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Quantification of pravastatin in human plasma and urine after solid phase extraction using high performance liquid chromatography with ultraviolet detection

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Abstract

A high performance liquid chromatography (HPLC) method for the estimation of pravastatin in human plasma and urine samples has been developed. The preparation of the samples was performed by automated solid phase extraction using clonazepam as internal standard. The compounds were separated by isocratic reversed-phase HPLC (C_{18}) and detected at 239 nm. The method was linear up to concentrations of 200 ng/ml in plasma and 2000 ng/ml in urine. The intra-assay variability for pravastatin in plasma ranged from 0.9% to 3.5% and from 2.5% to 5.3% in urine. The inter-assay variability ranged from 9.1% to 10.2% in plasma and from 3.9% to 7.5% in urine. The validated limits of quantification were 1.9 ng/ml for plasma and 125 ng/ml for urine estimation. These method characteristics allowed the determination of the pharmacokinetic parameters of pravastatin after administration of therapeutic doses.
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Keywords: Pravastatin; OATP-C; Pharmacokinetic

1. Introduction

Pravastatin, hexahydro-6-hydroxy-2-methyl-8-(2-methylbutyryloxy)-1-naphthyl-3,5-dihydroxyheptanoate, is a competitive inhibitor of HMG-CoA reductase (Fig. 1). In the past years, studies have shown that the organic anion transporter protein OATP-C is responsible for the uptake of pravastatin into human liver cells [1–3]. Existing variations in the gene of the transporter protein can influence this uptake resulting in the change of the pharmacokinetic parameters of pravastatin [4–6]. Various single nucleotide polymorphisms have been identified for OATP-C. To investigate the influence of some genetic variations in the OATP-C gene on pravastatin kinetics, we have developed a chromatographic method to quantify pravastatin concentrations in human plasma and urine.

Several methods have been developed for estimation of pravastatin with ultraviolet (UV) detection [7–9]. These methods are very sensitive but they only described the quantification in plasma not in urine. Other authors have published methods for plasma and urine using radioactive labeled pravastatin [10,11]. These methods require special equipment and safety conditions. In a few publications the quantification of pravastatin with LC/MS is described [12–16], however, mass-selective detection is cost intensive and often not available for routine analysis.

We therefore developed and validated a stable analytical method for quantification of pravastatin in both plasma and urine under similar conditions to estimate pharmacokinetic parameters after oral administration.

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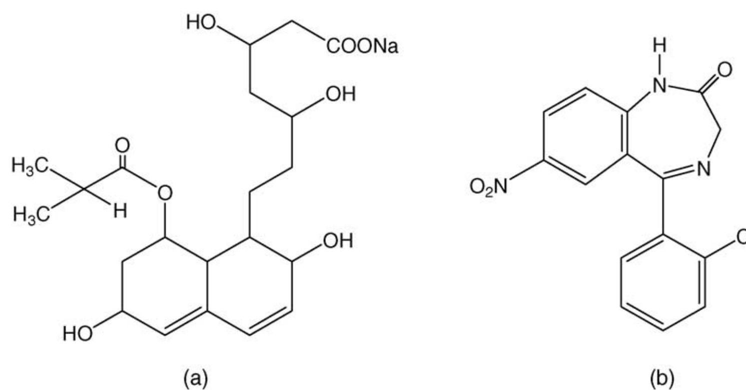

Fig. 1. Chemical structures of pravastatin (a), and clonazepam (internal standard) (b).

2. Experimental

2.1. Chemicals and reagents

Potassium dihydrogen phosphate, *ortho*-phosphoric acid (85%, extra pure), methanol, acetonitrile, and *n*-hexane, were of HPLC or analytical grade and were purchased from Merck (Darmstadt, Germany). Clonazepam was purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany).

2.1.1. Study

Thirty healthy male subjects were grouped according to their genetic determined OATP-C haplotypes (*1a, *1b and *5). After a single oral dose of 40 mg pravastatin, the pharmacokinetic parameters of pravastatin disposition in plasma were analyzed [6].

