in vivo jejunal P_{eff} of fluvastatin is high (2.4×10^{-4} cm/s) and accordingly the fraction dose absorbed of fluvastatin is more than 90%.[3−5] However, we have previously reported that the intestinal P_{eff} of fluvastatin is high throughout the rat intestine ($0.6-1.0 \times 10^{-4}$ cm/s), with the highest permeability obtained at the highest lumenal concentrations of fluvastatin.[6,7] Several mechanisms have been suggested to be the reason for this concentration-dependent intestinal P_{eff}, such as the involvement of intestinal efflux proteins or a physicochemical interaction between fluvastatin and the intestinal membrane.[6,7] Differences in the local intestinal absorption rate will probably influence the degree of liver selectivity due to potential nonlinearity of the hepatic extraction.

Another inhibitor of HMG-CoA reductase, atorvastatin, has recently been reported to be transported by P-glycoprotein (Pgp) in the Caco-2 cell model in vitro.[8] However, in situ experiments suggested that fluvastatin not was transported by Pgp in the rat intestine.[6] Another inhibitor of HMG-CoA reductase, pravastatin, is reported to be transported by the multidrug-resistance-associated protein 2 (mrp2) in the rat liver.[9] This protein is also expressed in the small and large intestine of the rat.[10] Results obtained from single-pass perfusion experiments in the rat in situ, with probenecid employed as an inhibitor of mrp2, suggested that mrp2 is not involved in the intestinal transport of fluvastatin to any significant extent.[11] However, the lack of effect of probenecid in these experiments might also be due to poor affinity of probenecid to mrp2 compared to fluvastatin, or unspecific binding of probenecid to other components present in the intestine in situ. In addition, probenecid is reported to be a less effective inhibitor of efflux by the mrp than indomethacin in vitro.[12,13] Therefore, to investigate the mechanism behind the concentration-dependent intestinal P_{eff} of fluvastatin, we wanted to further study the possible involvement of mrp2, using the less complex Caco-2 model and also a more potent inhibitor of mrp2, indomethacin.

The first aim of this study was to investigate the in vivo absorption, biliary secretion, and first-pass effect of fluvas-

(1) Lennernas, H.; Fager, G. Pharmacodynamics and pharmacokinetics of the HMG-CoA reductase inhibitors. Similarities and differences. *Clin. Pharmacokinet.* **1997**, *32*, 403−425.

(2) Sirtori, C. R. Tissue selectivity of hydroxymethylglutaryl coenzyme A (HMG CoA) reductase inhibitors. *Pharmacol. Ther.* **1993**, *60*, 431−459.

(3) Lindahl, A.; Sandstrom, R.; Ungell, A. L.; Abrahamsson, B.; Knutson, T. W.; et al. Jejunal permeability and hepatic extraction of fluvastatin in humans. *Clin. Pharmacol. Ther.* **1996**, *60*, 493−503.

(4) Tse, F. L.; Jaffe, J. M.; Troendle, A. Pharmacokinetics of fluvastatin after single and multiple doses in normal volunteers. *J. Clin. Pharmacol.* **1992**, *32*, 630−638.

(5) Tse, F. L.; Smith, H. T.; Ballard, F. H.; Nicoletti, J. Disposition of fluvastatin, an inhibitor of HMG-COA reductase, in mouse, rat, dog, and monkey. *Biopharm. Drug Dispos.* **1990**, *11*, 519−531.

(6) Lindahl, A.; Sandstrom, R.; Ungell, A. L.; Lennernas, H. Concentration- and region-dependent intestinal permeability of fluvastatin in the rat. *J. Pharm. Pharmacol.* **1998**, *50*, 737−744.

(7) Lindahl, A.; Persson, B.; Ungell, A. L.; Lennernas, H. Surface activity and concentration dependent intestinal permeability in the rat. *Pharm. Res.* **1999**, *16*, 97−102.

(8) Wu, X.; Whitfield, L. R.; Stewart, B. H. Atorvastatin transport in the Caco-2 cell model: contributions of P-glycoprotein and the proton-monocarboxylic acid co-transporter. *Pharm. Res.* **2000**, *17*, 209−215.

(9) Yamazaki, M.; Akiyama, S.; Ni'inuma, K.; Nishigaki, R.; Sugiyama, Y. Biliary excretion of pravastatin in rats: contribution of the excretion pathway mediated by canalicular multispecific organic anion transporter. *Drug Metab. Dispos.* **1997**, *25*, 1123−1129.

(10) Gotoh, Y.; Suzuki, H.; Kinoshita, S.; Hirohashi, T.; Kato, Y.; et al. Involvement of an organic anion transporter (canalicular multispecific organic anion transporter/multidrug resistance-associated protein 2) in gastrointestinal secretion of glutathione conjugates in rats. *J. Pharmacol. Exp. Ther.* **2000**, *292*, 433−439.

(11) Lindahl, A.; Frid, S.; Ungell, A. L.; Lennernas, H. No evidence for the involvement of the multidrug resistance-associated protein and/or the monocarboxylic acid transporter in the intestinal transport of fluvastatin in the rat. *PharmSci* **2000**, *2*, E26.

Table 1. Pharmacokinetic Parameters for Fluvastatin Following Iv Bolus Doses to Rats with an Intact Bile Flow (Not Bile Cannulated) and to Bile Cannulated Rats[a]

	not bile cannulated			bile cannulated
dose (μmol/kg):	2	10	20	2
n:	7	4	4	4
AUC (μM min)	79 ± 11	739 ± 171	1696 ± 99	83 ± 23
CL (mL/min/kg)	25.8 ± 3.0	14.2 ± 4.1[b]	11.8 ± 0.7[b]	25.8 ± 8.1
E_h (%)	66 ± 8	36 ± 11[b]	30 ± 2[b]	66 ± 20
V_{ss} (L/kg)	7.3 ± 0.3	2.7 ± 0.5[b]	2.1 ± 0.7[b]	6.4 ± 5.6
$t_{1/2}$ (min)	280 ± 60	218 ± 33	197 ± 24[b]	307 ± 283

[a] Mean ± SD from *n* rats. [b] Significantly different from the lowest dose ($p < 0.05$); no differences between the two highest doses were found.

tatin following administration of different doses into various intestinal regions in the rat. Second, we examined the membrane transport mechanisms of fluvastatin by using the Caco-2 cell model. Third, using physiology based simulation software we assessed the relative importance of various intestinal regions to the absorption of fluvastatin.

