Available online at www.sciencedirect.com
SCIENCE DIRECT
Journal of Chromatography B, 832 (2006) 256–261
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Chiral evaluation of fluvastatin in human plasma by high-performance liquid chromatography electrospray mass spectrometry

Giuliano Di Pietro [a], Eduardo Barbosa Coelho [b], Tufik Magalhães Geleilete [b], Maria Paula Marques [a], Vera Lucia Lanchote [a,*]

[a] *Faculdade de Ciências Farmacêuticas de Ribeirão Preto da Universidade de São Paulo, Ribeirão Preto, SP, Brazil*
[b] *Faculdade de Medicina de Ribeirão Preto da Universidade de São Paulo, Ribeirão Preto, SP, Brazil*

Received 11 February 2005; accepted 12 January 2006
Available online 9 February 2006

Abstract

The report describes for the first time the enantioselective analysis of fluvastatin in plasma using LC–MS–MS. The enantiomers of fluvastatin (FV) were extracted from plasma with diisopropyl ether at pH 5.0. The enantiomers were separated on a ChiralCel® OD-R column with a mobile phase consisting of a mixture of acetonitrile, methanol and water (24:36:40) containing 0.1% formic acid. The protonated ions and their respective product ions were monitored in two functions, 410.6 > 348.2 for FV enantiomers and 307.1 > 161.6 for the internal standard (warfarin). Recoveries were higher than 90% and the quantitation limit was 1.5 ng mL^{-1} plasma for both enantiomers. The coefficients of variation and the relative errors obtained for the validation of the intra- and interassay precision and accuracy were less than 10%. The method was applied to the investigation of the enantioselective pharmacokinetics of FV administered in a single dose of 40 mg (Lescol®, Novartis, São Paulo, SP, Brazil) to a patient with primary hypertension and hypercholesterolemia and genotyped as CYP2C9*1/*1. The data showed higher plasma concentrations of the (−)-3*S*,5*R*-fluvastatin enantiomer, with an AUC (−)/(+) of 1.84. Oral clearance values (CL/*F*) were 29.27 and 49.58 L/h, respectively, for the (−)-3*S*,5*R*- and (+)-3*R*,5*S*-fluvastatin enantiomers.

Keywords: Fluvastatin; Pharmacokinetics; Enantiomers; LC–MS–MS

1. Introduction

Fluvastatin (FV) is a 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitor used in the hypercholesterolemia treatment. Moderate reduction in serum cholesterol levels by about 20–30% is achieved with daily doses of 20–40 mg. FV is marketed as a racemic mixture of the (+)-3*R*,5*S* and (−)-3*S*,5*R* enantiomers. However, the therapeutic activity is 30-fold higher for the (+)-3*R*,5*S*-FV than (−)-3*S*,5*R*-FV enantiomer [3,4].

Regarding pharmacokinetic properties of FV, there is low and variable bioavailability (29 ± 18%) reported in healthy volunteers as a result of an extensive first-pass metabolism [1]. There are no data regarding the bioavailability of the individual enantiomers. The FV enantiomers are strongly and non-competitively bound to plasma proteins (>98%), especially albumin. The distribution volume of FV is low, with reported values of 0.42 L kg^{-1} [9]. The FV is mainly eliminated by metabolism. In vitro data have shown that approximately 50–80% of the metabolic clearance of FV depends on the polymorphic CYP2C9 [2,4].

Enantioselective analysis of FV in plasma was previously reported by [7,8]. In the first report, FV enantiomers were extracted from plasma at pH 6.0 with methyl *tert*-butyl ether, separated on a Chiralcel® OD-R column and detected by fluorescence. In the second report, the authors described a highly sensitive method for the analysis of FV enantiomers in plasma using solid phase extraction (C2), enantiomer separation through a Chiralcel® OD-R column and a fluorescence detection after post-column exposure of the eluate to UV light, which enhanced the sensitivity 10-fold. We have previously reported the enantioselective kinetic disposition of FV administered to a healthy volunteer [5], showing higher plasma concentrations for the (−)-3*S*,5*R*-FV enantiomer. Plasma samples (1 mL) were eluted into

* Corresponding author. Tel.: +55 16 36024699; fax: +55 16 36331936.
E-mail address: lanchote@fcfrp.usp.br (V.L. Lanchote).

