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Determination of fluvastatin and its five metabolites in human plasma using simple gradient reversed-phase high-performance liquid chromatography with ultraviolet detection

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Abstract

A simple gradient reversed-phase high-performance chromatographic method with ultraviolet detection for the determination of fluvastatin (FV) and its five metabolites, (M-2, M-3, M-4, M-5 and M-7) in human plasma was developed and validated. The limit of quantification of FV and its five metabolites in human plasma was 10 ng ml^{-1}. The assay had satisfactory selectivity, recovery, linearity and precision accuracy. Stability studies showed that FV and its five metabolites were stable in plasma up to at least 1 month of storage at −30°C.

Keywords: Metabolites; Fluvastatin; HMG CoA reductase

1. Introduction

Fluvastatin (FV, Fig. 1) is the first totally synthesized HMG-CoA reductase inhibitor and has clinical antihypercholesterolemic effects [1]. Further, the antiatherogenic properties of FV may not be limited to its hypocholesterolemic effect, but may also be related to its ability to reduce LDL oxidizability [2,3]. Antioxidant effects of FV in vivo have also been reported in humans [2–4] and animals [5,6].

After oral ^{3}H-FV administration to healthy volunteers, FV and its metabolites, 5-hydroxy FV (M-2, Fig. 1), 6-hydroxy FV (M-3, Fig. 1) and des-isopropylpropionic acid derivative of FV (M-4, Fig. 1), were detected in human plasma and M-2 (24% of dose), M-3 (24% of dose) and M-5 (11% of dose, Fig. 1) were predominant in feces [7]. In vitro data indicated that FV was metabolized by human liver microsomes to M-2, M-3 and desisopropyl-FV (M-5) [8]. In addition, previous animal studies showed that the highest level of radioactivity was found in the liver 2 h after administration of ^{14}C-FV to rats, about 50 times that in whole blood [9].

We previously reported the antioxidant effects of FV and its five metabolites on NADPH-induced lipid peroxidation using rat liver microsomes [10]. The inhibitory effects of M-2, M-3 and M-5 were

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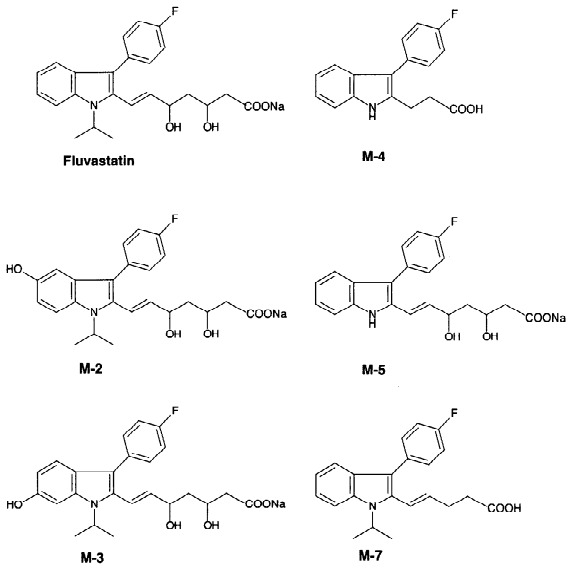

Fig. 1. Chemical structures of fluvastatin and its five metabolites.

stronger than that of FV. M-4 and M-7 (Fig. 1) also inhibit NADPH-induced lipid peroxidation, although these effects were weaker than those of FV.

Given the antioxidant effects of these metabolites, it is important to determine the concentrations of FV and its five metabolites in both in vivo and in vitro studies.

Analytical methods are described in the literature for the quantification of fluvastatin in human plasma (HPLC) [11] and for fluvastatin and its enantiomer in plasma [12,13]. However, a simple method for the determination of fluvastatin and its metabolites has not yet been developed and validated.

Therefore, we developed and validated two simple gradient reversed-phase high-performance liquid chromatographic methods with ultraviolet detection (HPLC–UV) for the determination of fluvastatin and its five metabolites (M-2, M-3, M-4, M-5 and M-7) in human plasma.