Materials and Methods

Fluvastatin sodium was provided by AstraZeneca R&D Mölndal, Sweden (former AstraHässle AB, Mölndal, Sweden). Antipyrin, probenicid, indomethacin, verapamil, and vinblastine were obtained from Sigma Chemicals (St. Louis). Dulbecco's modified Eagle medium (DMEM), fetal calf/bovine serum (FBS), minimal essential medium of nonessential amino acids (MEM), L-glutamine (200 mM), penicillin (100 units/mL)−streptomycin (100 μg/mL solution), phosphate-buffered saline (PBS), trypsin/EDTA, Hank's balanced salt solution (HBSS), and MES were obtained from Gibco, Life Technologies (Paisley, U.K.).

Animal Experiments. The pharmacokinetic animal experiments were performed in conscious male Sprague−Dawley rats (305 ± 20 g, Møllegaard, Denmark) after cannulation under general anesthesia (xylazine 10 mg/kg, ketamine 200 mg/kg intraperitoneal, ip) using surgical techniques described elsewhere.[14,15] In total 59 rats were used in this study; the numbers of animals in each treatment group are given in Tables 1 and 2. The rats were cannulated with polyethylene tubing in (a) the jugular vein and the carotid artery (for the intravenous study; 19 animals), (b) the jugular vein, the bile duct, the carotid artery, and the duodenum (for the bile study; 6 animals), and (c) the carotid artery and either the duodenum, the jejunum, the ileum, or the colon (for the intestinal absorption study; 38 animals). All cannulas were passed under the skin and externalized at the back of the neck of each rat. The animals had at least 2 days of recovery from the surgical procedure and were fasted for 16 h (water ad libitum) prior the pharmacokinetic experiments.

(A) Intravenous (Iv) Study. Fluvastatin sodium, dissolved in phosphate buffer (50 mM, pH 7.4), was administered into the jugular vein as a single bolus dose of 2, 10, or 20 μmol/kg (0.3 mL) over 30 s. Arterial blood samples (0.35 mL) were collected into tubes containing citrate buffer (0.13 M) just before dosing and 2, 5, 10, 20, 30, 60, 120, 180, 240, 300, 360, and 420 min after dosing. The collected blood was replaced with the same volume of saline. Following centrifugation for 10 min (5000*g*, 4 °C), the plasma was separated and frozen (at −20 °C) pending analysis.

(B) Biliary Secretion Study. The effect of bile secretion and enterohepatic circulation was investigated in two groups of rats. Both groups received a single iv bolus dose of fluvastatin (2 μmol/kg). In the first group ($n = 4$) the bile duct catheter was disconnected from the duodenal catheter at time zero when fluvastatin was given. In the second group ($n = 2$) the bile duct catheter remained connected to the duodenal catheter to study the effect of the bile duct cannulation per se. The bile was collected from the first group in 60 min intervals over 480 min and frozen (at −20 °C) pending analysis. Blood samples were collected from both groups and treated as described above. The two groups of bile cannulated rats were also compared with a group of uncannulated rats ($n = 4$) receiving the same iv dose (2 μmol/kg).

(C) Regional Intestinal Absorption Study. Fluvastatin sodium dissolved in a 0.1 M phosphate buffer (pH 8) was administered as a single bolus dose through the catheter placed in the duodenum, the jejunum, the ileum, or the colon. The number of animals in each study group is given in Table 2. The duodenal doses (1.46, 2.92, 7.32, and 14.6 μmol/kg) and the jejunal dose (14.6 μmol/kg) were given approximately 4−5 and 30 cm distal to the pylorus, respectively. The ileal and colonic doses (1.46 and 14.6 μmol/kg) were given approximately 20 cm proximal and 0.5 cm distal to the cecum, respectively. The volumes given were 1.2 mL/kg, and the concentrations of fluvastatin that entered the intestinal regions were 1.2−12 mM. Immediately after the administration, the catheters were flushed with 150 μL of the buffer (pH 8). Blood samples were collected from the carotid artery as described above at 0 (prior to dosing) and 5, 10, 15, 20, 30, 60, 120, 180, 240, 300, 360, and 420 min after dosing.

Caco-2 Cell Culture. Caco-2 cells were obtained from American Type Culture Collection (ATCC), Rockville, MD, and were maintained and cultivated as described by Neuhoff et al.[17] In short, Caco-2 cells of passage number 25−45 were used and were seeded on polycarbonate filters (12 mm diameter and 0.4 μm pore size, Transwell, Cat. No. 3401, Corning Costar Corporation, Cambridge, MA) at a density of 2.25×10^5 cells/cm^2. The cells were grown at 37 °C and

(12) Bakos, E.; Evers, R.; Sinko, E.; Varadi, A.; Borst, P.; et al. Interactions of the human multidrug resistance proteins MRP1 and MRP2 with organic anions. *Mol. Pharmacol.* **2000**, *57*, 760−768.

(13) Payen, L.; Courtois, A.; Vernhet, L.; Guillouzo, A.; Fardel, O. The multidrug resistance-associated protein (MRP) is overexpressed and functional in rat hepatoma cells. *Int. J. Cancer* **1999**, *81*, 479−485.

(14) Griffiths, R.; Lewis, A.; Jeffrey, P. Models of drug absorption in situ and in conscious animals. *Pharm. Biotechnol.* **1996**, *8*, 67−84.

(15) Sjostrom, M.; Lindfors, L.; Ungell, A. L. Inhibition of binding of an enzymatically stable thrombin inhibitor to lumenal proteases as an additional mechanism of intestinal absorption enhancement. *Pharm. Res.* **1999**, *16*, 74−79.

