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Short communication

Column-switching high-performance liquid chromatographic analysis of fluvastatin in rat plasma by direct injection

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Abstract

A column-switching high-performance liquid chromatographic (HPLC) method has been developed and validated for quantification of fluvastatin in rat plasma. Plasma samples were diluted with an equal volume of mobile phase, i.e. acetonitrile–5 mM potassium phosphate buffer (pH 6.8) (15:85, v/v), and the mixture was directly injected onto the HPLC system. The analyte was enriched in a pre-treatment column, while endogenous components were eluted to waste. The analyte was then back-flushed onto an analytical column and quantified with fluorescence detection (λ_{ex} = 305 nm; λ_{em} = 390 nm). The standard curve for the drug was linear in the range 0.5–100 ng mL^{-1} in rat plasma. The limit of quantitation for plasma was found to be 0.5 ng mL^{-1}. This method has been fully validated and shown to be specific, accurate and precise. The method is simple and rapid because of a minimized sample preparation and appears to be useful for the pharmacokinetic study of fluvastatin.

Keywords: Fluvastatin; Column-switching; High-performance liquid chromatography (HPLC); Rat plasma; Direct injection

1. Introduction

Fluvastatin sodium ([*R**,*S**,-(*E*)]-(±)-7-[3-(4-fluorophenyl)-1-(1-methylethyl)-1*H*-indol-2-yl]-3,5-dihydroxy-6-heptenoic acid, monosodium salt) is reversible, competitive and highly specific inhibitor of microsomal 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase and is used for the treatment of hypercholesterolemia (Fig. 1) [1–3]. The five HMG-CoA reductase inhibitors available presently are lovastatin, simvastatin, pravastatin, fluvastatin and atorvastatin. Lovastatin, simvastatin and pravastatin have similar structures while fluvastatin is a totally synthetic molecule and has structure distinct from the other statins. Analytical methods for the determination of fluvastatin in biological fluids have been reported based on HPLC methods using UV or fluorescence detection [4,5]. In addition, methods for the determination of plasma fluvastatin concentrations involve complex procedures such as liquid–liquid extraction (LLE) or solid-phase extraction (SPE).

Sample extraction procedure before chromatographic analysis in bioanalysis remains the major challenge for the determination of drugs in biological matrix. Numerous laborious and time-consuming off-line sample clean-up procedures based on LLE or SPE have been reported to get rid of some interferences. And besides, there are literatures introducing not only turbulent flow chromatography (TFC) which is a relatively new technique used for rapid extraction and analysis of drugs from bioanalytical fluids but also restricted-access media (RAM) using technique that is a relatively new one, uses interaction between RAM and small analytes and exclusion of big molecules [6–8]. Nowadays, several HPLC methods are described in literature for some drugs in various biological matrices based on direct injection of the samples without sample clean-up [9]. And especially, column-switching systems via a switching valve have great advantages in permitting the direct injection of protein rich samples such as plasma [10–12].

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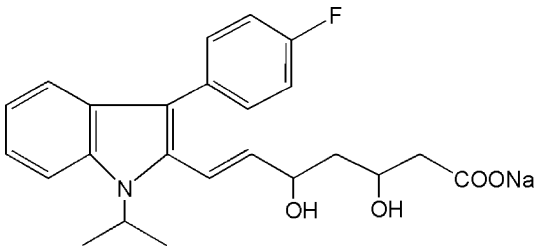

Fig. 1. The chemical structure of [R^*,S^*,-(*E*)]-(±)-7-[3-(4-fluorophenyl)-1-(1-methylethyl)-1*H*-indol-2-yl]-3,5-dihydroxy-6-heptenoic acid, monosodium salt (fluvastatin sodium).

The column-switching technique avoids off-line procedures for sample clean-up [10–13]. This method allows plasma samples to be directly injected onto a fully automated HPLC system, thereby resulting in an accurate assay without internal standards.

To minimize the possible introduction of analytical artifacts through excessive sample manipulation, an analytical method was developed and validated in our laboratory using high-performance liquid chromatography with column-switching by direct injection [10,12,14]. The purpose of the present study was to develop a simple, rapid and reliable HPLC method using column-switching for the direct analysis of fluvastatin in rat plasma without pre-purification step. The applicability of this method was proved by the pharmacokinetic study of fluvastatin after oral administration in rats.

