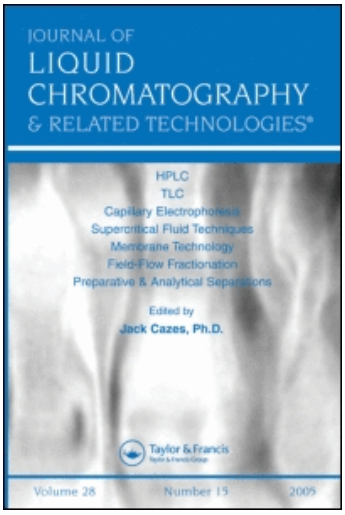

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Determination of Simvastatin in Human Plasma by Column-Switching HPLC with UV Detection

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ABSTRACT

The purpose of this study was to develop and validate a sensitive and specific analytical method for determination of simvastatin in human plasma by the column-switching high performance liquid chromatography (HPLC) system with UV detection. Simvastatin was extracted in diethyl ether from plasma. The residue was dissolved in mobile phase I [acetonitrile–20 mM potassium phosphate buffer (45 : 55, v/v, pH 5.6)] and the solution was injected into a pre-column. The analytes fractionated from the pre-column by a valve switching step were concentrated on the top of an intermediate C_{18} column. Then, the concentrated simvastatin was separated to the analytical column with a mobile phase

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II [acetonitrile–20 mM potassium phosphate buffer (65 : 35, v/v, pH 5.6)], using a UV detector at the wavelength of 238 nm. Simvastatin was eluted at 28.7 min without interference of endogenous material in plasma. The limit of quantification (LOQ) was 0.5 ng/mL of simvastatin. The calibration curve was linear in the concentration range of 0.5–20 ng/mL ($r^2 = 0.9986$). Moreover, the inter- and intra-day precisions of this method were less than 15%. The average recovery of extraction was 89.7% over the concentration. The assay was successful in measuring plasma concentrations of simvastatin in three volunteers after oral administration (80 mg simvastatin).

Key Words: Simvastatin; HPLC; Human plasma; Column-switching.

INTRODUCTION

Simvastatin, an analog 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″-oxo-butoxy)-1′-naphthaleneheptanoic acid, which lowers plasma cholesterol by inhibiting 3-hydroxy-3-methylglutaryl-CoA reductase. It is a highly effective cholesterol lowering agent, which is widely used in the treatment of hypercholesterolemia. Recently, it was reported that simvastatin is also effective in reducing lethality in coronary heart disease. Plasma levels of simvastatin following therapeutic oral doses were reported to be very low compared with levels observed after intravenous dosing,[1] probably because only 5% of the dosed simvastatin reaches the systemic circulation.[2] Therefore, sensitive and selective methods for the determination of simvastatin have been required for therapeutic drug level monitoring.

There are several methods for the determination of simvastatin in biological fluid, including liquid chromatography—mass spectrometry (LC–MS),[3] liquid chromatography-ultra violet detection (LC–UV),[4,5] and LC with fluorescence detection.[6] LC–MS methods are highly sensitive and selective enough to analysis the therapeutic plasma level of simvastatin, but the operation procedure is complicated.[3] Two LC–UV methods are not sensitive enough for determining the drug levels in plasma at therapeutic dose.[4,5] Although LC with fluorescence detection is a highly sensitive method, the samples need complex derivatization before their analysis, which is inconvenient,[6] Therefore, there is a need to develop a sensitive high performance liquid chromatographic method of simvastatin without time-consuming derivatization.

Here, the aim of the present study was to develop a sensitive, convenient, and accurate semi-micro high performance liquid chromatography (HPLC) method using column-switching for the determination of simvastatin in human

plasma. This assay method has adequate sensitivity and selectivity, enough to quantify plasma levels after oral administration of simvastatin to humans.

EXPERIMENTAL

Materials

Simvastatin [Fig. 1(a)] and lovastatin [Fig. 1(b)] were obtained from Dae Wha Pharm. Co. (Seoul, Korea). HPLC-grade methanol and acetonitrile were purchased from Burdick & Jackson, Inc. (Muskegon, MI). All other chemicals were analytical grade and used without further purification.

