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ORIGINAL RESEARCH

Determination of two HMG-CoA reductase inhibitors, pravastatin and pitavastatin, in plasma samples using liquid chromatography–tandem mass spectrometry for pharmaceutical study

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ABSTRACT: We developed a method for determining pravastatin or pitavastatin, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, in plasma using liquid chromatography and tandem mass spectrometry (LC–MS/MS). Pravastatin, pitavastatin and the internal standard fluvastatin were extracted from plasma with solid-phase extraction columns and eluted with methanol. After drying the organic layer, the residue was reconstituted in mobile phase (acetonitrile:water, 90:10, v/v) and injected onto a reversed-phase C_{18} column. The isocratic mobile phase was eluted at 0.2 mL/min. The ion transitions recorded in multiple reaction monitoring mode were m/z 423 → 101, 420 → 290 and 410 → 348 for pravastatin, pitavastatin and fluvastatin, respectively. The coefficient of variation of the assay precision was less than 12.4%, the accuracy exceeded 89%. The limit of detection was 1 ng/mL for all analytes. This method was used to measure the plasma concentration of pitavastatin or pravastatin from healthy subjects after a single 4 mg oral dose of pitavastatin or 40 mg oral dose of pravastatin. This is a very simple, sensitive and accurate analytic method to determine the pharmacokinetic profiles of pitavastatin or pravastatiny. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: pravastatin; pitavastatin; HMG-CoA; LC-MS/MS

INTRODUCTION

Pravastatin and pitavastatin are potent inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and were developed for the treatment of hyperchloesterolemia (Stewart *et al.*, 2000; Reid *et al.*, 2001; Mukhtar *et al.*, 2005). These two statins undergo little metabolism whereas other statins, such as simvastatin, lovastatin, atorvastatin, fluvastatin and cerivastatin, are metabolized by cytochrome P450 enzymes (Wang *et al.*, 1991; Prueksaritanont *et al.*, 1997; Lennernas, 2003; Transon *et al.*, 1995, 1996; Hatanaka, 2000; Fujino *et al.*, 2004; Muck, 2000). Cumulative *in vivo* and *in vitro* studies have revealed that organic anion transporting polypeptide 1B1 (OATP1B1), a sodium-independent bile acid transporter, is mainly involved in the disposition kinetics of pravastatin and pitavastatin (Nishizato *et al.*, 2003; Maeda *et al.*, 2006; Chung *et al.*, 2005; Hirano *et al.*, 2006). Clinical evidence suggests that the genetic variants of OATP1B1 lead to altered pharmacokinetics of OATP1B1 substrates, mainly pravastatin and pitavastatin. Therefore, pravastatin and pitavastatin may be used as useful probe substrates for study of OATP1B1 genetic polymorphism and OATP1B1-related drug–drug interaction.

Several methods have been described for the analysis of either pravastatin alone (Kawabata *et al.*, 2005; Zhu and Neirinck, 2003), or pravastatin with other statins such as atorvastatin, lovastatin, simvastatin and rosuvastatin (Pasha *et al.*, 2006; Miao and Metcalfe,

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Abbreviations used: HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A.

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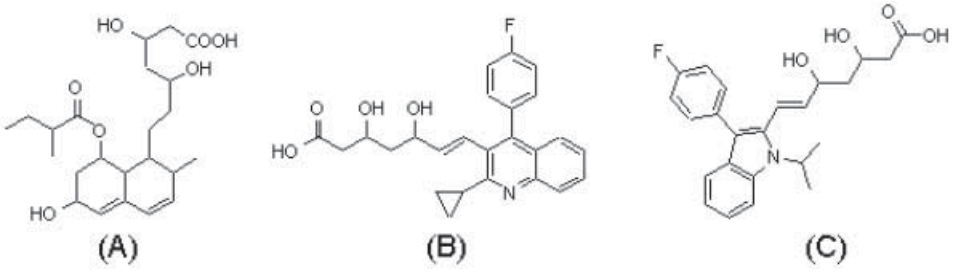

Figure 1. Chemical structures of (A) pravastatin, (B) pitavastatin and (C) fluvastatin (IS).

