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Simultaneous determination of simvastatin and its hydroxy acid form in human plasma by high-performance liquid chromatography with UV detection

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Abstract: Simvastatin (SV), an analogue of lovastatin, is the lactone form of 1′,2′,6′,7′,8′,8a′-hexahydro-3,5-dihydroxy-2′,6′-dimethyl-8′(2″,2″-dimethyl-1″-oxobutoxy)-1′-naphthalene-heptanoic acid (SVA) which lowers plasma cholesterol by inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase. A fast, simple and accurate method for determining SV and SVA concentrations in human plasma has been developed and validated for use in the analysis of plasma samples from patients and healthy volunteers. This method involves an extraction procedure using a mixture of acetonitrile–water and reversed-phase high-performance liquid chromatography with UV detection. The procedure was linear from 20 to 1000 ng ml^{-1} for SV and from 25 to 1000 ng ml^{-1} for SVA, respectively. The method was accurate with relative errors of 5.0, 2.1 and 3.2% for human plasma controls containing 50, 250 and 500 ng ml^{-1} of SV, respectively. The corresponding precision was 2.3, 1.8 and 1.0% (RSD %). Similarly, relative standard deviations less than 2.3% and relative errors of less than 5.2% were obtained from human plasma controls containing SVA at identical concentrations. The method is suitable for pharmacology and pharmacokinetic studies of simvastatin.

Keywords: *HPLC; simvastatin; hydroxy acid form of simvastatin; human plasma.*

Introduction

Simvastatin (SV, MK-0733) is an orally active cholesterol-lowering agent in animals and man [1, 2]. Its chemical name is [1*S*-[1α,3α,7β,8β(2*S**,4*S**),8aβ]]-2,2-dimethylbutanoic acid 1, 2, 3, 7, 8, 8a-hexahydro-3,7-dimethyl-8-[2-(tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl)-ethyl]-1-naphthalenyl ester (Fig. 1). It is obtained by the replacement of the 2-methyl-butyryl side chain of lovastatin with a 2,2-dimethyl-butyryl group. Upon hydrolysis, SV (a lactone) is converted to the β,δ-dihydroxy acid form SVA (Fig. 1), a potent competitive inhibitor of 3-hydroxyglutaryl-CoA reductase (HMG-CoA reductase) which catalyses the rate-limiting step of cholesterol biosynthesis [3]. Conversion of SV to the active inhibitor SVA *in vivo* is implicit in the observation that oral doses of SV are effective in lowering plasma cholesterol and LDL levels in rats, dogs, and humans [1, 4]. The absorption, excretion, and tissue distribution of SV and

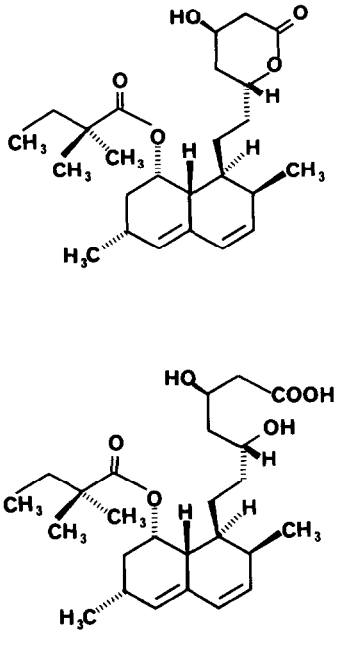

Figure 1
Structure of simvastatin and its hydroxy acid form.

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SVA have been reported [5, 6]. The metabolic processes result in a selective concentration of SVA in liver. This is a particularly convenient event, because the liver is the major organ involved in the homeostatis of cholesterol. Thus, SV is an example of a target organ-directed compound that is designed to biochemically control hypercholesterolemia. An elevated plasma cholesterol level has been recognized as a major risk factor for atherosclerotic disease, specifically for coronary heart disease [7]. This paper describes an HPLC method using a simple liquid–liquid extraction procedure for sample preparation prior to analysis of SV and SVA in human plasma. The method employed reversed-phase chromatography with a run time of approximately 20 min. This method has been shown to be sensitive, accurate, reproducible and suitable for analysing a large number of samples to support bioavailability and pharmacokinetic studies.