Preparation of Standards

Stock solutions of simvastatin and lovastatin, as an internal standard (IS), were prepared in methanol (1 mg/mL), respectively. The stock solution of simvastatin was subsequently diluted with acetonitrile to give concentrations of working standards of 0.05, 0.2, 0.5, 1, and 2 μg/mL. Standard solutions of simvastatin in human plasma were made by spiking each 10 μL of the diluted working standard solution. Lovastatin (IS) was diluted with acetonitrile to give a final concentration of 2 μg/mL solution.

Preparation of Samples

Each 1 mL of plasma was transferred into a glass test tube and spiked with 10 μL of lovastatin (IS, 2 μg/mL). Then, 10 N HCl was added to make simvastatin exist as a hydrochloride salt. It prevents simvastatin from volatilization during evaporation. After the plasma samples were extracted with 6 mL of diethyl ether, the mixtures were centrifuged at 3000*g* at 4°C for 10 min and the clear supernatants were transferred into other glass tubes. The supernatants were evaporated under a stream of nitrogen. The residue was reconstituted in 250 μL of mobile phase I [acetonitrile-20 mM potassium phosphate buffer (45 : 55, v/v, pH 5.6)]. An aliquot of 160 μL was injected into the HPLC system with the autosampler for analysis.

Column-Switching System and HPLC Conditions

The configuration of the column-switching system using three columns (Fig. 2) consisted of the Nanospace SI-1 series (Shiseido, Tokyo, Japan),

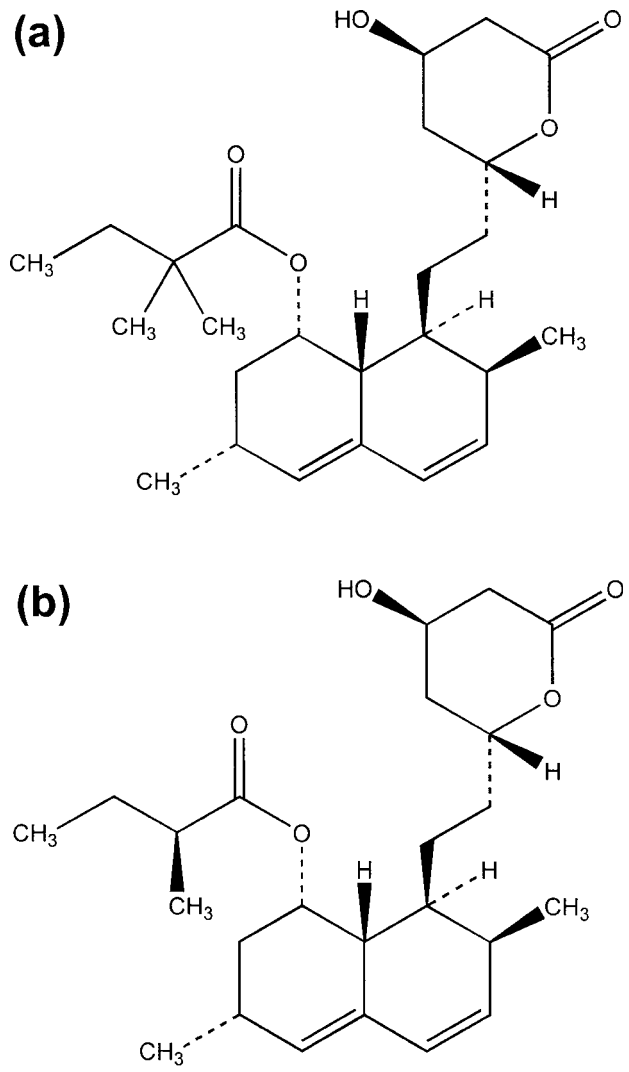

Figure 1. Chemical structures of (a) simvastatin and (b) lovastatin.

i.e., two 2001 pumps, a 2002 UV-VIS detector, a 2003 autosampler, a 2004 column oven, a 2012 high pressure switching valve, and a 2009 degassing unit. The signals were processed by dsChrom98 (Donam, Seoul, Korea).