2. Experimental

2.1. Chemicals

Fluvastatin was kindly donated by Novartis Korea (Seoul, Korea). HPLC grade acetonitrile was purchased from J.T. Baker (Deventer, The Netherlands). Analytical grade dipotassium hydrogenphosphate and potassium dihydrogenphosphate were obtained from Wako (Osaka, Japan). Water was purified with a Ultimate Reverse Osmosis system (Barnstead, Dubuque, USA). And the others used as reagents were of analytical or HPLC grade.

2.2. Equipments

The semi-microcolumn nanospace SI-2 HPLC system (Shiseido, Tokyo, Japan) consisted of two 3001 pumps, a 3023 autosampler with a sample cooler, a 3004 column oven, a fluorescence 3013 detector, a two-flow channel 3009 degasser and a high pressure six-port switching 3012 dual valve. The system included a pre-column, a trap column and an analytical column. The chromatography, switching valves and autosampler were controlled by a EZ Chrom elite (Scientific Software Inc., CA, USA) as a fully automated system. Data were acquired and processed on the software.

2.3. Chromatographic conditions

Chromatographic separation was achieved on a 150 mm × 1.5 mm, 5 μm Capcell pak C_{18} column (Shiseido, Tokyo, Japan) with a 50 mm × 4.6 mm, 5 μm Capcell pak mixed-functional (MF) ph-1 pre-column (Shiseido, Tokyo, Japan) and a 35 mm × 2.0 mm, 5 μm Capcell pak C_{18} trap column. The column temperatures of the pre-column and the analytical column were maintained at 40 °C and the trap column was operated at ambient temperature. The mobile phase A (Pump A) was acetonitrile–5 mM potassium phosphate buffer (pH 6.8) (15:85, v/v) and the mobile phase B (Pump B) was acetonitrile–5 mM potassium phosphate buffer (pH 6.8) (40:60, v/v). The flow rate of mobile phase A was 500 μL min^{-1} and mobile phase B was 150 μL min^{-1}. The autosampler was set with an injection volume of 25 μL, a rack temperature of 4 °C and a run time of 20 min. The fluorescence detector was operated at an excitation wavelength of 305 nm and an emission wavelength of 390 nm.

2.4. Column-switching procedure

Fig. 2 shows a schematic diagram of the automated system. The system was operated according to the following procedure where valve positions and switchover times are in parentheses.

Step 1 (valve A; 0–6 min): A plasma sample was injected onto the pre-column and the pre-column was washed by the mobile phase A at a flow rate of 500 μL min^{-1} in order to remove plasma proteins and other endogenous interferences. Data acquisition started in detector. Step 2 (valve B; 6–8 min): The valve was switched from position A to position B and the enriched compounds were eluted from the pre-column to the trap column in the back-flush mode. Step 3 (valve A; 8–20 min): Afterwards, the flow path of the mobile phase was returned to the initial condition and the enriched compounds were eluted from the trap column to the analytical column for separating the analyte in the analytical column from the co-eluted endogenous compounds with the mobile phase B.

2.5. Stock solution and calibration standards

About 1.0 mg of fluvastatin was accurately weighed into volumetric flask and dissolved in 10 mL of methanol to obtain the

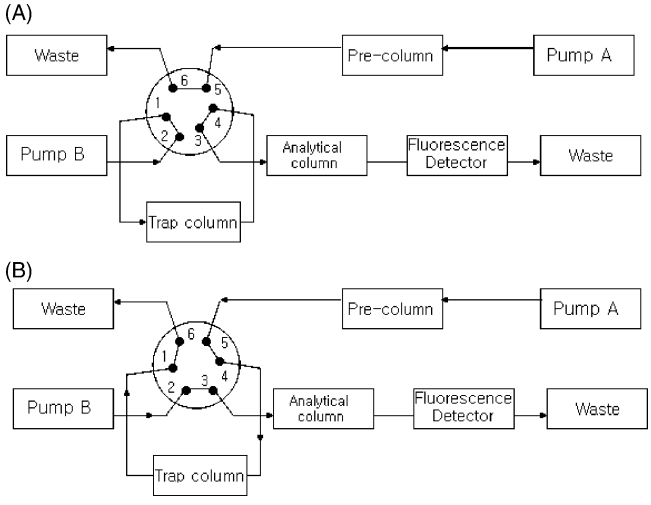

Fig. 2. Schematic diagram of the column-switching HPLC system using a six-port switching valve. Step1: valve A; step 2: valve B; step 3: valve A.

