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Determination of pravastatin in human plasma by high-performance liquid chromatography with ultraviolet detection

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Abstract

A sensitive high-performance liquid chromatographic (HPLC) method for the quantitation of the HMG-CoA reductase inhibitor pravastatin in human plasma is described. Sample preparation based on reversed-phase solid-phase extraction using triamcinolone acetonide as internal standard (I.S.). The compounds were separated on C_{18} reversed-phase analytical column and then determined by ultraviolet detection. The recovery of pravastatin from plasma was 69.2±6.7% (mean±S.D.). The limit of detection for pravastatin in aqueous solution was 0.4 ng, the limit of quantitation in plasma was 2 ng/ml. In a preliminary pharmacokinetic study with two healthy volunteers the $t_{1/2}$ of pravastatin in plasma was found to be 0.8 and 2.3 h. © 1998 Elsevier Science B.V.

Keywords: Pravastatin

1. Introduction

Pravastatin, hexahydro-6-hydroxy-2-methyl-8-(2-methylbutyryloxy)-1-naphthyl)-3,5-dihydroxyheptanoate (Fig. 1), a competitive inhibitor of HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, has been demonstrated to reduce effectively plasma cholesterol in animal studies [1] and in clinical trials [2,3].

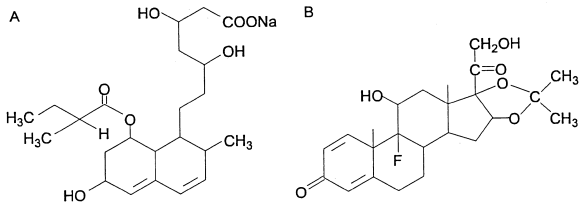

Fig. 1. Structures of pravastatin (A) and I.S. (B).

Pravastatin is the active compound, that is administered orally as the sodium salt and undergoes extensive first-pass extraction in liver. The main metabolite of pravastatin, a 3α-hydroxy isomeric compound (SQ 31 906), has approximately one-tenth to one-fortieth of the HMG-CoA reductase inhibitory activity of pravastatin [4], i.e. the enzyme inhibition is mainly attributable to the parent drug.

Methods of quantitative determination of pravastatin using radiochemistry [5,6], gas chromatography–mass spectrometry with negative-ion chemical ionisation [4] and HPLC–UV detection [7] in biological fluids (plasma, urine, and bile) have already been published. The aim of the present report is to describe a simple, fast and sensitive reversed-phase

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HPLC method, that can be used for pharmacokinetic studies and plasma level monitoring of pravastatin.

2. Experimental

2.1. Reagents and chemicals

Pravastatin was provided by Bristol-Myers Squibb (Regensburg, Germany). Triamcinolone acetonide (9-fluoro-11,21-dihydroxy-16,17-[1-methylethylidenebis(oxy)]pregna - 1, 4 - diene - 3, 20 - dione) (Fig. 1) was purchased by Synopharm (Barsbüttel, Germany). Water used was prepared by a Seralpur PRO 90 CN system (Seral, Ransbach-Baumbach, Germany). HPLC-grade acetonitrile and methanol were from Merck (Darmstadt, Germany). Orthophosphoric acid (85%), sodium dihydrogen phosphate monohydrate (Merck, Darmstadt, Germany) and dodecyl sulfate sodium salt (Aldrich, Steinheim, Germany) were all analytical-grade chemicals. Control plasma was obtained from healthy volunteers.

2.2. Preparation of solutions

2.2.1. Pravastatin

Pravastatin sodium was dissolved in water to prepare the stock solution (10 μg/ml) which was stored at 4°C in 500-μl aliquots. The stock solution was proved to be stable for at least 1 month. Working solutions (20–2000 ng/ml) were prepared by diluting the stock solution with water. Aliquots (100 μl) of the working solutions were added to blank human plasma (1 ml) to obtain calibration standards.

