ORIGINAL ARTICLE

Simultaneous quantitation of atorvastatin and its two active metabolites in human plasma by liquid chromatography/(–) electrospray tandem mass spectrometry

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Abstract   A sensitive, accurate and selective liquid chromatography–tandem mass spectrometry method (LC–MS/MS) was developed and validated for the simultaneous quantitation of atorvastatin (AT) and its equipotent hydroxyl metabolites, 2-hydroxy atorvastatin (2-AT) and 4-hydroxy atorvastatin (4-AT), in human plasma. Electrospray ionization (ESI) interface in negative ion mode was selected to improve the selectivity and the sensitivity required for this application. Additionally, a solid phase extraction (SPE) step was performed to reduce any ion-suppression and/or enhancement effects. The separation of all compounds was achieved in less than 6 min using a C_{18} reverse-phase fused-core® column and a mobile phase, composed of a mixture of 0.005% formic acid in water:acetonitrile:methanol (35:25:40, v/v/v), in isocratic mode at a flow rate of 0.6 mL/min. The method has lower limit of quantitation (LLOQ) of 0.050 ng/mL for all analytes. The method has shown tremendous reproducibility, with intra- and inter-day precision less than 6.6%, and intra- and inter-day accuracy within $\pm 4.3\%$ of nominal values, for all analytes, and has proved to be highly reliable for the analysis of clinical samples.

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1.   Introduction

Atorvastatin (AT) is a synthetic competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which catalyzes the conversion of HMG-CoA to mevalonate, an early and a rate limiting step in cholesterol biosynthesis. Among statins it has established a prominent place in therapeutics that can achieve

relatively large reduction in plasma cholesterol levels and, thereby, ameliorate vascular atherosclerosis and reduce cardiovascular morbidity and mortality [1,2]. It is administered in the active lipid-lowering acid form, atorvastatin acid, and gets completely absorbed upon oral administration [3,4]. It has low oral bioavailability of about 12–14% due to its rapid presystemic clearance in the gut wall as well as metabolism in the liver [5].

AT remains highly bound to plasma protein ($\geq 98\%$) and has the elimination half-life of approximately 14 h, which is considerably longer than that of most other statins [6]. It gets extensively metabolized to 2- and 4-hydroxylated derivatives and various β-oxidation products mediated through cytochrome P450 3A4 (CYP3A4) metabolism, and around 70% of the HMG-CoA reductase inhibition associated with AT has been ascribed to its 2- and 4-hydroxylated metabolites [7,8]. In-vivo AT and its major hydroxylated metabolites, 2-AT and 4-AT, are present in equilibrium with their corresponding inactive lactone forms (Fig. 1) and it is mainly the lactone form that undergoes metabolism by CYP3A4, because of a significant higher affinity for CYP3A4 compared with that of the acid form. Lactonization of the acid form and hydrolysis of the lactone form to the open-acid form of AT have been suggested to be catalyzed by uridine diphosphate (UDP)-glucuronosyltransferase (UGTs) and esterases (paraoxonases) enzyme, respectively [9,10]. This interconversion phenomenon also takes place in the samples during storage, sample preparation stages, detection, and seriously impacts the accuracy of results during conduct of pharmacokinetic studies. In order to characterize the pharmacokinetic parameters accurately, it is essential to avoid their ex-vivo interconversion. The use of an appropriate anticoagulant, lowering sample temperature and pH of the plasma, could significantly affect the interconversion between lactone and acid forms.

A variety of analytical methods including radioimmunoassay [11], high performance liquid chromatography coupled with electrochemical or ultra violet (UV) detection [12,13], enzyme inhibition bioassay [14], gas chromatography–mass spectrometry (GC–MS) [15], liquid chromatography–mass spectrometry (LC–MS) [16], liquid chromatography–tandem mass spectrometry (LC–MS/MS) [17–23], and ultra-high performance liquid chromatography–tandem mass spectrometric detection (UPLC–MS/MS) [24] have been reported for the analysis of AT alone and its metabolites in biological matrix. The published methods with UV or electrochemical detection, radioimmunoassay and enzyme inhibition assay were proved to be time consuming and have higher limit of quantification. Although the GC–MS methodology meets the desired LLOQ, it requires complex derivatization of analytes prior to analysis, which makes the sample preparation more laborious. Most of the reported LC–MS, UPLC–MS/MS and LC–MS/MS methods utilize electrospray ionization interface in positive ion mode towards the quantification of AT and its metabolites. These methods either require higher plasma aliquot volume (>300 μL), tedious and time-consuming extraction procedures and/or are not sensitive enough for the estimation of active metabolites concentration for pharmacokinetic study after administration of low dose AT. Macwan et al. [25] reported a method, comparable in terms of sensitivity with the present work, with LLOQ of 0.050 ng/mL for AT and its five metabolites using 50 μL of plasma. This method employed protein precipitation extraction with a chromatographic run time of 7 min.

In this research we report for the first time a stability indicating, sensitive and precise LC–MS/MS methodology for simultaneous determination of AT and its equipotent hydroxyl metabolites with an ESI interface in negative ion mode. The validated method was successfully applied to a clinical pharmacokinetic study following an oral administration of AT in healthy adult male volunteers with successful incurred sample reanalysis (ISR).

2. Experimental

2.1. Chemicals and materials

AT, 2-AT, 4-AT, and their respective deuterium labeled, -D5, analog (AT-D5, 2-AT-D5, and 4-AT-D5) were procured from TLC Pharma Chem. Canada, whereas standard compounds of AT lactone, 2-AT lactone, and 4-AT lactone were purchased from TRC Canada. Ammonium acetate, acetonitrile and methanol of LC–MS grade were obtained from FLUKA (Sigma-Aldrich, Steinheim, USA). Analytical grade ortho-phosphoric acid (OPA) and hydrochloric acid (HCL) were purchased from Fisher Scientific (Mumbai, India). Oasis® HLB (30 mg, 1 cc), solid phase extraction (SPE) cartridges were obtained from Waters (Milford, MA, USA). All aqueous solutions and buffers were prepared using water that was purified using Milli-Q® Gradient A10® (Millipore, Molscheim, France). Human plasma matrix lots for method development, validation, to prepare calibration standards and quality control (QC) samples were obtained from clinical unit of Ranbaxy Research Laboratories (Delhi, India).

2.2. LC–MS/MS instrumentation and operating conditions

The liquid chromatography separation was performed using a Shimadzu scientific instruments (Shimadzu Corporation; Kyoto, Japan) comprising of two LC-20AD pumps, a cooling autosampler (SIL 20AC), a column oven of temperature control (CTO-20AC) and a CBM 20A controller. Chromatography separation of analytes and their corresponding D5-ISTDs was accomplished within 6.0 min using an Ascentis® Express C$_{18}$ (75 mm × 4.6 mm, 2.7 μm; Supelco, Bellefonte, Pennsylvania, USA) column and a mobile phase consisting of 0.005% formic acid in water:acetonitrile:methanol (35:25:40, v/v/v) in isocratic mode at 0.6 mL/min. The column and autosampler temperature were kept at 45 °C and 5 °C, respectively.