In order to remove proteins from plasma samples, they were pre-separated on a Capcell Pak MF C_8 pre-column (150 mm $\times$ 4.6 mm I.D., Shiseido). The

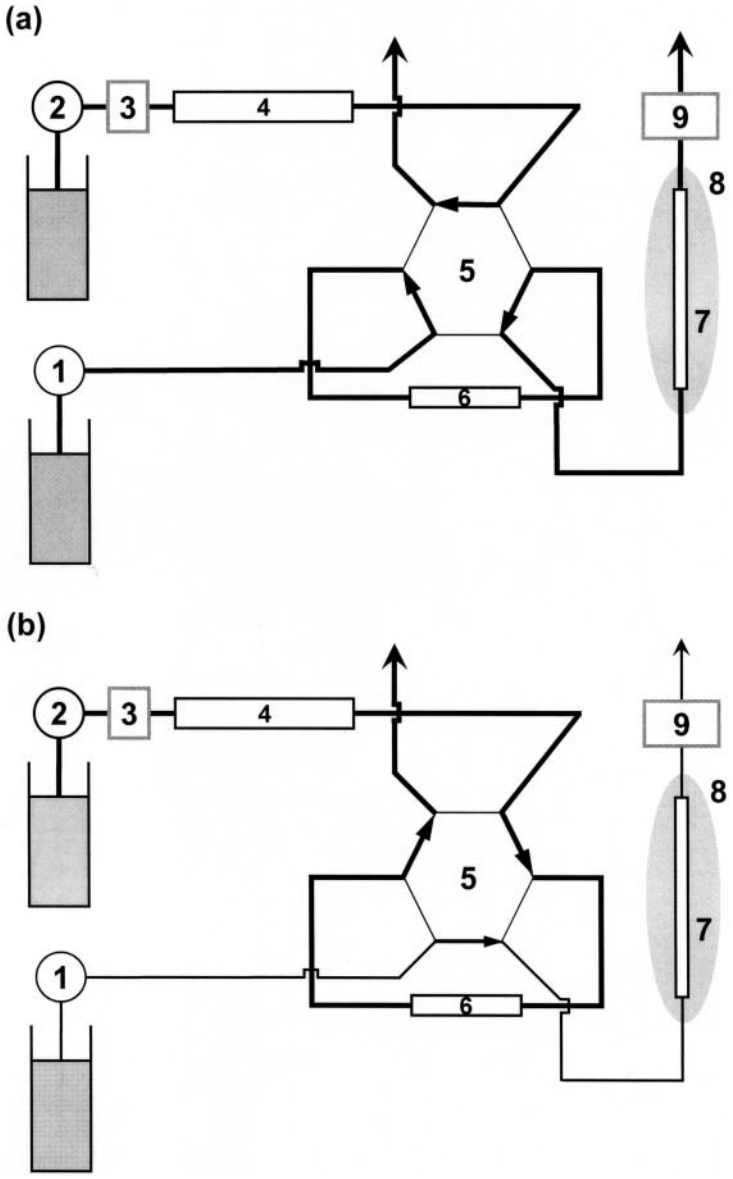

Figure 2. Schematic diagram of HPLC. (a) Removal position of proteins and separation position. (b) Concentration position. 1, Pump (for separation); 2, pump (for pre-treatment); 3, autosampler; 4, pre-column; 5, switching valve; 6, intermediate column; 7, analytical column; 8, column oven; 9, UV detector.

drug molecule fractions from the primary separation were transferred to an intermediate column (Capcell Pak C_{18} UG 120U, 35 mm × 2.0 mm I.D., Shiseido), and the final separation was performed on a Capcell Pak C_{18} analytical column (250 mm × 1.5 mm, I.D., 5 μm, Shiseido). The column temperature was 45°C and the effluent was monitored at 238 nm.