2.2. Chromatography

Pravastatin was quantified using a Shimadzu high performance liquid chromatography (HPLC) system (Duisburg, Germany) consisting of a pump LC 9A, an automatic sampler SIL 6A, a column heater CTO 6A and an ultraviolet detector SPD6AV. The Class LC10 software version 1.6 (Shimadzu) was used for data analysis and processing. The compounds were separated at 50 °C on a Luna C_{18} (2) column (5 μm, 250 × 4.6 mm I.D.) (Phenomenex, Aschaffenburg, Germany) with guard column and quantified by ultraviolet detection at 239 nm. For preparation of the mobile phase, 300 ml of acetonitrile was mixed with 700 ml of 0.05 M potassium dihydrogen phosphate puffer, adjusted to a pH of 2.3 with concentrated phosphoric acid. The mobile phase was prepared weekly and was delivered at a flow rate of 1.0 ml/min. The substances were quantified using their peak area ratio to the internal standard.

2.3. Sample preparation

2.3.1. Plasma

The sample preparation was performed by using of an automatic solid phase extraction workstation (Benchmate II, Zymark, Hopkinton, MA, USA) and 1 ml Clean-Up®-Extraction columns CUC18 (ICT Handels-GmbH, Bad Homburg, Germany). In a 2 ml polypropylene tube, 1.0 ml plasma was mixed with 50 μl internal standard solution (1 μg clonazepam/1 ml methanol) and 1 ml of methanol. The proteins were precipitated under agitation for 20 min at room temperature. Samples were spun for 10 min at 3000 × *g*, supernatants were transferred into a 10 ml glass tube and diluted with 2.5 ml of distilled water. After conditioning with 2 ml of methanol/acetonitrile (1:1, v/v) and 2 ml of distilled water the extracts were introduced onto the SPE columns. After washing with water, with 5% methanol in water and with *n*-hexane, the samples were eluted with 2 ml of methanol/acetonitrile (1:1, v/v). After evaporation to dryness at 40 °C under a stream of nitrogen, the dried extracts were resolved in 100 μl of 30% acetonitrile in water. The complete reconstituted extract was subjected to HPLC analysis.

2.3.2. Urine

In a 10 ml glass tube, 0.2 ml urine was mixed with 0.8 ml of blank plasma, 200 μl internal standard solution (1 μg clonazepam/1 ml methanol) and 1 ml of methanol. After protein precipitation and centrifugation, the supernatants were diluted with 2.5 ml of distilled water. Further sample preparation was identical to that described for plasma samples. For injection to the HPLC, only 5–10 μl were used.

2.4. Preparation of stock solutions, calibration standards and quality control samples

A stock solution was prepared by dissolving 40 mg pravastatin in 80% methanolic solution in a 50 ml volumetric flask. The working solution was obtained by dilution with methanol to a final concentration of 80 μg/ml. This solution was used for preparation of calibration standards and quality control samples. For the preparation of the internal standard solution, 10.0 mg clonazepam were dissolved in methanol in a 10 ml volumetric flask. The working solution was obtained by dilution with methanol to a final concentration of 1 μg/ml.

2.4.1. Plasma

For calibration standards, 20 μl working solution was evaporated to dryness and reconstituted in 20 ml blank human plasma yielding the highest calibration standard with a concentration of 240 ng/ml pravastatin, which was then used to generate standard samples with final pravastatin concentrations of 1.9, 3.8, 7.5, 15.0, 30.0, 60.0, and 120.0 ng/ml by serial dilution with blank plasma. For preparation of quality control samples, appropriate aliquots of the pravastatin working solution were evaporated to dryness and reconstituted in 20 ml blank human plasma. The final pravastatin quality control concentrations were 5.1, 12.8, 32.0, and 80.0 ng/ml. Calibration standards, blank plasma samples and quality control samples were stored in aliquots of 1 ml at −20 °C until analysis.