2003) in plasma using LC-MS/MS employing solid-phase extraction. However, the previously published HPLC methods for quantitation of pitavastatin require a large volume (1.0 mL) of plasma and a tedious liquid–liquid extraction step (Chung *et al.*, 2005; Kojima *et al.*, 1999). In addition, to date, no publication has appeared to measure the both compounds in human plasma samples.

Therefore, we developed a simple and accurate method for determining pravastatin or pitavastatin in human plasma using liquid chromatography with a tandem mass spectrometry. This method was successfully applied to characterize the pharmacokinetics of pravastatin or pitavastatin in humans.

EXPERIMENTAL

Reagents and column. Pravastatin, pitavastatin and fluvastatin (Fig. 1) were purchased from Toronto Research Chemicals Inc. (North York, Canada). High-performance liquid chromatography (HPLC)-grade acetonitrile and methanol were purchased from Merck (Darmstadt, Germany). All other chemicals and solvents were of the highest analytical grade available. The Sep-Pak C_{18} cartridge column was obtained from Waters (Milford, MA, USA).

Preparation of standards and quality controls. Pravastatin, pitavastatin and fluvastatin (internal standard, IS) were dissolved in methanol at 1 mg/mL, separately. The standard solutions of pravastatin and pitavastatin were serially diluted with methanol and added to drug-free plasma to obtain the concentrations of 1, 4, 10, 20, 50 and 100 ng/mL. Calibration graphs in plasma were derived from the peak area ratio of pravastatin and pitavastatin to IS with a linear regression. Quality controls were prepared in 0.3 mL of blank human plasma by adding 10 µL of different concentrations of standard solution, and finally they were prepared for 4 and 20 ng/mL to evaluate the inter- and intra-day precision and accuracy of this assay method.

Characterization of the product ions using tandem mass spectrometry. One micromolar pravastatin, pitavastatin and IS solutions were infused into the mass spectrometer separately at a flow rate of 10 µL/min to characterize the product ions of each compound. The precursor ions, $[M - H]^-$, and the pattern of fragmentation were monitored using negative ion mode. The major peaks observed in the production scan mode were used to quantify pravastatin and pitavastatin.

Analytical system. The plasma pravastatin or pitavastatin concentrations were quantified using liquid chromatography–mass spectrometry with a QTrap 4000 LC/MS/MS system (Applied Biosystems, Foster City, CA, USA) equipped with an electrospray ionization interface used to generate negative ions $[M - H]^-$. The compounds were separated on a reversed-phase column (Luna C_{18}, 50 × 2 mm i.d., 3 µm particle size; Phenomenex, Torrance, CA, USA) with an isocratic mobile phase consisting of acetonitrile and water containing 0.1% formic acid (90:10, v/v). The mobile phase was eluted using an Agilent 1100 series HPLC system (Agilent, Wilmington, DE, USA) at 0.2 mL/min.

The turboion spray interface was operated in the negative ion mode at −4500 eV and 500°C. The operating conditions were optimized by flow injection of a mixture of all analytes and were determined as follows: nebulizing gas flow, 8 L/min; curtain gas flow, 10 L/min; collision gas (nitrogen) pressure, medium; collision energy, −44 eV. Quantitation was performed by multiple reaction monitoring (MRM) of the deprotonated precursor ion and the related product ion for pravastatin or pitavastatin using the internal standard method with peak area ratios and a weighting factor of $1/x$. The mass transitions used for pravastatin, pitavastatin, and internal standard were m/z 423 → 101, 420 → 290 and 410 → 348, respectively (dwell time 200 ms). Quadrupoles Q1 and Q3 were set on unit resolution. The analytical data were processed by Analyst software (version 1.41, Applied Biosystems, Foster City, CA, USA).