An Applied Biosystems Sciex API 4000 (MDS-Sciex®, Concord, Canada) consisted of an ESI interface was operated in negative ion mode. Quantification was carried out using multiple reaction monitoring (MRM) mode of the transitions m/z 557.4→278.1 for AT, m/z 562.4→283.2 for AT-D5, m/z 573.5→278.1 for 2-AT, m/z 578.5→283.1 for 2-AT-D5, m/z 573.4→278.2 for 4-AT and m/z 578.5→283.2 for 4-AT-D5, with dwell time set at 150 ms per transition. Nitrogen was used as the nebulizer, auxiliary, collision and curtain gases. The main working source/gas parameters of the mass spectrometer were optimized and maintained as follows: collision activated dissociation (CAD) gas, 7; curtain gas (CUR), 20; gas 1 (nebulizer gas), 50; gas 2 (heater gas), 55; turbo ionspray (IS) voltage, -4500 V; and source temperature, 500 °C. Other optimized compound parameters for monitoring analytes were set as follows: declustering potential (DP), -70 V; entrance potential (EP), -14 V; collision energy (CE), -60 V; and collision cell exit potential (CXP), -18 V.

Calibration curves were constructed by calculating the analyte to internal standard (ISTD) peak area ratio (y) against analyte concentrations (x). Data acquisition and processing were performed using Analyst version 1.4.1 software (MDS-Sciex®, Canada).

2.3. Preparation of stock and working solutions, standard and quality control samples

Stock solutions of AT, 2-AT, 4-AT and their respective -D5 ISTDs were prepared separately by dissolving the accurately weighed compounds in methanol to obtain a final concentration of 1 mg/mL. The prepared stock solutions were stored at $-20\,^{\circ}C$ until use. Similarly, stock solutions of AT lactone, 2-AT lactone and 4-AT lactone were prepared as and when required in acetonitrile to obtain a final concentration of 1 mg/mL.

Two sets of working solutions were then prepared in methanol–water (50:50, v/v) for the preparation of calibration standards. One set contained a mixture of AT and 2-AT at nine concentration levels in the range 5.007–10,013.765 ng/mL for AT and 5.010–10,019.831 ng/mL for 2-AT, and another set contained 4-AT at nine concentration levels in the range 5.001–500.063 ng/mL. Calibrations standards containing a mixture of all three analytes were prepared by 1% addition of each working solution in human blank plasma (e.g., at each concentration level, 10 μL of each of the two working solutions was added to 1 mL human plasma). This resulted in the calibration range 0.050–100.138 ng/mL for AT, 0.050–100.198 ng/mL for 2-AT and 0.050–5.001 ng/mL for 4-AT. Simultaneously quality control samples were prepared in the same manner as that of calibration standards in bulk from the working solutions at four levels "High/HQC" (80.201 ng/mL for AT, 79.520 ng/mL for 2-AT, and 3.950 ng/mL for 4-AT), "Middle/MQC" (12.815 ng/mL for AT, 12.723 ng/mL for 2-AT and 1.101 ng/mL for 4-AT), "Low/LQC" (0.125 ng/mL for AT, 0.127 ng/mL for 2-AT, and 0.126 ng/mL for 4-AT), and "LOQQC" (0.050 ng/mL for AT, 2-AT, and 4-AT). In order to evaluate the stability in the presence of inactive lactone metabolites, lactone fortified QC samples were also prepared at two levels, high and low. The working solutions for the lactone metabolites were prepared in acetonitrile:water (50:50, v/v). The fortified low QC (F-LQC) samples contained AT lactone, 2-AT lactone, and 4-AT lactone at levels 3.0, 5.0 and 7.0 times higher than their respective acid form, whereas the fortified high QC (F-HQC) samples contained AT lactone, 2-AT lactone, and 4-AT lactone at levels 1.5, 3.0 and 4.0 times higher than their respective acid form. The amount of lactone metabolite was chosen so as to be representative of incurred/clinical samples [26].

A modified procedure towards stabilizing the analytes in the biological matrix was applied, wherein a 2% of plasma, from each spiked calibration standards, QC samples as well as incurred/clinical samples during sample collection, was replaced with the buffer solution (10% v/v, OPA in water). A maximum of 5% dilution of the plasma matrix was allowed. If not used, the calibration standards and quality control samples were stored in aliquots at $-50\,^{\circ}C$ until analysis. The stock solutions used for the preparation of quality control samples were different from the one used for the preparation of calibration curve standards.

A working solution containing the three ISTDs was prepared in methanol:water (50:50, v/v) at concentrations of 25.000 ng/mL for AT-D5 and 50.000 ng/mL for 2-AT-D5 and 4-AT-D5. All the above mentioned working solutions were prepared and stored under low light conditions in ice-cold water bath until use.

2.4. SPE procedure

Plasma samples frozen at $-50\,^{\circ}C$ were thawed on the day of extraction in ice-cold water bath followed by vortexing to ensure homogeneity. Eppendorf pipette was used to aliquot 200 μL of

plasma samples into appropriately labeled polypropylene tubes and plasma samples were mixed with 50 μL of ISTDs working solution as well as 400 μL of water titrated to pH 4.1 ± 0.1 with formic acid. After brief stirring with vortex mixer, the samples were centrifuged at 4000 rpm for 5 min. The supernatant was then loaded onto Waters; Oasis, HLB disposable extraction cartridge (30 mg, 1 cc). The cartridges were previously conditioned with 0.500 mL of methanol and then with 0.500 mL of water. Washing step was performed using 1 mL of 30% methanol in water and analytes were eluted with 0.500 mL of methanol twice. The eluents were then evaporated to dryness using a Zymark TurboVap LV evaporator (Caliper, Hopkinton, MA, USA) and reconstituted with 100 μL of reconstitution solution consisting of acetonitrile: methanol:water titrated to pH 4.1 ± 0.1 with formic acid (20:20:60, v/v/v). The reconstituted samples were transferred to autosampler glass vials. 20 μL of sample was injected into the LC–MS/MS system for analysis.

The whole sample preparation was carried out in ice-cold water bath (excluding vortex mixing, SPE and drying) and under low light conditions, to avoid temperature and light-induced degradation of the analytes.

2.5. Interconversion

The interconversion between the acid and lactone forms of the analytes was evaluated under different experimental conditions. Since this methodology involves estimation of acid form of analytes, the change in peak area response of quantifiable acids between the stability and comparison samples was attributed to the interconversion. The percent change in acid form of analyte was calculated as: % change $=(s-c)/c\times100$, in which s and c represent the average peak area response of the analytes obtained from stability and comparison samples ($n=4$), respectively.

2.5.1. Effect of plasma temperature
Two sets of plasma samples were prepared: set-1 contains all three acid compounds, whereas set-2 contains all three lactone compounds. The aliquots of spiked samples were taken and kept at room temperature as well as in ice cold water bath for about 4 h acting as stability samples and evaluated against the comparison samples prepared at the end of 4 h.

2.5.2. Effect of anticoagulants
The role of various anticoagulants like lithium heparin, citrate phosphate dextrose adenine (CPDA), sodium fluoride (NaF) and ethylenediaminetetraacetic acid tripotassium salt (K_3EDTA) towards restricting the conversion of lactone compounds to acid compounds was compared. Plasma sets containing the anticoagulant were taken separately and spiked with all three lactone compounds and kept on bench for about 4 h acting as stability samples and evaluated against the comparison samples prepared at the end of 4 h.