2.4.2. Urine

For preparation of calibration standards and quality control samples, appropriate aliquots of the pravastatin working solution were evaporated to dryness and reconstituted in blank human urine. The final standard sample concentrations were 0, 125, 250, 500, 1000, and 2000 ng/ml, and the quality control concentrations were 188, 375, 750, and 1500 ng/ml. Calibration standards, blank urine samples and quality control samples were stored in aliquots of 300 μl at −20 °C. After defrosting 200 μl were used for analysis.

3. Results

3.1. Separation and specificity

Pravastatin and the internal standard clonazepam were well separated under the HPLC conditions applied. Retention times were 14 min for pravastatin and 20 min for clonazepam. The retention times are shifted slightly with the aging of the column to 15 min for pravastatin and to 23 min for the internal standard. No important interferences were observed in blank plasma and urine samples. Fig. 2 shows the chromatograms of a blank plasma sample (a), a sample at LOQ (b), a quality control standard with a pravastatin concentration of 32.0 ng/ml (c), and plasma samples from a volunteer 1.5 h after administration of 40 mg of pravastatin (d), respectively. Fig. 3 shows the chromatograms of a blank urine sample (a), a sample at LOQ (b), a quality control standard with a pravastatin concentration of 750 ng/ml (c), and a urine sample from a 12 h collection after oral administration of 40 mg pravastatin (d).

3.2. Limit of detection (LOD) and limit of quantitation (LOQ)

The LOD determined as the amount of drug corresponding to a signal-to-noise ratio of 3:1 was 1 ng. The LOQ was determined as the lowest concentration of the analyte in plasma that could be quantified with an inter-assay coefficient of variation (CV) of <20% and an accuracy between 80% and 120%. The LOQ for pravastatin in plasma was 1.9 ng/ml with an inter-assay CV of 19.4% and an accuracy of 106%, for urine the lowest validated concentration was 125 ng/ml with a CV of 6.8% and an accuracy of 91%.

3.3. Recovery and linearity

The recovery of pravastatin after protein precipitation and solid phase extraction was approximately 90%, tested at plasma concentrations of 7.5, 15, and 60.0 ng/ml. Assay linearity was evaluated up to concentrations of 120 ng/ml in plasma and 2000 ng/ml in urine. The mean slopes, intercepts and r^2 values with SD and ranges are reported in Table 1.

3.4. Intra-assay and inter-assay variation

The intra-assay CV for pravastatin in plasma ranged from 0.9% to 3.5% and the inter-assay CV from 9.1% to 10.2%. In urine, the intra-assay CV ranged from 2.5% to 5.3% and the inter-assay CV from 3.9% to 7.5%. The results for variation are reported in Table 2.

3.5. Accuracy

The accuracy of the measurements was determined using the calibration standards and the four quality control samples for pravastatin in at least six runs. The results for accuracy of control samples are reported in Table 2.

Table 1
Assay linearity for the quantitation of pravastatin

		Mean	SD	Range	*n*
Plasma	Slope	0.016	0.0018	0.013–0.021	22
	Intercept	0.013	0.027	−0.026–0.089	22
	r^2	0.999	0.001	0.997–1.000	22
Urine	Slope	0.002	0.0002	0.001–0.003	10
	Intercept	−0.034	0.038	−0.090–0.034	10
	r^2	0.999	0.001	0.997–0.999	10

Table 2
Intra-assay and inter-assay variability and accuracy of the quantitation of pravastatin

	Concentration (ng/ml)	Intra-assay variability			Inter-assay variability		
		n	CV (%)	Accuracy (%)	*n*	CV (%)	Accuracy (%)
Plasma	5.1	6	2.7	95.3	20	9.4	100.1
	12.8	6	3.5	99.2	18	10.2	99.5
	32.0	6	2.6	107.7	19	9.1	98.4
	80.0	6	0.9	106.1	19	9.8	98.3
Urine	188	6	3.6	91.0	10	5.8	92.1
	375	6	5.3	91.1	10	6.2	97.9
	750	6	4.0	90.4	10	7.5	101.1
	1500	6	2.5	95.0	10	3.9	103.9