stock solution (100 μg mL^{-1}). The stock solution was made for the preparation of calibration standards. The concentrations of fluvastatin used for the preparation of calibration standards were 1, 2, 6, 10, 20, 50, 100 and 200 ng mL^{-1}. They were obtained from serial dilution of stock solution with mobile phase A. The diluted standard solutions were spiked into the plasma (50:50, v/v). Final concentrations for calibration standards were 0.5, 1, 3, 5, 10, 25, 50 and 100 ng mL^{-1}. After then, these mixtures were centrifuged at 1000 × *g* at 4 °C for 15 min. The supernatants were analyzed without sample clean-up procedure in off-line.

2.6. Validation study

The analytical method for determination of fluvastatin in rat plasma was developed according to the guidance of bioanalytical method validation [15]. The limit of quantitation, specificity, linearity, accuracy, precision and stability of fluvastatin in plasma samples were evaluated.

2.6.1. Accuracy and precision

The intra-day accuracy (%) and precision (coefficient of variation; CV) were estimated by analyzing five replicates containing fluvastatin at four different concentrations, i.e. 0.5, 3, 25 and 50 ng mL^{-1} in a day. The inter-day accuracy (%) and precision (CV) were determined by analyzing five replicates at the four different concentrations within a week. The acceptance criteria for intra-day and inter-day accuracy and precision are below 15% bias or CV except at the lower limit of quantitation (LLOQ), for which bias and precision should be below 20%.

2.6.2. Linearity

The linearity of the method was evaluated by analysis of calibration standards. The final concentrations of fluvastatin in the prepared samples were 0.5, 1, 3, 5, 10, 25, 50 and 100 ng mL^{-1}. Calibration curve was generated by plotting peak areas against drug concentrations. The coefficient of determination (r^2) was determined.

2.6.3. Stability

The stability tests of fluvastatin in plasma were carried out by evaluating the freeze and thaw stability, short-term temperature stability, long-term stability, stock solution stability and post-preparative stability. The tests of stability were assessed with two concentrations of samples, i.e. 3 and 50 ng mL^{-1} as low and high concentrations using at least three aliquots. In the freeze and thaw stability test, the samples were stored at −74 °C for 24 h and thawed at room temperature for approximately an hour with three freeze/thaw cycles. The short-term stability was assessed after storage of samples at room temperature for 4–24 h while the long-term stability was evaluated after storage of samples more than longer period of last sample analysis (4 weeks). Stock solution of fluvastatin was kept at room temperature for 6 h and then analyzed. The post-preparative stability was evaluated by determining the stability of sample in the autosampler.

The samples were prepared using the same procedure as described in the sample preparation section and considered to be stable if assay values were within the acceptable limits of accuracy (i.e. ±15%).

2.7. Animal study

Male Sprague-Dawley (SD) rats (body weight: 250–300 g) were kept under conventional conditions of animal house and experiments started after a week of acclimatization period. Rats were fasted overnight before the dosing day and had free access to food and water after 4 h of post-dosing. Animals were anesthetized with an inhalation of ether and the carotid artery was cannulated with a PE-60 tubing. Fluvastatin was administered to the rats orally at a dose of 1.0 mg kg^{-1} body weight as a suspension (in 0.9% sodium chloride). Approximately 0.2 mL of blood samples were collected at 0, 0.08, 0.25, 0.5, 0.75, 1, 1.5, 2, 4, 6, 8, 10, 12 and 24 h post-dosing. Heparinized blood samples were harvested by centrifugation (Micro12, Han-Il Science Industrial Co., Incheon, Korea) at 10,770 × *g* for 15 min and stored at −74 °C immediately until analysis. After plasma samples of rats were diluted with mobile phase A (50:50), the mixtures were centrifuged at 1000 × *g* at 4 °C for 15 min. The supernatants were analyzed without sample clean-up procedure.