2.5.3. Effect of plasma pH
Several different strengths of acidified buffer were assessed to restrict the interconversion between the acid and lactone forms of analytes. The buffer solutions tested include 10% OPA in water, 50% OPA in water and 1 M HCL in water. Two sets of plasma samples were prepared to evaluate the interconversion: set-1 contains all three acid compounds, whereas set-2 contains all

three lactone compounds. The spiked plasma samples were treated with these buffer solutions separately and kept at room temperature for about 4 h acting as stability samples and evaluated against the comparison samples prepared at the end of 4 h.

2.5.4. Effect of storage on bench top for different time and multiple freeze–thaw cycles

Three sets of plasma samples were prepared: set-1 contains all three acid compounds; set-2 contains all three acid and lactone compounds; set-3 contains all three acid and lactone compounds in buffered plasma. Four replicates of samples were prepared in each set, and analyzed after being kept at room temperature for different time and after exposing to multiple freeze–thaw cycles. The changing trends of analyte peak area response were plotted against respective variables.

2.6. Method validation

A full method validation was performed according to guidelines set by the USFDA [27]. The validation of this procedure was performed in order to evaluate the method in terms of selectivity, sensitivity, linearity, accuracy, precision, recovery, matrix effect, and stability of analyte during both short-term sample processing and long-term storage.

2.6.1. Selectivity

The selectivity of the assay was evaluated by analyzing blank plasma samples (six normal, one lipemic and one hemolyzed) from eight different donors and spiked plasma samples at LLOQ level. The peak area of the co-eluting components should be less than 20% and 5% those of the analytes and the corresponding ISTDs, respectively.

2.6.2. Linearity and sensitivity

The linearity of the method was determined by analysis of standard plots associated with a nine point calibration curve. Calibration curves from accepted three precision and accuracy batches were used to establish linearity. Peak area ratios of analyte/ISTD obtained from MRM were utilized for the construction of calibration curves, using weighted $(1/x^2)$ linear least squares regression of the plasma concentrations and the measured peak area ratios. Back-calculations were made from these curves to determine the concentration of analytes in each calibration standards and the resulting calculated parameters were used to determine concentrations of analyte in quality control or unknown samples. The correlation coefficient $(r) > 0.99$ was desirable for all the calibration curves. The lowest standard on the calibration curve was to be accepted as the LLOQ, if the analyte response was at least five times more than that of drug-free (blank) extracted plasma. In addition, the analyte peak of LLOQ sample should be identifiable, discrete, and reproducible with accuracy within $\pm 20\%$ and a precision $\leq 20\%$. The deviation of standards other than LLOQ from the nominal concentration should not be more than $\pm 15\%$.

2.6.3. Accuracy and precision

Intra- and inter-day accuracies were expressed as a percentage of deviation from the respective nominal value and the precision of the assay was measured by the percent coefficient of variation (%CV) at concentrations. Intra-day precision and accuracy was assessed by analyzing six replicates of the quality control samples

at four levels during a single analytical run. The inter-day precision and accuracy was assessed by analyzing 18 replicates of the quality control samples at each level through three precision and accuracy batches runs on 2 consecutive validation days. The deviation at each concentration level from the nominal concentration was expected to be within $\pm 15\%$ except limit of quantitation quality control (LOQQC), for which it should not be more than 20%. Similarly, the mean accuracy should not deviate by $\pm 15\%$ except for the LOQQC where it can be $\pm 20\%$ of the nominal concentration.

2.6.4. Recovery

Recovery was estimated at three QC concentration levels (low, medium, and high) by comparing the mean peak area of all the analytes in the QC samples $(n=6)$ with those of neat solutions $(n=3)$ containing analytes at concentrations equivalent to those obtained in the final extracted concentration in the QC samples. The recoveries of D5-ISTDs were measured in a similar manner using their corresponding medium QC samples as reference.

2.6.5. Matrix effect and matrix factor

Matrix effect was assayed at two concentration levels (LOQQC and HQC). Six different plasma lots (four normal, one hemolyzed, one lipemic) free of any significant interference at the retention time (RT) of analytes and ISTDs, were selected and spiked with the working solutions of LOQQC and HQC. Spiked samples from each plasma lot were processed in duplicate and quantitated against freshly spiked calibration curve. The matrix effect is validated to be nullified if the accuracy and precision does not deviate by $\pm 15\%$ for HQC and $\pm 20\%$ for LOQQC of the nominal concentration.

The matrix factor is defined as the peak area response in the presence of matrix ions versus the peak area response in the absence of matrix ions. Since this method involved terminal drying step, biological matrix samples were prepared by reconstituting the post-extracted blank plasma samples with neat solutions $(n=3)$ containing analytes and ISTDs at equivalent concentration representing the final extracted concentration for the analytes (low, medium, and high QC level) and ISTDs. The control samples were the same neat solutions prepared in reconstitution solution. Matrix factor was evaluated using six different blank plasma lots (four normal, one hemolyzed, one lipemic) and determined by measuring the respective mean peak area response (absolute matrix factor) and mean analyte/ISTD peak area ratio (ISTD normalized matrix factor) of biological matrix sample against the mean peak area response and mean analyte/ISTD peak area ratio of neat solutions.

2.6.6. Stability

Stability of analytes was evaluated using lactone fortified QC (L-QC) and unfortified QC samples. Bench-top stability was evaluated in ice cold water bath for ~ 6 h, which exceeds the residence time of the sample processing procedures. The freeze–thaw stability was evaluated after undergoing three freeze (at $-50\,^\circ$C)–thaw (ice cold water bath) cycles. Long-term stability was assessed after storage of the test samples at $-50\,^\circ$C for 103 days. The autosampler storage stability was determined by storing the reconstituted QC samples for ~ 49 h under autosampler condition (maintained at 5 $^\circ$C) before being analyzed. All stability exercises were performed against freshly spiked calibration standards processed along with freshly spiked unfortified comparison QC (UC-QC) samples at low and high concentrations $(n=4)$ for

determining the absolute stability of analytes. Absolute stability was calculated as follows: % absolute stability = (average concentration of stability samples/average concentration of comparison samples × C.F.) × 100; C.F. = concentration of stability sample/concentration of comparison sample. The analyte was considered stable at each concentration if the mean calculated concentration of stability samples does not deviate by ±15% of the mean calculated concentration of comparison quality control samples.

The working solutions and stock solutions of analytes and ISTDs were also evaluated for their stability at room temperature for about 9 h and at −20 °C for 17 days, respectively.