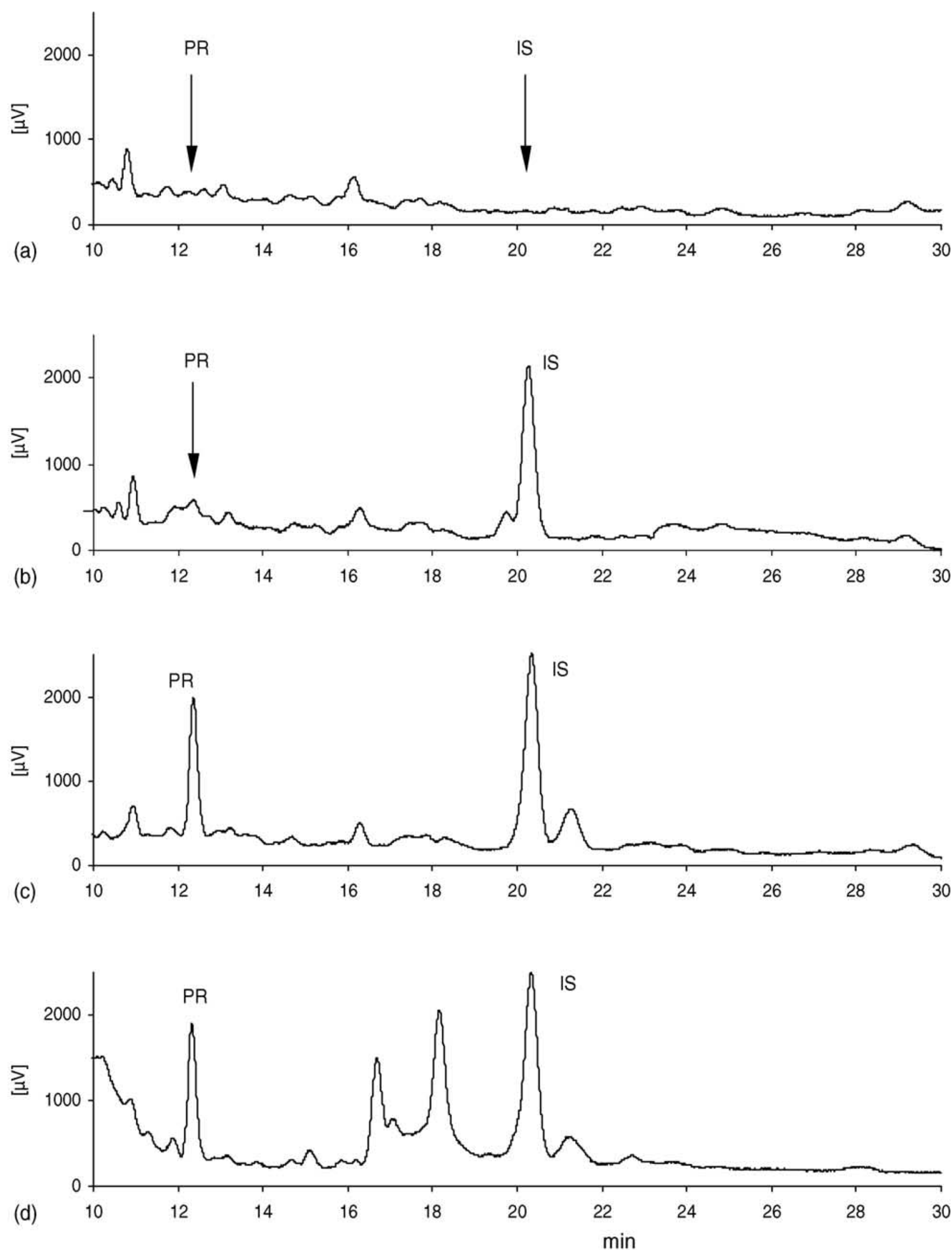

Fig. 2. HPLC trace of pravastatin (PR) and the internal standard clonazepam (IS) using ultraviolet detection at 239 nm: (a) blank plasma sample; (b) quality control sample (LOQ); (c) quality control sample (32 ng pravastatin/ml); (d) plasma sample 1.5 h post-administration of 40 mg pravastatin (plasma concentration 26.3 ng/ml).

