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Determination of fluvastatin enantiomers and the racemate in human blood plasma by liquid chromatography and fluorometric detection

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Abstract

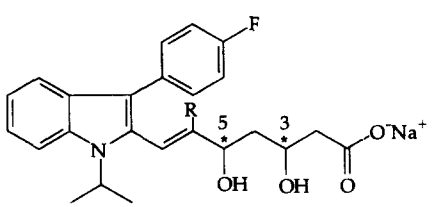
Liquid chromatographic methods for the determination of fluvastatin, as racemate and as separated enantiomers, are described. Fluvastatin was extracted at pH 6.0 from blood plasma into methyl *tert.*-butyl ether. The organic phase was evaporated and the extract redissolved into either a phosphate buffer solution of pH 6.0 containing tetrabutylammonium fluoride and methanol for the racemate determination, or in a mixture of acetonitrile and water for assaying the enantiomers. The absolute recoveries were 95 and 86% for the racemate and the enantiomers, respectively, and the limit of quantitation 0.5 nmol/l for the racemate, and 5 nmol/l for the enantiomers, when using half a millilitre of plasma sample. The samples were chromatographed on a C_8 column (racemate) and on a Chiralcel OD-R column (enantiomers), and monitored using fluorescence detection. In the achiral system, post-column exposure of the eluate to UV light enhanced the sensitivity by 4 to 5 times when compared with analysis based on the native fluorescence.

Keywords: Enantiomer separation; Fluvastatin

1. Introduction

Fluvastatin sodium ([*R**,*S**,-(*E*)-](±)-7-[3-(4-fluorophenyl)-1-(1-methylethyl)-1*H*-indol-2yl]-3,5-dihydroxy-6-heptenoic acid, monosodium salt, Fig. 1), is a potent inhibitor of hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase, the rate limiting enzyme in cholesterol biosynthesis. Fluvastatin has two asymmetric carbons, the 3- and 5- carbons of the heptenoic acid side-chain, and is a racemic mixture of two out of four possible stereoisomers (3*S*,5*R* and 3*R*,5*S*).

An LC method for the determination of the fluvastatin racemate in blood plasma samples has



	R
Fluvastatin	H
Internal standard	CH_3

Fig. 1. Structural formulas of fluvastatin and the internal standard, Sandoz compound 63-267.

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been described previously [1], using methyl *tert.*-butyl ether for the extraction and tetrabutylammonium fluoride in the mobile phase. LC methods for the determination of another statin, pravastatin, in plasma samples have also been reported [2,3]. For sample clean-up a C_2 column [2] or a column with an immobilized antibody [3] was used. The analyte was monitored by UV detection [2] or by laser-induced fluorescence after fluorogenic derivatization [3]. Carlucci et al. [4] reported an LC method with UV detection for the determination of simvastatin in human blood plasma. That method involves an extraction procedure using a mixture of acetonitrile and water.

This paper describes methods used for the determination of the two enantiomers of fluvastatin as well as of the racemate and they have been applied to the analysis of plasma samples from clinical pharmacology studies. The racemate has, up to now, been assayed in more than thousand samples and the enantiomers in about 10% of those samples. By optimizing the experimental conditions an extraction recovery of 95% was obtained. In the achiral chromatographic system, post-column exposure to UV light was done, making the method 4 to 5 times more sensitive when compared with monitoring the native fluorescence of fluvastatin. Irradiation by UV light, before fluorometric detection of rivastatin in blood plasma, has been described earlier by Krol et al. [5]. The chiral system enables a simple separation of the enantiomers without derivatization of the sample.

2. Experimental

2.1. Chemicals and reagents

Fluvastatin sodium and the internal standard (Sandoz compound 63-267; [*R**,*S**,-(*E*)-](±)-7-[3-(4-fluorophenyl)-1-(1-methylethyl)-1*H*-indol-2yl]-3,5-dihydroxy-6-methyl-6-heptenoic acid, monosodium salt, Fig. 1) were kindly supplied as reference substances from Sandoz (NJ, USA). Methanol, acetonitrile and methyl *tert.*-butyl ether (MTBE) were of HPLC grade from Rathburn (Walkerburn, UK) and tetrabutylammonium fluoride (TBAF), 75% in water, was from Aldrich (Milwaukee, WI, USA). The buffer substances were of analytical grade, Merck (Darmstadt, Germany). Water was from an ELGA purification system, ELGA (High Wycombe, Bucks, UK).

