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Determination of simvastatin and its active metabolite in human plasma by column-switching high-performance liquid chromatography with fluorescence detection after derivatization with 1-bromoacetylpyrene

Hisao Ochiai*, Naotaka Uchiyama, Kazuhide Imagaki, Shunsuke Hata, Toshio Kamei

Drug Metabolism, Development Research Laboratories, Banyu Pharmaceutical Co., Ltd., 810 Nishijo, Menuma-machi, Osato-gun, Saitama 360-02, Japan

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Abstract

By using a fluorescent derivatization and column-switching technique, a highly sensitive and selective high-performance liquid chromatographic (HPLC) method has been developed for the determination of simvastatin (I, β-hydroxy-δ-lactone form) and its active hydrolyzed metabolite (II, β,δ-dihydroxy acid form of I) in human plasma. A plasma sample spiked with internal standards was applied to a C_8 solid-phase extraction column. Compounds I and II were separately extracted from plasma into two fractions. Compound I in one of the fractions was hydrolyzed to II. A fluorescent derivative was prepared by esterification of II with 1-bromoacetylpyrene in the presence of 18-crown-6 for both fractions. The pyrenacyl ester of II thus obtained was purified on a phenylboronic acid (PBA) solid-phase extraction column, and was measured by column-switching HPLC with fluorescence detection. The calibration curves for both I and II were linear in the concentration range of 0.1–10 ng/ml. The intra-day coefficients of variation were less than 11.0%, and the accuracies were between 91.7% and 117% within the concentration range for both analytes. The limits of quantification (LOQ) for both analytes were set to 0.1 ng/ml. This assay method has adequate sensitivity and selectivity to measure the concentrations of I and II in human plasma from clinical studies.

Keywords: Simvastatin; 1-Bromoacetylpyrene

1. Introduction

Simvastatin (I, Fig. 1) [1], an inactive lactone, is converted to the corresponding, β,δ-dihydroxy acid (II, Fig. 1) after oral administration. Compound II is a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase which catalyzes the rate-limiting step of cholesterol biosynthesis [2]. Compound I is widely used in the treatment of hypercholesterolemia [3]. Recently, it was reported that I is also effective in reducing lethality in coronary heart disease [4]. Plasma levels of I and II following therapeutic oral doses are reported to be very low compared to levels observed after intravenous dosing [5,6], probably because only 5% of the dosed I reaches the systemic circulation [7]. Therefore, sensitive and selective methods for

*Corresponding author.

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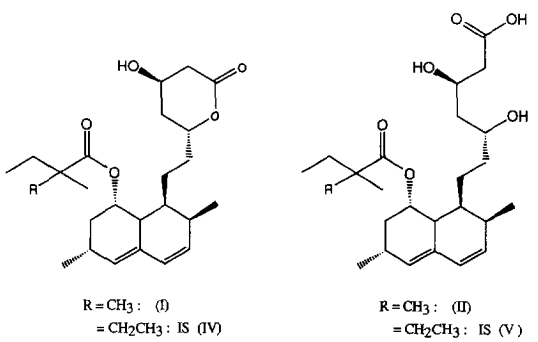

Fig. 1. Chemical structures of simvastatin, its active metabolite and internal standards fluorescence derivatization.

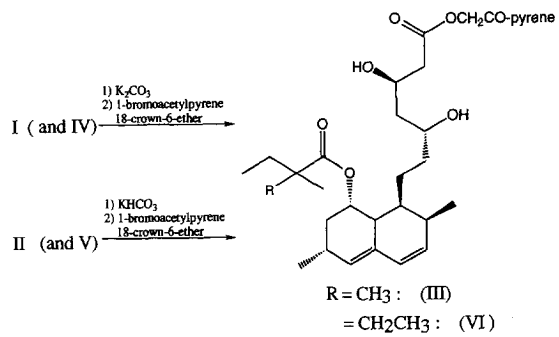

Fig. 2. Route of the fluorescence derivatization reaction.

the determination of I and II have been required for therapeutic drug level monitoring.

An enzyme assay to measure HMG–CoA reductase inhibitory activity is sensitive, but is not selective, because there are active metabolites in plasma of humans receiving an oral dose of I [8]. GC–MS methods are highly sensitive and selective enough to determine the therapeutic plasma levels of both I and II [5,6], but the operation and clean-up procedure prior to analysis seems to be complicated. The operation and pretreatment in high-performance liquid chromatographic (HPLC) methods by ultraviolet (UV) detection are simpler than those in the GC–MS method, but the HPLC method is not sensitive enough for determining the drug levels in plasma at therapeutic dose [9,10]. Because the intensity of UV absorption for I and II are insufficient for the detection of plasma levels of I and II in humans, a chromophore with sufficient intensity has to be introduced to these compounds. So, we developed a new alternative HPLC method by derivatization to highly sensitive compounds for the sensitive and selective analysis of I and II.

