Journal of Chromatography, 614 (1993) 307–313
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6750

High-performance liquid chromatographic method for the determination of fluvastatin in human plasma

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(First received October 6th, 1992; revised manuscript received January 8th, 1993)

ABSTRACT

The method outlined in this paper utilizes internal standardization and is simple, reliable, and sensitive for the determination of fluvastatin in plasma. Fluvastatin sodium and the internal standard are extracted from buffered plasma into methyl *tert.*-butyl ether, followed by evaporation of an aliquot of the organic phase. After reconstitution of the dried sample into a small volume of mobile phase (methanol–13 m*M* tetrabutylammonium fluoride, 3:2, v/v), the sample is chromatographed on an LC-18 column thermostated at 50°C. Fluorescence detection (excitation at 305 nm and emission at 380 nm) is used to monitor both fluvastatin (free acid) and the internal standard. The method can accurately detect 1 ng/ml fluvastatin using a 1.0-ml plasma sample. The precision and reproducibility over the linear range of the method are 5.57 and 7.32%, respectively. This method has been used to measure fluvastatin plasma concentrations in support of bioavailability/pharmacokinetic studies with no indication of interference.

INTRODUCTION

Fluvastatin sodium (Sandoz compound XU 62-320 na; [*R**,*S**,-(*E*)-](±)-7-[3-(4-fluorophenyl)-1-(1-methylethyl)-1*H*-indol-2-yl]-3,5-dihydroxy-6-heptenoic acid, monosodium salt; I; Fig. 1) is a new synthetic drug which has been shown to be a potent competitive inhibitor of hydroxymethylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the biosynthetic pathway of cholesterol [1,2]. Fluvastatin sodium is a racemic mixture of two enantiomers (3*S*,5*R* and 3*R*,5*S*).

Analytical methods for the determination of other drugs in this class [*i.e.*, lovastatin (mevinolin), pravastatin, simvastatin] from biological

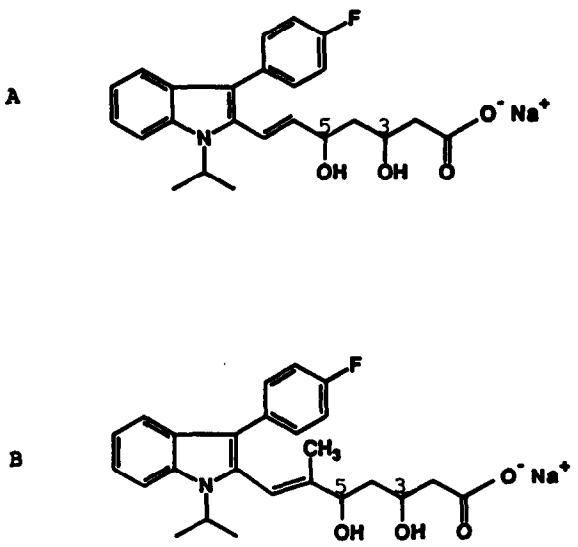

Fig. 1. Structures for (A) fluvastatin sodium (I) and (B) Sandoz compound 63-267 (II), the internal standard.

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0378-4347/93/$06.00

fluids have been reported previously [3–8]. Manning *et al.* [3] described a semi-automated radioenzymatic assay for pravastatin and lovastatin in human serum. Tsay *et al.* [4] developed and reported a specific radioimmunoassay for the measurement of pravastatin in human serum. Whigan *et al.* [5] reported an HPLC method using UV detection for the determination of pravastatin sodium in urine. A capillary GC–negative ion chemical ionization MS method for pravastatin sodium in human serum was developed by Funke *et al.* [6].

Methodology for the determination of simvastatin has been reported by Takano *et al.* [7]. This method quantifies simvastatin and its acid form in human plasma by a GC–MS selected ion monitoring method.

An HPLC method for the determination of mevinolin has been reported by Stubbs *et al.* [8]. This paper describes a reversed-phase HPLC method with UV detection. This method is reported to be suitable for the determination of mevinolin and mevinolinic acid in plasma and bile. The limit of detection for this method is 25 ng/ml.

This paper, unlike those mentioned above, describes a simple, reliable, and sensitive reversed-phase HPLC determination without sample derivatization of an new synthetic drug (I) from plasma with fluorescence detection.