Experimental

Chemicals

Simvastatin (SV) and its acid form (SVA) were supplied by Merck Sharp & Dohme Italia (Rome, Italy). HPLC-grade acetonitrile was obtained from Farmitalia Carlo Erba (Milan, Italy). Water was purified and deionized using a Milli-Q ion-exchange filtration system (Millipore, Bedford, MA, USA). Water was filtered through WCN 0.45 μm filters, while acetonitrile was filtered through WTP 0.5 μm filters (Whatman Ltd, Maidstone, UK). Sodium dihydrogen phosphate analytical grade was obtained from J.T. Baker (B.H. Schilling, Milan, Italy).

Chromatographic system and conditions

The chromatographic apparatus (Waters Assoc., Milford, MA, USA) consisted of a model 6000A solvent delivery system, a Lambda 481 LC-spectrophotometer connected to a CC-12 Computing Integrator (Perkin–Elmer, Rome, Italy). A Rheodyne model 7125 sample injector (Cotati, CA, USA) equipped with a 20 μl loop was used. The separation was performed on an analytical column (250 × 4.6 mm, i.d.), packed with the reversed-phase material 5 μm ODS Hypersil, supplied by HPLC Technology (Macclesfield, UK) connected to a 2 cm pelliguard (40 μm particle size) precolumn (Supelco, Bellefonte, PA, USA). The mobile phase consisted of a mixture of 0.025 M sodium dihydrogenphosphate (pH 4.5)–acetonitrile (35:65, v/v). The mobile phase was prepared daily, sonicated and filtered before use and delivered at a flow rate of 1.5 ml min^{-1}. The column was maintained at ambient temperature (20°C) and the compounds thus eluted were recorded by the detector at a constant wavelength of 238 nm.

Standard solutions

Stock solutions (1.0 mg ml^{-1}) of SV were prepared in acetonitrile, while for SVA solutions (1.0 mg ml^{-1}) a mixture of acetonitrile–water (80:20, v/v) was used as solvent. Calibration curves were derived from pooled human plasma by adding appropriate amounts of a mixture of SV and SVA. Calibration curves were obtained by plotting peak-height of each drug versus concentration of the drug. (The use of area measurements gave equivalent results.)

Control pools

Human plasma control pools for SV and SVA were prepared by diluting the SV and SVA stock solutions to 10 μg ml^{-1} with control human plasma. The solutions were further diluted with control human plasma to yield control pools containing 50, 250 and 500 ng ml^{-1} of SV and SVA, respectively.

Assay validation

Calibration curves were derived from pooled human plasma by adding appropriate amounts of a mixture of SV and SVA in acetonitrile–water (80:20, v/v). The assay was validated over the concentration range (20–1000 ng ml^{-1}) of SV and 25–1000 ng ml^{-1} for SVA in plasma by assaying seven calibration standards and three control pools in triplicate on three consecutive days. The best-fit line was determined by regression analysis. SV and SVA concentrations in seeded control pools and samples were calculated using the regression parameters.

Extraction efficiency of SV and SVA

The extraction efficiencies of SV and SVA were determined at concentrations of 50 and 250 ng ml^{-1}, by comparing peak-heights of solutions of extracts versus aqueous standards.

Biological samples

Patients affected by moderate hypercholesterolemia from whom informed consent had

been obtained were treated with a single oral dose of 10 mg of simvastatin. Plasma was collected at various time intervals afterwards and extracted for HPLC analysis.

Sample preparation

Heparinized blood samples from various patients were centrifuged and plasma collected and frozen at −20°C. Samples were thawed just prior to the extraction procedure, thoroughly agitated and centrifuged at 800 *g* for 10 min. Plasma (1.0 ml) was mixed with 50 μl of a mixture of acetonitrile–water (60:40, v/v) in a 3 ml plastic centrifuge tube and agitated for 2 min in a Dubnoff mechanical shaker (150 cycles min^{-1}). Separation of the phase from precipitate was achieved by centrifugation at 1500 *g* for 3 min. The supernatant was transferred to a second tube. Fresh acetonitrile (400 μl) was added to the first tube and the same extraction procedure was repeated twice. The supernatants collected from the extractions of the same sample were pooled. This fraction was finally centrifuged and evaporated to dryness with a nitrogen stream under vacuum. The samples were filtered through a Millipore filter (0.45 μm, SJHV) and then reconstituted with (200 μl) acetonitrile–water (25:75, v/v). Aliquots of each sample (20 μl) were chromatographed.