2.6.7. Method application

An open label, balanced, randomized, two-treatment, two-period, two-sequence, single-dose, crossover design was used for the assessment of pharmacokinetics and bioequivalence. Eighteen healthy adult male volunteers who gave written informed consent took part in this study. The study was approved by ethics committee of Institutional Review Board at Majeedia hospital (New Delhi, India). After an overnight fast of at least 10 h, all subjects were given a single oral dose of AT 80 mg tablet of Ranbaxy and Lipitor® tablet (containing AT calcium 80 mg) from Pfizer laboratories of Pfizer. Inc. USA during each period of the study. Blood samples were collected in pre-chilled K_3EDTA vacutainers at pre-dose (in-duplicate), 0.167, 0.333, 0.500, 0.750, 1.000, 1.250, 1.500, 1.750, 2.000, 2.250, 2.500, 2.750, 3.000, 4.000, 5.000, 6.000, 7.000, 8.000, 12.000, 16.000, 24.000, 36.000, 48.000, 60.000, 72.000 and 96.000 h post dose in each period. The plasma was immediately separated by centrifugation as well as stabilized with the buffer solution and stored at −50 °C until analysis. The pharmacokinetics parameters were calculated by a non-compartmental analysis using WinNonlin Professional software (Version 5.0, Pharsight Corp., Mountain View, CA, USA). The peak plasma concentration (C_{max}) and time to reach C_{max} (T_{max}) were read directly from the experimental data. The total area under the plasma concentration–time curve from time zero to infinity ($AUC_{0→∞}$) and from time zero to the last measurable concentration ($AUC_{0→t}$) was calculated using the trapezoidal rule-extrapolation method.

An incurred sample reanalysis was performed on ninety-six sample points selected randomly from the study population. The basic objective of ISR was to reconfirm the initial values and to demonstrate that the assay is reproducible. The conformity of the original result with the ISR sample is calculated as a % difference. The % difference should be within 20% for at least 67% or 2/3rd of the total reanalyzed incurred samples [28] [% difference = absolute (reanalyzed value − original value)/average of reanalyzed and original value × 100%].

3. Results and discussion

3.1. Method development

3.1.1. Mass spectrometry optimization

During the early stage of method development, both ESI and atmospheric pressure chemical ionization sources were investigated. ESI offered much higher signal intensities for all target analytes than the latter and was thus chosen as the ionization.

The first LC–MS/MS tests to select the optimum MS/MS parameters and the appropriate ions were carried out by syringe pump infusion of standard solution at a concentration of 25 ng/mL in mobile phase, with monitoring of MS intensity. The ionization efficiencies of AT and its active metabolites were almost similar.

These analytes contain an amino group that can receive protons and an aliphatic carboxylic group that can release proton. Hence, these analytes show responses in either ESI positive or ESI negative ion mode. It was found that the best results in terms of peak area and peak height were obtained in ESI negative ion mode than in ESI positive ion mode due to their readiness to lose proton from the carboxylic group. Moreover, the negative ion mode is usually considered as more specific, being relatively low the number of compounds undergoing negative ionization mode, and consequently less subjected to ion suppression [29,30]. In this context ESI negative ion mode was chosen. Fig. 2 shows the product ion spectra of the analytes and their deuterated ISTDs, as well as their tentative fragmentation profiles. The product ion is formed by the loss of 3,5-dihydroxy-heptanoic acid side chain along with phenylaminocarbonyl group. Deuterated ISTDs shared similar fragmentation patterns with their non-labeled counterparts. For MRM detection, the most stable and appropriate fragment ions produced by the analytes and ISTDs were selected. The lactone compounds are devoid of carboxylic functional group and thus show poor response in ESI negative ion mode compared to their acid counterparts (data not shown). Different additives of varying strength were added to the mobile phase, so as to obtain higher abundance of deprotonated parent ion of analytes and ISTDs. Use of formic acid over additives like acetic acid, ammonium formate, ammonium acetate, etc., in mobile phase enhances the occurrence of $[M−H]^−$ and eventually results in an optimal area response for analytes and ISTDs.

3.1.2. Liquid chromatography conditions

To select the starting conditions towards optimizing the LC parameters, we have paid attention to previous work, relating to the estimation of AT and its metabolites. In most of these studies, widely accepted C_{18} based analytical columns were employed to achieve good resolution with satisfying peak shape and peak symmetry. Thus a number of reversed-phase columns, such as Atlantis™ dC18, Ascentis® C_{18}, Symmetry® C_{18}, YMC basic C_{18}, and Ascentis® Express C_{18} were tested to achieve optimal resolution, selectivity, and efficiency with a short running time. Ascentis® Express C_{18} was chosen as an analytical column because it offers a really high separation power with modest operating pressure and involves the usual compromise of chromatographic characteristics. This technology of small superficially porous particles, also called as Fused-core®, gains wider popularity towards improving separation efficiencies and speed without reducing particle size. The very high efficiency of the fused-core particles is likely to be due to their extremely narrow particle size distribution, lower internal porosity, smaller diffusion distance, improved mass transfer, and better packing. Columns of 2.7 µm fused core particles show efficiencies almost equivalent to those containing totally porous sub-2 µm particles while maintaining robustness of the column [31].

The optimization procedure was focused on the mobile phase composition, column oven temperature, and injection volume. Firstly, several mobile phases composed of acidified water with appropriate organic phase (acetonitrile/methanol) in different ratios were tested. Initially, when acetonitrile was used, the acid metabolites exhibited satisfactory sensitivity; however, a good separation was not achieved between the acid and lactone compounds and matrix peaks affected the determination of 4-AT. Whereas the desired separation could be easily accomplished when methanol was used as organic phase; unfortunately, this leads to partial loss in
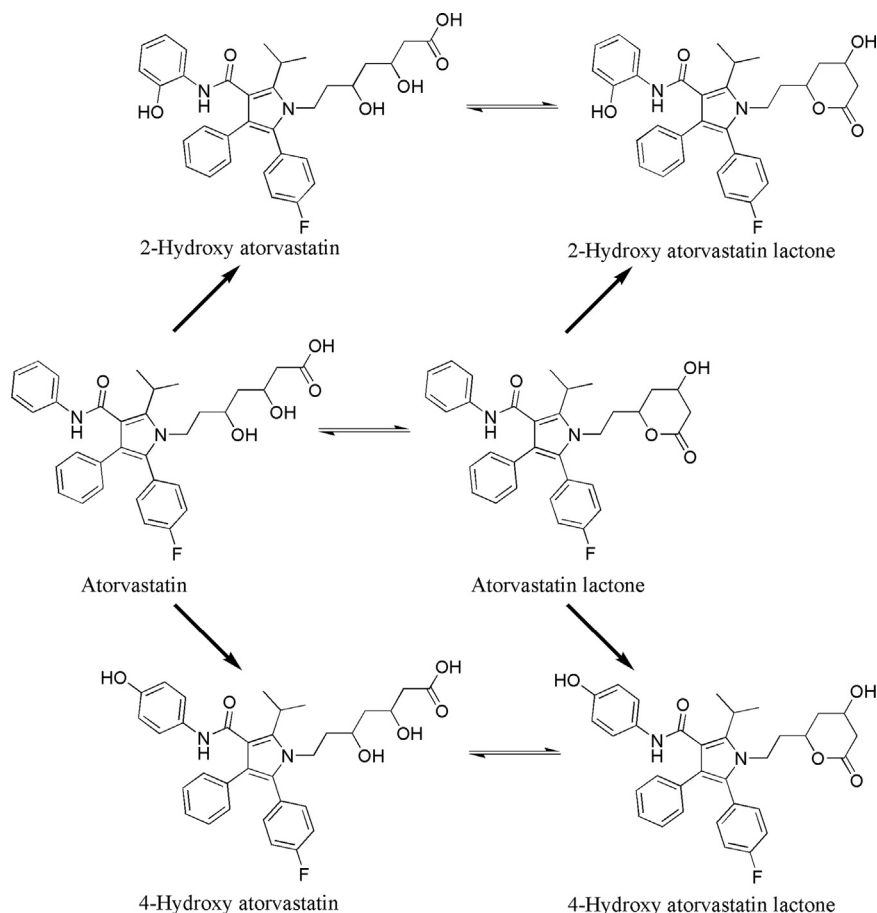
Fig. 1 Chemical structure and metabolic pathways of AT and its metabolites.