Reactions of carboxylic acids with 1-bromoacetylpyrene were reported to give fluorescent derivatives of carboxylic acid compounds and to be successful for their analysis in biological fluid [11,12]. To evaluate pharmacokinetics of I and II, we postulated that a limit of quantification (LOQ) as low as 0.1 ng/ml is required. Consequently, a thorough clean-up procedure was necessary to attain the LOQ. In the present study, compounds I and II were fractionated separately from plasma by C_8 solid-phase column extraction and were converted to the salt of carboxylic acid. The pyrenacyl ester of II (III, Fig. 2) was prepared with 1-bromoacetylpyrene in the presence of 18-crown-6. In order to effectively isolate the derivatives from interferences, a purification with a phenylboronic acid (PBA) solid-phase extraction column and measurement by a column-switching HPLC method were performed. This overall HPLC method described here was selective and sensitive enough to quantify plasma levels after oral administration of I to humans.

2. Experimental

2.1. Chemicals

Compound I ((+)-(1*S*,3*R*,7*S*,8*S*,8a*R*)-1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-[(2*R*,4*R*)-tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl]ethyl]-1-naphthyl 2,2-dimethylbutanoate), metabolite II, and lovastatin (2-methylbutanoate analog of I) were supplied by Merck Research Laboratories (Rahway, NJ, USA). Compounds IV and V (Fig. 1) used for internal standards were synthesized from lovastatin [1]. Reagent grade *N*,*N*-dimethylformamide (DMF), 18-crown-6 and 1-bromoacetyl-pyrene were purchased from Wako Pure Chemicals (Osaka, Japan). All other chemicals were of reagent grade from commercial sources. Water was purified with a Milli-Q system (Millipore-Japan, Tokyo, Japan). Bond Elut C_8 (500 mg, 2.8 ml), C_{18} (200 mg, 3 ml), and Bond Elut LRC PBA (100 mg, 10 ml) solid-phase extraction columns were purchased from Uniflex (Tokyo, Japan).

2.2. Preparation of standard solution

Standard solutions of I and II were prepared by dissolving them at 1 mg/ml in acetonitrile and acetonitrile–water (7:3, v/v), respectively. Further dilution of each solution was performed by dissolving in acetonitrile–water (7:3, v/v) at 20 μg/ml. These were stored below −20°C as stock solutions. Equal volumes of I and II were combined and subsequent appropriate dilutions were made in acetonitrile–water (1:9, v/v). The solution of internal standards (IV and V) was also prepared in acetonitrile–water (1:9, v/v) at 5 ng/ml each in the same manner. Plasma standards were prepared by the addition of known amount of standard solution of I and II in acetonitrile–water (0.5 ml, 1:9, v/v) to 1 ml of control plasma. The final concentrations of the analytes in plasma were 0.1, 0.5, 1, 5 and 10 ng/ml.

2.3. Sample preparation

The sample preparation procedure for the determination of I and II is described as follows. Plasma samples were stored at −80°C until assay. After addition of internal standards (IV and V, 2.5 ng each) to a plasma sample (1 ml), the mixture was applied to a C_8 solid-phase extraction column pre-conditioned with methanol (2 ml) and water (2.5 ml). The column was washed with acetonitrile–water (2 ml, 1:9, v/v) and then with methanol–water (1 ml, 3:7, v/v). Compounds II and V were eluted with methanol–water (2 ml, 6:4, v/v, acid fraction). Consecutively, I and IV were eluted with acetonitrile (2 ml, lactone fraction). Potassium carbonate (20 m*M*, 0.1 ml) was added to the lactone fraction containing I and IV. Potassium hydrogen carbonate (20 m*M*, 0.1 ml) was added to the acid fraction containing II and V. Both fractions were evaporated to dryness under reduced pressure at 40°C, and was further dried in vacuo for more than 30 min. Consequently, the lactones were hydrolyzed to the corresponding potassium carboxylates. Subsequently, both fractions were separately treated in the same way as follows. To the residue, 1-bromoacetylpyrene in DMF (10 m*M*, 0.1 ml) and 18-crown-6 in DMF (10 m*M*, 0.1 ml) were added. The reaction mixture was kept at room temperature for 30 min, and then triethylamine–acetonitrile (2 ml, 1:9, v/v) was added to effectively retain the derivatives on a PBA solid-phase extraction column. The resulting solution was applied to a PBA solid-phase extraction column. After washing with methanol (4 ml) and then with acetonitrile (2 ml), the derivatives were eluted with a mixture of propylene glycol and acetonitrile (2 ml, 4:6, v/v). The eluate was diluted with water (1 ml), and the solution was applied to a C_{18} solid-phase extraction column pre-conditioned with acetonitrile (1.5 ml) and water (2 ml). After the column was washed with acetonitrile–water (2 ml, 7:3, v/v), the derivatives were eluted with acetonitrile (3 ml). The eluate was concentrated to dryness, and the residue was reconstituted in acetonitrile–water (300 μl, 7:3, v/v) for an assay sample.