1570-0232/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2006.01.007

LC-18 Supelclean columns and the enantiomers were separated by HPLC on a Chiralcel OD-H chiral phase column and detected by fluorescence. The method was later used by [4] for the enantioselective analysis of FV administered to healthy volunteers with different CYP2C9 genotypes.

LC–tandem mass spectrometry (LC–MS–MS) has been widely used in pharmacokinetic studies mainly because of its high sensitivity and selectivity. The present study reports for the first time the enantioselective analysis of FV using LC–MS–MS. The method was applied to the investigation of enantioselectivity in the kinetic disposition of FV administered in a single dose to a patient with primary hypertension and hypercholesterolemia.

2. Materials and methods

2.1. Standard solutions and reagents

The racemic sodium fluvastatin (*rac*-FV) was kindly provided by Novartis Biociências S.A. (São Paulo, SP, Brazil). The stock solution of *rac*-FV was prepared in methanol at the concentration of 1 mg mL^{-1}. This solution was diluted with methanol at the concentrations of 20, 4, 2, 0.4, 0.2 and 0.06 μg for each FV enantiomer mL^{-1}. The FV solutions were prepared at 15-day intervals and stored at −20 °C protected from light. The internal standard solution, racemic warfarin (IS), was prepared with methanol at the concentration of 1 μg mL^{-1}.

The solvents acetonitrile, diisopropyl ether and methanol, chromatography grade, were obtained from Merck (Darmstadt, Germany). Formic acid (88%) was from J.T. Baker (Phillipsburg, NJ, USA). All water used was purified with the Milli-Q Plus system (Millipore, Bedford, MA, USA).

2.2. Equipment

The liquid chromatography system consisted of a Shimadzu (Kyoto, Japan) LC10ADVP pump and a CTO-10ASVP column oven. Resolution of the FV enantiomers was obtained with a Chiralcel® OD-R chiral column (Chiral Technologies Inc., Exton, PA, USA) (250 mm × 4.6 mm i.d., with a particle size of 10 μm) and a LiChrospher® 100 CN precolumn (4 mm × 4 mm i.d., with a particle size of 5 μm) from Merck. The mobile phase consisted of a mixture of acetonitrile, methanol and water (24:36:40, v/v/v) containing 0.1% formic acid used at a flow rate of 0.7 mL min^{-1}. The column was kept at a temperature of 30 ± 1 °C.

2.3. Conditions of detection by mass spectrometry

The mass spectrometry detection system (MS–MS) was Quattro micro™ LC triple quadrupole (Micromass, Manchester, UK) equipped with an electrospray interface (ESI). The analyses were performed in the negative ion mode.

The capillary voltage in the ESI probe was 3.0 kV. The source block and dessolvation temperatures were set at 120 and 200 °C, respectively. Nitrogen was used as nebulizing gas at 365 L h^{-1} and argon was used as collision gas at a pressure of approximately 2.1×10^{-3} mbar. The cone voltage was set at 30 V and the collision energy of 20 eV were used for FV and IS.

Optimization of MS conditions was obtained by direct infusion of standard solutions (10 μg mL^{-1}) prepared in the mobile phase and introduced with an infusion pump at a flow rate of 20 μL min^{-1}. The analyses were performed in the selected reaction monitoring (SRM) mode. Two transitions 410.6 > 348.2 for FV enantiomers and 307.1 > 161.6 for the IS were monitored (Fig. 1). Data acquisition and quantitation were performed using the MassLynx software, version 3.5 (Micromass, Manchester, UK).

2.4. Sample preparation

Plasma samples from healthy volunteers were obtained from the Blood Center of the University Hospital, Faculty of Medicine of Ribeirão Preto, University of São Paulo, Ribeirão Preto, SP, Brazil.

Twenty-five microliters of the IS solution, 1 mL of 0.75 M acetate buffer, pH 5.0, and 5 mL diisopropyl ether were added to 0.5 mL aliquots of human plasma. The samples were extracted in a horizontal shaker for 30 min and centrifuged at 2000 × *g* for 5 min. The organic phases were transferred to conical tubes and evaporated dry with a vacuum evaporation system (RCT90 and RC10.22 mode) from Jouan AS (St. Herblain, France) at 25 °C. The residues were reconstituted with 50 μl of the mobile phase and vortex mixed for 10 s and 20 μl were injected into the analytical column. All analytical procedures were performed under yellow light because of the photosensitivity of FV.