sensitivity. Considering the complementary advantages of acetonitrile and methanol in terms of signal sensitivity and separation efficiency, the mixture of methanol and acetonitrile was reasonably chosen as organic phase. After several tests had been performed, it was possible to obtain the desired resolution and sensitivity with the mobile phase composed of 0.005% of formic acid in water: acetonitrile:methanol (35:25:40, v/v/v). Simultaneously, tests were carried out to study the influence of the column temperature (between 30 and 65 °C, at an increment of 5 °C) on retention time and injection volume (from 10 to 50 μL) towards enhancing the sensitivity of the method. The best results were achieved when 20 μL was injected and column oven maintained at 45 °C. The set of samples prepared in reconstitution solution shows symmetric peak shape and produces higher signal–noise ratio (S/N) compared to those prepared in mobile phase.

Under the chromatographic conditions described above, the D5-ISTDs were eluted at the same retention time as their corresponding unlabeled analytes. The retention times were 1.8, 3.2 and 4.1 min for 4-AT, 2-AT, and AT, respectively (Fig. 3). The retention times were 2.1, 3.8 and 5.0 min for 4-AT lactone, 2-AT lactone, and AT lactone, respectively (data not shown). The difference in elution pattern achieved chromatographically benefited towards minimizing the potential interference to analytes, due to the ion-source fragmentation, from the labile lactone metabolites.

3.1.3. Sample preparation
During method development different options were evaluated to optimize sample cleanup so as to eliminate possible matrix inter-

ferences, concentrate the sample and obtain a sample as clean as possible to preserve the life of the analytical column. Firstly, the simplest and fastest protein precipitation (PPT) method for preparing samples was carried out; unfortunately, it did not result in a very clean extract and produced higher background noise with poor sensitivity. Secondly, liquid–liquid extraction was evaluated towards isolation of analytes from biological matrix that yields rather clean extracts compared to PPT, but the procedures involved was cumbersome and find multiple pitfalls. We needed to put in multiple extraction steps to increase analyte recovery and to get cleaner extracts; but failed to improve extraction efficiency for the polar acid metabolites specifically 4-AT. Finally, SPE was employed that always results in significant lower lipid levels, a significant source of matrix, compared to PPT and often produces excellent recoveries for polar and non-polar analytes. Taking this into account, we employed Oasis HLB SPE disposable cartridge to extract analytes from plasma samples. Use of acidified water during sample preparation helps in breaking the drug–protein binding partially alongside stabilizing the analytes in biological matrix. Moreover, 30% methanol in water during washing step helps to remove polar matrix interferences from cartridge bed. The extraction procedure described here offers a rapid way to isolate analytes and ISTDs from plasma matrix and provides scope for automation.

3.1.4. Interconversion
During the initial stages of method development, the influence of temperature on stability of the acid and lactone forms of analytes
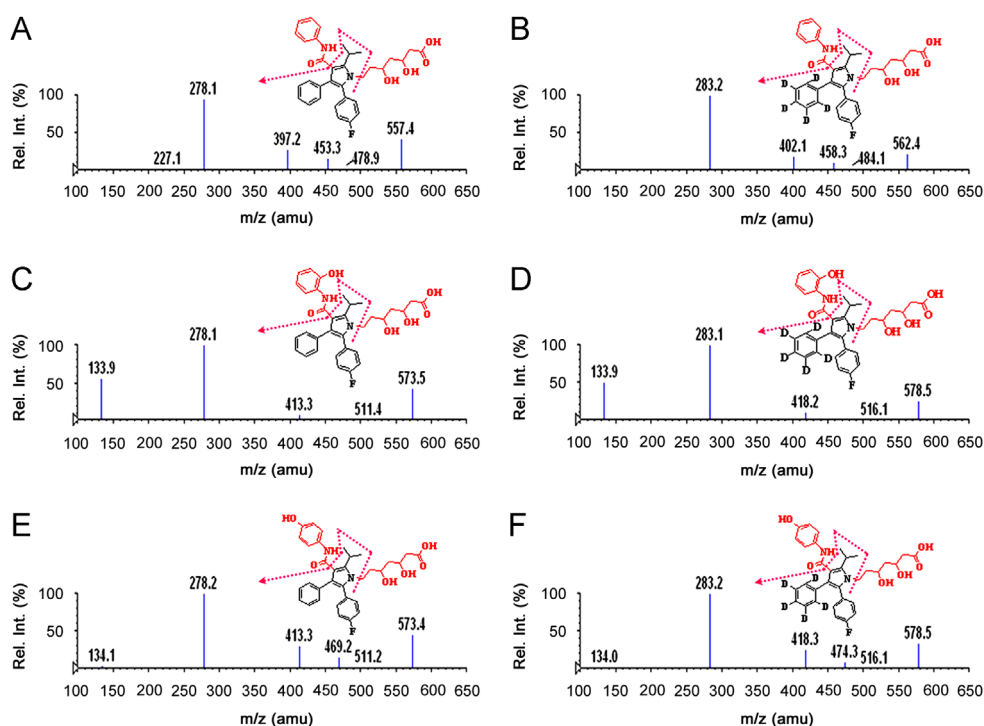
Fig. 2 The product ion spectra of: (A) AT, (B) AT-D5, (C) 2-AT, (D) 2-AT-D5, (E) 4-AT, and (F) 4-AT-D5.

was evaluated in plasma. The samples containing the three lactone compounds show a degradation of about 55% and 30% in 4 h when kept at room temperature and in ice-cold water bath, respectively, evidenced by an increased in peak area response of their respective acid in stability samples. On the contrary, samples containing the three acid compounds were found stable under both conditions demonstrated by an unchanged peak area response for acids in stability samples.

The influence of different anticoagulants on the conversion of lactone compounds to acid compounds was evaluated and it was observed that the use of anticoagulants like EDTA or NaF acts a good inhibitor of ester hydrolysis and restricts the conversion of lactone compounds to acid compounds certainly to a good extent compared to other tested anticoagulants. Simultaneously, the influence of different strengths of acidified buffer on the inter-conversion between acid and lactone compounds was investigated and shows that the addition of different acidified buffers to plasma could significantly affect the interconversion. When plasma pH was not controlled, i.e., at normal plasma pH the lactone compounds would easily get converted to acid compounds. However, if plasma pH was made too acidic a decrease in peak area response for the acid compounds would be observed; reasonably the acid compounds would have got converted to lactone compounds. The test result obtained therein (data not shown) validates that an immediate addition of 2% buffer solution (10% v/v, OPA in water) during sample collection in clinic as well as CC standard and QC sample preparation in lab, restricts the interconversion efficiently.

The test results of multiple freeze–thaw cycles and bench top storage for different time on the interconversion and/or stability of acid and lactone compounds (data not shown), indicated a prominent degradation of lactone compounds to acid compounds in the plasma samples (set-2) which were not stabilized with acidified buffer solution, evidenced by a significant increase in

the area response of acid compounds. Whereas the comparison between set-1 and set-3 data indicates that there was no conversion and/or degradation of lactone compounds to acid compounds seen in plasma samples treated with acidified buffer solution.