FV and its five metabolites were analyzed on two chromatographic systems, with different settings, due to the rather wide range of hydrophilic characteristics among these investigated compounds. In this study, the six compounds were divided into two groups. M-2, M-3 and M-5, the more hydrophilic compounds, were applied to the same assay system (system 1). M-4, M-7 and FV were applied to another assay system (system 2). As a result, development and validation of the two systems for the determination of these compounds were successful. The assays reported here proved to be useful for the evaluation of both in vivo and in vitro studies of the antioxidant effects of fluvastatin and its metabolites.

2. Experimental

2.1. Chemicals and reagents

FV, M-2, M-3, M-4, M-5 and M-7 were supplied by Novartis (East Hanover, NJ, USA), Tanabe Seiyaku (Osaka, Japan) and Daiichi Pure Chemicals (Japan). The two internal standards (I.S.) used for the two chromatographic methods, benzophenone and 9-cyanoanthracene, were purchased from Wako (Osaka, Japan) and Tokyo Kasei Kogyo (Tokyo, Japan), respectively. The chemical structures of benzophenone and 9-cyanoanthracene are shown in Fig. 2. All other chemicals were of the highest grade commercially available. Human blank plasma was obtained from healthy male volunteers.

2.2. Instruments and chromatographic conditions

2.2.1. System 1 (assay of M-2, M-3 and M-5)

System 1 consisted of a HPLC pump (2690 separation module, Waters, Millipore, Tokyo, Japan), an integrator (C-R4A, Shimadzu, Kyoto, Japan), an ultraviolet detector (UV-970, Jasco, Tokyo, Japan) operated at a wavelength of 305 nm and a reversed-phase L-column ODS (150×4.6 mm I.D., 5 μm particle size, Chemicals Evaluation and Research Institute, Fukuoka, Japan). The column temperature was kept at 40°C. Sample aliquots of 20 μl were injected automatically into the HPLC system by an

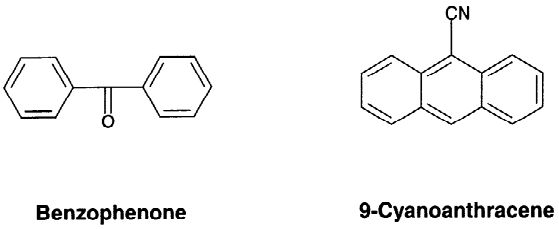

Fig. 2. Chemical structures of the two internal standards.

autosampler (2690 Separation module, Waters). Mobile phase A consisted of methanol and mobile phase B consisted of distilled water containing 0.2% phosphoric acid. A flow-rate of 1 ml min^{-1} was used over the entire gradient of 20 min. Gradient: 0 min, 55% A; 6 min, 55% A; 14 min, 70% A (linear gradient from 6 to 14 min); and 14.01 min, 55% A to return the column to initial conditions by 20 min.

2.2.2. System 2 (assay of M-4, M-7 and FV)

System 2 consisted of a HPLC pump (PU-980 Jasco), an integrator (C-R4A, Shimadzu), an ultraviolet detector (UV-970, Jasco) operated at a wavelength of 305 nm and a reversed-phase L-column ODS (150×4.6 mm I.D., 5 μm particle size, Chemicals Evaluation and Research Institute). The column temperature was kept at 40°C. Sample aliquots of 40 μl were injected automatically into the HPLC system by an autosampler (AS-950 Jasco). Mobile phase A consisted of methanol and mobile phase B consisted of distilled water containing 0.2% phosphoric acid. A flow-rate of 1 ml min^{-1} was used over the entire gradient of 25 min. Gradient: 0 min, 60% A; 15 min, 60% A; 20 min, 90% A (linear gradient from 15 to 20 min); and 20.01 min, 60% A to return the column to initial conditions by 25 min.

2.3. Preparation of standard solutions and standard samples

Stock solutions of standards were prepared by dissolving accurately weighed FV and its five metabolites in methanol in a volumetric flask. The two standard solutions, for system 1 (assay of M-2, M-3 and M-5) and for system 2 (assay of M-4, M-7 and FV), were diluted with methanol.

