Journal of Pharmaceutical & Biomedical Analysis
Vol. 7, No. 7, pp. 907–912, 1989
Printed in Great Britain

0731–7085/89 $3.00 + 0.00

Short Communication

Determination of pravastatin sodium and its isomeric metabolite in human urine by HPLC with UV detection

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Keywords: *Pravastatin sodium; solid-phase extraction; reversed-phase HPLC; UV detection; urine analysis.*

Introduction

Pravastatin sodium (**I**), (+)-sodium (3*R*,5*R*)-3,5-dihydroxy-7-[(1*S*,2*S*,6*S*,8*S*,8a*R*)-6-hydroxy-2-methyl-8-[(*S*)-2-methyl-butyryloxy]-1,2,6,7,8,8a-hexahydro-1-naphthyl] heptanoate, belongs to a new class of cholesterol-lowering agents which are inhibitors of HMG-CoA reductase [1, 2]. These agents are competitive inhibitors of 3-hydroxy-3-methylglutaryl coenzyme *A* reductase and have been reported to lower total cholesterol and low-density lipoproteins in patients with hypercholesterolemia [3, 4].

Pravastatin sodium is currently being developed for human clinical use. Its inhibitory activity is more potent than that of compactin and comparable to that of lovastatin [5]. It differs from other agents in that it is a tissue-selective inhibitor of HMG-CoA reductase [6].

Following oral human administration, one of its isomeric metabolites (**II**) is found in urine. An assay was required to determine the amounts of pravastatin sodium (**I**) and the metabolite (**II**) in human urine. A solid-phase extraction using a disposable cartridge was developed to isolate both **I** and **II** from urine. Measurement by high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection is described.

Experimental

Chemicals

Pravastatin sodium (**I**) and its metabolite (**II**) were supplied by Sankyo Co., Tokyo, Japan. The internal standard (**III**) was synthesized at the Squibb Institute for Medical Research (see Fig. 1). It is 11β, 16α, 17α-9-fluoro-11-hydroxy-16,17-[1-methylethyl-

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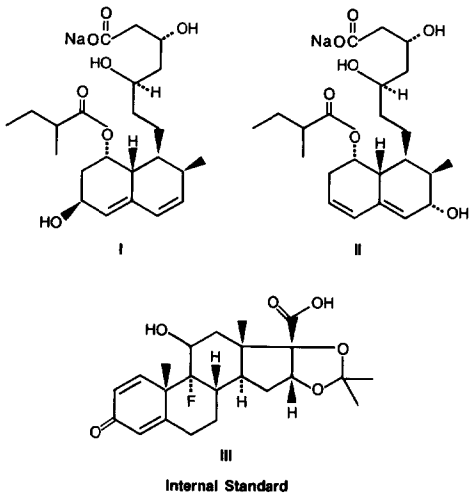

Figure 1
Structures of analytes and internal reference standard.

idenebis(oxy)]-3-oxoandrosta-1,4-diene-17-carboxylic acid. Potassium dihydrogen phosphate, HPLC grade, was obtained from Fisher Scientific Co. (Fairlawn, NJ, USA). All solvents used were HPLC grade.

Apparatus

The liquid chromatographic system consisted of the following components: Kratos Spectroflow 400 pump and Kratos Spectroflow 783 variable-wavelength detector (ABI Analytical, Ramsey, NJ, USA), Perkin–Elmer ISS-100 autosampler equipped with a 50-μl loop (Perkin–Elmer Corp., Norwalk, CT, USA). The autosampler was equipped with a refrigerated tray maintained at 15°C by circulating a mixture of ethylene glycol/water (20:80, v/v) using a Neslab Instruments model RTE-4 recirculating bath (Portsmouth, NH, USA). Chromatograms were recorded on a 10 mV strip chart recorder (Kipp and Zonen, model BD-40, Delft, Netherlands).

Chromatographic conditions

The isocratic separation was achieved using an alkyl phenyl column (25 cm × 4.6 mm, i.d. 5 μm packing) supplied by ES Industries (Marlton, NJ, USA). A pre-column, dry packed with 37–53 μm silica gel (Whatman, Clifton, NJ, USA), was inserted in front of the injector to protect the analytical column. The mobile phase consisting of an aqueous buffer, acetonitrile and isopropanol (62:30:8, v/v/v) was maintained at a flow rate of 0.8 ml min^{-1}. The aqueous buffer was 0.1 M acetic acid adjusted to pH 3.0 with dilute ammonium hydroxide solution. The analytes were detected at 239 nm using an amplifier sensitivity setting of 0.012 AUFS.