It is reported that apart from the enzymes in plasma, changing plasma pH which increases upon storage may results in degradation of drug and drug metabolites after samples have been collected and during sample processing [32]. Thus, it is supposed that use of an anticoagulant with esterase inhibitory property, simultaneously main-taining a stable pH in plasma alongside lowering sample temperature, is reasonable to restrict the instability problem completely as described in this research work.

3.2. Method validation

3.2.1. Selectivity
Fig. 3 shows the typical MRM chromatograms of blank plasma; plasma spiked with ISTDs; plasma spiked with AT, 2-AT and 4-AT at LLOQ (0.050 ng/mL for each analyte) and ISTDs; and plasma spiked with AT, 2-AT and 4-AT at ULOQ and ISTDs. Interfering peaks from endogenous components were not observed at the retention time of all the analytes and ISTDs.

3.2.2. Linearity and sensitivity
The linear regressions of the peak area ratios versus concentrations were fitted over the range of 0.050–100.138 ng/mL for AT, 0.050–100.198 ng/mL for 2-AT and 0.050–5.001 ng/mL for 4-AT in human plasma. The typical equations of the calibration curves were as follows: AT: $y=0.607x+0.0407$, $r=0.9991$; 2-AT: $y=0.356x+0.0283$, $r=0.9989$; 4-AT: $y=0.388x+0.0048$, $r=0.9989$; where y represents the ratio of analyte peak area to that of ISTD and x represents the nominal plasma concentration.

The LLOQ for this method was 0.050 ng/mL for AT and its hydroxyl metabolites. The achieved LLOQ was sufficient to study the pharmacokinetic profiles of AT, 2-AT and 4-AT following an oral administration of AT. Sensitivity of the method was established using LOQQC samples analyzed in three consecutive validation runs. Wherein, precision values obtained were below 6.6% and accuracy values were within ±4.3%.

3.2.3. Accuracy and precision

Eighteen replicates of the QC samples from three consecutive validation runs were used to evaluate precision and accuracy at each concentration level. The intra- and inter-day precision and accuracy values of the QC samples are summarized in Table 1. The intra-day precision for all the analytes was less than 5.2%, the inter-day precision was less than 6.6%, and the intra- and inter-day accuracy values were within ± 5.7%. The intra- and inter-day precision and accuracy values were within the acceptable range. The method was thus judged to be accurate and reproducible.

3.2.4. Recovery

At low, middle, and high QC concentration levels the mean recovery values were 76.3%, 78.0%, and 77.9% for AT; 73.1%, 75.1%, and 74.7% for 2-AT; and 75.7%, 72.6%, and 74.4% for 4-AT, respectively. In addition, the mean extraction recovery for AT-D5, 2-AT-D5, and 4-AT-D5 was 79.9%, 78.3%, and 71.1%, respectively. The result indicates that the extraction efficiency for analytes and ISTDs using SPE was satisfactory, consistent and was not concentration dependent.

3.2.5. Matrix effect and matrix factor

Matrix effect as well as matrix factor on the presented method was evaluated following the procedures described above. Results obtained therein indicate that no additional variations in plasma concentration due to the use of different plasma lots were observed as accuracy values at LOQQC and HQC levels were within the acceptable range (data not shown). Hence, the concentration of the analyte obtained from clinical study samples should therefore be considered as reliable.

The average absolute matrix factor values are 0.92, 0.95, and 0.94 for AT; 0.92, 0.97, and 0.98 for 2-AT, and 1.08, 1.09 and 1.05 for 4-AT at low, middle and high QC levels, respectively. Whereas the ISTD normalized matrix factor values are 1.01, 1.01, and 1.06 for AT; 0.99, 1.01, and 0.99 for 2-AT and 1.05, 0.98, and 0.96 for 4-AT at low, middle and high QC levels, respectively. Moreover, the CVs of absolute matrix factor and ISTD normalized matrix factor for all analytes from six lots of plasma samples were less than 4.9%. These data indicated that ion suppression or enhancement from the human plasma was negligible under the current conditions.

3.2.6. Stability studies

The results of stability tests evaluated following the procedures described above (data not shown) point out that all analytes spiked into human plasma were stable for at least 6.12 h in ice-cold water bath, in an autosampler post extraction for 49 h at 5 °C, in plasma placed at −50 °C for 103 days, and in plasma after three freeze–thaw cycles (−50 °C to ice-cold water bath). The stock solutions of all analytes and ISTDs were found stable at −20 °C for 17 days and the working solutions of all analytes and ISTDs were found stable for about 9 h at room temperature.

3.3. Method application

This developed method was applied to a pharmacokinetic study of AT in 18 healthy adult male volunteers following oral administration of 80 mg of AT tablet. The mean concentration–time profile of AT, 2-AT and 4-AT in these volunteers is shown in Fig. 4 and the mean estimated pharmacokinetic parameters derived from the plasma concentration profiles are summarized in Table 2. The bioequivalence parameters almost overlapped between the test and reference samples. The results of ISR, showing that 100.0%, 97.9%, and 95.7% of sample points for AT, 2-AT and 4-AT, respectively, were within ±20% of initial concentration value, further proving the proposed method is reproducible and suitable for pharmacokinetic evaluation of AT.

Table 1 Intra- and inter-day precision and accuracy data for the determination of AT, 2-AT, and 4-AT.