The I.S. stock solution A for system 1 (1000 μg/ml) was prepared by dissolving accurately weighed benzophenone in methanol in a volumetric flask. The I.S. for system 1 (200 μg/ml) was prepared by dilution of the stock solution with methanol. The I.S. stock solution for system 2 (1000 μg/ml) was prepared by dissolving accurately weighed 9-cyanoanthracene in methanol in a volumetric flask. The I.S. solution B for system 2 (200 μg/ml) was prepared by dilution of the stock solution with methanol.

Human plasma standards were prepared at concentrations of 10, 40, 100, 400 and 1000 ng/ml of two individual standard solution mixtures, one for system 1 (assay of M-2, M-3 and M-5) and the other for system 2 (assay of M-4, M-7 and FV), by spiking a 1% volume to human blank plasma.

2.4. Assay procedures

2.4.1. System 1 (assay of M-2, M-3 and M-5)

Sample pretreatment for the determination of M-2, M-3 and M-5 was as follows; To a 0.5-ml aliquot of human plasma sample, 0.5 ml of 0.1 mol/l phosphate buffer (pH 7) and 0.44 ml of methanol were added, mixed well and then applied to a conditioned (with 1 ml methanol, followed by 1 ml distilled water) Oasis HLB cartridge (30 mg/l ml). After loading the sample mixture on the cartridge, the resulting eluate was discarded. Then the cartridge was washed, in the following order, with 1 ml of 0.1 mol/l phosphate buffer (pH 7), 1 ml of a 0.1 mol/l phosphate buffer (pH 7)–methanol (95:5), 1 ml of a 0.1 mol/l phosphate buffer (pH 7)–methanol (70:30) and finally with 1 ml of distilled water. The compounds of interest were eluted with 1 ml of methanol, collected in a conical tube and subsequently 0.1 ml of 5% propylene glycol in methanol was added. The eluate was then evaporated to dryness under a gentle nitrogen gas stream at room temperature. The dry residue was dissolved with 0.02 ml of I.S. solution A and 0.2 ml of methanol–distilled water (1:1). The solution was shaken and then tested using the HPLC system 1 as described.

2.4.2. System 2 (assay of M-4, M-7 and FV)

Sample pretreatment for the determination of M-4, M-7 and FV was as follows: to a 0.5-ml aliquot of human plasma sample, 0.5 ml of 0.1 mol/l phosphate buffer (pH 7) was added and placed in a test tube with screwcap. After addition of 5 ml of diethyl ether, the mixture was shaken for 5 min and centrifuged at 1700 *g* for 10 min at 4°C. The upper (organic) layer was transferred to a conical tube. To the aqueous phase, another 5 ml of diethyl ether were added and treated in the same manner as described above. Then the upper (organic) layers were combined and 0.1 ml of 5% propylene glycol in methanol was added. The eluate was then evaporated to dryness under a gentle nitrogen gas stream at

room temperature. The dry residue was dissolved with 0.02 ml of I.S. solution B and 0.2 ml of methanol. The solution was shaken and then tested with the HPLC system 2 as described.

2.5. Calibration curves

The peak areas of FV and its five metabolites were divided by the peak area of the I.S. to determine peak area ratios. The calibration curves for FV and its five metabolites were obtained by weighted [1/peak area ratios^2] least-squares linear regression analysis of the peak area ratios of the standards versus the drug concentrations.

2.6. Recovery

The absolute recoveries of FV and its five metabolites from human plasma were estimated by comparing the peak areas obtained from injections of absolute recovery samples consisting of 25 μl of the standard solutions (0.2, 2 and 20 μg/ml), 175 pA of 50% methanol or methanol and 20 μl of I.S. solution, with those obtained from injections of extracted plasma samples spiked with known concentrations of FV and its five metabolites.

2.7. Selectivity

Blank plasma from three healthy male volunteers was assayed by the procedure described above to evaluate the selectivity of the method.

2.8. Precision and accuracy

Intra-day precision and accuracy of the method were evaluated by replicate analysis ($n=5$) of the plasma calibration standards.

