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Original Article

The quantitation of atorvastatin in human plasma by solid phase micro-extraction followed by LC-MS/MS and its application to pharmacokinetics study

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Abstract

Determination of atorvastatin in human plasma using LC-MS/MS has been widely accepted as an effective method owing to its practical applicability in routine drug analysis in pharmacokinetic and pharmacogenetic study. Nevertheless, the method sensitivity can be compromised when limited sample volume is available. This problem is typically encountered in pharmacokinetics studies for which only a small volume of plasma sample can be obtained. Drug extraction from human plasma was performed with the aid of reversed-phase C18 solid phase micro-extraction. The detection was accomplished by LC-MS/MS. The need of plasma volume of less than 250 µl for each sample made it possible to decrease the sample preparation time. The method was successfully validated and proved appropriate for the analysis of atorvastatin in human plasma and can be applied to pharmacokinetics and pharmacogenetics study.

Keywords: atorvastatin, LC-MS/MS, plasma, solid phase micro-extraction, pharmacokinetics

1. Introduction

Atorvastatin is a HMG-CoA reductase inhibitor, which has a widespread use in the prevention of cardiovascular event. Atorvastatin is rapidly absorbed after oral administration. However, due to pre-systematic clearance in gastrointestinal mucosa and metabolism in the liver, its absolute bioavailability is approximately 12 % and low plasma concentration is achieved following administration of the drug (Schachter, 2005). Thus, quantification methods of the drug in pharmacokinetic studies need to be sensitive and specific. For atorvastatin, several HPLC methods for the determination of drug in human plasma have been previously published. Most extraction methods use liquid-liquid extraction (LLE) for sample preparation and these methods have been applied in clinical studies, with atorvastatin dose regimen 40 and 80 mg (Pilli *et al.*, 2011; Nirogi *et al.*, 2006; Bullen *et al.*,1999; Jemal *et al.*, 1999). Other extraction methods such as solid

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phase extraction (SPE) have been used for atorvastatin plasma sample (Hermann *et al.*, 2005; Vlckova *et al.*, 2011; Shah *et al.*, 2011; Bahrami *et al.*, 2005).

However, sensitivity can be compromised when limited sample volumes are available, which is common in pharmacokinetics studies involving small volume of plasma from human or animal. To create a micro volume SPE device, a reversed phase C18 SPE (Omix® 100 µl C18) is packed in a 100 µl pipette tip. The monolithic nature of the SPE sorbent allows less restricted bi-directional flow of liquid though the bed, unlike traditional silica bed-based materials. Thus, the aim of this study is to develop a chromatographic method for determination the atorvastatin in human plasma, using solid phase micro-extraction and to apply it to pharmacokinetic study.

2. Methods and Materials

2.1 Reagents and chemical

Atorvastatin calcium (M.W.; 604.69) and Fluvastatin sodium (M.W.; 410.46) were purchased by Aldrich. Analytical HPLC grade methanol, HPLC grade acetonitrile and formic acid were obtained from J.T. Bakers, USA. Ammonium acetate and water were obtained from Merck, Germany.

2.2 Bioanalytical methods of atorvastatin

2.2.1 Preparation of standard solutions

Atorvastatin standard solutions were prepared from 0.1 mg/ml stock solution in 50% acetonitrile in water. The calibration and the quality control (QC) samples were in the range of 0.2–80 ng/ml. Fluvastatin was used as an internal standard (IS) at the concentration of 12 ng/ml prepared in the same diluent as for atorvastatin.

2.2.2 Sample preparation and solid-phase micro-extraction extraction

The plasma samples 250 µl were spiked with 50 µl of internal standard, 200 µl of 0.1 mM acetic acid (pH=3) and then vortexed and centrifuged at 31514 ×g for 10 min. After centrifugation, the total of 100 µl of supernatant was subsequently transferred to SPE. A reversed phase C18 SPE sorbent material was packed in a 100 µl pipette tip to create a micro volume SPE device (OMIX® 100 µl C18). Plasma samples of healthy volunteers were spiked with the IS solution before passing through the SPE step as the follows; To prepare sorbent condition, 100 µl of 100% methanol and 100 µl of 0.1 mM acetic acid, pH3 were twice pipette, respectively. The 100 µl of prepared sample was then loaded with slowly eluted sample out of the tip. The rinse step was done by loading 100 µl of 5% aqueous methanol followed by elution. The sorbent bed were dried by quickly force the air through the tip 2 to 4 times. Then, 100 µl of 95:5 methanol: 10 mM aqueous formic acid was loaded and eluted. This step was repeated for total elution volume of 500 µl. Next, total elution was evaporated to be dried under stream nitrogen and the residual was reconstituted in 100 µl of acetonitrile: water (50:50 V/V) and centrifuged at 31514 ×g, 10 min at 4°C, prior to transferal to HPLC vial and injection of 10 µl in the HPLC system.