Results and Discussion

Chromatograms of SV and SVA in human plasma samples are shown in Fig. 2. They do not contain any interfering peak with a retention time similar to that of SV and SVA, for which the average retention times were 7.2 and 3.6 min, respectively. The extraction efficiencies of SV added to human plasma at concentrations of 50 and 250 ng ml^{-1} were 95.2 (±1.3%) and 96.3 (±0.8%) whilst the extract-

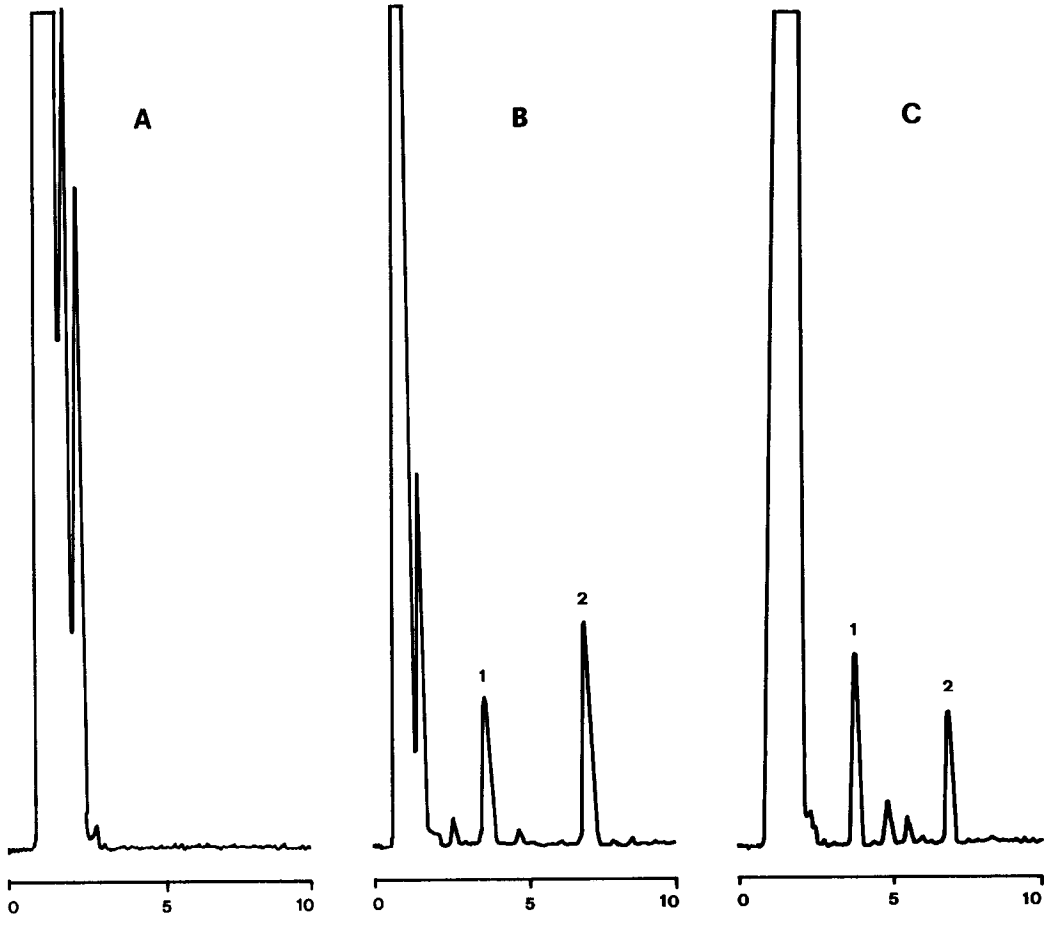

Figure 2
Chromatograms from human plasma extract. (A) Blank plasma; (B) blank plasma spiked with 200 ng ml^{-1} of SV (2) and 200 ng ml^{-1} of SVA (1); (C) plasma sample collected 1 h after a dose of 10 mg of simvastatin. All concentrations refer to sample extracts.