Reagents

Phosphate buffer (pH 7.0) was prepared by dissolving 6.8 g of potassium dihydrogen phosphate in 1000 ml of distilled water and adjusting the pH with sodium hydroxide. The pH of 0.1 M acetic acid solution was adjusted to 3.5 with dilute ammonium hydroxide solution. A wash solvent consisting of 0.1 M acetic acid, pH 3.5/acetonitrile/isopropanol (62:30:8, v/v/v) was employed.

Standard solutions

Stock solutions of **I**, **II** and **III** were prepared in methanol at a concentration of 200 μg ml^{-1}. These were prepared every 2 weeks. A secondary internal reference standard solution, prepared daily at 10 μg ml^{-1} in water, was used to spike the samples prior to extraction. An unextracted working standard solution was prepared daily to contain 1 μg each of **I**, **II** and **III** per ml of wash solvent.

Extraction cartridges

Bond Elut® cyclohexyl (CH) or octadecyl (C18) cartridges from Analytichem International (Harbor City, CA, USA) were used for sample extraction. Each cartridge contains 500 mg of sorbent with a total reservoir volume of 3 ml. Cartridges were processed on a Vac-Elut® processing station including an Analytichem vacuum manifold.

Sample preparation

The disposable extraction cartridges were conditioned with 5 ml of methanol, followed by 5 ml of acetonitrile, then 5 ml of pH 7 phosphate buffer. A suitable volume of urine, up to 0.5 ml, was transferred to a cartridge and 100 μl, containing 1 μg, of secondary internal standard, was added. The samples were passed through the cartridges using vacuum. The cartridges were sequentially washed with 5 ml of distilled water, 5 ml of phosphate buffer, 1 ml of wash solvent, discarding all washings. Each cartridge was eluted with 3 ml of acetone into a 20 ml scintillation vial. The eluate was evaporated to dryness at 50°C under a gentle stream of nitrogen. The residues were dissolved in 1 ml of wash solvent, mixed well and transferred into an autosampler vial for chromatography.

Measurement

Measurement was performed by comparing peak height ratios from extracted samples compared to non-extracted working standard solutions.

Results and Discussion

Specificity of the chromatography

Baseline resolution of **I**, **II** and **III** (Fig. 2a) was achieved following the recommended chromatographic conditions. Pravastatin, the metabolite, and the internal standard contain a UV chromophore with a λ_{max} of approximately 239 nm in the wash solvent. The pH of the acetic acid solution in the mobile phase should be 3.0 ± 0.1 for optimum resolution. The addition of isopropanol to the mobile phase resulted in sharper peaks and increased selectivity. The amount of isopropanol may vary from 7 to 9% but the total amount of organic solvent (acetonitrile and isopropanol) should be 38 ± 1%. The retention times of **I**, **II** and **III** were found to be 10.3, 11.4 and 15.8 min, respectively.

When the mobile phase was used to make up the working standard solution and dissolve the sample residues, degradation of both pravastatin and the metabolite was observed. Consequently, a wash solvent with similar composition but slightly higher pH was used instead of the mobile phase. The degradation of the analytes was further minimized by cooling the sample tray. Over a period of 24 h, degradation was <2% for each analyte and the internal standard.

The chromatogram of a drug-free urine extract (Fig. 2b) shows minimal endogenous interferences. The chromatogram in Fig. 2c shows the extract of a urine sample to which **I**, **II** and **III** were added.

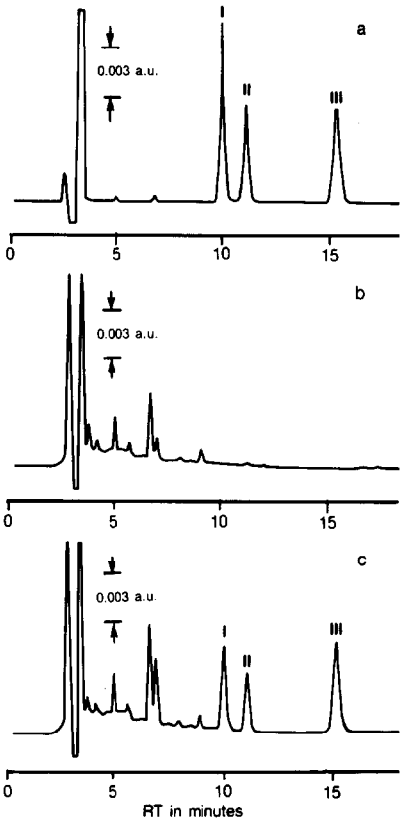

Figure 2
Representative chromatograms: a, unextracted standard solution containing 1 μg ml^{-1} each of **I**, **II** and **III**; b, blank urine; c, blank urine containing 1 μg ml^{-1} each of **I** and **II**, assayed as a sample.