3. Results and discussion

3.1. Method development

In this study, a sample clean-up procedure has not been used prior to analysis of fluvastatin in rat plasma. Although Al-Rawithi et al. [1], Kalafsky and Smith [4], Toreson and Eriksson [16] and Lanchote et al. [17] have proposed HPLC procedures with fluorimetric detection for the quantitative analyses of fluvastatin in human plasma, all of the published methods involved laborious and time-consuming off-line sample clean-up procedure.

The major advantage in the development of a direct injection column-switching method for quantifying fluvastatin in plasma was, considering the viscosity of blood sample and large size of endogenous components, to separate fluvastatin from endogenous components without sample pre-purification. Several different factors; such as, columns with high efficiency stationary phases and mobile phases at different pHs and with different percentages of organic solvent; were taken into account to optimize this parameter [10,14,18,19].

The column-switching system developed in this study provided on adequate clean up of plasma by the absence of interfering peaks in blank plasma sample, as shown in Fig. 3. The selectivity of the assay was checked by measurement of drug-free plasma. The reduced and focused transferring time also limited the transfer of unwanted late eluting interfering compounds from the trap column to the analytical column.

The lower limit of quantitation was 0.5 ng mL^{-1} (S/N = 5:1). This method is considered to be one of the most sensitive bioanalysis methods for fluvastatin among all the published methods. The analyte peak was identifiable, discrete and reproducible with a precision of 20% and an accuracy of 80–120%. Fig. 3 shows typical chromatograms obtained from samples of blank

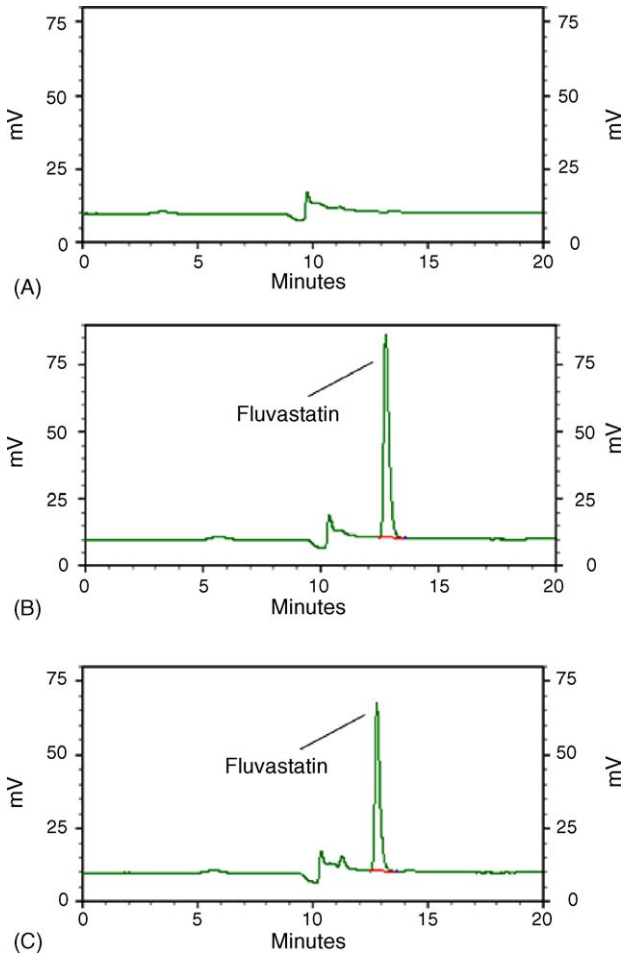

Fig. 3. Representative column-switching HPLC chromatograms of rat plasma samples. (A) Blank plasma; (B) plasma spiked with 100 ng of fluvastatin; (C) plasma obtained at 0.75 h after oral administration of 1 mg kg^{-1} fluvastatin to a rat.

rat plasma (A), plasma spiked with fluvastatin (B) and rat plasma obtained at 0.75 h (C) after oral administration of 1 mg kg^{-1} of fluvastatin. Visual examination of the HPLC-fluorescence chromatograms of blank samples obtained during the validation, including the rat plasma from five different sources, indicated high specificity. The analytes were chromatographically resolved and no significant interferences from endogenous materials were observed.