Sample preparation. The Waters C_{18} Sep-Pak cartridge column (100 mg), set on a Visiprep SPE vacuum manifold (Supelco, St Louis, MO, USA) connected to a vacuum pump, was conditioned with 1 mL of methanol and de-ionized water, respectively. Then, 0.3 mL of plasma spiked with 20 µL of IS (fluvastatin, 25 ng/mL) was applied to the column. After washing with 1 mL of de-ionized water, drugs were eluted with 1 mL of methanol. The eluate was evaporated to dryness at 40°C in a Speed-Vac (Savant, Holbrook, NY, USA); the residue was reconstituted with 100 µL of mobile phase and injected into the LC-MS/MS system.

Validation procedure. The validation parameters were selectivity, extraction recovery, precision and accuracy. Ten batches of blank heparinized human plasma were screened

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to determine the specificity. The results from drug-spiked plasma extracts were compared with blank plasma extracts with drug added after extraction to determine the extraction recovery. The precision and accuracy of the intra- and inter-day assay validation were estimated using the inverse prediction of the concentration of the quality controls from the calibration curve.

Clinical application. Ten healthy subjects who gave written informed consent took part in this study. Health problems, drug or alcohol abuse, and abnormalities in laboratory screening values were exclusion criteria. This study was approved by the Institutional Review Board of Busan Paik Hospital (Busan, Korea). After an overnight fast, all the subjects were given a single 40 mg oral dose of pravastatin or 4 mg oral dose of pitavastatin. Blood samples were taken before and 0.33, 0.66, 1, 1.5, 2, 3, 4, 6 and 8 h after drug administration. The plasma samples was separated by centrifugation at 1000***g*** for 10 min, and stored at –80°C until analysis.

Pharmacokinetic analysis. The pharmacokinetic analysis was performed using noncompartmental methods. The area under the plasma concentration vs time curve (AUC) was calculated using the trapezoidal rule and extrapolated to infinity. The time courses of the plasma pravastatin or pitavastatin concentration were used to determine the maximum plasma concentration (C_{max}) and the time (T_{max}) to reach C_{max}. The elimination rate constant (k_e) was obtained by the linear regression of the terminal phase and the calculated elimination half-life ($T_{1/2,\beta}$) was $0.693/k_e$.

RESULTS AND DISCUSSION

Determination of pravastatin and pitavastatin

There were no interfering peaks at the elution times for either analytes (pravastatin, 0.9 min; pitavastatin, 0.9 min) or IS (fluvastatin, 1.0 min). Figure 2 represents the typical chromatograms for blank plasma (A) and plasma spiked with 100 ng/mL for pravastatin and pitavastatin, and 1 ng/mL for IS (B), and a plasma sample from a volunteer (C).

Method validation

The calibration curve provided a reliable response from 1 to 100 ng/mL. The mean equation of the regression line for pravastatin and pitavastatin was $y = 0.073x - 0.038$ ($r^2 = 0.999$) and $y = 0.040x - 0.043$ ($r^2 = 0.999$), respectively. The limit of detection was 1 ng/mL for pravastatin and pitavastatin. The intra- and inter-day precision and accuracy of our method are listed in Table 1. The coefficients of variation of the precision of the intra- and inter-day validation were less than 12.4 and 8.6%, respectively. The accuracies of the intra- and inter-day validation were 100.5–106.7 and 89.5–115.4%, respectively. The extraction recoveries were 93.2–101.2% ($n = 3$, Table 2).