Analytical Procedure

Step 1 (0.0–7.0 min, Valve Position A)

The plasma sample (160 μL) was introduced onto a Capcell Pak MF C_8 pre-column where plasma proteins, simvastatin, and lovastatin were separated using mobile phase I at a flow-rate of 0.5 mL/min. The intermediate C_{18} column and analytical column were equilibrated using the mobile phase.

Step 2 (7.0–9.6 min, Valve Position B)

When the valve status was changed to B, the target drug-containing zone separated in Capcell Pak MF C_8 pre-column was focused onto the top of an intermediate C_{18} column using mobile phase I at a flow-rate of 0.5 mL/min. The analytical column was equilibrated using the mobile phase.

Step 3 (9.6–35 min, Valve Position A)

The analytes trapped in the intermediate C_{18} column were transferred to a Capcell Pak C_{18} analytical column, and separated by using 0.1 mL/min of mobile phase II [acetonitrile–20 mM potassium phosphate buffer (65 : 35, v/v, pH 5.6)] when the valve status was switched back to the position A. At the same time, the MF C_8 pre-column was equilibrated with a mobile phase I.

Method Validation

The chromatographic method was quantified to evaluate the recovery, linearity, precision [the coefficient of variation (CV) of replicate analysis], and accuracy (the bias between theoretical and actual concentration).

Application to Human Plasma Samples

Three healthy male volunteers received oral doses of four simvastatin tablets (80 mg). Blood samples (4 mL) were withdrawn from the forearm

vein at 0, 0.33, 0.67, 1.0, 1.5, 2, 2.5, 3, 4, 6, 8, and 12 hr post-dosing, transferred to Vacutainer® tubes, and centrifuged at 600*g* for 15 min (4°C). Then, plasma samples were transferred to eppendorf tubes and stored at −70°C prior to analysis. The peak concentration (C_{max}) and the time to peak concentration (t_{max}) of simvastatin were determined by visual inspection from each volunteer's plasma concentration–time plots for simvastatin. The area under the plasma concentration–time curves (AUC) was calculated by the linear trapezoidal method from 0 to 12 hr. Plasma elimination half-life ($t_{1/2}$) of simvastatin was determined from the descending slope of the concentration–time profiles after logarithmic transformation of the concentration data.

RESULTS AND DISCUSSION

Column-Switching Procedure

The column-switching technique is a useful sample preparation system that can analyze biological samples in the hundreds of microliters without any loss in the sensitivity increase and chromatographic efficiency obtained by semimicro columns.[7] In the column-switching technique, the choice of the pre-column packing, washing solvent, and valve switching time is crucial in order to obtain complete adsorption of simvastatin and lovastatin from biological samples, and to remove unwanted components from the pre-column.[8,9]

The Capcell Pak MF column, used as the pre-column in this study, has a property of a mixture of hydrophilic and hydrophobic phases on silicone polymer-coated silica beads[10,11] and, therefore, minimized interference by the biological hydrophilic components such as proteins or bilirubin.

The separation profile of simvastatin and lovastatin in plasma on the pre-column was evaluated using mobile phase I to obtain good recovery and to determine the appropriate valve switching time (Fig. 3). Simvastatin and lovastatin were retained in the pre-column during the exclusion of the plasma proteins, which was seen at the position of void volume. The peak of simvastatin and lovastatin appeared from 7.0 to 8.2 min and 8.2 to 9.6 min, respectively. Therefore, the eluate of the pre-column from 7.0 to 9.6 min after injection of the plasma sample was transferred to the intermediate C_{18} column by switching the valve to position B.