EXPERIMENTAL

Apparatus

Analyses were performed on an HPLC system consisting of a Model 110A pump (Beckman, San Ramon, CA, USA), a Model LP-21 pulse dampener (Scientific Systems, State College, PA, USA), an ISS-100 autosampler and LC-100 column oven (Perkin-Elmer, Norwalk, CT, USA), an RBC-3 refrigerated circulating bath (Neslab, Newington, NH, USA) and a Hitachi F1000 fluorescence detector (EM Science, Cherry Hill, NJ, USA). All peak-height measurements, baseline integrations and related calculations were performed by a computer-automated laboratory system (CALS) software package (Beckman, Waldwick, NJ, USA) operating on an HP-1000 computer system (Hewlett-Packard, Paramus, NJ, USA).

Reagents and solvents

Reagents and solvents used were I and Sandoz compound 63-267 na ([*R**,*S**)-(*E*)-](±)-7-[3-(4-fluorophenyl)-1-(1-methylethyl)-1*H*-indol-2-yl]-3,5-dihydroxy-6-methyl-6-heptenoic acid, monosodium salt; II; Fig. 1) (Sandoz Pharmaceuticals, East Hanover, NJ, USA), methanol, acetonitrile, and methyl *tert.*-butyl ether (MTBE, UV grade, distilled in glass; Burdick & Jackson Labs., Muskegon, MI, USA), HPLC-grade potassium phosphate monobasic anhydrous and sodium phosphate dibasic heptahydrate certified A.C.S.P. (Fisher Scientific, Fair Lawn, NJ, USA), tetrabutylammonium fluoride (TBAF, Aldrich, Milwaukee, WI, USA), and glass-distilled water. Human plasma (Sera-Tec Biologicals, North Brunswick, NJ, USA) was used for standard preparation.

Chromatographic conditions

A Supelcosil LC-18, 150 mm × 4.6 mm I.D. 5 μm particle size column (Supelco, Bellefonte, PA, USA) thermostated at 50°C was used for this reversed-phase separation. The column was equilibrated with methanol–13 m*M* tetrabutylammonium fluoride (3:2, v/v) which had been filtered/degassed using a 0.45-μm filter (Nylon-66, Rainin Instrument, Woburn, MA, USA) on a vacuum filter system (Millipore, Woburn, MA, USA).

Instrumental parameters

The excitation and emission wavelengths for fluorescence detection were set at 305 and 380 nm, respectively. A fixed band pass of 15 nm for each monochromator was used. The signal to the data system was unattenuated at 1 V and the time constant was 0.3 s.

Standard solutions

Both I and II are hygroscopic and sensitive to sunlight. Care should be taken to minimize exposure to moisture and sunlight while handling the drug substances and samples.

A 2 mg/ml stock solution of I was prepared by dissolving 21.0 mg of the sodium salt (equivalent to 20 mg free acid) into 10 ml of glass-distilled water. The 2 mg/ml stock solution was further diluted with water to 100 μg/ml and used to prepare individual plasma standards containing concentrations of fluvastatin ranging from 1 to 1000 ng/ml.

A 2 mg/ml stock solution of II was prepared by dissolving 5.26 mg of II (equivalent to 5.0 mg free acid) in 2.5 ml of glass-distilled water. The 2 mg/ml stock solution was further diluted with water to 300 ng/ml for use during sample preparation.

Sample preparation

Plasma (1.0 ml) was pipetted into screw-capped 125 mm × 25 mm disposable culture tubes (Fisher Scientific) to which was added 1.0 ml of acetonitrile by a Repipet (Labindustries, Berkeley, CA, USA). The sample was then mixed on a Maxi-Mix (Thermolyne, Dubuque, IA, USA) for a duration of 5 s. Using separate Repipets, 1.0 ml of internal standard (II), 2.0 ml of phosphate buffer and 10.0 ml of MTBE were added. The culture tube was then capped (polypropylene screw cap, Fisher Scientific) and shaken for 15 min at 200 cycles/min on a platform shaker (Eberbach, Ann Arbor, MI, USA) at a 0° (horizontal) angle. After shaking, the sample was centrifuged for 5 min at approximately 700 *g* to separate the phases, and the upper phase (8.5 ml) was transferred into a clean culture tube for evaporation *in vacuo* (Buchler Instruments, Fort Lee, NJ, USA). Immediately prior to chromatography 0.4 ml of mobile phase was added to each sample and transferred to a 1-ml amber glass sample vial. The vial was sealed with a crimp cap containing a PTFE seal, and 200 μl of the sample were injected onto the chromatographic system.