2.5. Validation of the analytical method

The calibration curves were constructed by spiking 0.5 mL aliquots of blank plasma with 25 μL of each standard solution of *rac*-FV, in duplicate, with resulting plasma concentrations of 1.5–500 ng of each FV enantiomer mL^{-1}. The linearity of the method was determined by the analysis of plasma samples spiked with FV at concentrations of 1.5–1000 ng of each enantiomer mL^{-1}. The recovery of the extraction process was determined by the analysis in triplicate of 0.5 mL aliquots of plasma spiked with three *rac*-FV concentrations (5, 50 and 500 ng of each enantiomer mL^{-1}). The samples were submitted to the extraction process and the area ratios were compared to those obtained by direct injection of FV and IS into the mobile phase.

The precision and accuracy of the method were assessed by the analysis of plasma samples spiked with three FV concentrations, 4, 40 and 400 ng mL^{-1}, for both enantiomers. Aliquots of the plasma samples were stored at −20 °C and analyzed in replicate ($n = 10$) using a single calibration curve for intra-day analysis and in duplicate for 5 consecutive days for inter-day analysis.

The quantitation limit was obtained by the replicate analysis ($n = 9$) of plasma samples spiked with *rac*-FV at concentrations lower than 5 ng mL^{-1} for both enantiomers. The quantitation