2.2.2. Internal standard

A 250 μg/ml stock solution was prepared by dissolving triamcinolone acetonide in methanol. This solution was further diluted with methanol to provide a working solution of I.S. with a concentration of 25 μg/ml. Aliquots of the working solution (50 μl) were added to the human plasma for assay.

2.3. Instrumentation

The chromatographic system consisted of an L-6200 Pump, an AS-2000 autosampler, and an L-4250 UV detector (Merck, Darmstadt, Germany). Separation was achieved on a Purospher RP-18 end-capped column (5 μm particle size, 250×4 mm I.D.) using a LiChrospher 100 RP-18 precolumn (5 μm particle size, 4×4 mm I.D.) from Merck (Darmstadt, Germany). The chromatographic data were collected and processed with D-6000 HPLC-manager software (Merck, Darmstadt, Germany).

2.4. Chromatographic conditions

The mobile phase was 20 m*M* sodium dihydrogen phosphate buffer containing 1 m*M* dodecyl sulfate and 35% (v/v) acetonitrile. The pH of the mobile phase was adjusted to 2.0 with phosphoric acid. The flow-rate was 1.0 ml/min and the column temperature was 40°C. Under these conditions the retention times for pravastatin and the I.S. were 6.8 and 11.0 min, respectively. The UV detector was set at 239 nm.

2.5. Extraction procedure

To 1 ml of human plasma, 50 μl of I.S. working solution was added and vortex-mixed. The samples were loaded onto Bond Elut C_{18} (Varian, Harbor City, CA, USA) solid-phase extraction cartridges, which were conditioned with 2 ml of methanol and 3 ml of water. The cartridges were then washed with 2 ml of water and allowed to dry for 2 min under vacuum. Finally, the cartridges were eluted with 300 μl of methanol and 100 μl were injected on the HPLC column. Standard samples were prepared by spiking blank plasma with known amounts of pravastatin and used for construction of calibration curves.

2.6. Recovery from human plasma

Recoveries of pravastatin from human plasma were determined at concentrations of 2, 5, 10, 20, 50, 100, and 200 ng/ml. Recovery of I.S. from human plasma was determined at the concentration used in the samples. The recoveries were measured by direct comparison of peak areas of non-extracted water standards vs. plasma extracts.

2.7. Storage stability

The storage stability of pravastatin in human plasma was evaluated by analyzing plasma samples, which were stored at 4 and −70°C. Frozen specimens were subjected to three freeze–thaw cycles.

2.8. Precision and accuracy

The precision and accuracy of the method were assessed by inter- ($n=6$) and intra-assay ($n=4$) validations. Control samples were freshly spiked with the corresponding amount of pravastatin to provide seven final concentrations in the range of 2–200 ng/ml. For each concentration in spiked human plasma the relative standard deviation (R.S.D.) of the mean served as measure of the precision. The accuracy was determined by comparing the measured concentrations to the expected concentrations of pravastatin in spiked blank human plasma and given as relative error.

2.9. Data analysis

To evaluate the linearity of the method, calibration curves (range of 2–200 ng/ml) were generated by plotting peak-area ratios (pravastatin/I.S.) versus pravastatin concentration using computer software InPlot (version 4.03, GraphPad Inc., San Diego, CA, USA).

2.10. Pharmacokinetic study

A single 40-mg dose of pravastatin sodium (Bristol-Myers Squibb, München, Germany) was administered orally to two volunteers. Blood samples were taken at 0, 0.33, 0.66, 1.0, 1.5, 2.0, 2.5, 3.5, 4.0, 6.0, 8.0, 10.0 and 12.0 h after ingestion using EDTA-vacutainer collection tubes (Monovette, Sarstedt, Nümbrecht, Germany). The tubes were centrifuged at 1000 *g* for 10 min and the plasma was collected.

The highest plasma concentration measured for each volunteer was the C_{max}, and the time at which C_{max} occurred was the t_{max}. The apparent plasma elimination rate constant (k_e) was estimated using linear regression of the ln(concentration)–time curve. The $t_{1/2}$ was calculated by $0.693/k_e$.