Afterward, the reconstituted plasma sample was analyzed by LC-MS/MS analysis. The QC sample was also treated in the same fashion as for the plasma samples.

2.2.3 LC-MS/MS conditions for the analysis of atorvastatin

Chromatographic conditions

The LC system consisted of an Algilent™ Serial 1100 LC binary pump and an auto-sampler. The analytes were separated through Higgins C18 (3.0 mm * 100 mm, 5 µM) at a flow rate of 0.6 ml/min, 30°C. The mobile phase composed of solvent A (10mM ammonium acetate) and solvent B (acetonitrile). The gradient was as follow: solvent B held at 40% for 2 min, linearly ramped from 40% to 60% in 1 min, ramp 60% to 90% in 1 min, remains 90% 3 min and bought back down to 40% for equilibration. Total run time was 10 min with flow rate of 0.6 ml/min acetonitrile. The retention time of atorvastatin and fluvastatin was about 4.5±0.5 min.

MS/MS conditions

Mass spectrometric detection was performed on an API 4000 triple quadrupole instrument (ABI-SCIEX, Toronto, Canada) using multiple reaction monitoring. A turbo electrospray interface in positive ionization mode was used. The main working parameters of the mass spectrometer are summarized in Table1. Data processing was performed on Analyst 1.4 software package (SCIEX). The quantitation was performed using multiple reaction monitoring (MRM) precursor-product ion transitions at *m/z* 559.2 to 440.1 for

Table 1. Tandem mass-spectrometer main working parameters

Parameter (Unit)	Value
Source temperature (°C)	400
Dwell time per transition (ms)	200
Ion source gas 1 (psi)	20
Ion source gas 2 (psi)	40
Curtain gas (psi)	25
Collision gas (psi)	5
Ion spray voltage (V)	5500
Entrance potential (V)	10
Declustering potential (V)	98 (analyte) and 80 (IS)
Collision energy (V)	50 (analyte) and 30 (IS)
Collision cell exit potential (V)	30 (analyte) and 13 (IS)
Mode of analysis	Positive
Ion transition for atorvastatin (*m/z*)	559.2/440.1
Ion transition for fluvastatin (*m/z*)	414.2/224.2

atorvastatin acid and *m/z* 412.2 to 224.2 for the IS, respectively.

2.3 Pharmacokinetics study

The WinNonlin professional edition version 3.1 wes used for noncompartmental analysis. The plasma concentration time profile for each subject was fitted using noncompartmental model of extravascular input. The λ_z was calculated according to the log-linear portion of terminal phase of plasma concentration time course. The time range for determining λ_z was selected to get log-linearity, then AUC_{last} was calculated. The T_{max} and C_{max} were observed directly from the plasma concentration time curve. The means of these parameters for atorvastatin tablets were calculated.

3. Results and Disscussion

3.1 Mass spectrometry

In order to develop a method with the desired sensitivity (< 1 ng/ml), it was necessary to use MS/MS detection, as the compound did not possess UV absorbance or fluorescence properties needed to achieve this limit. The inherent selectivity of MS-MS detection was also expected to be beneficial in developing a selective and sensitive method. The positive ion TurboIonspray Q1 mass spectrum and product ion mass spectrum of atorvastatin are shown in Figure 1 and Figure 2, respectively. The $[M + H]^+$ was the predominant ion in the Q1 spectrum, and was used as the precursor ion to obtain product ion spectra. The most sensitive mass transition was from *m/z* 559.2 to 440.1 for atorvastatin and *m/z* 414.2 to 224.2 for fluvastatin (internal standard; IS) (Figures 3 and 4). However, another mass transition from *m/z* 559.2 to 250.1 for atorvastatin was detected in the same times.

The ion spray voltage, declusturing potential, entrance potential, collision energy and collision cell exit potential were optimized to deliver effective fragmentation of the $[M + H]^+$ without excessive fragmentation, which would have reduced sensitivity. The parameters are presented in Table 1.