Inter-day precision and accuracy was determined by assaying the plasma calibration standards on three separate days. The limit of quantification was chosen to be the concentration of the lowest calibration standard with an acceptable limit of variance (15%).

2.9. Stability

The stabilities of FV and its five metabolites in human plasma were investigated by preparing plasma quality control (QC) samples of FV and its five metabolites at two different concentrations (100 and 1000 ng/ml). Freeze–thaw stability was tested by exposing the QC samples to two cycles of freezing–thawing before processing. Long-term stability was monitored by assaying QC samples, which had been stored at −30°C for 0 days (just after preparation) or 1 month.

2.10. Effect of dilution

The effect of dilution was evaluated by analysis of samples exceeding the calibration range (>1000 ng/ml) by 2-fold dilution with control plasma.

3. Results and discussion

3.1. Chromatography and selectivity

Determination of FV and its five investigated metabolites was attempted by using one chromatographic system. However, the analysis time exceeded a reasonable time frame, resulting in a time of ≈120–130 min due to a wide range of hydrophilic characteristics among these investigated compounds. To achieve a shorter run time, FV and its five investigated metabolites were divided into two groups according to their hydrophilic characteristics. M-2, M-3 and M-5, the more hydrophilic compounds, were applied to the same assay system (system 1). M-4, M-7 and FV were applied to another assay system (system 2). As a result, development and validation of the two systems with shorter analytical time for the determination of these compounds were successful.

3.1.1. System 1 (assay of M-2, M-3 and M-S)

After characterization of the ultraviolet spectra of M-2, M-3 and M-5 in the mobile phase, the wavelength for ultraviolet detection was set at 305 nm. Well-defined chromatographic peaks for M-2, M-3 and M-5 and the I.S. benzophenone were obtained on the L-column ODS (150×4.6 mm I.D., 5 μm particle size), with the free silanol groups almost completely end-capped. A representative chromatogram of extracts from control plasma is shown in Fig. 3A. This chromatogram indicated that no endogenous com-