2.4. HPLC system and operating conditions

Fig. 3 shows a schematic diagram of the column-switching apparatus in the HPLC system. This system consisted of Shimadzu (Kyoto, Japan) LC-10A HPLC system equipped with three pumps (LC-10AD), a column oven (CTO-10A), an autosampler (SIL-10A), a degasser (DGU-3A), a six-port switching valve (FCV-12AH), a data processor (C-R7A), a signal cleaner (SC77, SIC, Tokyo, Japan), a system controller (SCL-10A), and a fluorescence detector (Waters 474, Milford, MA, USA).

The column-switching HPLC separation was performed with two different columns. A sample (150 μl) was injected onto a Varian Bondesil CH column (150×4.6 mm I.D., particle size 5 μm), and the column was washed with a mobile phase consisting of methanol and water (80:20, v/v) at a flow-rate of 1 ml/min. From 12.5 to 17.5 min, the flow path was switched to the analytical column, Capcell Pak C_{18}

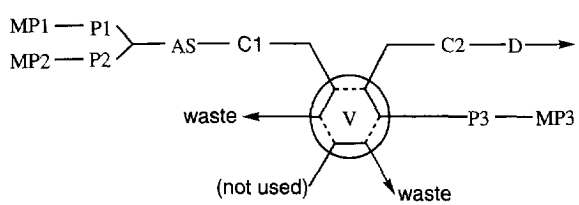

Fig. 3. Schematic diagram of the column-switching apparatus in the HPLC system. MP: mobile phase, P1-3: pump, AS: autosampler, V: switching valve. C1: Bondesil CH column, C2: Capcell pak C_{18} column, D: fluorescence detector.

(UG120, 150×4.6 mm I.D., particle size 5 μm, Shiseido, Tokyo, Japan), by valve switching. Consequently, the heart-cut fraction containing III and VI was transferred. After the valve was switched back to the initial position, the final separation of the derivatives was achieved on the analytical column by using a mixture of acetonitrile and water (80:20, v/v) as a mobile phase at a flow-rate of 1 ml/min. Meanwhile the pre-column was washed with methanol at a flow-rate of 1 ml/min for 10 min to remove late-eluting substances, and then equilibrated at the initial conditions for 6 min. The column oven was maintained at 40°C. The fluorescence detector was set at an excitation wavelength of 360 nm and an emission wavelength of 430 nm. The total analysis time was less than 35 min.

2.5. Assay validation

Calibration standards in human plasma were prepared by adding known amounts of I and II to control plasma (1 ml) in order to prepare final concentrations at 0.1, 0.5, 1.0, 5.0, and 10 ng/ml. The calibration curves for I and II were generated by measuring peak height ratio of the derivative of these analytes to that of the respective internal standard.

The intra-day coefficient of variation (C.V.) and accuracy of the quantification were assessed for the determination of I and II by analysis of plasma samples ($n=5$) spiked with the analytes at three different concentrations (0.1, 1.0, and 10 ng/ml). The C.V. and accuracy for inter-day assay were evaluated by analysis of samples ($n=2$) at the same concentrations, repeated for three different days.

3. Results and discussion

3.1. Derivatization

A pre-column fluorescence derivatization was adopted due to low intensity of UV absorption for I and II. It is known that 1-bromoacetylpyrene was a favorable derivatization reagent for carboxylic acid and has a pyrene chromophore which has high fluorescence intensity. Reaction of the potassium salt of II with 1-bromoacetylpyrene in the presence of 18-crown-6 as a catalyst gave III. Fluorescence spectrum of this derivative showed a maximum excitation wavelength at 360 nm and an emission wavelength at 430 nm. In order to obtain the optimal condition for derivatization, various factors were examined. The reaction temperature and time were tested at room temperature, 60°C and 100°C within 2 h. The reaction proceeded rapidly and adequately even at room temperature. At room temperature the peak height of derivative was almost maximal after 30 min and then constant until 2 h. At 60°C, the maximal peak height was obtained within 30 min, and gradually decreased after 30 min. The derivative was not detected at 100°C after 60 min, probably because the decomposition of the derivative was rapid. Therefore, the reaction time was fixed at 30 min and the reaction temperature was set at room temperature. As a reaction solvent, DMF was chosen since the solvent afforded slightly higher yield than any other solvent tested, such as acetonitrile, tetrahydrofuran and acetone. The effect of the concentration of 1-bromoacetylpyrene and 18-crown-6 on the yield of the reaction was examined in the concentration range 0 to 100 m*M*. Concentrations equal to and more than 10 m*M* of both reagents gave good yields. The derivative in the final reaction mixture was stable for at least 2 h at room temperature.

