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Stereoselective analysis of fluvastatin in human plasma for pharmacokinetic studies

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Abstract

Fluvastatin, an inhibitor of cholesterol biosynthesis, is commercialized as a racemic mixture of the (+)-3*R*,5*S* and (−)-3*S*,5*R* stereoisomers, although inhibition of HMG-CoA reductase mainly resides in the (+)-(3*R*,5*S*)-fluvastatin isomer. The aim of the present study was to analyze fluvastatin isomers in human plasma with application to studies on kinetic disposition. Plasma samples of 1 ml were eluted into 3 ml LC-18 Supelclean (Supelco) columns equilibrated with methanol and water. The columns were washed with water and acetonitrile and then eluted with methanol containing 0.2% diethylamine. The (+)-3*R*,5*S* and (−)-3*S*,5*R* isomers were separated by HPLC on a Chiralcel OD-H chiral phase column and detected by fluorescence (λ_{ex} 305 nm; λ_{em} 390 nm). The quantification limit was 0.75 ng for each isomer/ml plasma and linearity was observed up to 625 ng/ml. The relative standard deviations obtained for intra- and inter-assay precision were lower than 10% and the recovery was higher than 80% for both enantiomers. Application of the method to a stereoselective study on the pharmacokinetics of fluvastatin administered as a single oral dose (Lescol, 20 mg) to a healthy volunteer revealed stereoselectivity, with the highest plasma concentrations being observed for the (−)-3*S*,5*R* isomer (C_{max} 92.4 vs. 60.3 ng/ml, $AUC^{0-\infty}$ 133.3 vs. 97.4 ng h/ml, Cl/f 150.2 vs. 205.2 l h^{-1} and V_d/f 4.4 vs. 6.0 l/kg).

Keywords: Enantiomer separation; Pharmacokinetics; Fluvastatin

1. Introduction

Fluvastatin {the monosodium salt of [*R**,*S**,-(*E*)]-(±)-7-[3(4-fluorophenyl)-1-(methylethyl)-1H-indole-2yl]-3,5-dihydroxy-6-methyl-6-heptanoic acid} is a reversible, competitive and highly specific inhibitor of microsomal 3-hydroxy-3 methylglutaryl-coenzyme A (HMG-CoA) reductase and is used for the treatment of hypercholesterolemia. The presence of the asymmetric carbons 3 and 5 in the heptanoic acid side chain of fluvastatin results in two possible diastereoisomers, i.e., *erythro* and *threo*. The drug commercialized as Lescol (Novartis Biociências, São Paulo, Brazil) presents *erythro* configuration and consists of a racemic mixture of two stereoisomers, (+)-3*R*,5*S* and (−)-3*S*,5*R*; however, the inhibitory

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activity of HMG-CoA reductase is 30 times higher for the (+)-3*R*,5*S*-fluvastatin enantiomer [1–6].

The uptake of fluvastatin into rat primary cultured hepatocytes may have some features in common with the bile acid transport system while fluvastatin may be taken up by human aortic endothelial cells via nonspecific simple diffusion [7]. The results demonstrate that the uptake of fluvastatin enantiomers into the target tissue (liver) may be stereoselective.

About 90% of an oral dose of fluvastatin is absorbed by the intestine but bioavailability is low in healthy volunteers, with values of the order of 19 (2 mg dose) to 29% (10 mg dose), due to extensive presystemic elimination. The dose-dependent effect on bioavailability can be explained by a saturation effect of the first passage [1,4,8,9].

Binding of the fluvastatin enantiomers to normal human plasma proteins is higher than 99%, the binding of each being unaffected by the presence of the other [10]. In healthy volunteers, the distribution volume of fluvastatin is 0.42 l/kg and the elimination half-life is 0.7 to 1.0 h [4,9].