RESULTS AND DISCUSSION

Linearity

Daily analysis of duplicate plasma standards at concentrations of 0, 1.0, 5.0, 25, 100, 250, 500, and 1000 ng/ml was used to assess the linearity of the method. A relative response factor (RRF), defined as drug concentration (ng/ml)/peak-height ratio, was calculated for each standard analyzed with a mean RRF being obtained for each analysis day. The daily mean RRFs obtained ranged from 50.3 to 56.1 ng/ml. The small coefficient of variation (<8%) about the mean RRF on each analysis day supports a linear concentration–response relationship.

A linear regression analysis using two separate weighting schemes (wt. = 1 and wt. = $1/y^2$) for the peak-height ratio *versus* concentration was performed on each analysis day. The coefficients of determination (r^2) were greater than 0.99, for both weightings, on each analysis day. These results also support a linear concentration–response relationship.

Precision, reproducibility, accuracy and method error

The precision (within-day variation) and reproducibility (day-to-day variation) are best demonstrated using the data in Table I, which represents the analysis of a set of prepared "unknowns" presented blinded to the analyst. The precision (within-day) ranged from 0.699 to 15.9% (median = 5.57%; n = 27) at concentrations at and above 1 ng/ml while the reproducibility (across-day) ranged from 3.86 to 12.9% (median = 7.32%; n = 9) over the same concentrations. At a concentration of 0.75 ng/ml the precision ranged from 28.9 to 104% (median = 30.0%; n = 15) and the reproducibility was 56.4%.

The accuracy of the method was also evaluated using the data in Table I. Using the mean and variance of the observed absolute differences and the t-value from a one-tailed Student's t-distribution table, the 95% confidence intervals for a single determination were calculated (Table I). At and above a concentration of 1 ng/ml, the results indicate that the result of any single analysis will fall within 6.73 and 25.3% of the true value. At the 0.75 ng/ml concentration the result of any single analysis will fall within 97.6% of the true value.

TABLE I

VALIDATION OF THE METHOD FOR I IN PLASMA SPIKED AT VARIOUS CONCENTRATIONS

Values in parentheses are coefficients of variation (%).

Theoretical concentration (ng/ml)	Mean found concentration (ng/ml)								Absolute difference from theoretical value (mean ± S.D.) (ng/ml)	95% Confidence limit (ng/ml ± %)
	Day 1[a]		Day 2[a]		Day 3[a]		Days 1–3[b]			
0.75	0.951	(28.9)	0.866	(30.0)	0.297	(104)[d]	0.705	(56.4)[e]	0.310 ± 0.234	0.75 ± 97.6
1.0	0.991	(6.65)[c]	0.977	(6.35)	1.12	(8.30)	1.03	(9.45)[f]	0.072 ± 0.071	1.0 ± 19.7
1.5	1.55	(7.80)	1.52	(5.60)	1.42	(6.47)	1.49	(7.32)	0.083 ± 0.069	1.5 ± 13.6
2.0	2.08	(10.9)	2.00	(8.84)	1.78	(15.9)	1.95	(12.9)	0.205 ± 0.146	2.0 ± 23.1
5.0	5.52	(6.76)	5.13	(7.05)	5.76	(9.63)	5.47	(8.91)	0.529 ± 0.418	5.0 ± 25.3
10	10.2	(3.00)	9.64	(2.36)	9.70	(3.86)	9.86	(3.86)	0.350 ± 0.184	10 ± 6.73
50	49.3	(1.81)	43.7	(3.64)	51.1	(5.57)	48.0	(7.72)	3.15 ± 2.70	50 ± 15.8
200	207	(2.66)	185	(1.50)	213	(0.972)	202	(6.40)	11.6 ± 5.08	200 ± 10.3
450	451	(1.78)	403	(2.15)	467	(3.17)	441	(6.76)	23.1 ± 20.3	450 ± 13.1
750	753	(0.699)	668	(1.37)	760	(6.54)	727	(7.06)	41.1 ± 37.2	750 ± 14.2

[a] $n = 5$ unless otherwise noted.
[b] $n = 15$ unless otherwise noted.
[c] $n = 4$.
[d] $n = 3$.
[e] $n = 13$.
[f] $n = 14$.