Table 2. Pharmacokinetic Parameters for Fluvastatin after Regional Intestinal Bolus Doses Given to Rats

	duodenum				jejunum	ileum		colon	
dose (μmol/kg):	1.46	2.92	7.32	14.6	14.6	1.46	14.6	1.46	14.6
n:	4	3	4	4	4	3	6	4	6
AUC (μM min)	42 ± 14	71 ± 7	252 ± 48	691 ± 84	597 ± 54	29 ± 2	506 ± 239	38 ± 5	261 ± 215
C_{max} (μM)	0.26 ± 0.10	0.47 ± 0.12	1.86 ± 0.29	7.06 ± 0.99	6.19 ± 0.27	0.04 ± 0.01	1.80 ± 1.80	0.15 ± 0.07	1.74 ± 2.01
C_{max}/AUC$_{lqc}$ (h^{-1})	0.59 ± 0.11	0.55 ± 0.06	0.58 ± 0.04	0.71 ± 0.06	0.71 ± 0.07	0.22 ± 0.07	0.30 ± 0.13	0.39 ± 0.12	0.53 ± 0.13
T_{max} (min)	5 ± 0	5 ± 0	15 ± 0	20 ± 0	10 ± 0	102 ± 123	32 ± 44	10 ± 6	5 ± 0
F (%)	69 ± 20	62 ± 6	47 ± 9	64 ± 8	55 ± 5	50 ± 3	53 ± 17	65 ± 8	48 ± 20
$t_{1/2}$ (min)	252 ± 89	300 ± 49	259 ± 77	213 ± 69	165 ± 15	592 ± 58	365 ± 135	349 ± 35	295 ± 86

90% relative humidity in a 5% CO_2 atmosphere using culture medium consisting of DMEM supplemented with 10% (v/v) heat inactivated FBS, 1% (v/v) MEM and 1% (v/v) penicillin-streptomycin, 1.5% (v/v) L-glutamine. The medium was changed every second day, and the cells were allowed to grow for 14−18 days before being used in the transport experiment.

Drug Transport Studies across Caco-2 Cell Monolayers. The transepithelial electric resistance (TEER) of the monolayers was checked routinely at 37 °C before and after the experiment by using the EndOhm (World Precision Instruments Inc., Sarasota, FL). All monolayers showing TEER below 150 Ω·cm^2 (corrected for the resistance of empty filters) before and after the absorption experiment were omitted. Hanks' balanced salt solution (HBSS) was used in all experiments after adjustment of the pH to 6.5 (apical side) and 7.4 (basolateral side) with MES and HEPES, respectively. Prior to transport experiments, the culture medium was replaced with the transport medium (0.5 mL at the apical side and 1.5 mL at the basolateral side). Following a 30 min preincubation at 37 °C, the TEER was measured.

Transport studies of fluvastatin (16 μM) were made in apical-to-basolateral and basolateral-to-apical direction, with and without various efflux inhibitors (all in excess, 160 μM), and samples were withdrawn at 0, 60, 120 min and 0, 15, 30, 60, 120 min from the donor and receiver chambers, respectively. Verapamil and vinblastine were employed as inhibitors of Pgp, whereas probenecid and indomethacin were used as inhibitors of mrp2. The inhibitor was added to both compartments at the start of the experiment. ^{14}C-Mannitol (New England Nuclear, Boston, MA) was used as a marker for monolayer integrity in all experiments, which were made in triplicate.

Simulations in Silico. The human in vivo gastrointestinal absorption of fluvastatin from an oral immediate release (IR) tablet (40 mg dose, fasted state) and an oral extended release (ER) tablet (40 mg dose, fasted state, integrated tablet, zero order release during 12 h) were simulated in GastroPlus 4.0 (Simulations Plus Inc., CA). Input parameters were obtained from a previously reported absorption study in healthy volunteers: human jejunal permeability of 2.38×10^{-4} cm/s, clearance 8.7 mL/min/kg, first pass extraction 67%, half-life 1 h (one-compartment model), molecular weight 411, solubility 80 mg/mL, pK_a 4.6 (acid), and log *P* 3.8 (octanol/water).[3] The transit times used for the stomach, the small intestine, and the colon were 0.25, 3.3 and 18 h, respectively. The stomach volume was set to 50 mL, the small intestinal length to 300 cm, and the small intestinal radius to 1.2 cm. It was assumed that the dosage form was administered together with a glass of water (250 mL). The total simulation time was 24 h.

Bioanalytical Method. The samples taken during transport studies across Caco-2 cell monolayers, plasma, and bile were analyzed for their content of fluvastatin with a previously described and validated HPLC method with fluorescence detection.[16] The limit of quantification was 20 nM.

Data Analysis. (A) Animal Experiments. Multiexponential equations were fitted to the observed plasma concentration−time data obtained following the intravenous bolus administration with use of the nonlinear regression program MINIM.[18] The discrimination between different pharmacokinetic models was based on (a) visual inspection of the data and the fitted equation, (b) trends in residuals between the observed and the predicted plasma concentrations, (c) the standard error of the estimated pharmacokinetic parameters, and (d) the Akaike criterion. The area under the plasma concentration−time curve (AUC) from time zero to time infinity following intravenous administration was calculated from the pharmacokinetic macroconstants of the fitted equations.[19] The plasma clearance (CL) was calculated by dividing the intravenous dose by the AUC. The volume of distribution at steady state (V_{ss}) was calculated from eq 1:

$$V_{ss} = \frac{(\text{Dose}_{iv}) \times (\text{AUMC})}{\text{AUC}^2} \quad (1)$$

where AUMC is the area under the curve time × concentration vs time from zero to infinity.

The hepatic extraction (E_h) following the intravenous doses was estimated from

(16) Toreson, H.; Eriksson, B. M. Determination of fluvastatin enantiomers and the racemate in human blood plasma by liquid chromatography and fluorometric detection. *J. Chromatogr. A* **1996**, *729*, 13−18.

(17) Neuhoff, S.; Ungell, A. L.; Zamora, I.; Artursson, P. pH-dependent bidirectional transport of weakly basic drugs across Caco-2 monolayers: implications for drug-drug interactions. *Pharm. Res.* **2003**, *20*, 1141−1148.

(18) Purves, R. D. Anomalous parameter estimates in the one-compartment model with first-order absorption. *J. Pharm. Pharmacol.* **1993**, *45*, 934−936.