2.2. Chromatographic system

The LC system consisted of an LKB Model 2248 pump (Bromma, Sweden), a Perkin Elmer ISS-200 refrigerated autosampler (Überlingen, Germany) and a Jasco FP-920 fluorescence detector (Tokyo, Japan). The excitation and emission wavelengths were set at 305 and 390 nm, respectively. Data were monitored and processed using a Multichrom Chromatographic Data System (VG Data Systems, Altrincham, UK).

Achiral system

The achiral chromatographic system consisted of a Zorbax Rx-C8 analytical column (150×4.6 mm I.D., 5 μm) from Rockland Technologies (Newport, DE, USA) and a guard column, Brownlee CN, (15×3.2 mm I.D., 7 μm) from Brownlee, (Santa Clara, CA, USA). The mobile phase contained 0.1 *M* aqueous TBAF–phosphate buffer pH 6.0 (ionic strength, I= 0.1)–methanol (15:25:60). The flow-rate through the analytical column was 1.0 ml/min and the column temperature was maintained at 40°C. A Beam Boost photochemical reaction unit from ICT (Frankfurt, Germany) was placed between the analytical column and the detector. It contained a UV lamp with a nominal wavelength of 254 nm and a Teflon coil (10 m×0.3 mm I.D.).

Chiral system

The chiral column was a Chiralcel OD-R (250× 4.6 mm I.D, 10 μm) from Daicel Chemical Industries (Japan) and the guard column, Brownlee Si (15×3.2 mm I.D., 7 μm) was from Brownlee. The mobile phase contained acetonitrile–phosphate buffer pH 2.5 (I=0.04) (40:60). The flow-rate through the column was 0.5 ml/min and the column temperature was maintained at 15°C.

2.3. Sample preparation procedure

Both fluvastatin and the internal standard are hygroscopic and light sensitive, so care was taken to minimize exposure to moisture and light. Venous

blood samples were collected into heparin tubes and separated promptly by centrifugation for 5 min at 1500 *g*. The plasma samples were transferred to disposable polypropylene tubes and frozen at −20°C. The frozen blood plasma samples were thawed at room temperature, homogenized by mixing and centrifuged for 5 min at 1200 *g*. Aliquots of 500 μl of the samples were pipetted into 10-ml centrifuge tubes, mixed with 100 μl of internal standard solution (500 nmol/l), 500 μl acetonitrile and 500 μl phosphate buffer pH 6.0 (I=0.5). The samples were extracted with 5.0 ml MTBE by shaking for 30 min. After centrifugation for 5 min at 1200 *g* the lower aqueous phase was frozen in a dry-ice ethanol bath, and the organic phase was transferred to a 10-ml conical centrifuge tube and evaporated under nitrogen. The extracts were dissolved in 400 μl of mobile phase, vortex-mixed 3 times for 30 s each time, transferred to glass vials and placed in the autosampler. Aliquots of 20–100 μl of the samples were injected into the LC system. Reference samples were prepared by mixing 100 μl of the standard solution of fluvastatin in water (500 nmol/l) with 500 μl drug-free blood plasma and were then prepared according to the described procedure.

The same procedure was used for the analysis of the enantiomers, except that no internal standard was used. Reconstitution of the samples after evaporation was done using a mixture of acetonitrile and water (40:60) instead of mobile phase.

3. Results and discussion

3.1. Extraction

Absolute recoveries of fluvastatin and internal standard were determined by comparing the extracted plasma samples containing known amounts of fluvastatin and internal standard with direct injections of reference solutions containing the same concentrations of the substances. The extraction recoveries of fluvastatin and internal standard were influenced by the pH of the sample. The best recoveries were obtained at a sample pH of 6.0. At a pH above 6.5 the extraction recoveries decreased since fluvastatin is charged at higher pH and at a lower pH there was a risk of decomposition.