The elimination of fluvastatin is almost exclusively dependent on metabolism and the main enzyme involved is CYP2C9. The main reactions are the hydroxylation of the indole ring at positions 5 and 6 and the loss of the isopropyl group (Fig. 1). The quantitatively most important metabolite in human plasma is *N*-diisopropyl propionic acid, which is inactive. 5-Hydroxy and 6-hydroxy fluvastatin are able to inhibit HMG-CoA but their plasma concentrations are extremely low [1,4,5,11].

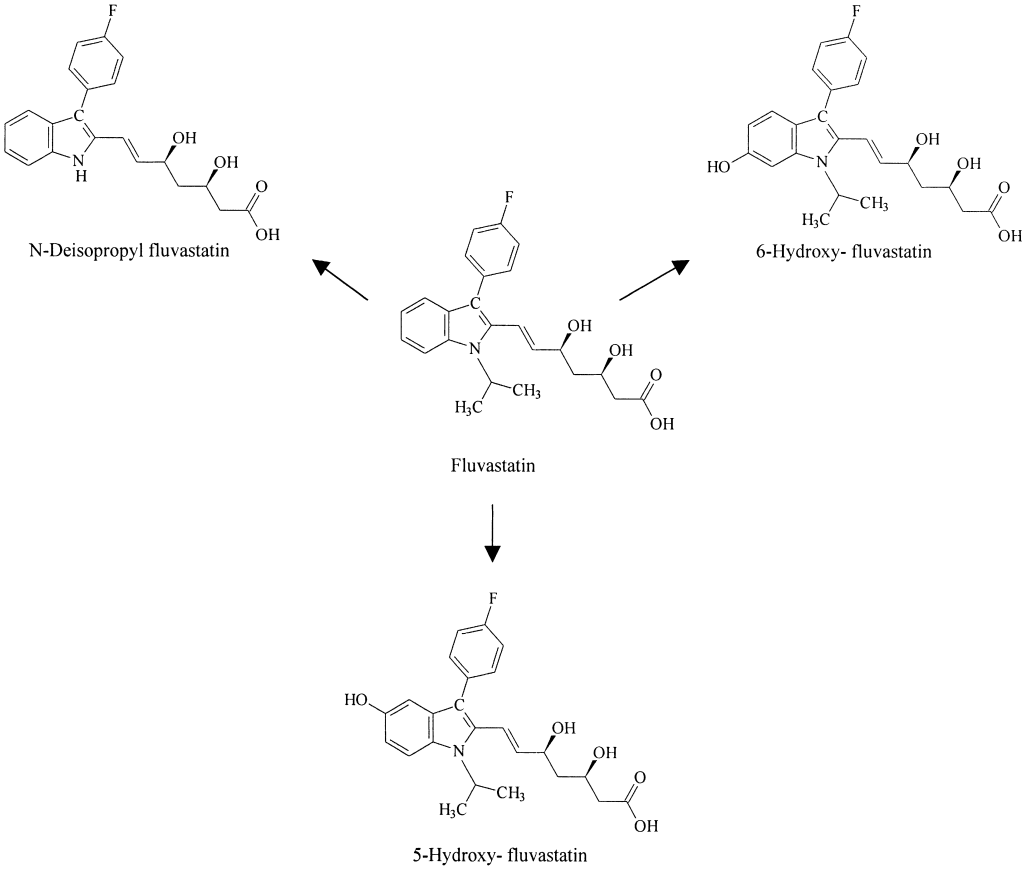

Fig. 1. Main metabolic pathways of fluvastatin in man [5].

Only Toreson and Eriksson [12,13] performed stereoselective analysis of fluvastatin in human plasma. The authors used a high-performance liquid chromatography (HPLC) system with fluorescence detection after liquid–liquid or solid-phase extraction and separation of the enantiomers on a stationary chiral phase Chiralcel OD-R column [cellulose Tris(3,5-dimethylphenyl)carbamate on silica gel]. There are no data regarding the fluvastatin enantiomer ratio in plasma from healthy volunteers or from patients treated with fluvastatin.