3.2. Purification by the PBA solid-phase extraction column

A number of interfering peaks were eluted very close to III. These peaks were assumed to be undesired products of the reaction of endogenous carboxylates with 1-bromoacetylpyrene. Attempts to remove these interferences by conventional solid-phase extraction prior to HPLC analysis were unsuccessful. It is known that PBA forms a cyclic boronate with bifunctional group like as 1,3-diol compound [13,14]. When the reaction mixture was applied to the PBA solid-phase extraction column, an excess of 1-bromoacetylpyrene and compounds which could not form the cyclic boronate were selectively washed to waste with methanol and acetonitrile. While washing, derivatives having 1,3-diol moiety were retained on PBA solid-phase extraction column. Then the derivatives were quantitatively eluted with a mixture of propylene glycol and acetonitrile (40/60, v/v). Propylene glycol was

used for elution of the derivative from the column without hydrolysis of the ester moiety. It is thought that propylene glycol cleaves cyclic boronate bonding between III and phenyl boronic acid moiety of PBA solid-phase extraction column. However, this eluate seems to be difficult to evaporate due to high boiling point of propylene glycol (bp 187°C). Consequently, the C_{18} solid-phase extraction column was used for the removal of propylene glycol.

3.3. Column-switching HPLC

Several analytical columns such as C_{18}, PH, CH, and CN were examined for HPLC separation in combination with mobile phases of methanol–water or acetonitrile–water. Above all, a combination of C_{18} column with acetonitrile–water gave a sharp peak for III, but complete separation of the derivative from interfering peaks could not be attained. On the other hand, the use of CH column with methanol–water made it possible to effectively separate the derivative from the interferences; however, the peak of derivative became broad. In order to exploit advantageous characters of these two different chromatographic systems, the separation by CH column was combined with the separation by C_{18} column using a column–switching technique. The effluent containing the derivative of analytes and internal standards from the CH column was introduced onto the C_{18} column in the heart-cut mode. As a result, III was completely separated from endogenous interfering peaks, and showed a sharp peak.

3.4. Calibration curves, coefficient of variation, and accuracy

The calibration curves exhibited linearity over the range of 0.1–10 ng/ml with the correlation coefficients greater than 0.999. The mean calibration curve for I was given by the equation including the S.D. ($n=4$), $y=0.5945(\pm 0.0082)\ x-0.0183(\pm 0.0032)$, and for II, $y=0.6609(\pm 0.0137)\ x+0.0276(\pm 0.0102)$, where y indicates the peak height ratio, x represents the concentration of respective analyte in ng/ml. The calibration curves were drawn singularly on each analysis day. Fig. 4(1)-a and (1)-b show the chromatograms of the lactone fraction obtained from drug-free plasma and plasma

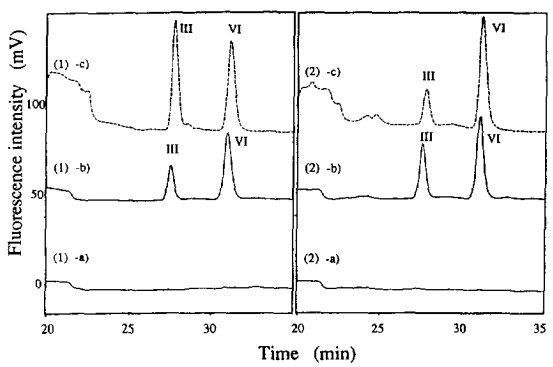

Fig. 4. Typical chromatograms obtained from lactone fraction (1) and acid fraction (2); (a) drug-free plasma, (b) drug-free plasma spiked with I (or II) at 1 ng/ml, (c) plasma collected at 2 h after oral administration of I (10 mg per man).