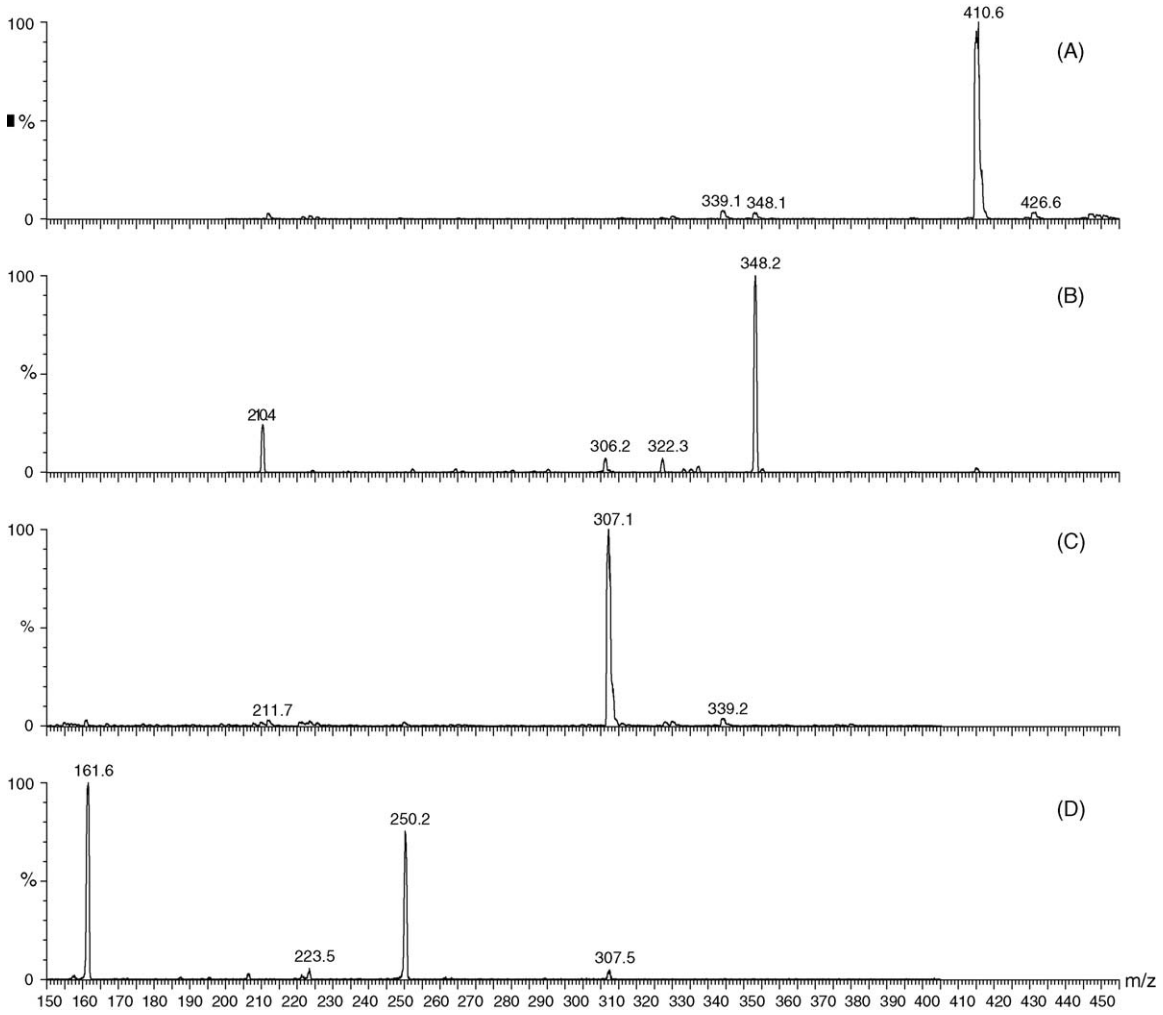

Fig. 1. Mass spectra of the protonated molecular ion of FV (A), product ion of FV (B), protonated molecular ion of warfarin (C) and product ion of warfarin (D).

limit was defined as the lowest plasma concentration of the FV enantiomers quantified with an error of less than 20%.

2.6. Method application

The study was conducted on a patient with primary hypertension and hypercholesterolemia (total cholesterol, 240 mg dL^{-1}; HDL cholesterol, 35 mg dL^{-1}), genotyped as CYP2C9*1/*1 using a restriction fragment length polymorphism (RFLP) method previously described by [6] and, treated with a single 40 mg dose of FV. The protocol was approved by the Ethical Committee of the University Hospital of Faculty of Medicine of Ribeirão Preto-USP and the patient (a 64-year-old woman, weighing 81.8 kg and measuring 157 cm in height) was informed in detail about the study and gave written informed consent to participate. After clinical and biochemical examination have been done to confirm normal hepatic, renal and cardiac functions, the patient received a single 40 mg tablet of *rac*-FV p.o. (Lescol®, Novartis) after a 12 h fast. Blood samples were collected at zero, 15, 30 and 45 min and at 1.0, 1.50, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6.0, 8.0 and 11.0 h after FV administration. The blood samples were transferred to tubes protected from light containing heparin (Liquemine®, 5000 IU, Roche, São Paulo, SP, Brazil). The samples were centrifuged at 1800 × *g* for 10 min and the plasma samples were stored protected from light at −75 °C until the time for chromatographic analysis.

Maximum plasma concentrations (C_{max}) and time to reach C_{max} (t_{max}) were directly determined from the data obtained. The area under the curve for plasma concentrations versus time ($AUC^{0-\infty}$) was calculated by the trapezoidal method and extrapolated to infinity. Total clearance (CL/*F*) was obtained using the equation $CL/F = Dose/AUC^{0-\infty}$, where *F* is the bioavailability. The elimination (β) and distribution (α) constants were estimated using the $0.693/t_{1/2}$ equation.

The enantioselective kinetic disposition of FV was determined using the WinNonlin software, version 4.0 (Pharsight Corp, Mountain View, CA, USA).

3. Results and discussion

The FV enantiomers were separated on a Chiralcel OD-R chiral column as reported by [7,8]. The mobile phase used in the present investigation, a mixture of acetonitrile, methanol and water (24:36:40, v/v/v) containing 0.1% formic acid, resulted in the separation of the FV enantiomers at shorter retention