The objective of the present study was to develop and validate an analytical method for the determination of fluvastatin enantiomers in human plasma with application to enantioselective studies on the kinetic disposition of the statin administered in the racemic form.

2. Materials and methods

2.1. Standard solutions and reagents

The stock solution of sodium fluvastatin (Novartis Biociências) was prepared at a concentration of 100 μg [(*R**,*S**)-(*E*)-](±)-fluvastatin per ml water. The solutions diluted in water were prepared at concentrations of 50, 40, 10, 4, 2, 0.4, 0.2 and 0.06 μg [(*R**,*S**)-(*E*)-](±)-fluvastatin/ml. All fluvastatin solutions were stored at −20°C in the dark.

The solvents used for solid-phase extraction and as components of the mobile phase of the HPLC system were purchased from Merck (Darmstadt, Germany) and were of chromatography grade.

2.2. Equipment

An Aspec XL solid-phase extractor (Gilson Medical Electronics, Villiers-le Bel, France) and 3-ml LC-18 Supelclean columns (Supelco, Bellefonte, PA, USA) were used for the extraction procedure.

The HPLC system consisted of a Shimadzu chromatograph (Kyoto, Japan) equipped with an LC-10 AS pump with a 50-μl sampler and an FR 551 fluorescence detector, operating at an excitation wavelength of 305 nm and an emission wavelength of 390 nm. A Model CR 6A integrator was used for recording and integration of the chromatogram peaks.

2.3. Solid-phase extraction and chiral chromatography

The LC-18 extraction columns were equilibrated with 2.0 ml methanol eluted at a flow-rate of 6.0 ml/min, followed by 2.0 ml water eluted at the same rate. Plasma samples were centrifuged (10 min at 1800 *g*) and 1.0-ml aliquots were eluted at a flow-rate of 0.5 ml/min. The columns were then washed twice, first with water and then with acetonitrile, using volumes of 2.0 ml eluted at a flow-rate of 3.0 ml/min. Fluvastatin was eluted from the columns with 2.0 ml of a 0.2% diethyl amine solution in methanol at a flow-rate of 0.5 ml/min, using a volume of 0.6 ml air released at a flow-rate of 3.0 ml/min during each step. Aliquots of 1.3 ml of the eluates were evaporated to dryness and the residues were dissolved in 100 μl of hexane–ethanol (9:1, v/v) and 50 μl was chromatographed.

Separation of fluvastatin enantiomers was carried out on a 150×4.6 mm Chiralcel OD-H column (Chiral Technologies, Exton, PA, USA). The mobile phase consisted of hexane–ethanol (9:1, v/v) plus 0.2% trifluoroacetic acid and the flow-rate was 1.0 ml/min.

Sample preparation and chromatographic analyses were carried out under yellow light as the only light source.

2.4. Determination of the elution order of the enantiomers

For the determination of the elution order of the fluvastatin enantiomers, 25 μl of [(*R**,*S**)-(*E*)-](±)-fluvastatin solution was evaporated to dryness and submitted to chromatography on a Chiralcel OD-H chiral phase column as described above. Fractions of the eluate corresponding to the separated enantiomers were collected, concentrated dry and submitted to chromatography as described by Toreson and Eriksson [12], using a Chiralcel OD-R chiral phase column and a mobile phase consisting of acetonitrile–0.1 *M* phosphate buffer, pH 3 (40:60, v/v).

2.5. Validation

For construction of the calibration curves 1.0-ml plasma samples enriched with 25 μl of each standard fluvastatin solution were submitted to extraction and chromatography (0.75–625 ng of each fluvastatin enantiomer/ml plasma).

2.5.1. Recovery

Recovery of plasma fluvastatin was calculated by comparing the height of the peaks obtained after the extraction of fluvastatin from plasma with those obtained after direct injection of the standard solution. Recovery was analyzed in triplicate at concentrations of 5, 50 and 500 ng of each enantiomer/ml plasma.