The calibration curve was generated by plotting peak areas against the concentrations of fluvastatin at 0.5–100 ng mL^{-1}. The coefficient of determination (r^2) was greater than 0.9999.

Table 2
Stability of fluvastatin in rat plasma[a]

Test of stability	Accuracy of fluvastatin (%)	
	Low QCs[b]	High QCs[c]
Freeze and thaw	99.3	103.5
Short-term temperature	94.8	97.9
Long-term	107.9	95.1
Stock solution[d]	91.5	
Post-preparative	99.5	97.8

[a] Number of aliquots = 3.
[b] Low QCs of low quality control samples are 3.0 ng mL^{-1}.
[c] High QCs of high quality control samples are 50.0 ng mL^{-1}.
[d] Stock solution sample is 100.0 μg mL^{-1}.

3.2. Accuracy and precision

The accuracy and precision of HPLC data collected begin with a well-behaved chromatographic system. The system suitability specifications and tests were evaluated for providing assistance in achieving this purpose [15].

Table 1 shows the results of inter- and intra-day accuracy and precision of fluvastatin. All the accuracy and precision of inter-day and intra-day were within the specified ranges and therefore acceptable [15,20].

3.3. Stability

Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix and the container system. The analyte was stable during sample collection and handling, after short- and long-term storage, after going through freeze and thaw cycles, and during the analytical process. Conditions used in the stability experiments reflected situations likely to be encountered during actual sample handling and analysis. The stability of analyte in the stock solution was also proven [15].

The analyte was found to be stable and the range of accuracy was from 90.9 to 110.2%. Fluvastatin was shown to be stable in rat plasma at room temperature for at least 4 h and the post-preparative samples were stable in autosampler for at least 24 h (Table 2).

3.4. Application of the method

This method has been successfully applied to the analysis of rat plasma samples proving the advantage of the method developed in this study. Fig. 4 shows a plasma concentration–time

Table 1
Accuracy and precision for the determination of fluvastatin in rat plasma[a]

Concentration of fluvastatin (ng mL^{-1})	Intra-day			Inter-day		
	Found (ng mL^{-1})	Accuracy (%)	Precision CV (%)	Found (ng mL^{-1})	Accuracy (%)	Precision CV (%)
0.5	0.6	110.2	12.6	0.6	112.2	11.1
3.0	3.0	99.6	13.6	2.9	98.1	6.9
25.0	24.8	99.2	8.2	24.7	98.9	4.7
50.0	49.9	99.8	9.2	50.2	100.4	4.8

[a] Number of replicates = 5.

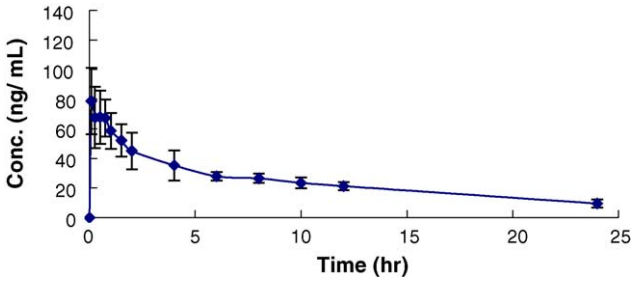

Fig. 4. Plasma concentration–time curve of fluvastatin after oral administration of 1 mg kg^{-1} of fluvastatin to male SD rats. Mean values (±S.D.) are given ($n = 5$).

curve obtained from animal studies. The plasma concentration of fluvastatin was ranged from 9.49 to 78.67 ng mL^{-1} over a 24 h period.

4. Conclusion

For the direct analysis of fluvastatin from rat plasma samples without pre-purification procedures, an automated HPLC method using column-switching has been developed. The method was very simple and sample preparation process was minimized. The method has been fully validated and shown to be sensitive, accurate, precise and precise.

This analytical method might be useful for therapeutic plasma level monitoring and human pharmacokinetic studies of fluvastatin.

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