With MTBE, the extraction solvent used by Kalafsky et al. [1], an extraction recovery of 95% was obtained, but if no acetonitrile was added to the sample prior to extraction the recovery was only 60%. The precipitation of proteins by acetonitrile facilitated the extraction of fluvastatin. Other extraction agents tested, dichloro-methane, diethyl ether, ethylacetate and mixtures of MTBE and dichloromethane or hexane, gave lower recoveries, which ranged from 50 to 90%. Recoveries of fluvastatin at three different concentrations (151, 38.4 and 2.3 nM) and of internal standard at a concentration of 149 nM were measured (n=8). The observed mean recoveries of fluvastatin and of internal standard were 95 and 98%, respectively.

When assaying the enantiomers no internal standard was used since the second fluvastatin peak (3*R*,5*S*) interfered with the first peak of the enantiomers of the internal standard. However, the second peak (stereoselectively isolated) would be a possible alternative as internal standard.

3.2. Chromatographic conditions

Achiral system

In the achiral system, column efficiency and the retention times of fluvastatin and internal standard were influenced by the concentration of TBAF in the mobile phase. The retention times for both fluvastatin and internal standard increased 2.5 times and the column efficiency, expressed as plate number (N), improved 1.5 times when the concentration of the TBAF solution was increased over the range of 0–0.1 mol/l.

Increasing the column temperature (from 20 to 50°C) or the mobile phase pH (from 3 to 8) did not influence the resolution but the retention times for both fluvastatin and internal standard decreased. A pH of 6.0 and a temperature of 40°C were chosen to give suitable retention times.

A Beam Boost photochemical reaction unit was placed between the analytical column and the detector. The column eluate was irradiated by UV light as it passed through a transparent knitted reaction coil composed of Teflon that was inside the Beam Boost. This post-column exposure to UV light enhanced the detection signal 4 to 5 times, when using the prescribed mobile phase containing 60% methanol in

phosphate buffer pH 6.0 containing TBAF (Fig. 2). The presence of TBAF did not seem to have any influence on the detection signal, whereas by lowering of the mobile phase pH or if acetonitrile was used instead of methanol, the increase in the detector signal was less pronounced. The mobile phase used for the enantiomeric separation, containing 40% acetonitrile in phosphate buffer pH 2.5, gave the same detection signal irrespective of the usage of Beam Boost. Two metabolites (1 and 2, see Fig. 2) could only be detected without exposure to UV light. Irradiation by UV light is a simple and convenient procedure used to enhance the sensitivity of the fluvastatin racemate. In Fig. 2 chromatograms from an authentic plasma sample containing 58.8 nmol/l of fluvastatin and 149 nmol/l of internal standard is shown.

Chiral system

In the chiral system both the column temperature and the mobile phase pH influenced the selectivity and the column efficiency. A decrease in temperature from 35 to 10°C increased the separation factor (α) from 1.06 to 1.11, while the efficiency decreased from 9000 to 7000 theoretical plates. The retention times increased only slightly. The separation of the two enantiomers improved with decreasing the mobile phase pH and a change in pH from 6.0 to 1.9 increased the separation factor from 1.00 to 1.10 (see Fig. 3). A pH of 2.5 and a temperature of 15°C were