3. Results and discussion

The HPLC method reported earlier by Whighan et al. [7] offered a limit of quantitation of only 100 ng/ml for pravastatin from urine samples, being too insensitive for determination of the plasma concentration of pravastatin after administration of therapeutic doses. Therefore, in order to investigate plasma kinetics of pravastatin it was necessary to improve the efficiency of a method for the quantitation of the compound. The present method provides a shorter sample preparation procedure and different chromatographic conditions than described earlier [7].

3.1. HPLC conditions

The resolution of several HPLC columns, such as a Purospher RP-18 endcapped column (5 μm particle size, 250×4 mm I.D.), a LiChrospher RP-select B (5 μm particle size, 250×4 mm I.D.) and a Nova-Pak C_{18} (4 μm particle size, 250×4.6 mm), was compared. Only the Purospher RP-18 column enabled a good quality of the peaks of interest and their separation from unidentified plasma peaks.

Optimisation of the chromatographic conditions was also carried out with respect to mobile phase composition. Acetonitrile was preferred, because a poor peak symmetry was observed when using methanol. Adding 1 m*M* dodecyl sulfate to the mobile phase modified the retention time of endogenous plasma peaks, that otherwise had interfered with the peaks of interest. The best separation of pravastatin from plasma peaks was obtained when the pH of the mobile phase was adjusted to 2.0.

After plasma extraction, no co-eluting peak was detectable in control samples at the retention time of pravastatin. Pravastatin was also completely resolved from the I.S. The run-time of one sample was 13 min. Typical chromatograms are shown in Fig. 2.

The performance of the HPLC column was acceptable even after 500 injections of plasma extracts prepared according to the described method.

3.2. Extraction procedure

Solid-phase extraction was used because of the short duration of the clean-up procedure. The effica-