Method bias

The bias of the method can be estimated from the individual data in Table I. These data indicate that there is no apparent bias (consistent deviation from the theoretical concentration) in the method above 1 ng/ml.

Limit for quantification

Based upon the assessment of data (95% confidence limits, method error, precision, and reproducibility) in Table I the limit of quantification has been set at 1 ng/ml. In general, it is the practice in our laboratories to specify a level of confidence defined to be within 30% of the true value (95% confidence interval), in the absence of significant bias, as a minimal requirement for quantitation.

Selectivity

This method provides for the quantitation of I in plasma using fluorescence detection. The enantiomers of I (3*S*,5*R* and 3*R*,5*S*) are not separated utilizing this method but the *erythro* and *threo* diastereomers are well separated (Fig. 2). The drug (I) is chromatographically separated from the internal standard (II) and endogenous fluorogenic materials observed in human plasma (Fig. 2). Separation of the components of interest from any endogenous material is optimized on each analysis day through minor changes (1–5%) in the organic composition of the mobile phase. The addition of TBAF to the mobile phase resulted in improved column efficiency, symmetrical peak shapes, and better selectivity. These advantages enhanced the separation of the diastereomers. The chromatograms presented in Fig. 2 were generated on separate analysis days resulting in differences in retention times. The differences in retention times observed in Fig. 2A and C are a reflection of the different columns (new and used) and slight variations of the mobile phase used on each day of analysis. The re-