2.5.2. Quantification limit

The quantification limit was defined as the lowest plasma concentration of each fluvastatin enantiomer analyzed, with an error of 20% or lower. Drug-free plasma samples spiked with concentrations as low as 0.75 ng of each enantiomer/ml were analyzed in quintuplicate.

2.5.3. Linearity

Drug-free plasma samples spiked with fluvastatin in the range of 0.75–625 ng of each enantiomer/ml were analyzed. The method was considered to be linear up to the highest concentration of each enantiomer analyzed, with an error lower than 15%.

2.5.4. Selectivity

Different drugs possibly used in combination with fluvastatin were analyzed. Standard drug solutions were prepared at concentrations of 1.0 mg/ml methanol and stored at −20°C. For chromatography 25 μl of the standard solution was evaporated to dryness and reconstituted in 100 μl of the mobile phase. The retention times of the fluvastatin enantiomers were compared with those obtained for the experimental drugs.

2.5.5. Precision and accuracy

Precision and accuracy were determined for plasma samples enriched with fluvastatin at concentrations of 5 and 500 ng of each enantiomer/ml. Aliquots of plasma samples were stored at −20°C and analyzed in replicate experiments ($n=5$) using a single calibration curve for intra-assay evaluation, and in duplicate on 5 consecutive days for inter-assay evaluation.

2.6. Clinical study

Volunteer F.H.M. (a 22-year-old man, 68 kg, 175 cm tall) was informed about the study and gave written consent to participate. After clinical examination and biochemical tests for the confirmation of normal hepatic, renal and cardiac functions the volunteer received one tablet of 20 mg fluvastatin (Lescol, Novartis) after a 12-h fast. Blood samples were collected through an intravenous catheter at times 0 and 0.25, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 6, 8 and 9 h after fluvastatin administration. The blood samples were transferred to tubes containing heparin (Liquemine, 5000 IU, Roche) and centrifuged at 1800 *g* for 10 min. The plasma samples were stored at −20°C until chromatographic analysis.

The enantioselective kinetic disposition of fluvastatin was determined based on an open bicompartmental model. The absorption ($t_{1/2}a$) and distribution ($t_{1/2}\alpha$) half-lives were determined after correction of the respective phases by the residue method. The elimination half-life ($t_{1/2}\beta$) was directly determined by the graphic method (log c vs. t). The absorption (k_a), distribution (α) and elimination (β) rate constants were calculated using the $0.693/t_{1/2}$ equation. The maximum plasma concentration (C_{max}) and the time to reach C_{max} (t_{max}) were directly calculated from the plasma concentrations of the enantiomers obtained. The plasma concentration versus time area under the curve ($AUC^{0-\infty}$) was calculated by the trapezoid method with infinite extrapolation. This parameter was used for the calculation of apparent total clearance ($Cl/f=dose/AUC^{0-\infty}$; where f means bioavailability factor) and apparent distribution volume ($V_d/f=Cl/f/\beta$) [8,9].

3. Results and discussion

The enantioselective study of the kinetic disposition and metabolism of drugs requires analytical methods able to discriminate the chiral forms and

having confidence limits compatible with the low plasma concentration of the enantiomers [14].

The enantiomers of plasma fluvastatin were directly separated on a Chiralcel OD-H column (Fig. 2). The elution order of the enantiomers through this column, in the sequence (−)-(3*S*,5*R*) and (+)-(3*R*,5*S*), was derived from the study of Toreson and Eriksson [12]. The enantiomers eluted from the Chiralcel OD-H column were separated and injected into a Chiralcel OD-R column, resulting in the same enantiomer elution order, i.e., the (−) and (+) sequence. The enantiomers were eluted on a Chiralcel OD-R column with retention times of 29.7 and 31.4 min for the (−)-(3*S*,5*R*) and (+)-(3*R*,5*S*)-fluvastatin enantiomers, respectively [12]. The developed method was found to be fast, with retention times of 10 and 12 min for the elution of the enantiomers through the Chiralcel OD-H column. Analysis of the pool of blank plasma samples collected from different healthy volunteers did not show interference between endogenous compounds and the fluvastatin enantiomers.