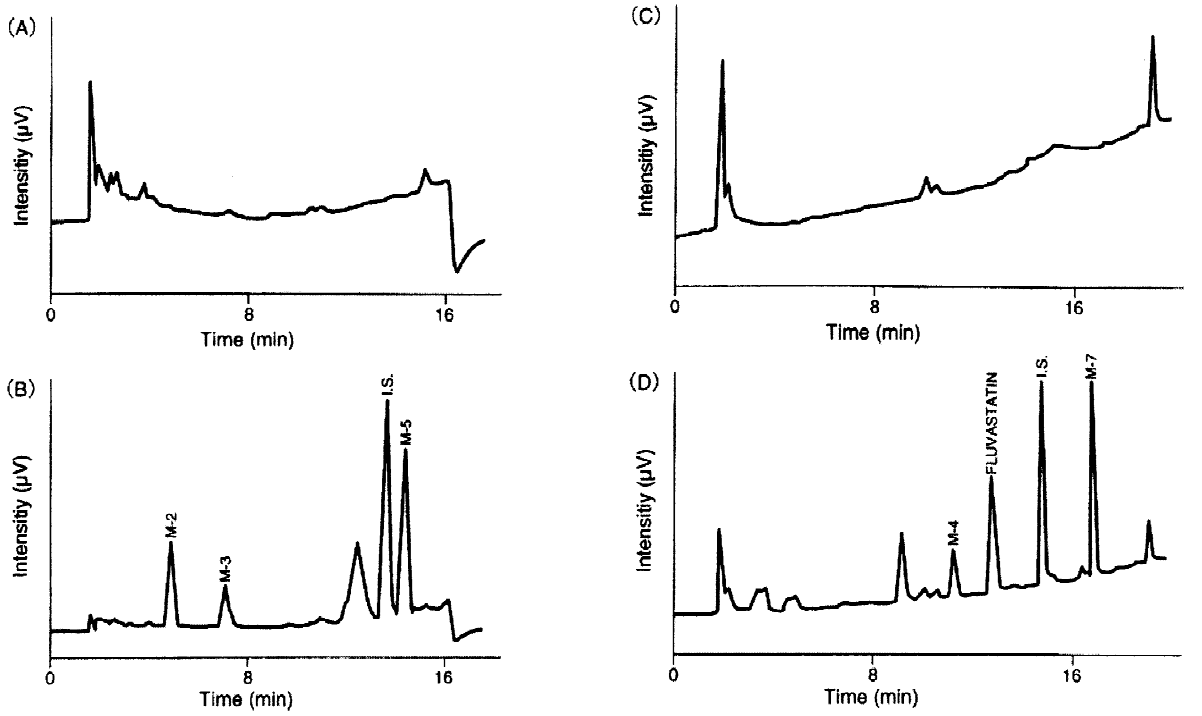

Fig. 3. (A) Typical chromatograms of human plasma extracts. (A) Blank human plasma for System 1(assay for M-2, M-3 and M-5); (B) spiked human containing M-2, M-3, I.S. and M-5 (400 ng/ml each); (C) blank human plasma for System 2 (assay for M-4, FV and M-7); (D) spiked human plasma containing M-4, FV, I.S. and M-7 (400 ng/ml each).

pounds interfered at the retention times of M-2, M-3, M-5 and I.S., as depicted in Fig. 3B. No interference was observed in plasma collected from three healthy volunteers. M-2, M-3, M-5 and the 1.S. were well-resolved, with retention times of approximately 4.7, 7.0, 14.3 and 13.6 min, respectively. The overall chromatographic run time cycle was 20 min. Applications of M-4, M-7 and FV did not cause any interference on this system.

3.1.2. System 2 (assay of M-4, M-7 and FV)

After characterization of the ultraviolet spectra of M-4, M-7 and FV in mobile phase solution, the wavelength for ultraviolet detection was set at 305 nm. Well-defined chromatographic peaks for M-4, M-7 and FV and the I.S. 9-cyanoanthracene were obtained on a L-column ODS (150×4.6 mm I.D., 5 μm particle size). A representative chromatogram of extracts from control plasma is shown in Fig. 3C. The chromatogram indicated that no endogenous compounds interfered at the retention times of M-4, M-7, FV and the I.S. (Fig. 3D). No interference was observed in plasma collected from three healthy volunteers. M-4, M-7, FV and the I.S. were well-resolved, with retention times of approximately 11.3, 17.1, 12.9 and 14.9 min, respectively. The overall chromatographic run time cycle was 25 min. Applications of M-2, M-3 and M-5 did not cause any interference in this system.

3.2. Calibration curves

Calibration curves for plasma obtained on 3 different days ($n=5$) were linear and reproducible for all investigated compounds (M-2, M-3, M-5, M-4, M-7 and FV). For all analysis, a weighting factor of $(1/\text{conc}^2)$ was used to improve homogeneity of variance. The calibration curves for all compounds spiked to human plasma exhibited linearity over the concentration range of 10–1000 ng/ml, with resulting correlation coefficients (r^2) >0.995.

3.3. Recovery on extraction

The absolute recoveries of all compounds from plasma were determined by comparing the peak areas of extracted standards with those of injected standards (Table 1). The recoveries of all compounds from spiked human plasma were evaluated at the concentrations of 10, 100 and 1000 ng/ml in quintruplicate. The recoveries ranged from 92.7 to 102.5% for M-2, from 94.4 to 142.3% for M-3, from 94.2 to 100.5% for M-5, from 67.8 to 102.8% for M-4, from 67.8 to 102.8% for M-7 and 89.4 to 97.3% for FV. In addition, the recoveries of the two I.S.s, benzophenone and 9-cyanoanthracene, from human plasma were 103.0–105.5% and 101.4–108.9%, respectively ($n=5$). I.S. compounds were applied to normalize the change of retention times of all compounds tested. Propylene glycol (5%) in methanol was added in order to avoid any possible loss of powder-like residue during the evaporation step.