4. Discussion

We introduced a method for the determination of pravastatin in human plasma and urine combining an automated solid phase extraction procedure with isocratic reversed-phase HPLC analysis with UV detection. Limits of quantitation of 1.9 ng/ml for pravastatin in plasma and 125 ng/ml in urine are sufficient to analyze samples from a pharmacokinetic study. The solid phase extraction procedure allowed simple and automated sample preparation. This confirmed the results of previously described methods [8,12,16].

The quantification of pravastatin in urine samples with UV detection had not yet been sufficiently described. Since concentrations of pravastatin in urine are high, compared to plasma, it is possible to extract lower urine volume or to dilute urine samples with blank urine or with water. In both cases, it is necessary to validate a new extraction procedure. From our experiences with other analytical problems, we know that the dilution of small amounts of urine with blank plasma results in similar extraction conditions like plasma samples (e.g. protein content, pH). The proportion of the two matrices after this dilution enabled us to extract both kinds of samples

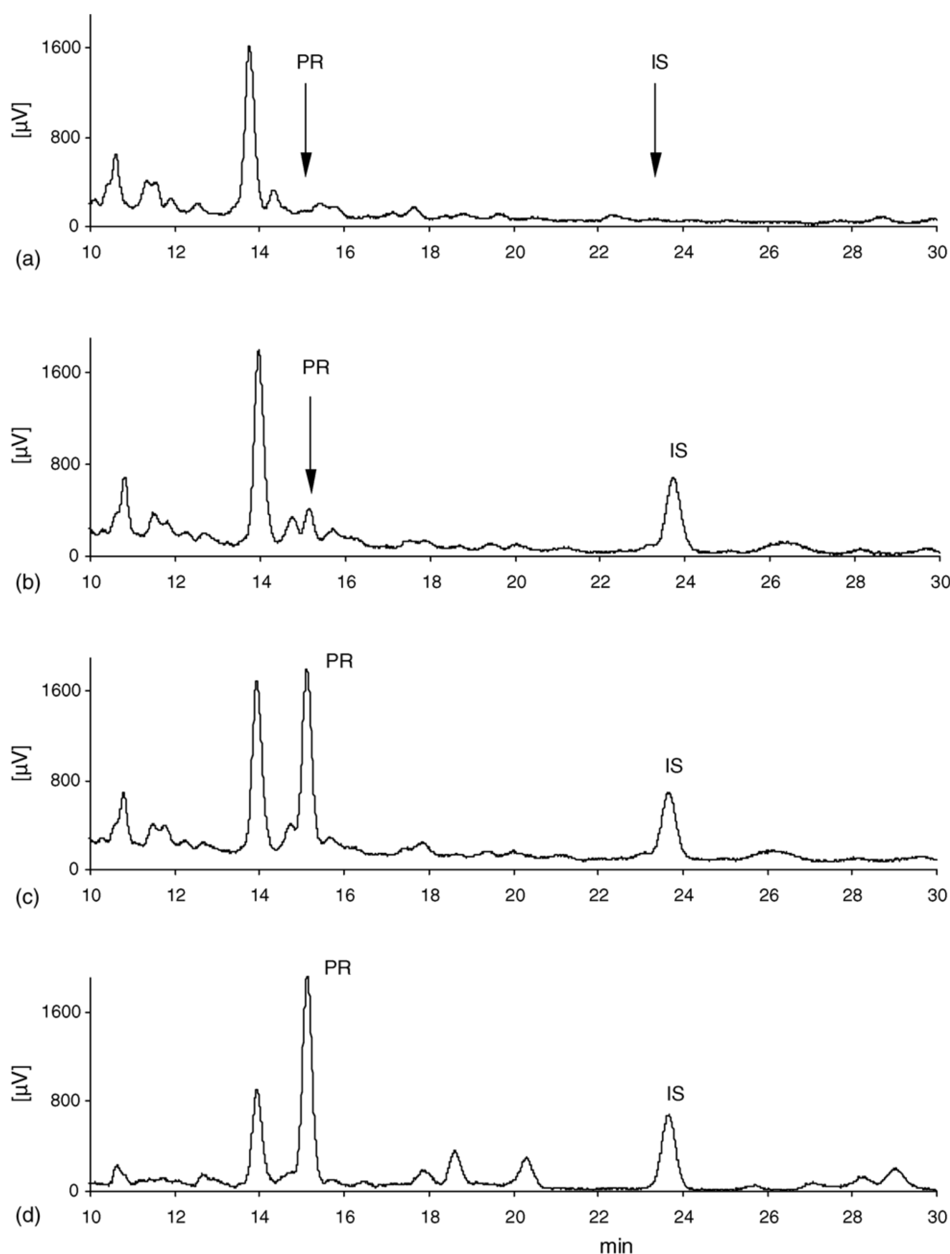

Fig. 3. HPLC trace of pravastatin (PR) and the internal standard clonazepam (IS) using ultraviolet detection at 239 nm: (a) blank urine sample; (b) quality control sample (LOQ); (c) quality control sample (750 ng pravastatin/ml); (d) urine sample collected within 12 h after administration of 40 mg pravastatin (urine concentration 772 ng/ml).