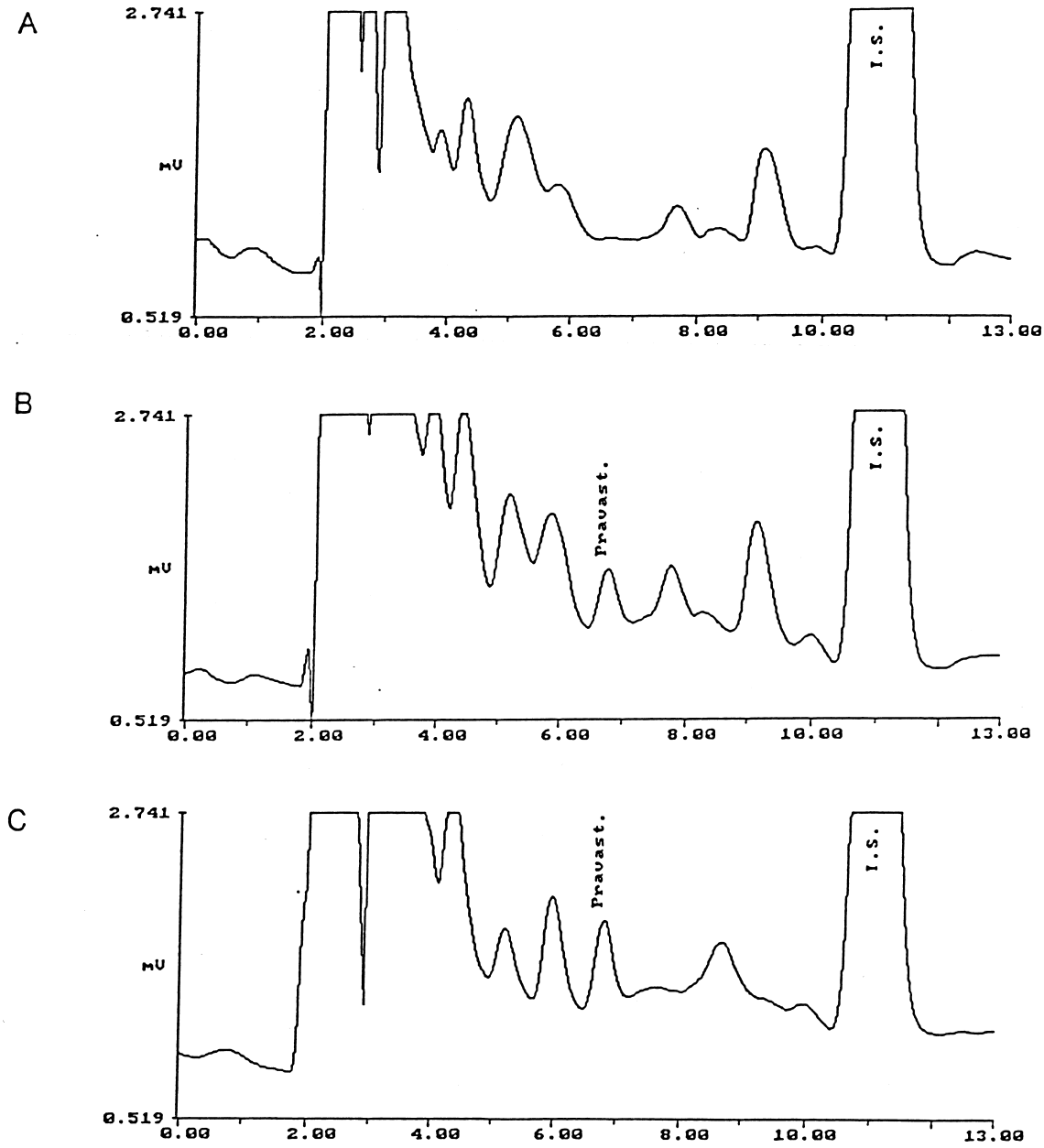

Fig. 2. Chromatograms of pravastatin and I.S. in human plasma after extraction. (A) Blank plasma; (B) human plasma standard with 20 ng/ml pravastatin; (C) plasma of one healthy volunteer 2 h after oral administration of 40 mg pravastatin.

cy of C_{18} solid-phase extraction cartridges from three different producers was compared. Due to the higher recovery of pravastatin, Bond Elut C_{18} extraction cartridges were chosen. Mixtures of water with methanol or acetonitrile, containing different portions of the organic solvent, were used to wash

the extraction column and discard the endogenous components retained in the sorbent. It was found that only the use of pure water did not affect the recovery of the drug.

The extraction cartridges were eluted with only 300 μl of methanol, thereby avoiding the necessity for evaporation and later dissolving of the residues, as described earlier [7].

3.3. Limits of detection and quantitation

The detection limit of pravastatin was determined by direct injection of aqueous standard into the HPLC column. The present validation measurements revealed a limit of detection (signal-to-noise ratio of 3) of 0.4 ng.

The limit of quantitation, defined as the lowest sample concentration detectable in plasma extract with adequate analytical precision (R.S.D≤20%), was 2 ng/ml for pravastatin, corresponding to about 0.5 ng injected.

3.4. Recovery from human plasma

Recovery (mean±S.D.) from pooled human plasma was 69.2±6.7% for pravastatin (Table 1), as determined in three replicates of seven different concentrations (n=21) within the range of the calibration curve. The recovery studies were performed also with unpooled plasma from four individuals (data not shown), but any significant differences in comparison to the pooled plasma was found. The recovery of I.S. in human plasma was 80.4±4.5% (n=21) at the concentration used in plasma samples.

Table 1
Recovery of pravastatin (n=3, mean±S.D.) from human plasma at different concentrations

Concentration (ng/ml)	Recovery (%)
2.0	67.3±2.5
5.0	68.0±7.0
10.0	63.7±5.7
20.0	66.7±6.4
50.0	72.5±4.9
100.0	77.7±6.4
200.0	70.0±1.7

The differences in chemical structure of pravastatin and I.S. are suggested to be responsible for the difference in the recoveries of the two substances.