Recovery values (Table 1) were higher than 80%, regardless of the concentrations of the fluvastatin

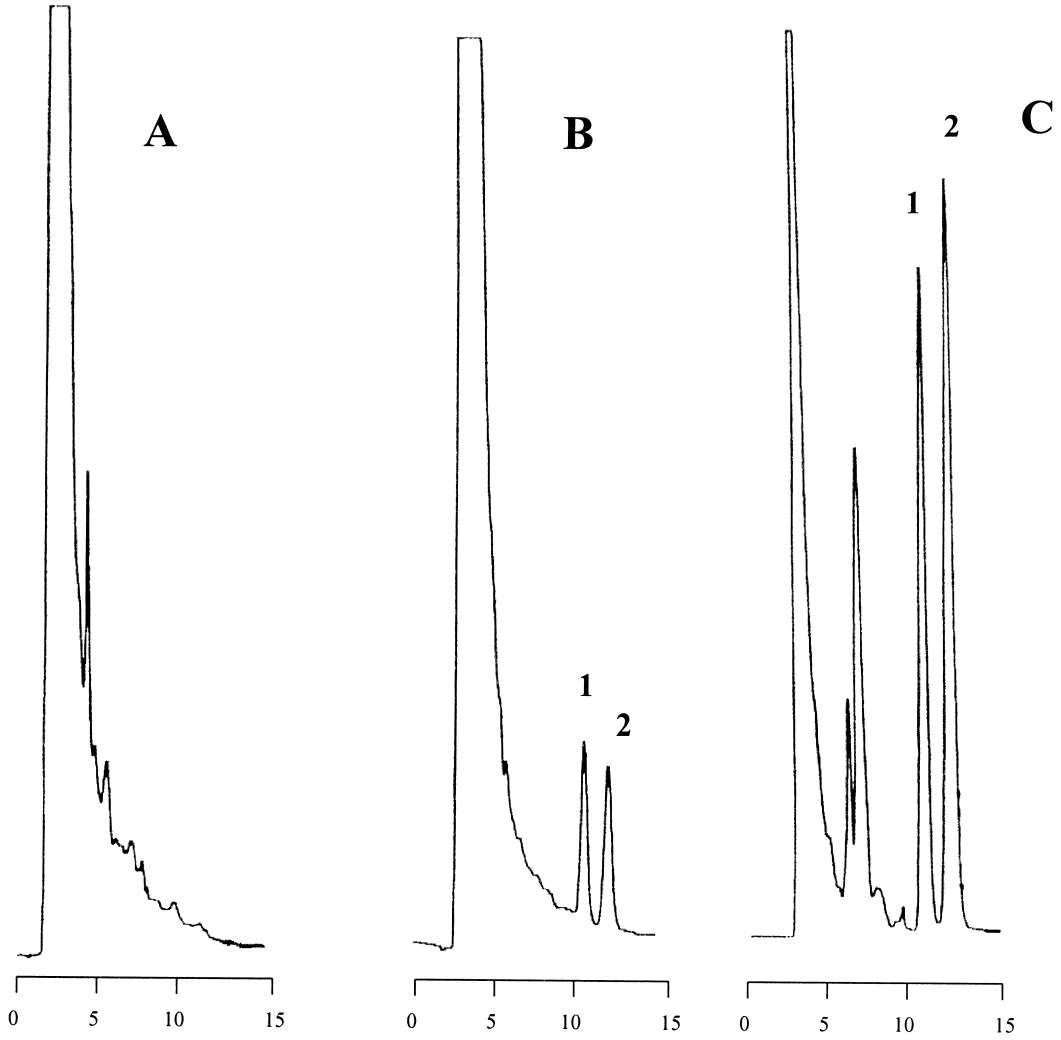

Fig. 2. Chromatograms of: (A) blank human plasma, (B) plasma sample spiked with 50 ng/ml *rac*-fluvastatin, and (C) human plasma obtained 1.5 h after a single oral dose of *rac*-fluvastatin. Peaks: (1) (−)-(3*S*,5*R*)-fluvastatin and (2) (+)-(3*R*,5*S*)-fluvastatin.