The analytes isolated from MF C_8 pre-column by the valve switching step to B position were transferred to the top of the intermediate C_{18} column. The intermediate C_{18} column was used for obtaining sharp peaks in the final separation, protecting a analytical column from high pressure and saving the analysis time.[12] Since the Capcell Pak C_{18} analytical column's diameter was 1.5 mm, the flow-rate was reduced to 0.1 mL/min during analysis.[13]

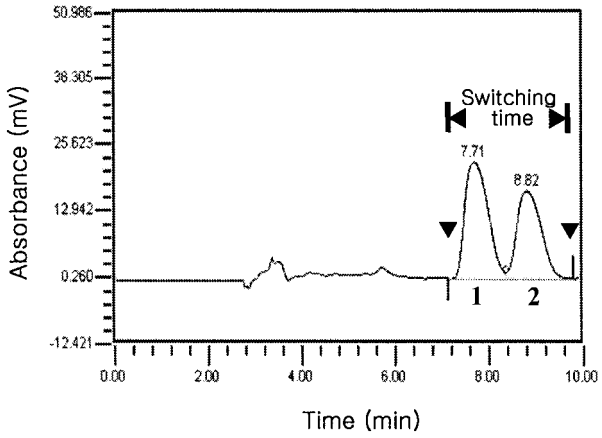

Figure 3. Separation of simvastatin and lovastatin-spiked mobile phase on MF C_8 pre-column (150 mm × 4.6 mm I.D.). Conditions: mobile phase I [acetonitrile–20 mM potassium phosphate buffer (45 : 55, v/v, pH 5.6)], flow-rate, 0.5 mL/min; injection volume, 160 μL. Peaks: 1, IS (lovastatin); 2, simvastatin.

Extraction Recovery

Recovery of the extraction procedure was determined for simvastatin at low (2 ng/mL), middle (5 ng/mL), and high (20 ng/mL) concentrations on the calibration curve. The recovery of IS (lovastatin) was determined at its working concentration of 20 ng/mL. Recovery was calculated by comparing the absolute peaks of the standards in human plasma, prepared as per the assay procedure, to neat standards evaporated and reconstituted in the same fashion as the extracted samples. The results are listed in Table 1. Recovery for simvastatin was higher than 86% at all tested concentrations. The recovery for the IS (lovastatin) was 83%.

Table 1. Extraction recovery for the determination of simvastatin in human plasma ($n = 5$).

Simvastatin concentration (ng/mL)	Recovery (%)
2	86.9 ± 4.7
5	91.5 ± 3.5
20	90.7 ± 2.8

Selectivity

Figure 4 shows chromatograms of extracted blank plasma and a plasma standard spiked to contain simvastatin and IS (lovastatin) at 20 ng/mL each, respectively. The selectivity of the assay is illustrated by the fact that no endogenous peaks are present at the retention times of simvastatin or IS (lovastatin) in either the control or pre-dose chromatograms.

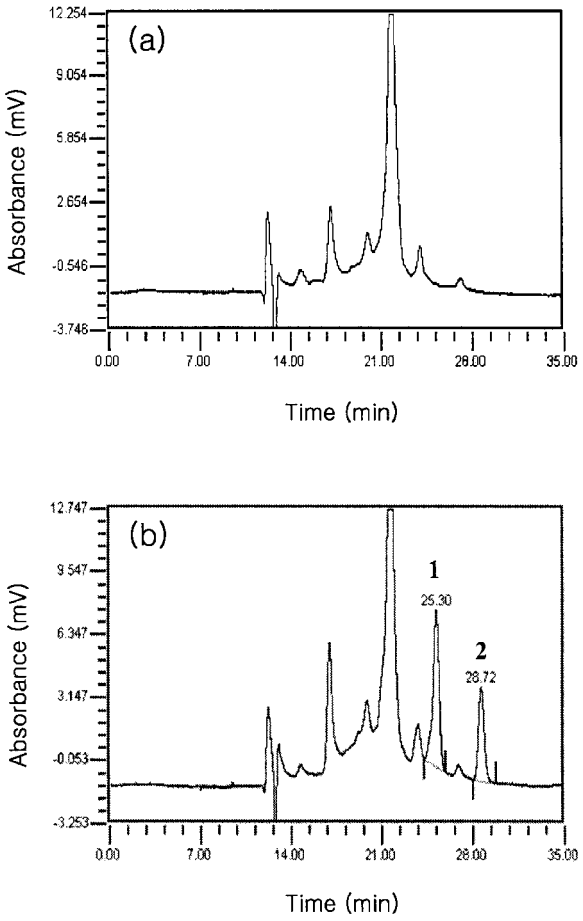

Figure 4. Representative chromatograms of (a) blank plasma and (b) plasma standard containing 20 ng/mL simvastatin and 20 ng/mL IS (lovastatin). Peaks: 1, IS (lovastatin); 2, simvastatin.