3.2 Performance of LC

The HPLC conditions were optimized such that the retention time was kept short at about 4.5±0.5 min in order to assure high throughput (Figure 5). The total runtime for each sample was 10 min by using mixture of 50 mM ammonium acetate buffer/acetonitrile (60:40). The Higgen C18 HPLC column (5µm, 100 mm × 3 mm) was chosen based on peak

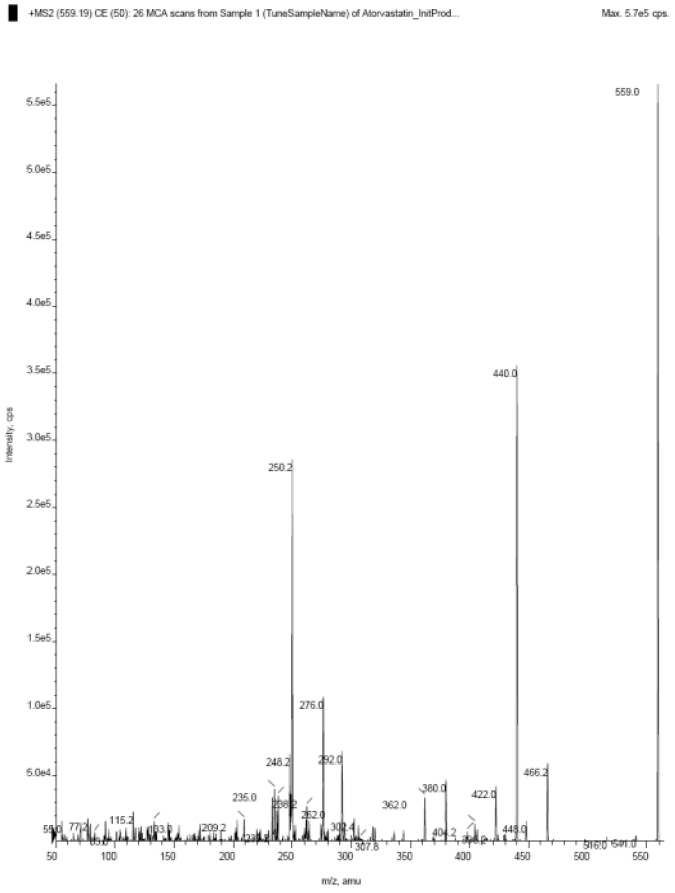

Figure 1. Full scan positive ion TurboIonspray Q1 mass spectra of atorvastatin.

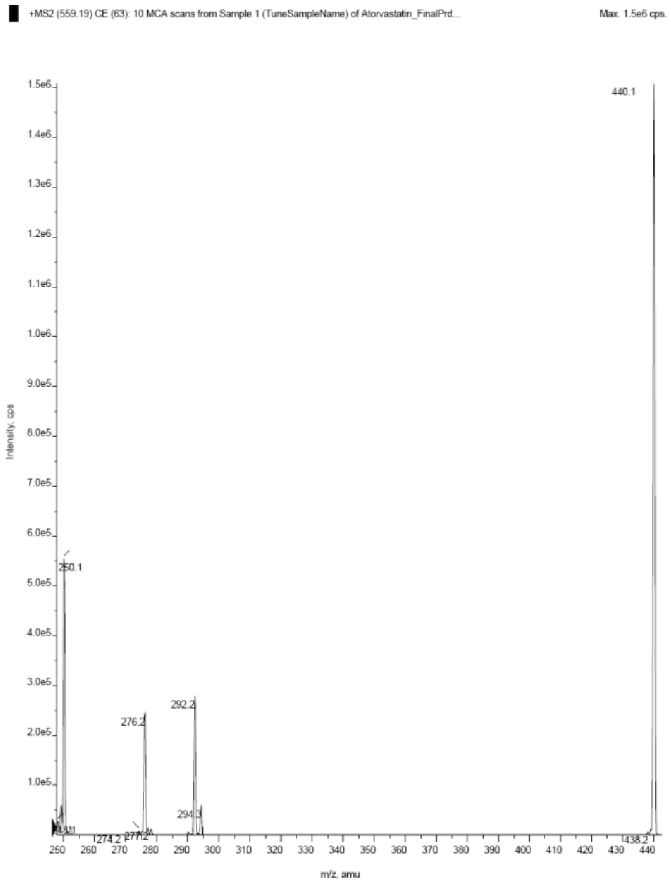

Figure 2. Full scan positive ion TurboIonspray product ion mass spectra of atorvastatin.

shape and the best response for atorvastatin and fluvastatin with acceptable analysis time.

3.3 Bioanalytical method validation

The method validation of bioanalysis was performed as stated in US-FDA guideline. Specificity, selectivity, % recovery, linearity, limit of detection, limit of quantitation, stability, accuracy and precision were analyzed.

3.3.1 Specificity and selectivity

The specificity/selectivity of the method was investigated by analyzing six blank human plasma extract (n=6) (Figure 5) and extract of spiked drug (Figure 6). No significant interference in the blank plasma traces was seen from endogenous substances in drug free human plasma at the retention time of the analyte. Under the chromatographic conditions described, atorvastatin and the internal standard were eluted with nearly the same retention time.