3.5. Stability

The mean concentration of pravastatin did not change substantially when plasma samples were stored at 4°C for up to 24 h. The difference from control was ≤5%. Measured concentrations of pravastatin in quality control samples (stored at −70°C) after three freeze–thaw cycles were within ±10% of the original values, indicating that the substance is stable in human plasma after repeated freezing and thawing.

3.6. Linearity

The calibration curve was linear in the range of 2–200 ng/ml of pravastatin (coefficient of correlation 0.9997–1.0000). The least-squares plot gave the equation $y=1254.83x+0.63$ for pravastatin.

3.7. Precision and accuracy

The intra- and inter-assay reproducibility data are summarized in Tables 2 and 3. In the intra-assay study, the R.S.D. varied from 0.4 to 5.2%. In the inter-assay study, this parameter did not exceed 7.3% on the calibration points, with the exception of 16.5% for the lowest concentration. On the whole, the accuracy of the plasma method was acceptable for both intra- and inter-assay with errors of ≤12%. However, the accuracy of the plasma sample spiked with 2 ng/ml was acceptable with errors of 20 and 15%, respectively.

3.8. Application

The validated method has been utilized to provide pharmacokinetic data in two volunteers following oral administration of 40 mg pravastatin. Analysis of plasma samples collected prior to drug administration demonstrated that endogenous components did not interfere with the peak of pravastatin on the

Table 2
Inter-assay ($n=6$) precision and accuracy of pravastatin

Added (ng/ml)	Found (ng/ml)	R.S.D. (%)	Relative error (%)
2.0	2.3	16.5	15.0
5.0	4.8	6.3	−4.0
10.0	9.3	6.0	−7.0
20.0	20.4	7.3	2.0
50.0	51.3	2.7	2.6
100.0	100.5	1.6	0.5
200.0	199.8	0.2	−0.1

Table 3
Intra-assay ($n=4$) precision and accuracy of pravastatin

Added (ng/ml)	Found (ng/ml)	R.S.D. (%)	Relative error (%)
2.0	2.4	3.3	20.0
5.0	4.4	5.2	−12.0
10.0	8.9	2.1	−11.0
20.0	19.7	1.6	−1.5
50.0	51.0	3.3	2.0
100.0	100.8	2.0	0.8
200.0	199.4	0.4	−0.3

chromatogram. Plasma concentration–time profiles, as well as data about the pharmacokinetics of pravastatin in human plasma, are given in Fig. 3.

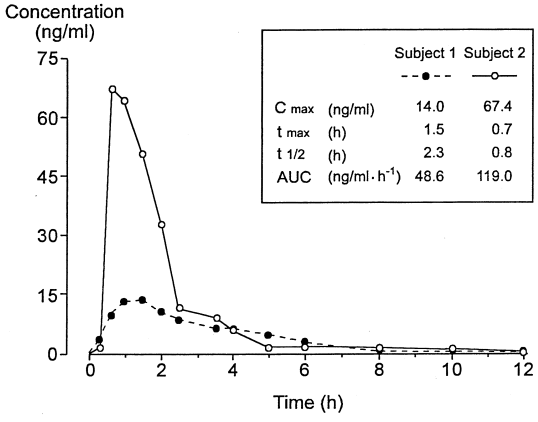

Fig. 3. Time–concentration profiles of pravastatin after a single oral dose of 40 mg. Inset: pharmacokinetic data of pravastatin in the plasma of two volunteers.

4. Conclusion

The reported method for the determination of pravastatin in human plasma includes a rapid and simple sample preparation. It provides a specific, sensitive and reproducible assay for the quantification of pravastatin in plasma. The method will be of interest for pharmacokinetic studies and drug monitoring in hospital use.

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