Table 1
Confidence limits of the analysis of fluvastatin enantiomers in plasma

	(−)-3*S*,5*R*-Fluvastatin	(+)-3*R*,5*S*-Fluvastatin
Recovery (%)	82	89
Quantification limit (ng/ml)	0.75	0.75
Precision (RSD, %)	5.8	4.5
Accuracy (% bias)	9.5	12.9
Linearity (ng/ml)	0.75–625.0	0.75–625.0
r	0.9947	0.9953
Intra-assay precision (RSD, %)		
500 ng/ml (n=5)	4.3	4.6
5 ng/ml (n=5)	6.2	8.3
Inter-assay precision (RSD, %)		
500 ng/ml (n=10)	4.7	8.2
5 ng/ml (n=10)	8.1	2.9
Intra-assay accuracy (% bias)		
500 ng/ml (n=5)	−11.5	−9.6
5 ng/ml (n=5)	−14.4	−9.4
Inter-assay accuracy (% bias)		
500 ng/ml (n=10)	−8.4	−7.3
5 ng/ml (n=10)	−5.4	−7.0

enantiomers, extracted with a C_{18} solid-phase extraction cartridge and eluted with methanol containing 0.2% diethyl amine. These recovery values are equivalent to those obtained by Toreson and Eriksson [12], who used liquid–liquid extraction at pH 6.5 and methyl *tert*.-butyl ether as solvent after protein precipitation with acetonitrile. The procedure of liquid–liquid extraction used by these authors has been previously described by Kalafsky and Smith [3]. Toreson and Eriksson [13] described the extraction of fluvastatin on a C_2 cartridge connected on-line with the analytical column with a recovery of 90%.

The quantification limit of 0.75 ng/ml obtained for both enantiomers led us to conclude that the method is more sensitive than that reported by Toreson and Eriksson [12], permitting its application to single-dose studies on kinetic disposition. It should be noted that the method described by Toreson and Eriksson [13], which included a step of sample irradiation with ultraviolet light in order to increase fluorescence detection, resulted in the quantification of concentrations as low as 0.2 ng of each enantiomer/ml plasma.

The present method can be considered highly selective as a result of the use of a procedure including solid-phase extraction and fluorescence detection, permitting its application to studies on the kinetic disposition of fluvastatin in combination with other drugs. This study confirms that no interferences occurs between fluvastatin enantiomers and other therapeutic compounds commonly given in combination such as analgesic–antipyretic, and nonsteroidal anti-inflammatory, anti-hypertensive, anti-arrhythmic, anti-microbials, drugs acting on the central nervous system and agents used in the treatment of asthma, as indicated in Table 2. In addition, the method presents great advantages compared to that described in the literature [13] in terms of the preparation and chromatographic analysis of the samples since an automated and thus less exhaustive extraction process is used and less than half the time is required for elution of the enantiomers, i.e., 13 vs. 31 min.

Table 2
Selectivity study: analysis of fluvastatin enantiomers in plasma

	Concentration (μg/ml)	Retention time (min)
(−)-(3*S*,5*R*)-Fluvastatin	25.0	10.15
(+)-(3*R*,5*S*)-Fluvastatin	25.0	12.38
Benzydamine	25.0	30.93
Clomipramine	25.0	8.7
Metoclopramide	25.0	25.31
Propranolol	25.0	7.30 and 33.85
Quinidine	25.0	6.21

Not detected during the 0–60 min interval: acetaminophen, aminopyrine, amiodarone, atenolol, captopril, carbamazepine, clobazam, chlorpromazine, dapsone, digoxin, etidocaine, phenacetin, lidocaine, nitrazepam, propafenone, sotalol, theophylline, trimipramine, verapamil.