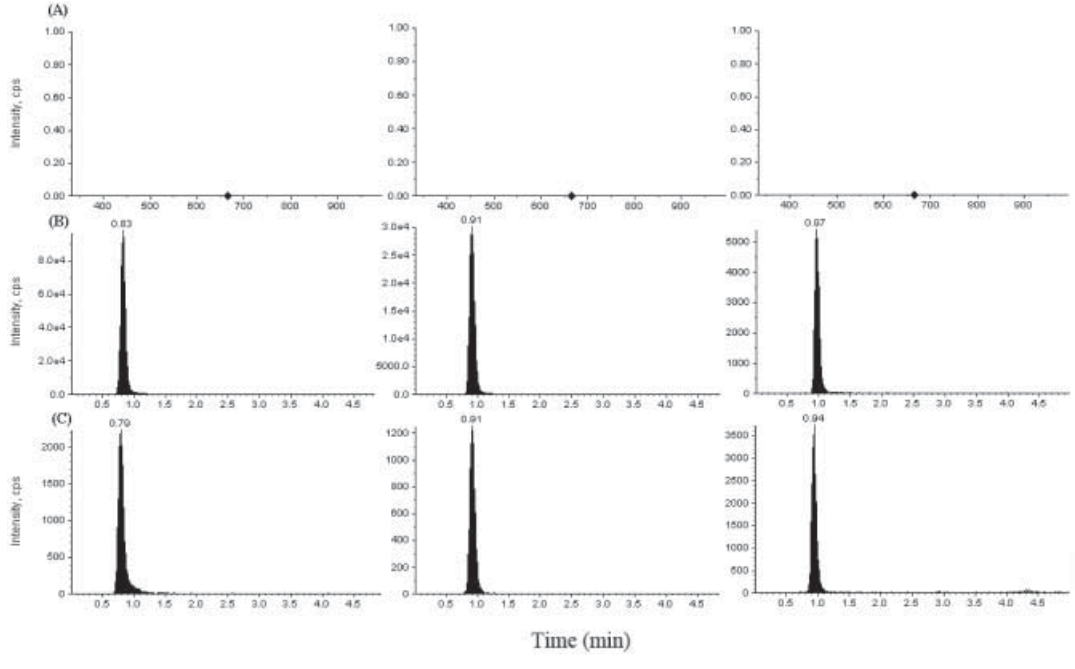

Figure 2. Chromatograms of pravastatin (left), pitavastatin (middle) and fluvastatin (IS, right). (A) blank plasma; (B) plasma spiked with 100 ng/mL pravastatin, 100 ng/mL pitavastatin and 1 ng/mL fluvastatin (IS); (C) plasma sample equivalent to 3.51 and 5.41 ng/mL for pravastatin and pitavastatin, respectively, from a volunteer 10 h after the oral dose of 40 mg pravastatin or 4 mg pitavastatin, respectively.

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Table 1. The precision and accuracy of the inter- and intra-day assay of pravastatin and pitavastatin (n = 5)

Compound	Added concentration (ng/mL)[a]	Inter-day Observed concentration (ng/mL)[a]	Inter-day Accuracy (%)[a]	Inter-day CV (%)[b]	Intra-day Observed concentration (ng/mL)[a]	Intra-day Accuracy (%)[a]	Intra-day CV (%)[b]
Pravastatin	4	4.4 ± 0.3	104.4 ± 15.1	6.2	4.1 ± 0.3	101.3 ± 8.0	7.9
	20	23.1 ± 1.6	115.4 ± 7.9	6.9	21.3 ± 0.7	106.7 ± 3.3	3.1
Pitavastatin	4	3.8 ± 0.3	89.5 ± 7.0	8.6	4.0 ± 0.5	100.5 ± 12.4	12.4
	20	20.5 ± 1.4	102.4 ± 6.8	6.6	20.6 ± 0.9	103.2 ± 4.7	4.5

[a] Mean ± standard deviation.
[b] CV, coefficient of variance (%).

Table 2. The recovery of pravastatin and pitavastatin assay (n = 3)

Compound	Concentration (ng/mL)	Recovery (%)[a]
Pravastatin	10	90.1 ± 4.0
	50	98.9 ± 3.1
Pitavastatin	10	93.2 ± 5.7
	50	101.2 ± 5.2

[a] Mean ± standard deviation.