spiked with I (1 ng/ml), respectively. Fig. 4(2)-a and (2)-b show the chromatograms of the acid fraction obtained from drug-free plasma and plasma spiked with II (1 ng/ml), respectively. No significant interference peak from endogenous substances in plasma was observed in the chromatograms. The results of intra-day and inter-day analyses of I and II are summarized in Table 1 and Table 2, respectively. The intra-day coefficients of variation did not exceed 11.0%, and the intra-day accuracies were between 91.7% and 117% within the concentration range of the calibration curve for both analytes. The inter-day coefficients of variation did not exceed 12.8%, and the inter-day accuracies were between 90.9% and 116% within the concentration range of the calibration curve for both analytes. The limits of quantification for both analytes were set to 0.1 ng/ml, which is the lowest concentration of the analyte that can be measured with a coefficient of variation and an accuracy from theory of less than 20%. The limits of detection were 0.02 ng/ml; at this concentration the signal-to-noise ratio was better than 5:1 due to use of the noise cleaner. The overall mean recoveries of I and II at 10 ng/ml were almost same, approximately 40%.

The derivative prepared from I or II in plasma after sample pretreatment was stable for at least 3 days in reconstituted solution within an autosampler. In plasma, hydrolysis of I was not observed for at least 40 min [15]. Actually, I and II in plasma were stable for at least 60 min at room temperature.

Table 1
Intra-day coefficient of variation and accuracy of measurement of I and II in human plasma

Spiked concentration (ng/ml)	I			II		
	Found concentration (ng/ml)	Coefficient of variation (%)	Accuracy (%)	Found concentration (ng/ml)	Coefficient of variation (%)	Accuracy (%)
10.000	10.397±0.131	1.3	104	10.317±0.105	1.0	103
1.000	0.917±0.007	0.8	91.7	0.982±0.020	2.1	98.2
0.100	0.117±0.003	2.6	117	0.105±0.012	11.0	105

Each value was obtained from five determinations within an assay.
The value of found concentration represents mean±S.D.

Table 2
Inter-day coefficient of variation and accuracy of measurement of I and II in human plasma

Spiked concentration (ng/ml)	I			II		
	Found concentration (ng/ml)	Coefficient of variation (%)	Accuracy (%)	Found concentration (ng/ml)	Coefficient of variation (%)	Accuracy (%)
10.000	10.400±0.265	2.5	104	10.267±0.208	2.0	103
1.000	0.909±0.019	2.1	90.9	1.001±0.009	0.9	100
0.100	0.116±0.007	6.2	116	0.108±0.014	12.8	108

Each value was obtained from assays on three different days.
The value of found concentration represents mean±S.D.

Compounds I and II in plasma were also stable after three repeated freezing and thawing cycles.

Interconversions between I and II during sample preparation process were examined using plasma spiked with I or II (10 ng/ml) in the absence of the other. As a result, no significant interconversion was observed in the chromatograms.

3.5. Application to clinical samples

The described method was applied to the determination of plasma levels of I and II in humans after oral administration of I (10 mg). Fig. 4 (1)-c and (2)-c show chromatograms obtained from 2 h post-dose plasma of the volunteer. The measured concentrations of I and II in this sample were 2.05 and 0.411 ng/ml, respectively. Mean plasma concentration–time curves of I and II after a single oral dose of I (20 mg) to three volunteers are depicted in Fig. 5. The mean C_{max} values for I and II were 4.14 and 4.11 ng/ml, respectively.

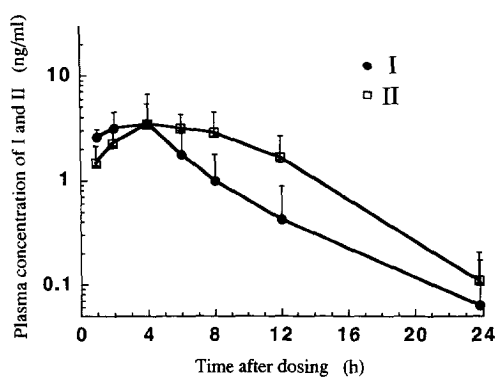

Fig. 5. Plasma concentration of I and II after single oral administration of I (20 mg per man) in three healthy male volunteers.

4. Conclusion

A selective and highly sensitive column-switching HPLC method for the quantification of I and II in human plasma was developed using precolumn derivatization with 1-bromoacetylpyrene and its fluo-

rescence detection. In the purification process prior to HPLC analysis, the use of PBA solid-phase extraction column drastically removed interferences originating from endogenous substances. Further HPLC separation using the column-switching technique enabled selective detection of pyrenacyl ester of analytes. This method has the adequate quantification limits (0.1 ng/ml) for both analytes, and was applied to the determination of I and II in plasma after oral administration of I to humans.

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