Table 1
Recoveries of M-2, M-3, M-5, M-4, M-7 and FV spiked human plasma

Concentration (ng/ml)	Recovery (%) Mean±SD	C.V.[a] (%)
M-2		
10	100.7±2.8	2.8
100	102.5±2.1	2.0
1000	92.7±6.1	6.6
M-3		
10	142.3±3.5	8.8
100	95.7±3.5	3.7
1000	94.4±6.4	6.8
M-5		
10	100.5±5.2	13.7
100	97.9±2.5	2.6
1000	94.2±5.6	5.9
IS		
10	105.5±5.2	4.9
100	104.8±2.8	2.7
1000	103.0±0.6	0.6
M-4		
10	67.8±3.4	5.0
100	95.2±6.6	6.9
1000	102.8±2.3	2.2
M-7		
10	67.8±3.4	5.0
100	95.2±6.6	6.9
1000	102.8±2.3	2.2
Fluvastatin		
10	99.8±7.3	7.3
100	97.3±6.9	7.1
1000	89.4±1.5	1.7
I.S.		
10	101.4±3.4	3.4
100	103.2±5.1	4.9
1000	108.9±1.3	1.2

[a] C.V., coefficient of variation.

3.4. Precision and accuracy

The intra-day precision and accuracy of the method for plasma were evaluated by analysis of human plasma spiked with all compounds at concentrations of 10, 100 and 1000 ng/ml in replicates of five (Table 2). Precision was based on calculation of the C.V. Accuracy was based on calculation of the relative error (R.E.) of the concentration found

Table 2
Intra-day precision and accuracy for the determination of M-2, M-3, M-5, M-4, M-7 and FV in human plasma

Theoretical concentration (ng/ml)	Mean found concentration (ng/ml)	C.V.[a]	R.E.[b]
M-2			
10	10.8	5.6	8.0
100	107.7	4.4	7.7
1000	1046.2	6.4	4.6
M-3			
10	10.3	14.6	3.0
100	93.5	3.5	−6.5
1000	1027.5	6.7	2.8
M-5			
10	10.0	12.0	0.0
100	109.2	2.5	9.2
1000	1098.4	5.8	9.8
M-4			
10	9.5	4.2	−5.0
100	99.5	2.7	−0.5
1000	1002.0	1.9	0.2
M-7			
10	9.8	5.1	−2.0
100	101.9	1.7	1.9
1000	1011.6	1.7	1.2
Fluvastatin			
10	10.7	5.6	7.0
100	104.5	3.8	4.5
1000	919.4	1.7	−8.1

$n=5$
[a] C.V., coefficient of variation.
[b] R.E., relative error.

compared to the theoretical concentration. The C.V. ranged from 4.2 to 6.5% for M-2, from 3.5 to 14.6% for M-3, from 2.5 to 12.0% for M-5, from 1.9 to 4.2% for M-4, from 1.7 to 5.1% for M-7 and from 1.7 to 5.6% for FV at concentrations of 10, 100 and 1000 ng/ml. The R.E. ranged from 4.6 to 8.0% for M-2, from −6.5 to 3.0% for M-3, from 0.0 to 9.8% for M-5, from −5.0 to 0.2% for M-4, from −2.0 to 1.9% for M-7 and from −8.1 to 7.0% for FV at concentrations of 10, 100 and 1000 ng/ml.

The inter-day precision and accuracy of the method for plasma were assessed by analysis of calibration standards at concentrations of 10, 100 and 1000 ng/ml of plasma in triplicate on 3 different days (Table 3). The C.V. ranged from 5.6 to 7.4% for M-2, from 5.6 to 13.8% for M-3, from 2.7 to 5.3% for M-5, from 4.0 to 7.3% for M-4, from 2.1 to 6.1% for M-7 and from 4.9 to 8.9% for FV at concentrations of 10, 100 and 1000 ng/ml. The R.E. ranged from 0.7 to 8.0% for M-2, from −6.0 to −0.6% for M-3, from 1.9 to 7.1% for M-5, from 1.0 to 6.5% for M-4, from 3.0 to 7.3% for M-7 and from 0.6 to 7.3% for FV at concentrations of 10, 100 and 1000 ng/ml.