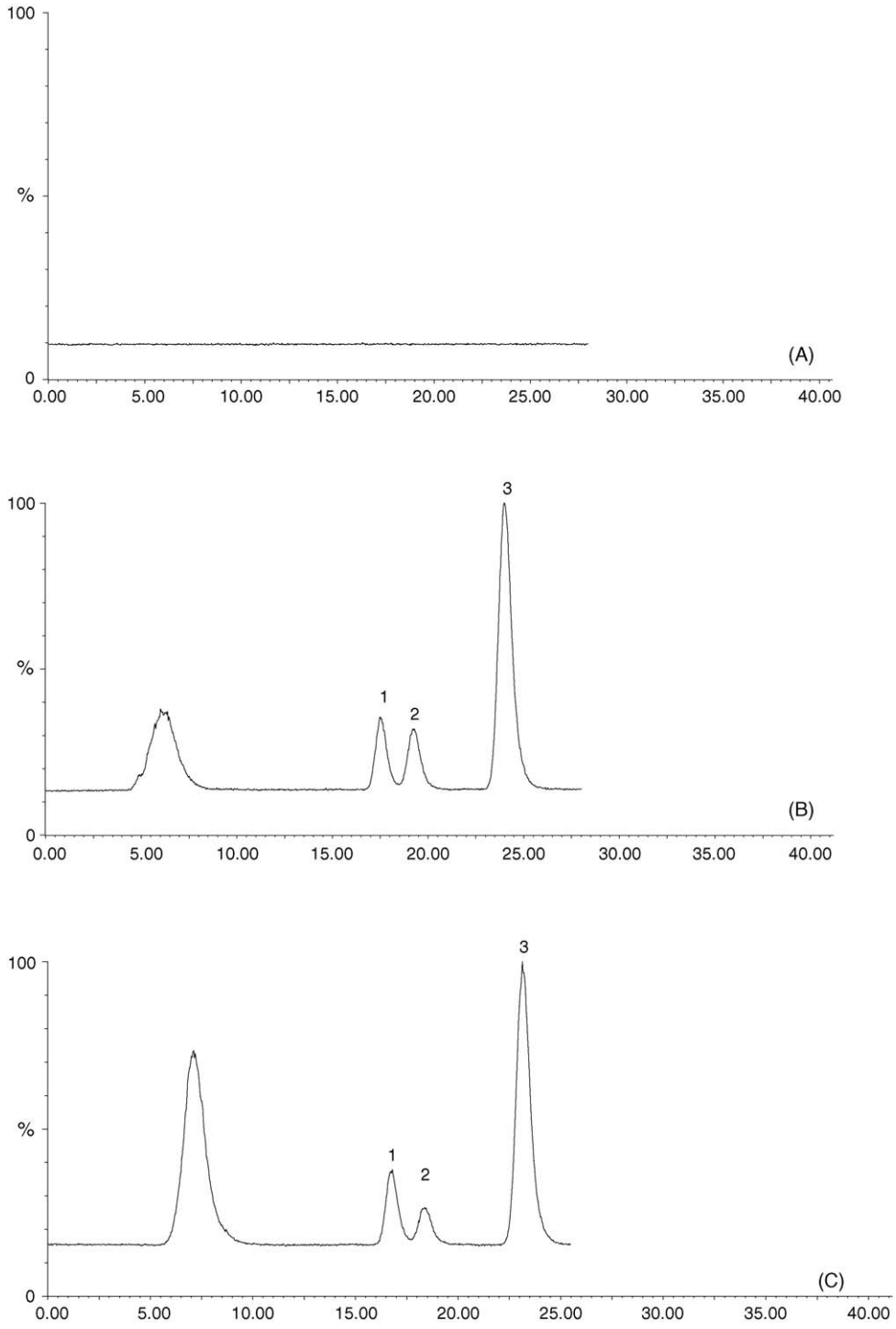

Fig. 2. Chromatograms: (A) blank plasma, (B) plasma sample spiked with 500 ng mL^{-1} of *rac*-FV and IS, and (C) plasma sample obtained 1.0 h after a single oral dose of *rac*-FV. Peaks: (1) (−)-3*S*,5*R*-FV, (2) (+)-3*R*,5*S*-FV, and (3) warfarin.

times than those reported by the above authors (Fig. 2). The elution order of the fluvastatin enantiomers was obtained by the analysis of individual enantiomers, previously separated and collected from the Chiralcel OD-R column according to the method described by [7]. The elution order was (−)-(3*S*,5*R*) and (+)-(3*R*,5*S*)-fluvastatin. The elution order was also determined using a circular dichroism detector to identify the two resolved enantiomers. As can be observed in Fig. 3, the first enantiomer [(−)-(3*S*,5*R*)] eluted from the Chiralcel OD-R column has a negative sign and the second enantiomer [(+)-(3*R*,5*S*)] has a positive sign in the 235 nm CD chromatogram.