(19) Gibaldi, M.; Perrier, D. *Pharmacokinetics*, 2nd ed.; Marcel Dekker: New York, 1982.

$$E_h = \frac{(CL)\times(1 - f_e)\times(C_p/C_b)}{Q_h} \quad (2)$$

where the plasma/blood-concentration ratio (C_p/C_b) of 1.53, the fraction eliminated in the urine (f_e) of 0.08, and the hepatic blood flow (Q_h) of 55.2 mL/min/kg in rats were taken from the literature.[5,20,21]

The maximum plasma concentration after the intestinal doses (C_{max}) and the time taken to reach C_{max} (T_{max}) were extracted manually from plasma concentration−time plots. As C_{max} depends not only on the rate of absorption but also on the extent, we calculated the ratio of C_{max} to the area under the plasma concentration−time curve to the last quantified concentration (AUC_{lqc}). C_{max}/AUC_{lqc} is essentially independent of changes in the extent of absorption and has been shown to be a more specific measure of the rate of absorption than C_{max}.[22,23] The AUC following the regional intestinal doses was calculated with the linear trapezoidal rule for ascending concentrations and the logarithmic trapezoidal rule for descending concentrations. The residual AUC was estimated by dividing the last predicted concentration by the terminal rate constant, obtained by linear regression analysis of the three to five last concentration−time points. The systemic bioavailability (F) from the intestinal doses was estimated from the ratio of the dose-corrected AUC of fluvastatin after intestinal (AUC_{gut}) and iv (AUC_{iv}) doses:

$$F = \frac{AUC_{gut}}{AUC_{iv}} \frac{Dose_{iv}}{Dose_{gut}} \quad (3)$$

As a consequence of the dose-dependent CL, each intestinal dose was matched against an iv dose that gave a plasma concentration−time profile in a similar range as the intestinal dose.

The influence of dose on the iv pharmacokinetic parameters was evaluated with analysis of variance (ANOVA) followed by Fisher's least-squares difference to identify significantly different groups (StatView, Abacus Concepts, Inc., Berkeley, CA). A probability level <0.05 was considered to be statistically significant. The effect of dose and intestinal region on the pharmacokinetics following the intestinal doses was evaluated for the 1.46 and 14.6 μmol/kg doses in the duodenum, ileum, and colon (two-way ANOVA). The four duodenal doses were analyzed separately (one-way ANOVA), and the jejunal dose was compared with an identical duodenal dose with a two-sided t-test. The C_{max} values were log-transformed prior to the statistical analysis. The T_{max} values were not evaluated statistically as the variances among the groups were not homogeneous. All data are given as mean values ± standard deviation, SD.

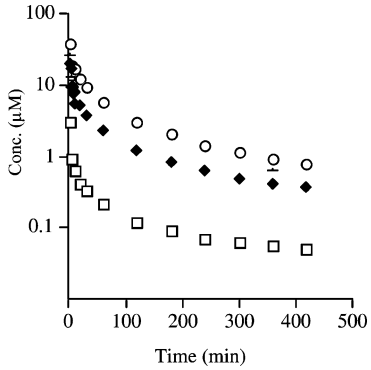

Figure 1. Mean plasma concentration−time profiles for fluvastatin following single iv administration of 2 (□), 10 (◆), and 20 μmol/kg (○). The bars indicate ±SD, $n = 4-7$.

(B) Caco-2 Experiments. Apparent permeability coefficients (P_{app}) of fluvastatin and mannitol in the two different directions in the absence and presence of the inhibitors were calculated according to[17]

$$P_{app} = \frac{dQ}{dt}\frac{1}{AC_0} \quad (4)$$

where dQ/dt is the steady-state appearance rate of the compound in the receiver compartment, A is the exposed cell monolayer area, and C_0 is the donor concentration.

The efflux ratio (Er), calculated from the mean P_{app} measured apical-to-basolateral (a-to-b) and basolateral-to-apical (b-to-a), respectively, was calculated using eq 5. The

$$Er = \frac{P_{app(b-a)}}{P_{app(a-b)}} \quad (5)$$

possible effect of the various inhibitors on the transport of fluvastatin was evaluated statistically by ANOVA-Tukey (Astute). Permeability values for fluvastatin in the presence and absence of the inhibitors were regarded as statistically different when $p < 0.05$. All data are given as mean values ± standard deviation, SD.

Results

Intravenous Dosing. The plasma concentration−time profiles of fluvastatin following the three different iv doses were all described by a three-exponential function (Figure 1). The three phases of the iv plasma concentration−time profiles corresponded to 10 ± 4%, 30 ± 9%, and 61 ± 12% of the total area, respectively, with no significant differences among the three different iv doses. The pharmacokinetic parameters calculated from the iv doses are given in Table 1.

The systemic clearance and the hepatic extraction of fluvastatin decreased from 25.8 ± 3.0 to 11.8 ± 0.7 mL/min/kg ($p < 0.05$), and from 66 ± 8% to 30 ± 2% ($p <$

(20) Tse, F. L.; Nickerson, D. F.; Yardley, W. S. Binding of fluvastatin to blood cells and plasma proteins. *J. Pharm. Sci.* **1993**, *82*, 942−947.

(21) Davies, B.; Morris, T. Physiological parameters in laboratory animals and humans. *Pharm. Res.* **1993**, *10*, 1093−1095.

(22) Bois, F. Y.; Tozer, T. N.; Hauck, W. W.; Chen, M. L.; Patnaik, R.; et al. Bioequivalence: performance of several measures of rate of absorption. *Pharm. Res.* **1994**, *11*, 966−974.

(23) Tothfalusi, L.; Endrenyi, L. Without extrapolation, Cmax/AUC is an effective metric in investigations of bioequivalence. *Pharm. Res.* **1995**, *12*, 937−942.

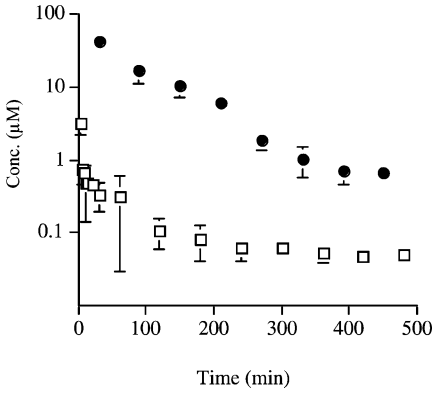

Figure 2. The total concentration of fluvastatin was approximately 10−100 times higher in the bile (●) than in the plasma (□) following an iv bolus dose (2 μmol/kg) to four rats (despite a possible plasma protein binding of 98%). This suggests that fluvastatin is actively transported into the bile. (Mean ± SD.)