Table 3. Pharmacokinetic parameters (mean ± SD) of pravastatin or pitavastatin in seven healthy subjects after a single 40 or 4 mg oral dose, respectively

Parameters	Pravastatin (n = 7)	Pitavastatin (n = 7)
AUC_{inf} (ng h/mL)	310.0 ± 130.9	361.8 ± 176.1
C_{max} (ng/mL)	149.2 ± 53.4	114.6 ± 90.1
$T_{1/2,\beta}$(h)	2.0 ± 0.7	6.6 ± 3.3
T_{max}(h)	0.9 ± 0.2	1.2 ± 0.9

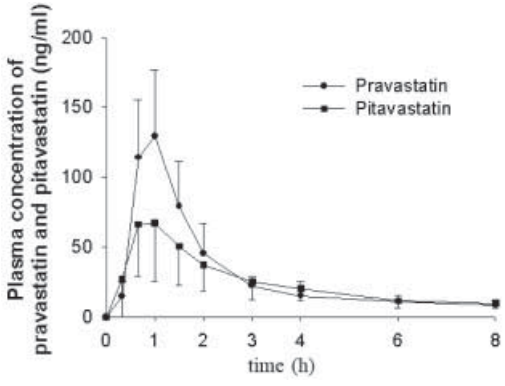

Figure 3. Time course of the plasma pravastatin (●) or pitavastatin (■) concentration in healthy subjects after a single 40 mg oral dose of pravastatin or a single 4 mg oral dose of pitavastatin. Each point represents the mean ± SD (n = 7).

Pharmacokinetics of pravastatin and pitavastatin

Figure 3 shows the time course of the pravastatin and pitavastatin plasma concentration after a single 40 mg oral dose of pravastatin or a single 4 mg oral dose of pitavastatin. Pharmacokinetic parameters of pravastatin and pitavastatin were calculated by non-compartmental analysis techniques using the WinNonlin software. The peak plasma concentration (C_{max}) values were estimated directly from the observed plasma concentration–time data. The area under the plasma concentration–time curve (AUC_{inf}) was calculated according to the linear trapezoidal rule and extrapolation to infinity. The pharmacokinetic parameters are listed in Table 3 (n = 7). For pravastatin, the C_{max} and AUC_{inf} were 140.9 ± 41.0 ng/mL and 275.2 ± 95.6 ng h/mL, respectively. For pitavastatin, the C_{max} and the AUC_{inf} were 84.6 ± 42.1 ng/mL and 281.3 ± 102.5 ng h/mL, respectively. The half-life of pravastatin and pitavastatin calculated from the terminal phase were 2.6 ± 0.7 and 5.3 ± 3.0 h, respectively. These values are comparable to those previously reported after a single oral dose of pravastatin or pitavastatin (Nishizato *et al.*, 2003; Maeda *et al.*, 2006; Chung *et al.*, 2005; Niemi *et al.*, 2004; Bauer *et al.*, 2005).

In previous studies, for analysis of pitavastatin, liquid–liquid extraction (Chung *et al.*, 2005; Kojima *et al.*, 1999) or column switching (Kojima *et al.*, 1999) methods have been used for cleaning up pitavastatin in plasma samples, whereas a solid-phase extraction method has been reported for cleaning of pravastatin (Kawabata *et al.*, 2005; Zhu and Neirinck, 2003; Miao and Metcalfe, 2003; Mulvana *et al.*, 2000). We found that pitavastatin could be also purified by solid-phase extraction method, and that this was much simpler than the pretreatment procedure used previously (Chung *et al.*, 2005; Kojima *et al.*, 1999). This method was successfully applied to characterize the pharmacokinetics of pravastatin or pitavastatin in the clinical treatment process since dosage in this study is similar to the dosage in clinical application.

CONCLUSIONS

The described method is simple and selective for the analysis of the pravastatin or pitavastatin in plasma

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after administration of drugs to humans. The linearity, extraction recovery, accuracy and precision data confirmed the reliability and reproducibility of this assay. The concentration range covered the concentrations expected from clinical studies. The method could be useful for the clinical pharmacokinetic study of pravastatin or pitavastatin in human plasma. The method could also be used for the evaluation of drug interaction potential of various possible OATP1B1 inhibitors or inducers because pravastatin and pitavastatin are good probe substrates for OATP1B1 phenotype.

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