The FV enantiomers were extracted from plasma at pH 5.0 using diisopropyl ether as the extracting solvent. The recoveries obtained for both FV enantiomers were more than 90% and were independent of the concentration in the 5–500 ng mL^{-1} range (Table 1). [8] reported recoveries of approximately 95% for both

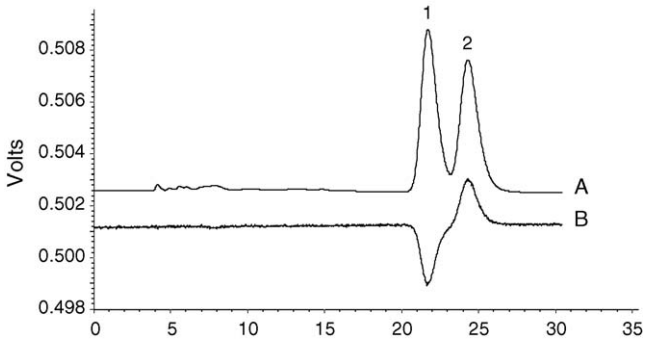

Fig. 3. Racemic FV resolved on a Chiralcel OD-R column and detected using CD/UV at 235 nm. (−)-(3*S*,5*R*)FV (21.5 min; negative peak at CD) and (+)-(3*R*,5*S*)-FV (24.0 min; positive peak at CD).

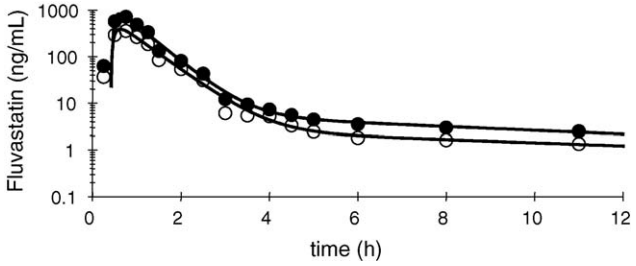

Fig. 4. Plasma concentrations vs. time curves for (−)-3*S*,5*R*-FV (●) and (+)-3*R*,5*S*-FV (○).

FV enantiomers using acetonitrile for protein precipitation and extraction with *tert*-butyl methyl ether with an aqueous phase at pH 6.0. [7] and [5] used solid-phase extraction, obtaining recoveries of more than 80%.

The quantitation limit of 1.5 ng mL^{-1} for both enantiomers was obtained with the extraction of plasma aliquots of only 500 μL. This value is acceptable although it is higher than reported values using LC with fluorescence detection. For instance, [5] reported quantitation limits of 0.75 ng mL^{-1} in the extraction of 1 mL plasma and [8] reported quantitation limits of 2 ng mL^{-1} for each enantiomer in the extraction of 500 μL plasma aliquots. The lowest quantitation limits of 0.4 ng mL^{-1} for each enantiomer were reported by [7] with irradiation of the post-column eluate with UV light.

The data presented in Table 1 show that the method is precise and accurate. The coefficients of variation and the relative errors obtained were lower than 10% for all concentrations tested. It should be pointed out that all experiments were performed using amber glassware and yellow light as the only source of illumination.

The enantioselective pharmacokinetics of FV was reported only for healthy volunteers by [4,5]. The results presented in Table 2 and Fig. 4 show higher plasma concentrations of the

Table 1
Confidence limits for the enantioselective analysis of fluvastatin by LC–MS–MS

	(−)-3*S*,5*R*-FV	(+)-3*R*,5*S*-FV
Recovery, % ($n=3$)		
5 ng mL^{-1}	98.9	94.2
50 ng mL^{-1}	95.5	92.0
500 ng mL^{-1}	96.4	97.8
Linearity		
Range (ng mL^{-1})	1.5–1000	1.5–1000
Equation	$y=-0.000626563+0.00544132x$	$y=+0.000774981+0.00543450x$
Coefficient of determination (r)	0.992272	0.992208
Quantitation limit ($n=9$)		
Concentration (ng mL^{-1})	1.50	1.50
Intra-assay precision (CV %)	14.00	11.60
Intra-assay accuracy (%)	−5.85	−4.89
Precision and accuracy		
Intra-assay precision; coefficient of variation (%); $n=10$		
4 ng mL^{-1}	6.27	7.29
40 ng mL^{-1}	6.82	6.07
400 ng mL^{-1}	12.90	8.73
Inter-assay precision; coefficient of variation (%); $n=5$		
4 ng mL^{-1}	7.66	6.03
40 ng mL^{-1}	6.01	1.99
400 ng mL^{-1}	4.73	1.91
Intra-assay accuracy; relative error (%); $n=10$		
4 ng mL^{-1}	−3.75	−4.75
40 ng mL^{-1}	−7.67	−4.30
400 ng mL^{-1}	−1.07	−2.70
Inter-assay accuracy; relative error (%); $n=5$		
4 ng mL^{-1}	2.00	5.30
40 ng mL^{-1}	−3.00	−1.70
400 ng mL^{-1}	3.80	−0.12