0.05), respectively, when the iv dose was increased from 2 to 20 μmol/kg. In addition, the volume of distribution at steady state decreased from 7.3 ± 0.3 to 2.1 ± 0.7 L/kg as the iv dose increased ($p < 0.05$). The terminal half-life was relatively long, with a mean value of 280 ± 60 min at the lowest dose and 197 ± 24 min at the highest dose ($p < 0.05$). Cannulation of the bile duct and drainage of the bile flow did not affect the pharmacokinetic variables following iv administration (Table 1). In addition, the pharmacokinetics of fluvastatin following a 2 μmol/kg iv dose was not altered in two bile cannulated rats in which the bile was allowed to enter the duodenum (data not shown). The concentration of fluvastatin was significantly higher in the bile than in the plasma at all times, indicating carrier-mediated transport of fluvastatin into the bile (Figure 2). The bile clearance for fluvastatin could not be calculated in the current study, as the collection of bile was incomplete. However, assuming the bile flow to be in the normal range of 48−92 mL/day/kg,[21] the mean bile clearance could be estimated to 3.4−6.8 mL/h/kg, which is approximately 13−26% of the total clearance for fluvastatin.

Intestinal Dosing. The mean plasma concentration−time profiles following administration of fluvastatin at different duodenal doses (1.46, 2.92, 7.32, and 14.6 μmol/kg), jejunal dose (14.6 μmol/kg), ileal doses (1.46 and 14.6 μmol/kg), and colonic doses (1.46 and 14.6 μmol/kg) are shown in Figure 3. Neither the dose nor the intestinal region influenced the bioavailability of fluvastatin significantly (Table 2). The rate of drug absorption was, however, affected by both the dose and the site of administration. After duodenal administration, the T_{max} increased from 5 to 20 min and the C_{max}/AUC_{lqc} from 0.59 ± 0.11 to 0.71 ± 0.06 h^{-1} ($p < 0.05$ between doses 14.6 and 7.32 μmol/kg). In parallel, the mean C_{max} increased from 0.26 ± 0.10 to 7.06 ± 0.99 μM when the dose was increased from 1.46 to 14.6 μmol/kg. This increase was nonlinear when the dose was increased from 7.32 to 14.6 μmol/kg ($p < 0.05$). The half-life was shorter

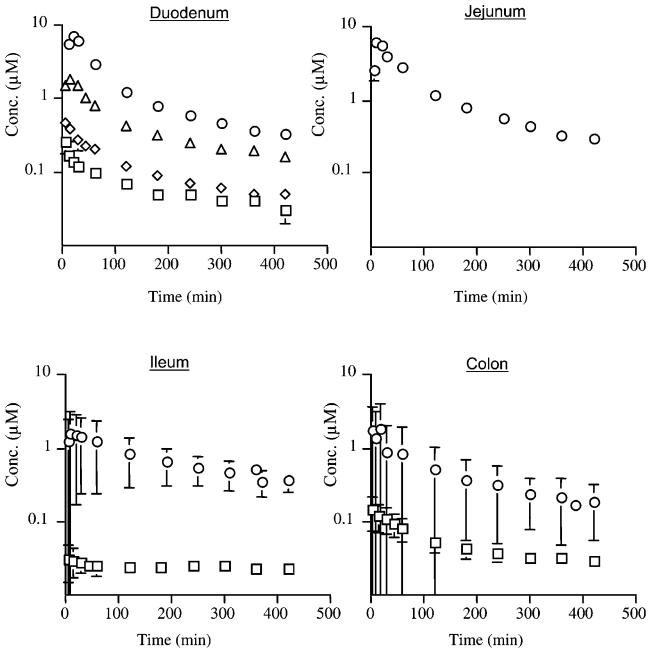

Figure 3. Plasma concentration−time profiles for fluvastatin following regional intestinal bolus doses of 1.46 (□), 2.92 (◇), 7.32 (△), and 14.6 μmol/kg (○) into the duodenum, the jejunum, the ileum, and the colon in rats. (Mean ± SD, n = 3−6.)

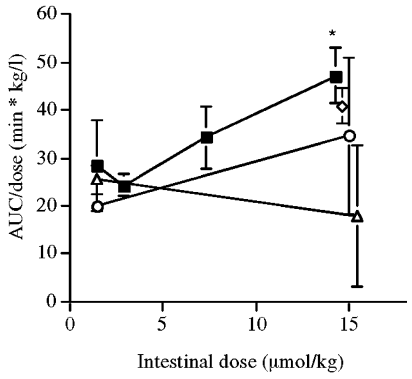

Figure 4. The ratio AUC/dose following various intestinal doses (1.46−14.6 μmol/kg) in the duodenum (■), jejunum (◇), ileum (○), and colon (△). The results from the various intestinal regions are separated slightly following the dose of 14.6 μmol/kg, to make the figure clearer. The asterisk (*) indicates a statistically significant difference compared to the corresponding value after administration of the lowest dose in the same intestinal region ($p < 0.05$, mean ± SD, n = 3−6).

following intestinal administration of the high dose compared to the low dose ($p < 0.05$).

The mean bioavailability (F, adjusted for the nonlinear systemic clearance) from the duodenum was approximately 60% (range of mean values 47−69%) and was not significantly affected by the different intestinal doses (Table 2). The ratio AUC/intestinal dose increased with increasing dose in the duodenum ($p < 0.05$) but not in the other intestinal regions (Figure 4). The half-lives of fluvastatin were similar after duodenal and intravenous administration (Tables 1 and

2). The dose administered into the jejunum resulted in values for the C_{max}, C_{max}/AUC_{lqc}, bioavailability, and half-life that were similar to the values resulting from the duodenal dose of the same strength, whereas the T_{max} was considerable shorter (Table 2). The mean C_{max} and C_{max}/AUC_{lqc} following ileal dosing were lower ($p < 0.05$), and the mean T_{max} longer with higher interindividual variability compared to the duodenal and jejunal dosing (Table 2). This suggests slower and more erratic absorption from the distal small intestine, which is supported by the increased mean half-life following ileal dosing in comparison to jejunal and duodenal administration ($p < 0.05$).

The C_{max}/AUC_{lqc} following colonic administration was higher compared to ileal administration, suggesting a more rapid absorption from the colon than the ileum (Table 2). The colonic C_{max}/AUC_{lqc} was, however, lower than following jejunal and duodenal administration (Table 2). The half-lives after the colonic doses were similar to those from the upper gastrointestinal tract (Table 2). The residual area (last sampling time to infinity) in the AUC calculations was higher after ileal ($47 \pm 17\%$) and colonic ($36 \pm 12\%$) administration than after duodenal ($23 \pm 14\%$) and jejunal ($12 \pm 2\%$) administration ($p < 0.05$).