3.3.2 Extraction recovery

The average recoveries for atorvastatin at three different concentrations (low, medium and high QC samples) are presented in Table 2. The recovery of the internal standard was 84.16% at the concentration used in the assay (24 ng/ml).

3.3.3 Calibration curve

Calibration curve was linear over the concentration range of 0.2-80 ng/ml for analyte. The calibration curve was fitted to a $1/x^2$ weighted linear regression (where x is the concentration of the analyte) as this was judged to be the weighting which made the assay most robust. The mean linear regression equation of calibration curve for the analyte was $y = 0.0312(\pm 0.0286) + 1.8250(\pm 0.2668)\ x$, where y was the peak area ratio of the analyte to IS and x was the concentration of the analyte. The mean correlation coefficient of the weighted calibration curves generated during the validation was 0.9965 for the analyte.

Table 2. The recoveries of atorvastatin

Sample concentration (ng/ml)	Recovery (%) (mean ± SD, n=6)
0.25 (QC A)	84.16±7.99
10 (QC B)	96.82±6.14
40 (QC C)	102.84±6.73

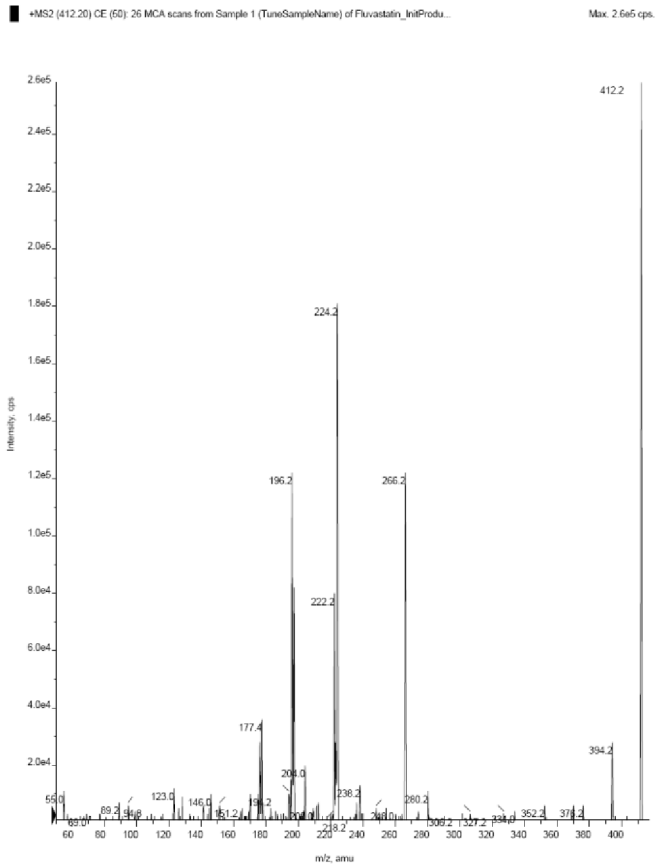

Figure 3. Full scan positive ion TurboIonspray Q1 mass spectra of internal standard (fluvastatin).

3.3.4 Limit of detection and quantitation

The lower limit of detection (LOD), estimated as the amount of atorvastatin furnishing a signal three time greater than the noise (S/N=3:1), was shown to be 0.05 ng/ml. The lower limit of quantitation (LLOQ) was defined as the lowest concentration that can be measured with acceptable accuracy and precision, and found to be 0.2 ng/ml in human plasma. The mean response for analyte peak at the assay sensitivity limit (0.2 ng/ml) was about 5 fold greater than the mean response for peak in six blank human plasma samples at the retention time of the analyte. The between-batch precision at the LLOQ was 3.475% and between-batch accuracy was 99.18% (Table 3).

3.3.5 Accuracy and precision

The within-batch for QC samples precision were 3.48-9.28% and accuracy were 99.18-115% (Table 3). The results showed that for within run and between run experiments the precision and accuracy for the analyte were within the acceptance criteria (Table 3).

3.3.6 Stability

In case of stability, accuracy and precision of analysis wer in the acceptable range according to the US-FDA guideline implying that atorvastatin in plasma was stable under the various condition: short-term 24 h, three freeze-thaw cycles, auto sampler 24 h, and long-term for 3 months at -20°C (Table 4).