Table 2
Enantioselective kinetic disposition of fluvastatin in a patient with primary hypertension and hypercholesterolemia

Parameters	(−)-3*S*,5*R*-FV	(+)-3*R*,5*S*-FV
C_{max} (ng mL^{-1})	779.45	423.59
t_{max} (h)	0.64	0.64
AUC$^{0-\infty}$ (ng h mL^{-1})	667.43	403.40
$t_{1/2}\alpha$ (h)	0.36	0.42
α (h^{-1})	1.94	1.64
$t_{1/2}\beta$ (h)	3.25	3.72
β (h^{-1})	0.21	0.19
CL/*F* (L h^{-1})	29.97	49.58
Vd/*F* (L)	16.69	32.20
AUC$^{0-\infty}$ (−)/(+)	1.84	

(−)-3*S*,5*R*-FV enantiomer, with an AUC (−)/(+) ratio of 1.84. [4] reported AUC (−)/(+) ratios from 1.1 to 1.6 in a study on healthy volunteers with different genotypes, except for those with the CYP2C9 *3/*3 genotype, who showed AUC ratios close to 2.0. It should be pointed out that the volunteer investigated in the present study was genotyped as CYP2C9*1/*1.

The oral clearance values obtained for the patient investigated, 29.97 L h^{-1} for (−)-3*S*,5*R*-FV and 49.58 L h^{-1} for (+)-3*R*,5*S*-FV, are within the range of clearance variability according to the CYP2C9 genotype demonstrated by [4]. These authors reported total oral clearance values of 17–95 L h^{-1} for (−)-3*S*,5*R*-FV and of 37.5 to 111 L h^{-1} for (+)-3*R*,5*S*-FV.

In conclusion, MS–MS detection proved to be a system of high specificity, precision and accuracy for FV enantiomers analysis. The method can be applied to studies of kinetic disposition of FV enantiomers in patients treated with a single dose of this anti-hypercholesterolemic agent.

Acknowledgements

The authors are grateful to FAPESP (Fundação de Amparo a Pesquisa do Estado de São Paulo) for financial support and to CNPq (Conselho Nacional de Desenvolvimento Científico and Tecnológico) and CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for research fellowships.

References

[1] J.G. Dain, E. Fu, J. Gorski, J. Nicoletti, T.J. Scallen, Drug Metab. Dispos. 21 (1993) 567.
[2] V. Fischer, L. Johanson, F. Heitz, R. Tullman, E. Graham, J.P. Baldeck, W.T. Robinson, Drug Metab. Dispos. 27 (1999) 410.
[3] F.G. Kathawala, Med. Res. Rev. 11 (1991) 121.
[4] J. Kirchheiner, D. Kudlicz, C. Meisel, S. Bauer, I. Meineke, I. Roots, Brockmöller J. Clin. Pharmacol. Ther. 74 (2003) 186.
[5] V.L. Lanchote, A. Rocha, F.U.V. Albuquerque, E.B. Coelho, P.S. Bonato, J. Chromatogr. B 765 (2001) 81.
[6] T.H. Sullivan-Klose, B.I. Ghanayem, D.A. Bell, Z.Y. Zhang, L.S. Kaminsky, G.M. Shenfield, J.O. Miners, D.J. Birkett, J.A. Goldstein, Pharmacogenetics 6 (1996) 341.
[7] H. Toreson, B.M. Eriksson, Chromatographia 45 (1997) 29.
[8] H. Toreson, B.M. Eriksson, J. Chromatogr. A 729 (1996) 13.
[9] F.L.S. Tse, J.M. Jaffe, A. Troendle, J. Clin. Pharmacol. 32 (1992) 630.