Caco-2 Experiments. The in vitro permeability for fluvastatin was high (Biopharmaceutical Classification System), both in the absence and in the presence of indomethacin, probenecid, verapamil, and vinblastine (Figure 5). The basolateral-to-apical (b-to-a) permeability was higher than the apical-to-basolateral (a-to-b) permeability in the control experiment and in the presence of probenecid, verapamil, or vinblastine (Er 4.7, 4.1, 4.8, and 4.5, respectively). However, in the presence of indomethacin, the a-to-b permeability increased and the b-to-a permeability decreased significantly ($p < 0.002$ and $p < 0.0001$, respectively, Er 1.3), compared to the control experiment. In fact, in the presence of indomethacin, the transport of fluvastatin in the a-to-b direction and that in the b-to-a direction were not statistically different from each other.

Simulations in Silico. By using physiology based simulation with Gastroplus we were able to assess the relative importance of different gastrointestinal regions after single dose administration of fluvastatin as an IR or ER formulation. When fluvastatin was given as an IR tablet, 93% of the dose was absorbed from the small intestine and only 6% from the colon (total fraction dose absorbed 99%). On the other hand, the relative importance between small and large intestine switched when an ER formulation with 12 h release was simulated, as only 21% of the dose was absorbed from the small intestine and 74% from the colon (total fraction dose absorbed 95%) (Figure 6).

Discussion

Our main objective was to investigate the in vivo absorption, biliary secretion, and first-pass effect of fluvastatin following regional intestinal dosing in the rat. We also examined the membrane transport mechanisms and made in silico predictions of the relative importance of various intestinal regions to the human absorption of fluvastatin. Fluvastatin has been shown to have a high intestinal permeability in human jejunum, rat intestine, and Caco-2 monolayers.[6] A possible involvement of efflux transporters in the absorption of fluvastatin is therefore expected to have a larger impact on the rate of absorption than the extent of absorption. The absorption rate of fluvastatin, as judged from C_{max}, T_{max}, and C_{max}/AUC_{lqc}, was lower in the ileum and the colon compared to the duodenum and the jejunum. Thus, the region-specific in vivo absorption rates in rats were ranked; duodenum = jejunum > colon > ileum. The rate of absorption of fluvastatin was not only region-dependent, but also dose-dependent, with a more rapid absorption following the higher intestinal dose of 14.6 μmol/kg compared to the low dose of 1.46 μmol/kg. This is in line with the previously

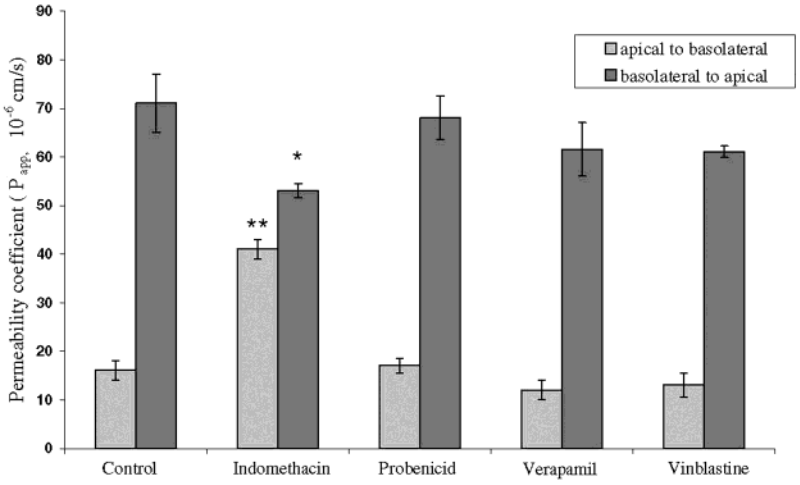

Figure 5. Bidirectional transport of fluvastatin (16 μM) across Caco-2 monolayers in the absence or presence of various transporter inhibitors. The single asterisk (*) indicates a statistically significant change in secretory transport ($p < 0.002$) and the double asterisk (**) indicates a statistically significant change in absorptive transport ($p < 0.0001$) compared to the corresponding transport without inhibitor.

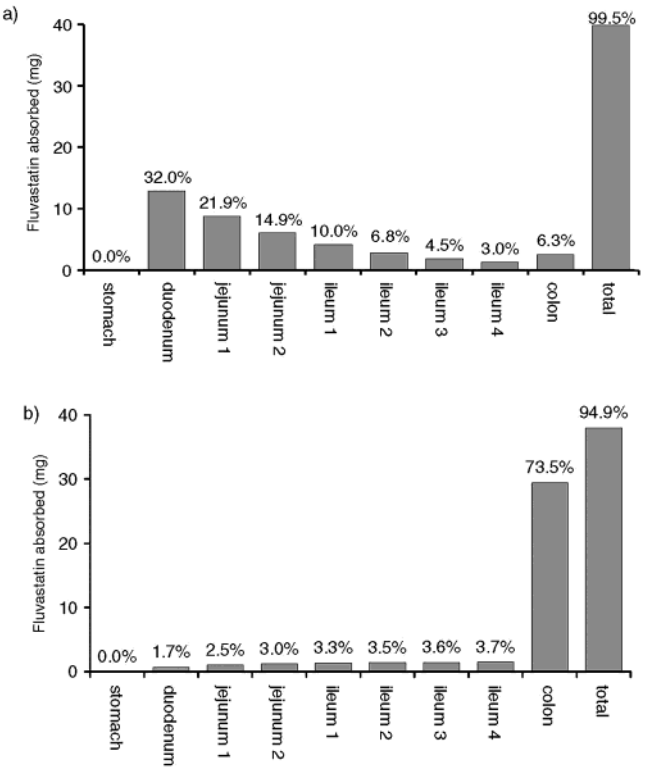

Figure 6. In silico simulated absorption of fluvastatin from the human gastrointestinal tract following the administration of 40 mg of fluvastatin as (a) an oral immediate release tablet and (b) an extended release integrated tablet with zero order release for 12 h.

reported concentration-dependent intestinal permeability of fluvastatin in the rat, with higher permeability at higher lumenal concentrations of fluvastatin.[6] The mechanism(s) explaining this nonlinear membrane passage has not been fully explained.