3.4 Pharmacokinetics of atorvastatin in healthy volunteers

Mean values of pharmacokinetic parameters of atorvastatin were obtained from the plasma-concentration time curve for each subject in the study. The atorvastatin plasma concentration-time data from the study were analyzed using WinNonlin® professional edition version 3.1, using the non-compartmental model. Four parameters were studied C_{max}, $AUC_{0\text{-last}}$, $AUC_{0\text{-inf (observe)}}$ and T_{max} (Table 5). These parameters are commonly used in pharmacokinetics analysis. The plasma concentrations of atorvastatin, at specified sampling time, from 24 healthy Thai subject following oral administration of 40 mg dose were presented as plasma-concentration time. Spaghetti plots for plasma concentration time profile of atorvastatin for all subjects are shown in Figure 8A, 8B. The mean and SD value of atorvastatin at specified sampling time obtained from 24 healthy Thai subjects are presented in Table 5. For most subjects, atorvastatin was rapidly absorbed. It reached a maximum concentration within 1-2 hour. The largest maximum plasma concentration was 36

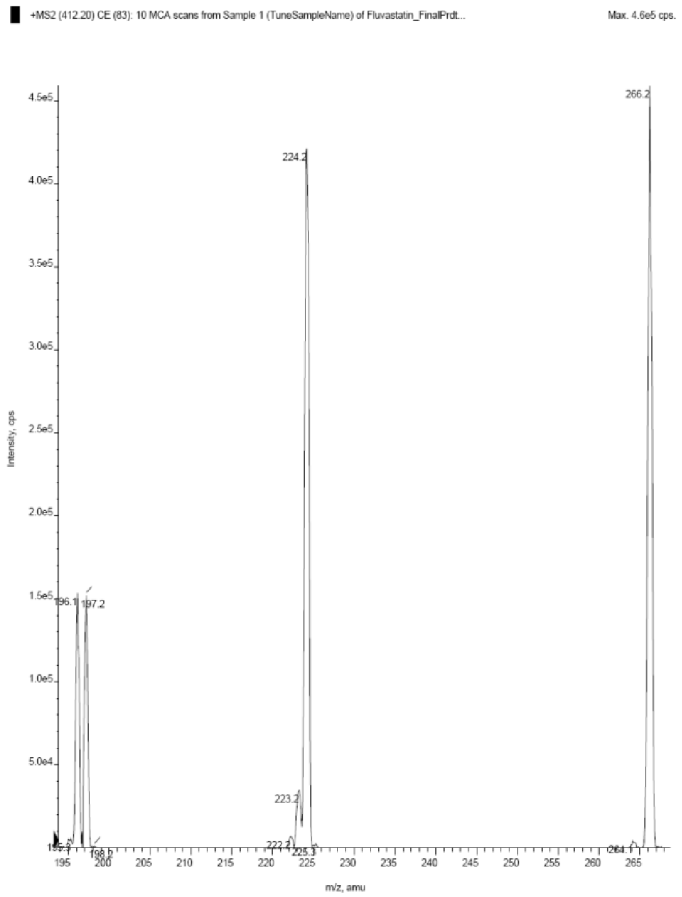

Figure 4. Full scan positive ion TurboIonspray product mass spectra of internal standard (Fluvastatin).

Table 3. Precision and accuracy of the method for determining atorvastatin concentrations in

Accuracy and precision	QC samples (ng/ml)	Concentration added (mean ± SD $n = 6$) (ng/ml)	Concentration found (%)	CV (%)	Accuracy
Intra-day	LLOQ	0.2	0.099±0.003	3.47	99.18
	Low	0.25	0.250±0.022	8.87	99.95
	Mid	10	11.37±0.082	0.72	113.67
	High	40	45.18±0.646	1.43	113
Inter-day	LLOQ	0.2	0.1068±0.006	6.226	106.8
	Low	0.25	0.2408±0.062	5.579	96.32
	Mid	10	8.169±0.1164	2.348	99.11
	High	40	0.474±1.29	3.235	101.19

ng/ml.

Data of maximum plasma concentration (C_{max}), area under the plasma-concentration time curve from time zero to last time ($AUC_{0\text{-last}}$), area under the plasma-concentration time curve from time zero to the infinity time from observed ($AUC_{0\text{-inf(observed)}}$) and time to reach maximum plasma concentration (T_{max}) of atorvastatin after single dose oral administration of 40 mg atorvastatin tablet of the atorvastatin product were compared to the result described by Koytchev *et al.* (Koytchev *et al.*, 2004)

The geometric mean values of area under the plasma-concentration time curve from time zero to the last time ($AUC_{0\text{-inf}}$) for atorvastatin, between 33.5-149.3 ng*ml/h, reflect the high variability of the pharmacokinetics of atorvastatin may occur from pharmacogenetic variations such as Transporters, CYP450 etc. (Shitara and Sugiyama, 2006; Kameyama *et al.*, 2005; Thompson *et al.*, 2005; Kajinami *et al.*, 2004). The mean values were approximately the same values obtained by LC-MS/MS after administration of single dose of atorvastatin (Kantola, Kivisto and Neuvonen, 1998; Borek-