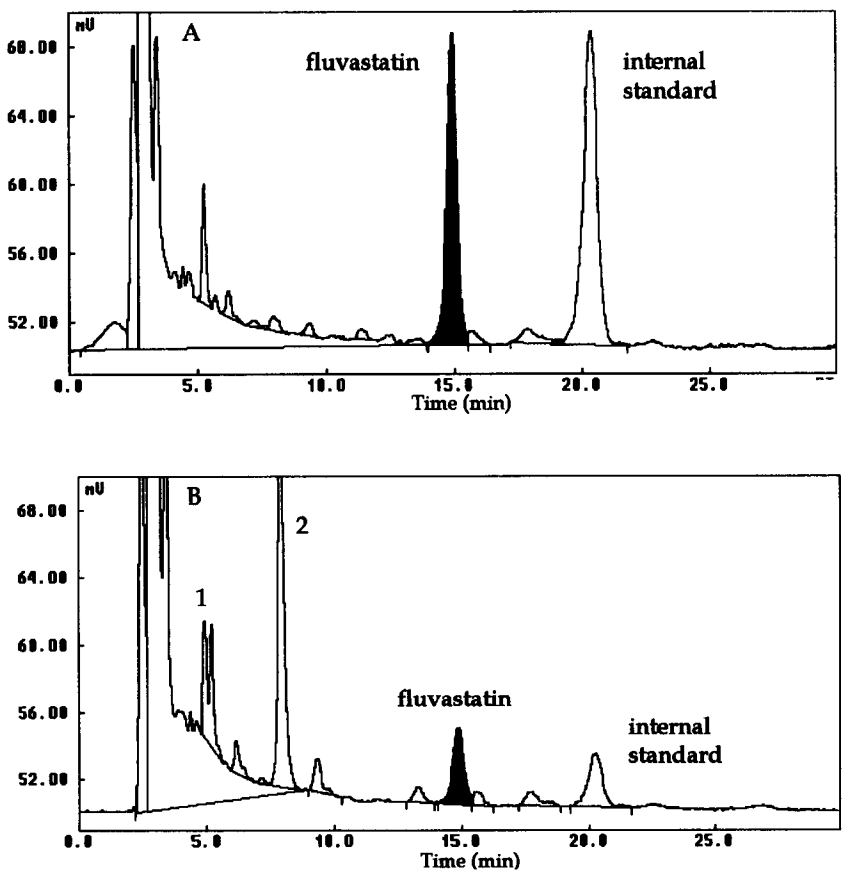

Fig. 2. Chromatograms of an authentic human plasma sample containing 58.8 n*M* of fluvastatin and 149 n*M* of internal standard, (A) with Beam Boost and (B) without Beam Boost. Column: Zorbax Rx-C8 (150×4.6 mm I.D., 5 μm). Mobile phase: 0.1 *M* TBAF–phosphate buffer pH 6.0 (I=0.1)–methanol (15:25:60). Column temperature 40°C. Injection volume 50 μl.

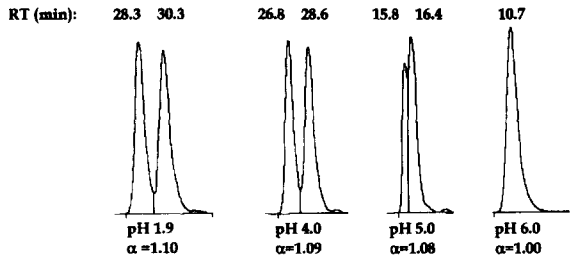

Fig. 3. Influence of mobile phase pH on the enantiomer separation. Column: Chiralcel OD-R (250×4.6 mm I.D., 10 μm). Mobile phase: acetonitrile–phosphate buffer (I=0.04) (40:60). Column temperature 15°C. Injection volume 100 μl.

chosen. A chromatogram of the same sample as in Fig. 2, analysed with the enantioselective method is shown in Fig. 4. Assays performed on drug-free plasma samples did not show any interfering endogenous peaks.

3.3. Quantitation and accuracy

Racemate

The ratios of the peak height of fluvastatin to that of the internal standard in the reference plasma samples were measured and used for the calculation of the racemate in the unknown samples. Within-day precision was assessed using spiked samples at different concentration levels. The coefficients of variation (n=8) were 2.9, 1.9 and 6.1% for samples containing 1150, 105 and 0.5 nM, respectively. Between-day precision was studied using blood plasma samples containing 54.2 nM of fluvastatin. Analyses performed on 34 different occasions within a 3-month period gave a coefficient of variation of 3.8%.

Calibration curves for fluvastatin in plasma at eight different concentration levels were obtained by plotting the peak height ratio of fluvastatin to that of the internal standard versus the concentrations. The curves were found to be linear over the concentration range studied 1.0–5000 nmol/l (R^2=0.9999). The limit of quantitation was 0.5 nmol/l.