| Compound	Spiked concentration (ng/mL)	Intra-day ($n=6$)			Inter-day ($n=18$)		
		Mean (ng/mL)	Accuracy (%)	CV (%)	Mean (ng/mL)	Accuracy (%)	CV (%)
AT	0.050	0.049	98.3	4.9	0.049	98.7	3.5
	0.125	0.126	100.7	4.6	0.126	101.1	3.0
	12.815	13.313	103.9	2.0	13.202	103.0	1.5
	80.201	78.703	98.1	1.7	78.534	97.9	1.8
2-AT	0.050	0.053	105.7	2.8	0.051	101.8	4.9
	0.127	0.129	101.2	5.2	0.126	99.3	4.0
	12.723	12.704	99.8	1.3	12.525	98.4	1.9
	79.520	77.709	97.7	1.2	77.154	97.0	2.1
4-AT	0.050	0.048	95.7	4.7	0.051	102.0	6.6
	0.126	0.121	96.2	2.6	0.124	98.4	3.5
	1.101	1.083	98.3	3.4	1.068	97.0	3.1
	3.950	3.935	99.6	2.7	3.901	98.7	2.2
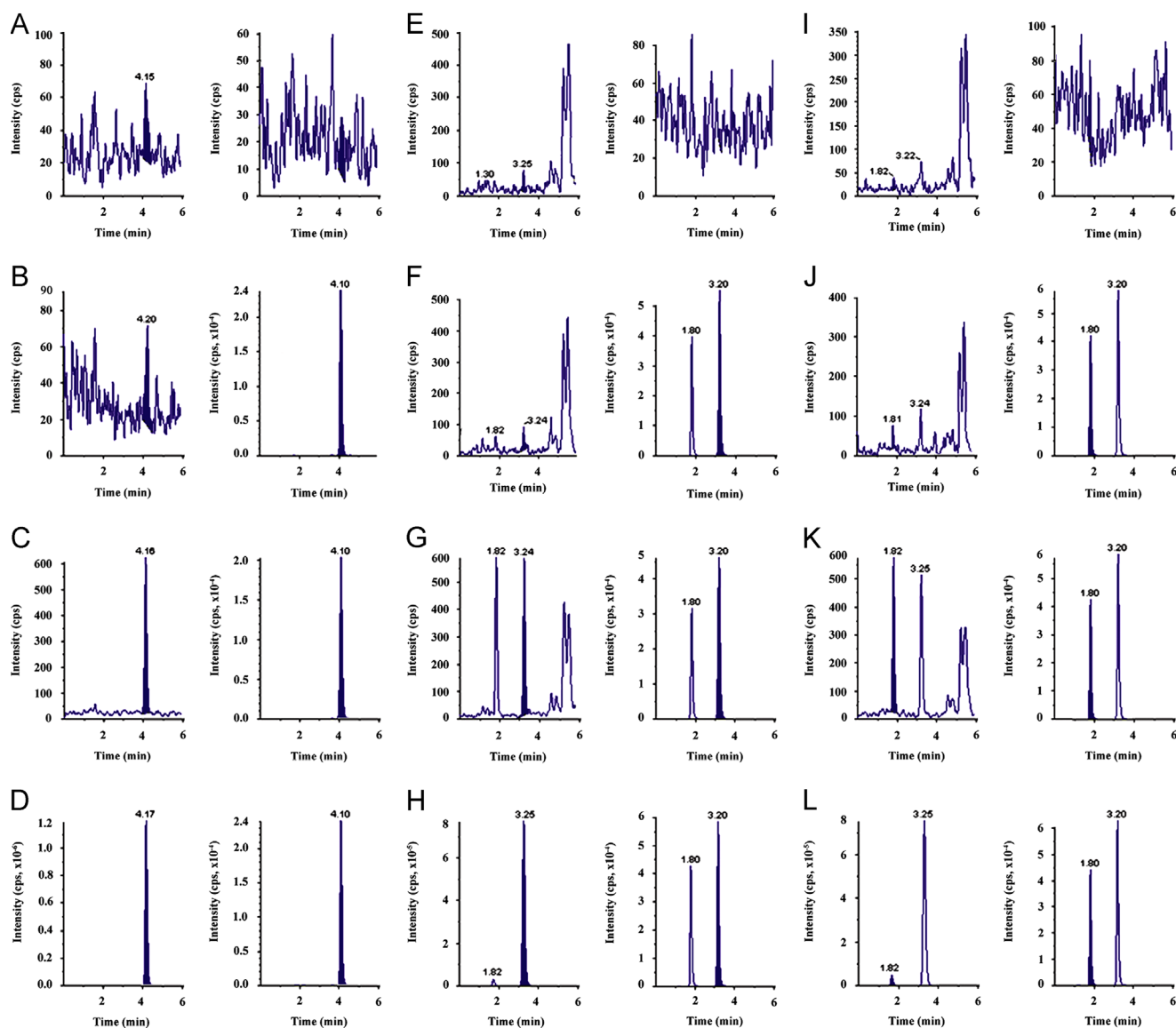
Fig. 3 Representative chromatograms of (1) AT, (2) 2-AT, and (3) 4-AT in human plasma: (A, E, I) blank; (B, F, J) blank+ISTD; (C, G, K) LLOQ; and (D, H, L) ULOQ. AT and AT-D5 (left panels, A–D); 2-AT and 2-AT-D5 (middle panels, E–H); 4-AT and 4-AT-D5 (right panels, I–L).
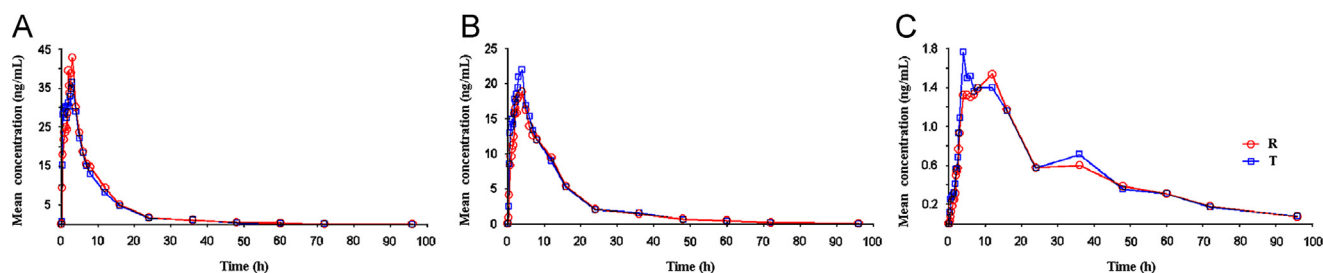

Fig. 4 The linear plasma mean concentration versus time profile of (A) AT, (B) 2-AT, and (C) 4-AT. (R: reference formulation; T: test formulation).

4. Conclusions

We developed and validated an LC–MS/MS method with ESI interface using negative ion mode for simultaneous determination of AT and its two hydroxyl metabolites (i.e., 2-AT and 4-AT) in human plasma. Use of deuterated ISTDs guaranteed the success of the assay by eliminating the impact of matrix effects. Fused-core® C$_{18}$ column chemistry, together with methanol–acetonitrile mixture as eluent in mobile phase, enhanced the separation capability of the positional isomers, improved the assay sensitivity, and

Table 2 Pharmacokinetic parameters (mean ± SD) of atorvastatin, after the administration of an oral dose of 80 mg test or reference formulations to healthy Indian male volunteers.

Parameters	AT		2-AT		4-AT	
	Reference	Test	Reference	Test	Reference	Test
T_{max} (h)	2.13 ± 1.20	1.85 ± 1.18	2.46 ± 1.20	2.55 ± 1.73	8.01 ± 3.93	10.06 ± 8.10
C_{max} (ng/mL)	74.94 ± 56.01	70.60 ± 35.89	27.78 ± 14.88	31.75 ± 21.59	1.85 ± 1.52	2.30 ± 2.65
$AUC_{0 \rightarrow t}$ (ng h/mL)	329 ± 229	314 ± 134	255 ± 151	267 ± 132	47 ± 48	49 ± 35
$AUC_{0 \rightarrow \infty}$ (ng h/mL)	334 ± 229	319 ± 135	259 ± 151	272 ± 132	68 ± 64	64 ± 39
$t_{1/2}$ (h)	10.96 ± 2.81	12.36 ± 3.38	15.51 ± 10.15	12.74 ± 3.35	19.45 ± 4.25	23.30 ± 7.88

shortened the running time. In addition proposed SPE procedure was simple, efficient and easy to automate. Moreover, our established method proved applicable to pharmacokinetic study, thus providing an efficient and robust support for further clinical studies.

Acknowledgments

References

[1] H. Lennernäs, Clinical pharmacokinetics of atorvastatin, Clin. Pharmacokinet. 42 (2003) 1141–1160.

[2] A. Poli, Atorvastatin: pharmacological characteristics and lipid-lowering effects, Drugs 67 (2007) 3–15.

[3] A.S. Kearney, L.F. Crawford, S.C. Mehta, et al., The interconversion kinetics, equilibrium, and solubilities of the lactone and hydroxyacid forms of the HMG-CoA reductase inhibitor, CI-981, Pharm. Res. 10 (1993) 1461–1465.