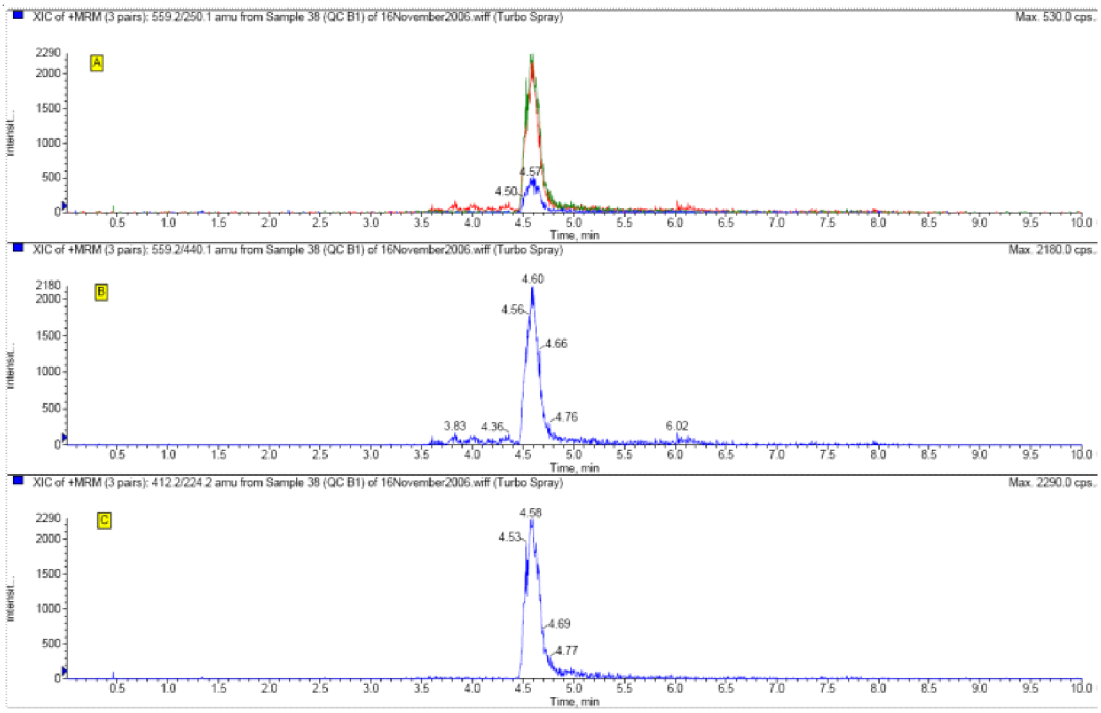

Figure 5. MRM ion-chromatograms resulting from the analysis of QC sample human plasma for atorvastatin (5A, 5B) and internal standard (5C).