The observed differences in the absorption rate might be due to physiological differences along the intestinal tract. For instance, the surface area available for absorption is significantly larger in the small intestine than in the colon, suggesting more efficient absorption from the upper gastrointestinal tract.[24] Several efflux proteins, e.g., Pgp and multidrug-resistance-associated protein (mrp), are expressed to a various extent in the different regions of the gastrointestinal tract. Makhey et al. reported that the in vitro efflux by these transporters was region-dependent in the rat, with the highest efflux being in the ileum and the colon.[25] Thus, if fluvastatin is effluxed by Pgp and/or mrp, this might explain the slower absorption rate from the ileum and the colon, as well as the dose-dependent absorption. To further investigate this hypothesis we studied the in vitro transport of fluvastatin across Caco-2 monolayers in the presence and absence of various substrates/inhibitors of Pgp and mrp. Verapamil and vinblastine were employed to indicate transport by Pgp, whereas indomethacin and probenecid were used to indicate transport by mrp. The transport of fluvastatin was significantly affected, both in the absorptive direction (a-to-b) and in the secretory direction (b-to-a), in the presence of indomethacin, but was not affected in the presence of probenecid, verapamil, or vinblastine. The absence of effect on the transport of fluvastatin in the presence of probenecid is in line with our previously reported results from intestinal in situ perfusions in the rat.[11] Taken together, the results from the Caco-2 experiments suggest that fluvastatin is transported by mrp2, but not by Pgp. Thus, the observed region- and dose-dependent rate of absorption in vivo may be due to the involvement of mrp2 and/or organic anion transport system(s) in the absorption process.

The intestinal P_{eff} of fluvastatin has previously been reported to be high throughout the rat intestine in situ, with the colon being the most permeable region.[6] In these perfusion experiments the concentration of fluvastatin in the perfusion solution was 1.6−160 μM, compared to the concentrations of 1.2 and 12 mM that were administered into the intestinal regions in the present study. At these high initial concentrations of fluvastatin the mrp2-mediated intestinal efflux (and/or other transporters) should be saturated, and therefore a dose-dependent rate of absorption less likely. However, on the basis of the results from the Caco-2 experiments, involvement of mrp2 cannot be excluded as a possible explanation for the regional and nonlinear absorption rate observed in the present in vivo pharmacokinetic study.

Fluvastatin (log *D* octanol/buffer pH 7.4 = 1.4, weak acid; pK_a = 4.6) was administered to the intestinal regions as a solution buffered to pH 8 in the present study.[26] The reason for this was the limited solubility of fluvastatin in physiological saline and at lower pH. The normal lumenal pH of the rat gastrointestinal tract increases distally from approximately 6.5 in the upper small intestine to 7.1 in the ileum, and then decreases to 6.6 in the colon.[24] Accordingly the local differences in pH along the rat intestine in vivo might have affected the absorption rate through both precipitation and/or passive intestinal permeability of fluvastatin. However, the rapid absorption of fluvastatin in the duodenum and the jejunum, where the pH is lowest, and the increase in C_{max} and C_{max}/AUC_{lqc} at higher doses suggest that precipitation is probably not the reason for the low rate of absorption observed from the ileum and the colon.

Simulations in silico were performed in order to estimate the regional human intestinal absorption of fluvastatin. Two 40 mg dosage forms were simulated: first an IR tablet and

(24) Kararli, T. T. Comparison of the gastrointestinal anatomy, physiology, and biochemistry of humans and commonly used laboratory animals. *Biopharm. Drug Dispos.* **1995**, *16*, 351−380.

(25) Makhey, V. D.; Guo, A.; Norris, D. A.; Hu, P.; Yan, J.; et al. Characterization of the regional intestinal kinetics of drug efflux in rat and human intestine and in Caco-2 cells. *Pharm. Res.* **1998**, *15*, 1160−1167.

(26) Winiwarter, S.; Bonham, N. M.; Ax, F.; Hallberg, A.; Lennernas, H.; et al. Correlation of human jejunal permeability (in vivo) of drugs with experimentally and theoretically derived parameters. A multivariate data analysis approach. *J. Med. Chem.* **1998**, *41*, 4939−4949.

then an ER integrated tablet with a zero order release profile for 12 h. Due to the high solubility (at higher pH) and high permeability of fluvastatin almost the whole dose was absorbed from the small intestine with only 6% being absorbed from the colon when an IR tablet was simulated. Following administration of the ER tablet, on the other hand, 73% of the dose was absorbed from the colon. Thus, these results suggest that fluvastatin should be well absorbed when given as an ER formulation to man. In fact, Sabia and co-workers reported that a fluvastatin ER formulation (12 h release) was generally safe and well tolerated by 40 hypercholesterolemic patients at doses up to 320 mg/day.[27] They observed plasma concentrations (80 mg dose: C_{max} 61 ± 16 ng/mL, T_{max} 3−5 h) that were in the same magnitude as those we observed in the simulations in silico in the present study (dose-adjusted C_{max} 30 ng/mL reached after about 10 h). The slight underestimation of C_{max} in the simulations is probably due to differences in release profiles for the ER formulations. Sabia and co-workers did not describe the release profile of their ER tablet more than " a 12-hour-release", whereas we used a 12 h zero order release formulation for the simulations. A limitation of the simulations is that no efflux proteins were present in the intestine in silico. However, the similarities between the results from the simulations and the results from Sabia and co-workers might suggest that the involvement of efflux proteins is not an important factor for the overall absorption of fluvastatin.

Fluvastatin exhibited dose-dependent plasma pharmacokinetics in the rat. The volume of distribution, the systemic clearance, and the terminal half-life all decreased when the intravenous bolus dose was increased from 2 to 10 and 20 μmol/kg in the present study. As the urinary excretion of unchanged fluvastatin accounts for less than 8% of the total clearance in the rat,[5] it is most likely that the saturation of the clearance occurred in the liver, although the specific mechanism is not known. Which enzyme or enzymes are involved in the biotransformation of fluvastatin in the rat has not been reported in the literature. In humans, on the other hand, fluvastatin is mainly metabolized by CYP2C9.[28,29]