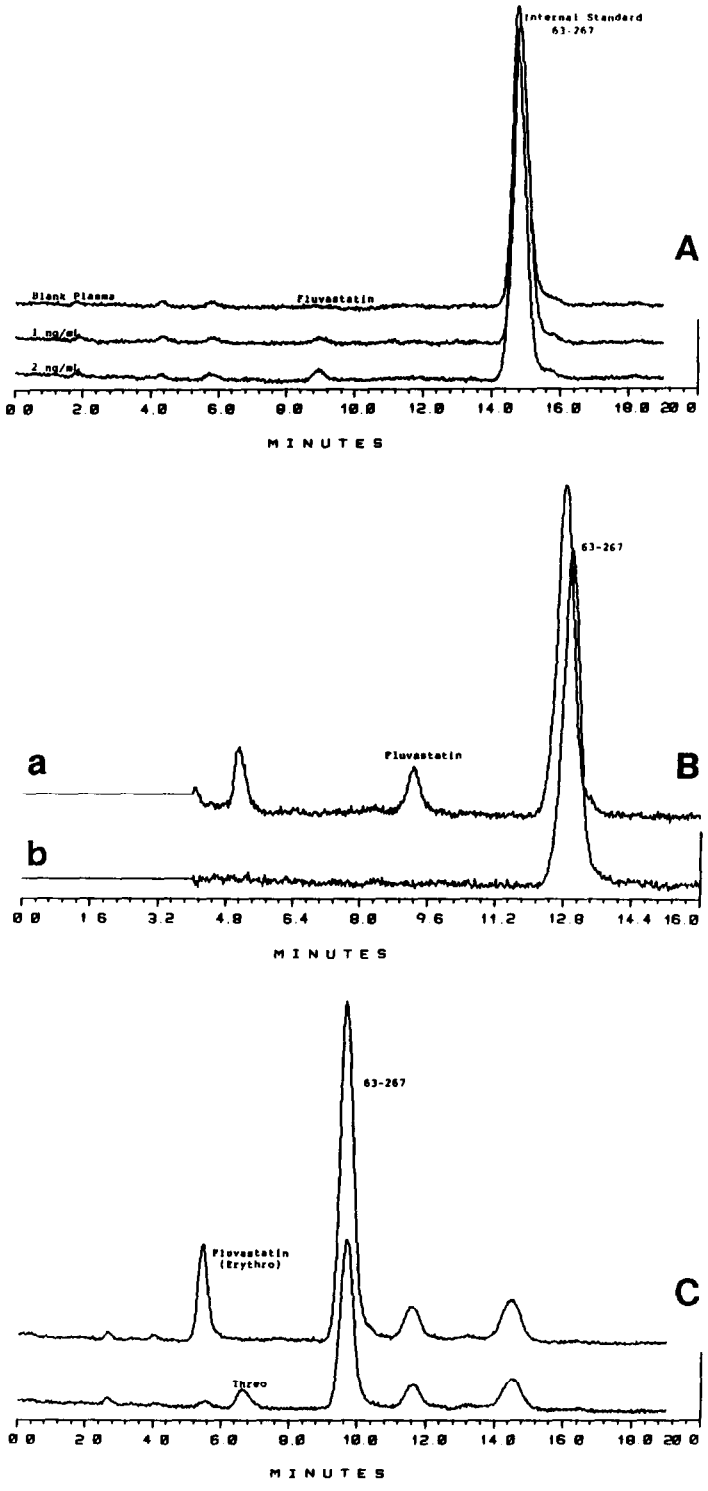

Fig. 2. Sample chromatograms showing I and II: (A) 1 and 2 ng/ml plasma standard *versus* blank human plasma; (B) 1-h post-dose plasma sample (20-mg dose) (a) *versus* pre-dose plasma sample (b); (C) *erythro versus threo* diastereomers in plasma.

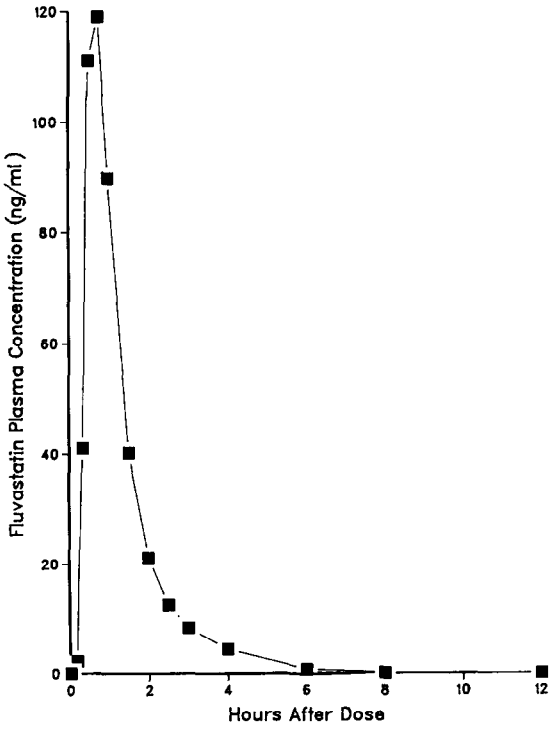

Fig. 3. Mean plasma concentrations obtained for I after a 20-mg oral dose given as a capsule to 34 healthy male volunteers.

tention times in Fig. 2B are offset due to a delayed acquisition start time. The method has been successfully applied to the analysis of over 16 000 samples from bioavailability/pharmacokinetic studies without any evidence of interference, from endogenous plasma components or metabolites of I.

Stability

The stability of I in plasma was assessed under both refrigerated and frozen conditions. Refrigerated (4°C) plasma samples containing 100 ng/ml I were found to be stable for at least a one-month period. Plasma samples prepared at various concentrations (1, 50, 100, 500 and 1000 ng/ml) and stored frozen (−10 to −20°C) were found to be stable for at least two years.

Application of the method

This method has been used on a routine basis for analysis of I in seventeen clinical studies. As a general rule in our laboratories, each analysis day is initiated with a standard curve (extending over the working range of the method) followed by unknown subject samples. Quality control and mid-range reference standards are interspersed throughout the unknown samples in order to monitor any temporal changes that may occur during the analysis period. The mean plasma concentrations obtained following administration of a single 20-mg oral dose of I given as a capsule to healthy male volunteers are shown in Fig. 3. Measurable plasma concentrations were observed out to 4 h after dosing with a maximal plasma concentration observed at 0.75 h.

CONCLUSION

This method provides a specific HPLC method for the determination of I in plasma utilizing an internal standard. The method has been shown to separate both I and the internal standard (II) from endogenous and drug-related fluorogenic material in human plasma. This method offered both the necessary sensitivity and the reliability to analyze over 16 000 samples during the routine analysis of human clinical studies in support of an NDA.

ACKNOWLEDGEMENTS

The authors wish to thank Mr. José Castello for his efforts while performing the validation of this method and Mr. Joseph J. Davis and Mr. Joseph J. Iuzzolino for their support and diligence in the application of this procedure during clinical studies.

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