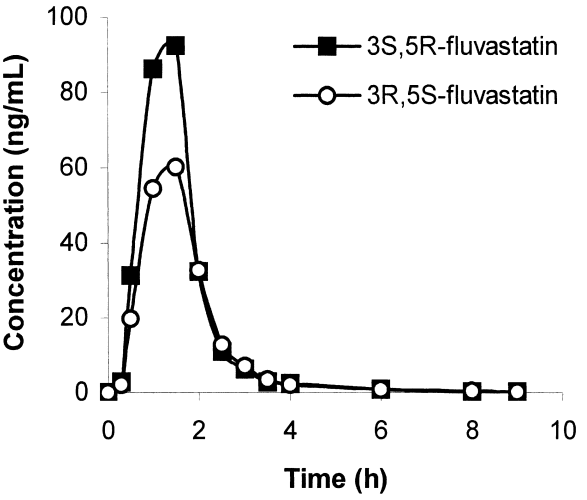

Fig. 3. Plasma concentration versus time curves for the (−)-(3*S*,5*R*)- and (+)-(3*R*,5*S*)-fluvastatin enantiomers after oral administration of 20 mg racemic fluvastatin.

There are no data in the literature about the stereoselectivity of the kinetic disposition of fluvastatin administered to healthy volunteers or hypercholesterolemic patients. The developed and validated method was employed in the enantioselective study on the kinetic disposition of fluvastatin administered in the racemic form to the volunteer F.H.M as a single p.o. dose of 20 mg (Table 3). The pharmacokinetic parameters obtained for both fluvastatin enantiomers are compatible with those described in the literature for enantiomer mixtures. The elimination half-life of 1.4 h obtained for the (−)-3*S*,5*R*- and (+)-3*R*,5*S*-fluvastatin enantiomers is compatible with the 0.5 and 2.4 h reported for enantiomer mixtures by Tse et al. [9], Smit et al. [15], Kivistö et al. [16] and Kantola et al. [17]. Desager and Horsmans [2] reported an apparent clearance for fluvastatin ranging from 70 to 435 l/h. In the present study, an apparent clearance of 150 and 205 l/h was obtained for the (−)-3*S*,5*R* and (+)-3*R*,5*S* enantiomers, respectively. The plasma concentration versus time curves showed higher C_{max} and $AUC^{0-\infty}$ values and lower total clearance for the (−)-(3*S*,5*R*) enantiomer (Fig. 3). The enantiomer ratio of plasma concentration versus time area under the curve ($AUC^{0-\infty}_{(-)}/AUC^{0-\infty}_{(+)}$) of 1.4 indicates higher concentrations of the (−)-(3*S*,5*R*) enantiomer, which is considered to have lower HMG-CoA inhibitory activity.

In conclusion, the method described in the present study is simple, rapid and showed to be highly reproducible and accurate. The quantification limit of 0.75 ng/ml for both enantiomers permits the use of the method in studies of clinical kinetic disposition.

Table 3
Enantioselective kinetic disposition of fluvastatin in a healthy volunteer

	(−)-(3*S*,5*R*)-Fluvastatin	(+)-(3*R*,5*S*)-Fluvastatin
C_{max} (ng/ml)	92.4	60.3
t_{max} (h)	1.5	1.5
$AUC^{0-\infty}$ (ng h ml^{-1})	133.3	97.4
V_d/f (l kg^{-1})	4.4	6.0
$t_{1/2}$ β (h)	1.4	1.4
β (h^{-1})	0.5	0.5
Cl/f (l h^{-1})	150.2	205.2
$AUC^{0-\infty}$ (−)/(+)	1.4	

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