[4] D.D. Cilla Jr., L.R. Whitfield, D.M. Gibson, et al., Multiple-dose pharmacokinetics, pharmacodynamics, and safety of atorvastatin, an inhibitor of HMG-CoA reductase, in healthy subjects, Clin. Pharmacol. Ther. 60 (1996) 687–695.

[5] A.P. Lea, D. McTavish, Atorvastatin. A review of its pharmacology and therapeutic potential in the management of hyperlipidaemias, Drugs 53 (1997) 828–847.

[6] M. Igel, T. Sudhop, K. von Bergmann, Pharmacology of 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors (statins), including rosuvastatin and pitavastatin, J. Clin. Pharmacol. 42 (2002) 835–845.

[7] A.E. Black, R.N. Hayes, B.D. Roth, et al., Metabolism and excretion of atorvastatin in rats and dogs, Drug Metab. Dispos. 27 (1999) 916–923.

[8] H.S. Malhotra, K.L. Goa, Atorvastatin: an updated review of its pharmacological properties and use in dyslipidaemia, Drugs 61 (2001) 1835–1881.

[9] W. Jacobsen, B. Kuhn, A. Soldner, et al., Lactonization is the critical first step in the disposition of the 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor atorvastatin, Drug Metab. Dispos. 28 (2000) 1369–1378.

[10] T. Prueksaritanont, R. Subramanian, X. Fang, et al., Glucuronidation of statins in animals and humans: a novel mechanism of statin lactonization, Drug Metab. Dispos. 30 (2002) 505–512.

[11] E.L. Posvar, L.L. Radulovic, D.D. Cilla Jr., et al., Tolerance and pharmacokinetics of single-dose atorvastatin, a potent inhibitor of HMG-CoA reductase, in healthy subjects, J. Clin. Pharmacol. 36 (1996) 728–731.

[12] G. Bahrami, B. Mohammadi, S. Mirzaeei, et al., Determination of atorvastatin in human serum by reversed-phase high-performance liquid chromatography with UV detection, J. Chromatogr. B 826 (2005) 41–45.

[13] A. Zarghi, A. Shafaati, S.M. Foroutan, et al., A simple and rapid HPLC method for the determination of atorvastatin in human plasma with UV detection and its application to pharmacokinetic studies, Arzneimittelforschung 55 (2005) 451–454.

[14] P.H. Siedlik, S.C. Olson, B.B. Yang, et al., Erythromycin coadministration increases plasma atorvastatin concentrations, J. Clin. Pharmacol. 39 (1999) 501–504.

[15] D.M. Gibson, R. Stern, R.B. Abel, Absolute bioavailability of atorvastatin in man [abstract], Pharm. Res. 14 (1997) S253.

[16] L. Ma, J. Dong, X.J. Chen, et al., Development and validation of atorvastatin by LC–ESI-MS and application in bioequivalence research in healthy Chinese volunteers, Chromatographia 65 (2007) 737–741.

[17] W.W. Bullen, R.A. Miller, R.N. Hayes, Development and validation of a high-performance liquid chromatography tandem mass spectrometry assay for atorvastatin, ortho-hydroxy atorvastatin, and para-hydroxy atorvastatin in human, dog, and rat plasma, J. Am. Soc. Mass Spectrom. 10 (1999) 55–66.

[18] M. Jemal, Z. Ouyang, B.C. Chen, et al., Quantitation of the acid and lactone forms of atorvastatin and its biotransformation products in human serum by high-performance liquid chromatography with electrospray tandem mass spectrometry, Rapid Commun. Mass Spectrom. 13 (1999) 1003–1015.

[19] M. Hermann, H. Christensen, J.L. Reubsaet, Determination of atorvastatin and metabolites in human plasma with solid-phase extraction followed by LC–tandem MS, Anal. Bioanal. Chem. 382 (2005) 1242–1249.

[20] V. Borek-Dohalský, J. Huclová, B. Barrett, et al., Validated HPLC–MS–MS method for simultaneous determination of atorvastatin and 2-hydroxyatorvastatin in human plasma—pharmacokinetic study, Anal. Bioanal. Chem. 386 (2006) 275–285.

[21] R.V. Nirogi, V.N. Kandikere, M. Shukla, et al., Simultaneous quantification of atorvastatin and active metabolites in human plasma by liquid chromatography–tandem mass spectrometry using rosuvastatin as internal standard, Biomed. Chromatogr. 20 (2006) 924–936.

[22] D. Liu, J. Jiang, H. Zhou, et al., Quantitative determination of atorvastatin and para-hydroxy atorvastatin in human plasma by LC–MS–MS, J. Chromatogr. Sci. 46 (2008) 862–866.

[23] D. Guillén, F. Cofán, E. Ros, et al., Determination of atorvastatin and its metabolite ortho-hydroxyatorvastatin in human plasma by on-line anion-exchange solid-phase extraction and liquid chromatography tandem mass spectrometry, Anal. Bioanal. Chem. 394 (2009) 1687–1696.

[24] L. Nováková, H. Vlčková, D. Satínský, et al., Ultra high performance liquid chromatography tandem mass spectrometric detection in clinical analysis of simvastatin and atorvastatin, J. Chromatogr. B 877 (2009) 2093–2103.

[25] J.S. Macwan, I.A. Ionita, M. Dostalek, et al., Development and validation of a sensitive, simple, and rapid method for simultaneous quantitation of atorvastatin and its acid and lactone metabolites by liquid chromatography–tandem mass spectrometry (LC–MS/MS), Anal. Bioanal. Chem. 400 (2011) 423–433.

[26] M. Hermann, M.P. Bogsrud, E. Molden, et al., Exposure of atorvastatin is unchanged but lactone and acid metabolites are

increased several-fold in patients with atorvastatin-induced myopathy, Clin. Pharmacol. Ther. 79 (2006) 532–539.

[27] Guidance for Industry: Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research, Rockville, MD, 2001. Available from: ⟨http://www.fda.gov/CVM⟩.

[28] C.T. Viswanathan, S. Bansal, B. Booth, et al., Workshop/conference report—quantitative bioanalytical methods validation and implementation: best practices for chromatographic and ligand binding assays, AAPS J. 9 (2007) E30–E42.

[29] J.M. Marín, E. Gracia-Lor, J.V. Sancho, et al., Application of ultra-high-pressure liquid chromatography–tandem mass spectrometry to the determination of multi-class pesticides in environmental and wastewater samples. Study of matrix effects, J. Chromatogr. A 1216 (2009) 1410–1420.

[30] W.M. Niessen, P. Manini, R. Andreoli, Matrix effects in quantitative pesticide analysis using liquid chromatography–mass spectrometry, Mass Spectrom. Rev. 25 (2006) 881–899.

[31] A.J. Alexander, T.J. Waeghe, K.W. Himes, et al., Modifying conventional high-performance liquid chromatography systems to achieve fast separations with fused-core columns: a case study, J. Chromatogr. A 1218 (2011) 5456–5469.

[32] A. Fura, T.W. Harper, H. Zhang, et al., Shift in pH of biological fluids during storage and processing: effect on bioanalysis, J. Pharm. Biomed. Anal. 32 (2003) 513–522.