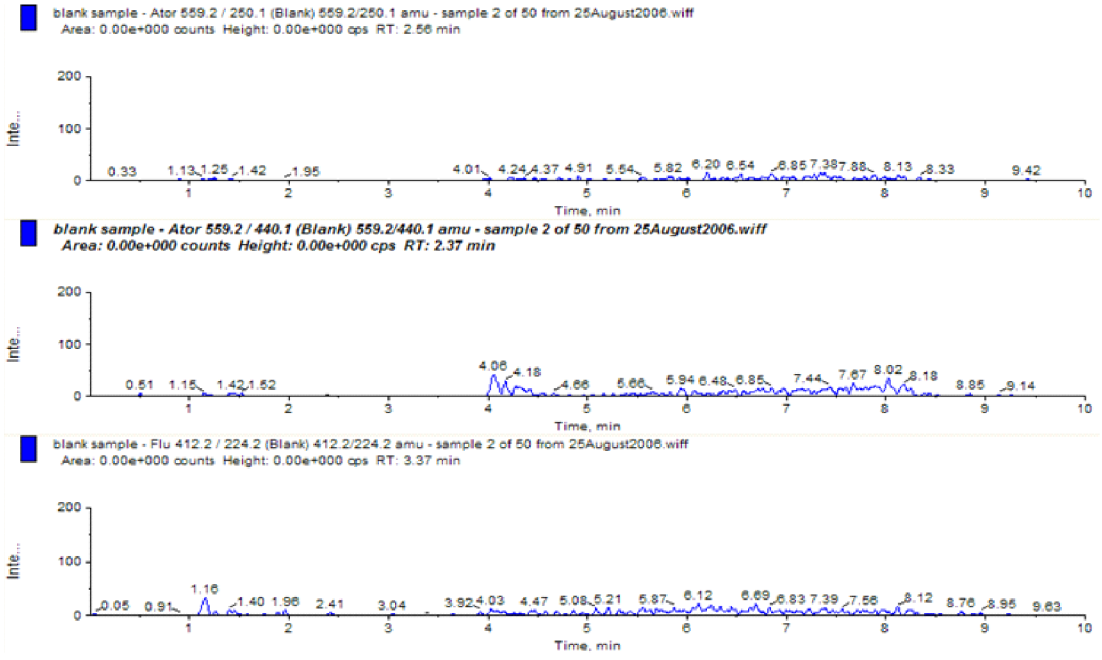

Figure 6. MRM ion-chromatograms resulting from the analysis of blank (drug and internal standard free) human plasma for atorvastatin and internal standard.

Dohalsky *et al.*, 2006) (Table 6). In contrast, results obtained by HPLC UV methods were different (Nirogi, Mudigonda and Kandikere, 2007). The comparative dose proportionality of the pharmacokinetics of atorvastatin and the peak concentration (C_{max}) of atorvastatin was in agreement with reported data. The observed median values and range of atorvastatin (T_{max}) were 1.25 hours and 0.2-6 hours, respectively. Comparing to the previous reported values, the median was not different, while the range was wider. Because foods considerably reduce the rate of atorvastatin absorption, the difference

Table 4. Stability of atorvastatin in human plasma

Sample concentration (ng/ml)	Concentration found (mean ± S.D. *n*=6)(ng/ml)	CV (%)	Accuracy (%)
Short-term stability for 24 h in plasma			
0.25	0.27±0.023	8.377	109.12
40	38.24±1.940	5.058	95.91
Three freeze–thaw cycles			
0.25	0.25±0.006	2.212	100.11
40	36.44±0.935	2.566	91.12
Auto sampler stability for 24 h			
0.25	0.27±0.015	5.34	109.07
40	44.30±1	2.26	110.75
Long-term stability for 3 month in plasma (-20°C)			
0.25	0.26±0.026	10.04	105.28
40	38.37±1.99	5.18	95.95

Table 5. Descriptive statistical of pharmacokinetics parameters

Descriptive statistical value	Pharmacokinetic parameters			
	C_{max} (ng/ml)	$AUC_{0\text{-last}}$ (min*ng/ml)	$AUC_{0\text{-inf (predict)}}$ (min*ng/ml)	T_{max} (min)
Mean	16.8	4660	4820	113
SD	7.42	1890	1950	62.4
SEM	1.51	386	397	12.7
Variance	55.0	3580000	3790000	3890
Min	4.48	1970	2010	30
Median	17.0	4500	4610	120
Max	33.5	8820	8970	240
%CV	44.1	40.6	40.3	55.2
Geometric mean	15	4300	4450	94.2

T_{max}, time to maximum concentration; C_{max}, maximum concentration; AUC, area under the concentration time curve; $T_{1/2}$, elimination half-life.

may also be because of the longer fasting period in this study, at least 8 hours rather than the 1 to 2 hours report by Kantola et al. and Lilja et al., respectively. The median elimination half-life was 7.9 hours (range 1.2 to 21 hours) which was consistent with several reports.

4. Conclusion

The bioanalytical methodology described in this report was specific, sensitive, accurate and precise enough to be successfully applied to pharmacokinetics study. The method employed sample preparation by solid phase microextraction (SPME) appeared to be adequate recovery including less time consuming, and was followed by gradient HPLC coupled with tandem mass spectrometric detection (LC-MS/MS). The LC-MS/MS method could be used to determination of atorvastatin in human plasma, in the concentration range 0.2-80 ng/ml. There were no interferences from endogenous plasma component or other sources. The need for plasma volume less than 250 ml for each sample made it possible to decrease volume of sample preparation per time. The method was successfully validated and proved appropriate for the analysis of atorvastatin in human plasma and can be applied for pharmacokinetics studies. The analytical method presented here has proved to be useful for investigation of the characteristics of atorvastatin in human plasma in pharmacokinetic and pharmacogenetic studies.