Linearity

The calibration curves were linear in the studied range. The mean equation of the calibration curve consisting of five points was $y = 0.0246(\pm 0.0005)x - 0.0002(\pm 0.0075)$ with correlation coefficient $r^2 = 0.9992(\pm 0.0005)$, where y represents the ratio of simvastatin peak area and the IS (lovastatin) one and x represents the simvastatin concentration (ng/mL).

Precision and Accuracy

Assessments of the intra- and inter-day variability of the assay were conducted in five different lots of human plasma spiked with simvastatin over the calibration range of 0.5–20 ng/mL. The resulting assay precision and accuracy data are presented in Table 2. The intra- and inter-day precisions of the assay, as measured by the CV (%), were 14.75% or better for all points on the calibration curves. Assay accuracy was found to be within 12.9% of nominal for all standards.

Limit of Quantification

The limit of quantification (LOQ) of the assay, defined as the lowest concentration that yielded a intra-day CV of less than 15% and an intra-day accuracy of between 85% and 115% of nominal concentration, was 0.5 ng/mL of simvastatin. This LOQ was sufficient to support human studies with the highest recommended oral dose of simvastatin (80 mg).

Table 2. Reproducibility of simvastatin determination in human plasma ($n = 4$).

Simvastatin concentration (ng/mL)	Precision[a] (%)		Accuracy[b] (%)
	Intra-day	Inter-day	
0.5 (LOQ)	12.57	14.75	112.9
2	10.58	13.79	96.51
5	7.82	8.16	101.6
10	6.18	9.96	97.31
20	5.72	7.39	101.0

[a]CV of peak area ratio of simvastatin/IS (lovastatin).
[b]Expressed as [(actual concentration)/(theoretical concentration)] × 100.

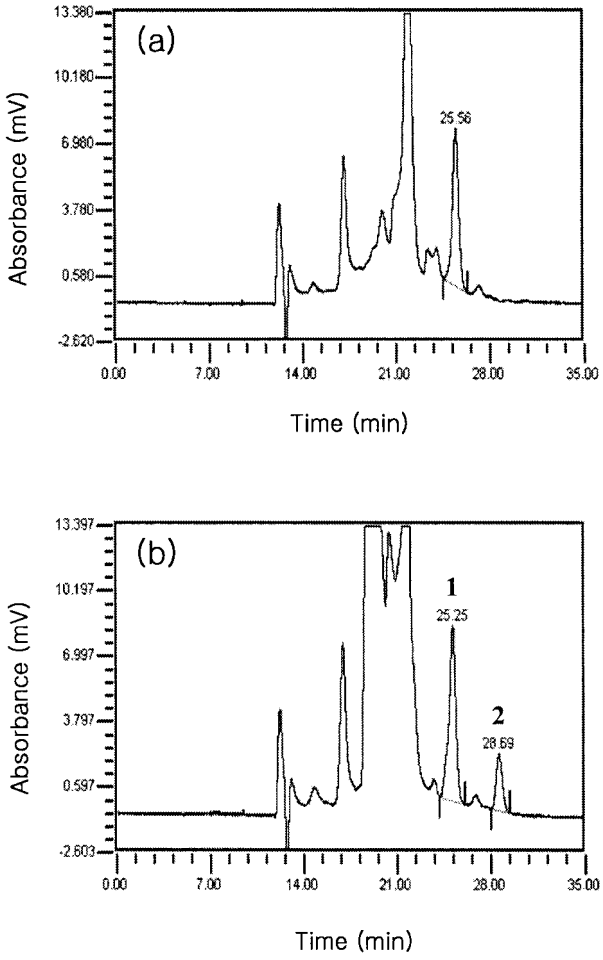

Figure 5. Representative chromatograms of plasma samples obtained from a subject receiving oral administration of simvastatin (80 mg). (a) Pre-dose sample; (b) sample at 1.5 hr after oral administration 80 mg of simvastatin. Peaks: 1, IS (lovastatin); 2, simvastatin.