Fluvastatin is actively secreted into the bile, as the concentration of fluvastatin in the bile was 10−100 times higher than the total plasma concentration at all times. If the high degree of protein binding (>98%) also is taken into account, the evidence for active transport into bile is even stronger.[20] Yamazaki et al. reported that the biliary excretion of pravastatin, another inhibitor of HMG-CoA reductase, is mediated mainly by the ATP-dependent canalicular multispecific organic anion transporter (mrp2) in the rat.[9] It has been shown that pravastatin, lovastatin, simvastatin, and atorvastatin are transported by rat isoform oatp1 and the human OATP2.[30] It has also been reported that fluvastatin is partly transported by a carrier such as OATP in human hepatocytes and that the transport could be inhibited by pravastatin.[31] Saturation of these sinusoidal and canalicular located membrane transporters at higher plasma concentrations should result in lower biliary clearance and might be one explanation for the observed dose-dependent hepatic clearance of fluvastatin. However, collection of the bile did not affect any of the pharmacokinetic parameters significantly, which indicates that bile secretion is of minor importance for the overall clearance of fluvastatin itself. This is in line with the previously reported 13% recovery of fluvastatin in the feces following an intravenous bolus dose of 0.6 μmol/kg in rat.[5] This leads to the conclusion that saturated biliary excretion of parent drug in the present study is not the explanation for the observed 54% decrease in the systemic clearance when the iv dose was increased 10 times. Instead saturated metabolic liver extraction is more plausible if it is only due to saturation of the CYP enzymes. It may also be due to decreased hepatic metabolism as a consequence of a saturated transport across the sinusoidal membrane.[32] Another statin, atorvastatin, has also been shown to exhibit a nonlinear increase in systemic exposure most likely as a consequence of capacity limited metabolism and/or saturable liver uptake and/or biliary excretion.[33] The AUC/intestinal dose ratios for the various doses shown in Figure 4 suggest nonlinear first-pass extraction and/or systemic clearance since the intestinal permeability seems to be high at all doses. The regional and expected dose-dependent differences in the true local absorption rate may still contribute to the variability seen in the plasma exposure due to different degree of saturation. The nonlinear decrease in clearance after iv administration of fluvastatin suggests that the dose-dependent increase in the AUC/intestinal dose ratios is also explained by saturable systemic clearance and not only saturable hepatic first-pass extraction. In fact, the estimated bioavailability was not dependent on the dose size

(27) Sabia, H.; Prasad, P.; Smith, H. T.; Stoltz, R. R.; Rothenberg, P. Safety, tolerability, and pharmacokinetics of an extended-release formulation of fluvastatin administered once daily to patients with primary hypercholesterolemia. *J. Cardiovasc. Pharmacol.* **2001**, *37*, 502−511.

(28) Transon, C.; Leemann, T.; Vogt, N.; Dayer, P. In vivo inhibition profile of cytochrome P450TB (CYP2C9) by (±)-fluvastatin. *Clin. Pharmacol. Ther.* **1995**, *58*, 412−417.

(29) Transon, C.; Leemann, T.; Dayer, P. In vitro comparative inhibition profiles of major human drug metabolising cytochrome P450 isozymes (CYP2C9, CYP2D6 and CYP3A4) by HMG-CoA reductase inhibitors. *Eur. J. Clin. Pharmacol.* **1996**, *50*, 209−215.

(30) Hsiang, B.; Zhu, Y.; Wang, Z.; Wu, Y.; Sasseville, V.; et al. A novel human hepatic organic anion transporting polypeptide (OATP2). Identification of a liver-specific human organic anion transporting polypeptide and identification of rat and human hydroxymethylglutaryl-CoA reductase inhibitor transporters. *J. Biol. Chem.* **1999**, *274*, 37161−37168.

(31) Ohtawa, M.; Masuda, N.; Akasaka, I.; Nakashima, A.; Ochiai, K.; et al. Cellular uptake of fluvastatin, an inhibitor of HMG-CoA reductase, by rat cultured hepatocytes and human aortic endothelial cells. *Br. J. Clin. Pharmacol.* **1999**, *47*, 383−389.

(32) Chandra, P.; Brouwer, K. L. The complexities of hepatic drug transport: current knowledge and emerging concepts. *Pharm. Res.* **2004**, *21*, 719−735.

(33) Lennernas, H. Clinical pharmacokinetics of atorvastatin. *Clin. Pharmacokinet.* **2003**, *42*, 1141−1160.

or the intestinal region when the calculations were adjusted for the nonlinear systemic clearance.

The decrease in volume of distribution by approximately 70% when the iv dose was increased from 2 to 20 μmol/kg suggests that the tissue binding of fluvastatin is saturated. In tissues, the potential binding sites include enzymes, receptors, and nonspecific binding proteins.[34] Fluvastatin, like other inhibitors of HMG-CoA reductase, exhibits liver-specific tissue distribution, resulting in very high concentrations in the liver compared to other tissues.[2,5] In 1994 Xu and Lin reported that the pharmacologically active forms of simvastatin and lovastatin exhibit a liver-tissue binding that decreases steeply at increasing steady-state concentrations in the rat liver during in vitro perfusions.[34] It was suggested that the binding site was HMG-CoA reductase; it was almost completely saturated at 5 μM.[34] As the plasma concentrations after the two highest iv doses of fluvastatin in the present study had this level of concentration, we suggest that the decreased volume of distribution was due to saturation of the binding to HMG-CoA reductase in the liver. However, further studies need to be undertaken in order to clarify the mechanisms behind the nonlinear decrease of the volume of distribution and clearance, and also the impact of the suggested saturated binding in the liver on the nonlinear metabolism of fluvastatin.

In summary, the results of this study show that the rate of absorption of fluvastatin from the intestinal tract of the rat in vivo is region- and dose-dependent. The rate of absorption was slower from the lower intestine. The results from the Caco-2 experiments show that a possible involvement of mrp2 cannot be excluded as one membrane transport mechanism in the intestine, even if it probably is of more quantitative importance in the liver. These intestinal absorption properties have to be considered in the development of an extended release formulation of fluvastatin. The bioavailability was approximately 50−70% from all intestinal regions, and was not dependent on the dose size or the intestinal region when the calculations were adjusted for the nonlinear systemic clearance. Fluvastatin was secreted into the bile, probably by active transport. Furthermore, we found that the systemic clearance, the hepatic extraction, and the volume of distribution all decreased following higher iv doses. A complex process including both saturated hepatic transporters/enzymes and liver-tissue binding probably causes this.

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(34) Lin, J. H. Dose-dependent pharmacokinetics: experimental observations and theoretical considerations. *Biopharm. Drug Dispos.* **1994**, *15*, 1−31.