Sample extraction

Cartridges with different bonded phases were tried for sample clean-up. Quantitative recoveries were obtained with either cyclohexyl (CH) or octadecyl (C_{18}) cartridges. Recoveries with phenyl (PH) cartridges were low for all three compounds. The analytical data were all processed with the CH cartridges. The final treatment with the wash solvent reduced the background. Since the composition of the wash solvent is similar to the mobile phase, too much wash solvent would elute the analytes from the cartridges, resulting in low recoveries. Adequate sample clean-up with quantitative recoveries were obtained when 0.8–1.3 ml of wash solvent was used.

Standard curves

The peak height ratios of analyte to internal reference standard are directly proportional to the concentration of analyte. Typical standard curves (concentration range 0–1.5 μg ml^{-1}) resulted in the following least-squares regression equations:

$$Y = 0.597x - 0.001 \qquad r = 1.000 \text{ for } \mathbf{I}$$
$$Y = 0.926x - 0.003 \qquad r = 1.000 \text{ for metabolite } \mathbf{II}.$$

The small interference from endogenous components with metabolite **II** shown in Fig. 2b

accounts for the higher intercept in the linear calibration curve. However, the higher intercept does not affect the results.

Recoveries and precision

The absolute recoveries of **I** and **II** were estimated by comparing peak height ratios obtained from extracts of urine samples to which **I** and **II** were added with the ratios obtained for the non-extracted standard solution injected directly onto the column. Recoveries in the range of 95–105% were obtained for both **I** and **II**. The peak heights of **I**, **II** or **III** were the same for both extracted or non-extracted samples.

To determine the accuracy and precision of the assay, replicate samples, spiked with different concentrations of **I** and **II**, were analysed. The results (Tables 1 and 2) show the assay to be both accurate and precise over the concentration range studied.

The minimum concentration that could be determined, defined as the concentration where the standard deviation is 10–15%, is 0.1 μg ml^{-1} urine for both **I** and **II**.

Table 1
Intra-day variation for the assay

Added concentration (μg ml^{-1})	Mean concentration (μg ml^{-1})	Recovery (%)	*N*	RSD (%)
Pravastatin sodium				
0.10	0.11	110	5	9.1
0.20	0.20	100	4	5.0
0.50	0.51	102	4	3.9
1.00	1.01	101	6	4.0
2.00	2.00	100	4	3.5
5.00*	5.00	100	4	2.2
Metabolite				
0.10	0.11	110	5	9.1
0.20	0.20	100	4	10.0
0.50	0.49	98	4	4.1
1.00	0.99	99	6	3.0
2.00	2.00	100	4	4.0
5.00*	5.02	100	4	2.2

*Spiked sample was diluted with water prior to assay.

Table 2
Inter-day variation for the assay

Added concentration (μg ml^{-1})	Mean concentration (μg ml^{-1})	Recovery (%)	*N*	RSD (%)
Pravastatin sodium				
0.10	0.11	110	4	9.1
0.50	0.51	102	4	5.9
1.00	1.01	101	4	3.0
5.00*	4.98	99.6	4	3.8
Metabolite				
0.10	0.10	100	4	10.0
0.50	0.51	102	4	5.9
1.00	0.98	98	4	5.1
5.00*	4.99	99.8	4	4.2

*Spiked sample was diluted with water prior to assay.

Table 3
Concentrations of analytes in urine fractions collected after a 20-mg oral dose of pravastatin sodium to human subjects

Time (h)	Pravastatin sodium ($\mu g\ ml^{-1}$)	Metabolite ($\mu g\ ml^{-1}$)
Subject 1		
0–4	3.46	1.74
4–8	2.48	0.70
8–12	0.78	0.25
12–24	0.20	<0.10
Subject 2		
0–4	2.69	0.62
4–8	1.77	0.33
8–12	0.93	0.10
12–24	0.10	<0.10

Assay applicability

The method has been successfully used to assay human urine samples from pharmacokinetics and clinical studies. Assay results from urine samples collected following a 20-mg oral administration to human subjects are shown in Table 3. Depending upon the time of collection and the volume of urine excreted, analyte concentrations were as high as 8 $\mu g\ ml^{-1}$. Samples containing analyte concentrations >3 $\mu g\ ml^{-1}$ were diluted with water prior to analysis.

Acknowledgements — The authors thank Dr G. A. Brewer for critically reviewing the manuscript, Dr E. Delaney for the preparation of the internal reference standard and Mrs E. Puritz for her technical assistance.

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[*Received for review 30 November 1987; revised manuscript received 12 August 1988*]