under identical conditions. So, it was possible to analyze urine as well as plasma samples without switching the method.

An important observation during the method validation was the instability of pravastatin in the mobile phase. In analytical batches with more than 8 h total run time, pravastatin peak areas were decreased in the latest samples if the extracts were reconstituted in the mobile phase. As a result of this observation, the reconstitution solvent was changed to 30% acetonitrile in water and no degradation of pravastatin was observed over a time period of more than 48 h.

The method has been successfully applied to a clinical study in our department. Plasma concentration curves obtained over 12 h post-administration of 40 mg pravastatin to healthy volunteers show that a reliable determination of pharmacokinetic parameters (i.e. area under the curve, $t_{1/2}$, clearance) does require quantitation of pravastatin at concentrations below 5 ng/ml (Fig. 4). This method has been found to be reliable and reproducible for that purpose. Fig. 4 shows the mean concentration time profile of pravastatin in plasma from the 10 volunteers, analyzed at first. The relatively high SD is caused by the different haplotypes of OATP-C gene, resulting in different individual pharmacokinetic profiles of pravastatin.

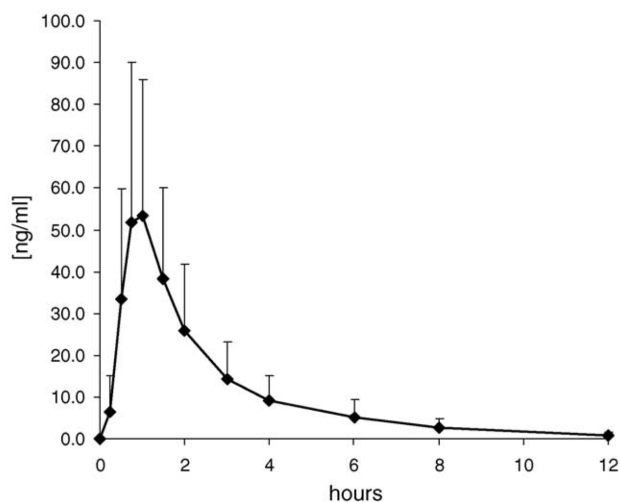

Fig. 4. Mean plasma concentration-time curve of pravastatin, 0–12 h after oral administration of 40 mg to healthy volunteers. Data represent the mean ± SD of 10 individuals.

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