Table 1
Precision and accuracy of SV and SVA calibration standards in human plasma

Simvastatin				Hydroxy acid form			
Conc. (ng ml^{-1})	Conc. found* (ng ml^{-1})	RSD (%)	RE (%)	Conc. (ng ml^{-1})	Conc. found (ng ml^{-1})	RSD (%)	RE (%)
20	19.0 ± 1.1	5.63	5.0	25	24.2 ± 0.7	3.1	5.4
25	24.0 ± 0.7	3.08	4.0	50	48.7 ± 1.1	2.3	2.6
50	49.4 ± 1.1	2.27	2.1	100	97.7 ± 1.4	1.4	2.3
100	98.8 ± 1.4	1.42	1.2	200	195.5 ± 3.0	1.5	2.3
200	197.2 ± 3.0	1.53	1.4	500	487.4 ± 5.0	1.0	2.6
500	488.2 ± 5.0	1.03	2.3	1000	979.8 ± 7.5	0.7	2.0
1000	985.5 ± 7.6	0.77	1.4				

*Mean of five assays ± SD.

Table 2
Precision and accuracy of the method during a 3-day period

	Simvastatin			Hydroxy acid form		
Conc. added (ng ml^{-1})	50	250	500	50	250	500
Conc. found (ng ml^{-1})	47.6	244.8	484.2	47.5	242.5	483.8
RSD (%)	2.3	1.8	1.0	2.3	1.9	1.2
RE (%)	5.0	2.1	3.2	5.2	3.0	3.3

RE = relative error (%).

ing efficiencies from SVA were 92.8 (±1.4%) and 95.1 (±1.1%) at identical concentrations. The assay was validated by analysing seven SV and SVA standards. Peak-heights were proportional to SV and SVA concentrations over the ranges described. The best-fit line was determined by regression analysis of data for the standard solutions previously reported. The results of a typical regression were: peak-height = 0.162 x + 0.005 (r = 0.999) for SV; while for SVA the parameters were: peak-height = 0.241 x + 0.003 (r = 0.998). The mean slope and intercept data from a 3-day period are 0.160 (±0.003) and 0.004 (±0.002) for SV and 0.239 (±0.004) and 0.002 (±0.003) for SVA, respectively. The resulting equations were used to calculate the concentrations of SV and SVA in the test samples. The accuracy and precision of the calibration curves were determined from the variation of the standards ranged from the regression line. The precision for the SV calibration standard ranged from 3.1 to 5.5% (RSD) with relative errors of 5.4–2.7%, while for SVA calibration standards ranged from 3.0 to 6.1% with relative errors of 2.9–4.8% (Table 1).

Based on these results, the method is linear from 20 to 1000 ng ml^{-1} for SV and from 25 to 1000 ng ml^{-1} for SVA. Plasma samples were stable for at least 1 week when stored at −20°C. An internal standard was not added due to the simplicity of the sample preparation and the excellent precision of the data. The detection limit (signal-to-noise ratio of 3) for SV was 15 ng ml^{-1}, while that for SVA was 20 ng ml^{-1}. Assay precision and accuracy were determined by analysing three control pools in triplicate on three consecutive days. Assay precision was ±2.3 based on RSDs of 2.3, 1.8 and 1.0% for human plasma containing 50, 250 and 500 ng ml^{-1} of SV. The accuracy of the method was ±5.0%, with relative errors of 5.0, 2.1 and 3.2% for the same three human control pools for SV (Table 2). Similarly, an assay precision of ±2.3% with relative errors of ±5.2% was obtained for human plasma control pools containing SVA at identical concentrations (Table 2). A specific liquid chromatographic method has been developed for the selective monitoring of SV and SVA in human plasma samples. Data obtained from this method have allowed precise and accurate pharmacological, toxicological and pharmacokinetic interpretations.

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