Table 3
Inter-day precision and accuracy for the determination of M-2, M-3, M-5, M-4, M-7 and FV in human plasma

Theoretical concentration (ng/ml)	Mean found concentration (ng/ml)	C.V.[a]	R.E.[b]
M-2			
10	10.6	5.7	6.0
100	108.8	5.6	8.0
1000	1007.2	7.4	0.7
M-3			
10	9.4	13.8	−6.0
100	99.4	10.4	−0.6
1000	968.4	5.6	−3.2
M-5			
10	10.5	3.8	5.0
100	107.1	2.7	7.1
1000	1019.3	5.3	1.9
M-4			
10	10.1	4.0	1.0
100	102.6	6.0	2.6
1000	1064.6	7.3	6.5
M-7			
10	10.3	5.8	3.0
100	103.2	2.1	3.2
1000	1073.3	6.1	7.3
Fluvastatin			
10	10.2	4.9	2.0
100	107.3	6.6	7.3
1000	1005.7	8.9	0.6

$n=5$.
[a] C.V., coefficient of variation.
[b] R.E., relative error.

3.5. Stability

All compounds exhibited acceptable stability in human plasma when exposed to up to two freeze–thaw cycles, ranging from −15.7 to 18.7 96 (Table 4) compared to analysis of samples without previous exposure to freezing.

The long-term stability of all compounds in plasma was assessed and confirmed for up to at least 1 month of storage at −30°C (Table 5).

These studies suggested that human plasma samples containing the above-described compounds could be handled under normal laboratory conditions without significant loss of compounds.

Table 4
Freeze–thaw stability of M-2, M-3, M-5, M-4, M-7 and FV in human plasma

Initial concentration (ng/ml)	Percent difference from the initial concentration at the indicated cycle[a]		
	0-cycle	1-cycle	2-cycle
M-2			
100	0.0	−15.7	2.5
1000	0.0	−0.9	8.6
M-3			
100	0.0	−2.6	14.5
1000	0.0	−1.9	3.5
M-5			
100	0.0	−14.6	−1.3
1000	0.0	4.2	9.3
M-4			
100	0.0	14.6	0.3
1000	0.0	12.6	7.1
Fluvastatin			
100	0.0	15.5	9.5
1000	0.0	18.7	12.3
M-7			
100	0.0	12.0	11.6
1000	0.0	1.2	5.0

[a] Results are reported as the mean percent difference of three determinations.

Table 5
Stability of M-2, M-3, M-5, M-4, M-7 and FV in human plasma stored at −30°C

Initial concentration (ng/ml)	Percent difference from the initial concentration at the indicated cycle[a]	
	0 week	4 week
M-2		
100	0.0	5.2
1000	0.0	18.5
M-3		
100	0.0	15.4
1000	0.0	15.5
M-5		
100	0.0	4.2
1000	0.0	8.3
M-4		
100	0.0	9.4
1000	0.0	9.4
Fluvastatin		
100	0.0	13.5
1000	0.0	17.7
M-7		
100	0.0	9.2
1000	0.0	9.4

[a] Results are reported as the mean percent difference of three determinations.

Table 6
Dilution of human plasma spiked with M-2, M-3, M-5, M-4, M-7 and FV into the calibration range

Initial concentration (ng/ml)	Percent difference form the theoretical concentration[a]
	Twofold R.E.
M-2	
1500	15.4
M-3	
1500	11.6
M-5	
1500	15.0
M-4	
1500	3.1
Fluvastatin	
1500	−7.6
M-7	
1500	1.8

[a] Results are reported as the mean percent difference of three determinations.

3.6. Sample dilution

The intra-batch R.E. of all compounds in QC plasma samples at a concentration of 1500 ng/ml were −7.6 to 15.4% after 2-fold dilution (Table 6). There was no effect of sample dilution observed on absolute concentration for any of the investigated compounds in human plasma.

4. Conclusion

A simple gradient reversed-phase HPLC–UV method for the determination of fluvastatin and its five metabolites in human plasma was successfully developed and validated. The method was shown to have satisfactory selectivity, recovery, linearity, precision and accuracy.

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