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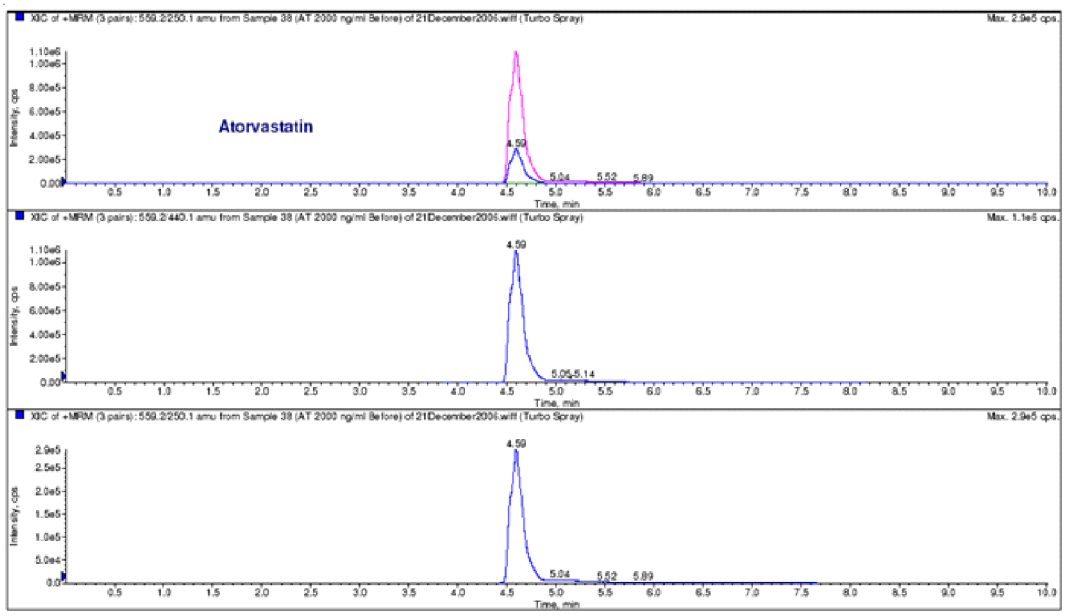

Figure 7. MRM ion chromatograms resulting from the analysis of blank plasma spiked with drug only.

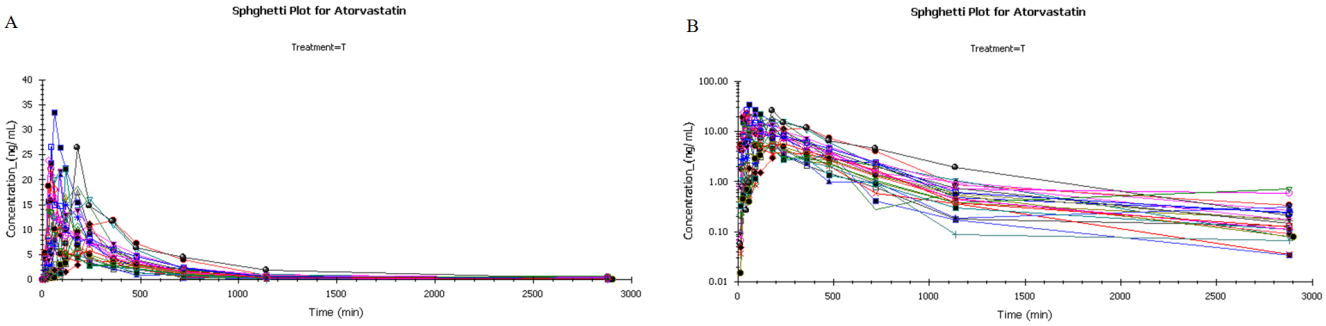

Figure 8. Spaghetti plot for plasma concentration time profile of test treatment atorvastatin for all subjects normal plot (8A) and semilog plot (8B) (n=24).

Table 6. Result from pharmacokinetic study of atorvastatin

Characteristic	$AUC_{0\text{-last}}$ (hr*ng/ml)	$AUC_{0\text{-inf}}$ (hr*ng/ml)	C_{max} (ng/ml)	t_{max} (hr)	$t_{½}$ (hr)
Obtained for reference product					
Dose 40 mg	71.66±35.7	75.17±38.5	15.9±6.97	2 (0.5-4)	7.9±4.8
Kantola *et al.*					
Dose 40 mg (Kantola *et al.*, 1998)	54.2±24.2	-	13.4±9.5	1 (0.5-3)	7.0±3.7
Lilja et al					
Dose 40 mg (Borek-Dohalsky *et al.*, 2006)	61.4±36.2	-	12.7±7.8	1.5 (1-3)	9.7±2.8
Bahrami *et al.*					
Dose 40 mg (Bahrami *et al.*, 2005)	787.7±548.5	1349.2±694.5	50.1±30.7	3.2 (-)	35.7±15.1
Borek-Dohalsky *et al.*					
Dose 80 mg (Borek-Dohalsky *et al.*, 2006)	91.9±42.9	95.1±43.8	23.2±10.4	0.5 (0.25-3)	12.4±7.0

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