Application to Human Plasma Samples

The suitability of this method was proven in the pharmacokinetic study of simvastatin after oral dosing of four simvastatin tablets (80 mg) to healthy male volunteers. The plasma chromatogram of a volunteer administered

simvastatin is shown in Fig. 5(b), which shows a typical chromatogram of simvastatin in plasma collected at 1.5 hr after oral administration of 80 mg to human subjects. The plasma concentration of simvastatin at 1.5 hr was 11.05 ng/mL.

Figure 6 shows the mean plasma concentration–time profiles of simvastatin in three subjects. C_{max}, t_{max}, AUC, and $t_{1/2}$ of simvastatin were 11.2 $\pm$ 0.28 ng/mL, 1.5 hr, 37.65 $\pm$ 0.83 ng hr/mL, and 3.05 $\pm$ 0.23 hr, respectively. These values are comparable to the corresponding parameters obtained by single oral doses of 20 or 40 mg simvastatin in the previous reports.[3,6] From these results, it is suggested that the present column-switching semi-micro HPLC analysis can be applied to the determination of simvastatin in human plasma.

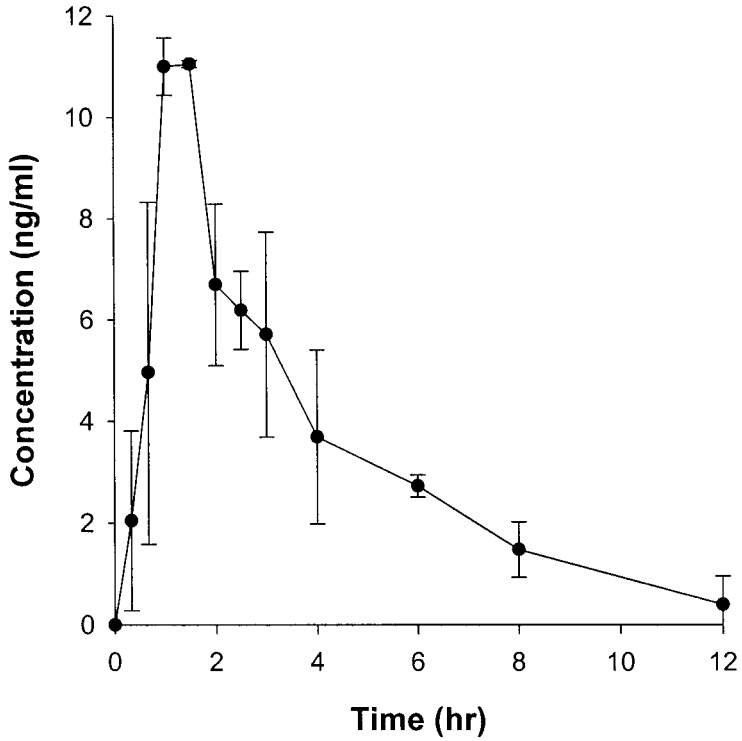

Figure 6. Plasma concentration–time profiles of simvastatin following oral administration of four simvastatin tablets (80 mg) in three male volunteers. The results represent the mean value $\pm$ SD ($n = 3$).

CONCLUSION

A semi-micro HPLC method using column-switching was efficiently developed, validated, and applied for assaying simvastatin in human plasma. It showed excellent sensitivity, reproducibility, and specificity for the sample analysis of simvastatin. The method was successfully applied to evaluate the pharmacokinetic study of simvastatin in human plasma. The use of the column-switching semi-micro HPLC allows for accurate, precise, and reliable measurement of simvastatin concentrations in human plasma for up to 12 hr after oral administration.

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