Enantiomers

The peak heights of the enantiomers in plasma samples spiked with fluvastatin were used for the calculation of the enantiomers in the unknown samples. Within-day precision was assessed using spiked samples at different concentration levels. The coefficients of variation (n=8) for the enantiomer 3S,5R were 2.4, 3.6 and 6.1%, and for 3R,5S 2.9, 3.5 and 5.0% for samples containing 239, 47.8 and 4.8 nM of each enantiomer.

Calibration curves at five different concentration levels of each enantiomer in plasma were obtained by plotting the peak heights of the two enantiomers versus the concentrations. The curves were found to

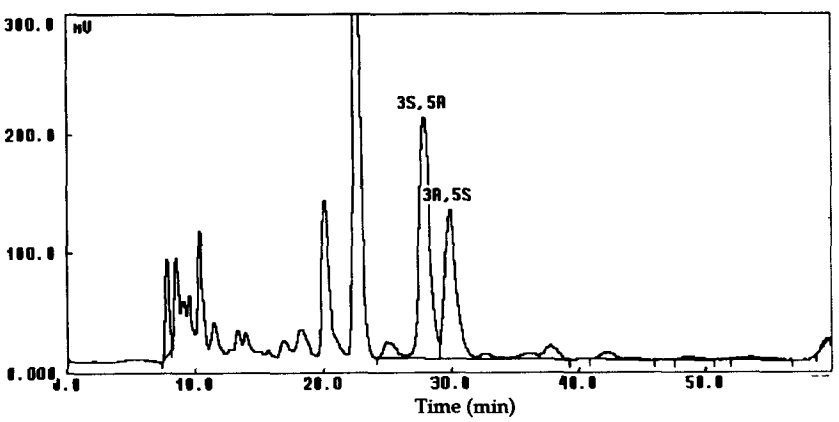

Fig. 4. Chromatogram of a human plasma sample containing 39.2 nM and 24.9 nM of the enantiomers 3S,5R and 3R,5S, respectively. Column: Chiralcel OD-R (250×4.6 mm I.D., 10 μm). Mobile phase: acetonitrile–phosphate buffer pH 2.5 (I=0.04) (40:60). Column temperature 15°C. Injection volume 100 μl.

be linear over the concentration range studied: 5.0–1200 n*M* (R^2=0.9999). The limit of quantitation was 5.0 nmol/l for each enantiomer.

Stability

Both fluvastatin and the internal standard are sensitive to light. Reference solutions in water were stable for at least one day if stored in darkness, but decreased 20 and 50% after 1 h on exposure to incandescent light and sunlight, respectively. In plasma fluvastatin was stable up to at least 2 h in incandescent light. Reference solutions were protected from light by wrapping the flasks with aluminium foil and keeping them in darkness in a refrigerator.

The stability of fluvastatin in stored plasma samples was studied using spiked samples (54.2 n*M*). Fluvastatin was stable for 5 months at −20°C and for at least one day at room temperature, when protected from light. Processed samples were stable in the refrigerated autosampler (10°C) for more than 2 days and at −20°C for at least 5 days.

4. Conclusions

The described methods for determination of fluvastatin enantiomers and the racemate in blood plasma yielded excellent recoveries and precision. The enantiomers were simply and reliably assayed without sample derivatization, and for the achiral chromatographic system a five-fold enhancement of the fluorescence was observed after post-column exposure of the eluate to UV light.

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References

[1] G. Kalafsky, H.T. Smith and M.G. Choc, J. Chromatogr., 614 (1993) 307.
[2] I. Iacona, M.B. Regazzi, I. Buggia, P. Villani, V. Fiorito, M. Molinaro and E. Guarnone, Ther. Drug Monit., 161 (1994) 191.
[3] C. Dumousseaux, S. Muramatsu, W. Takasaki and H. Takahagi, J. Pharm. Sci., 83 (1994) 1630.
[4] G. Carlucci, P. Mazzeo, L. Biordi and M. Bologna, J. Pharm. Biomed. Anal., 10 (1992) 693.
[5] G.J. Krol, G.W. Beck, W. Ritter and J.T. Lettieri, J. Pharm. Biomed. Anal., 11 (1993) 1269.