Quantitative Determination of Atorvastatin and Para-hydroxy Atorvastatin in Human Plasma by LC–MS–MS

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Abstract

A specific, sensitive, and fast method based on high-performance liquid chromatography coupled to tandem mass spectrometry was developed for the determination of atorvastatin and para-hydroxy atorvastatin in human plasma. Solid-phase extraction was used to isolate the compounds from human plasma followed by injection of the extracts onto a C_{18} column with isocratic elution. The lower limits of quantitation was 0.229 and 0.202 ng/mL for atorvastatin and para-hydroxy atorvastatin in human plasma, respectively. The method was then successfully applied to the pharmacokinetic study of atorvastatin in healthy Chinese male subjects.

Introduction

Atorvastatin (AT) is a potent synthetic inhibitor of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis. The drug has been extensively used in many countries for the treatment of hyperlipidemia because of its high efficacy and safety (1). The drug is administered in an inactive form of calcium salt and is converted into active atorvastatin acid which then metabolized into two other active metabolites, para-hydroxy atorvastatin (p-AT), and orth-hydroxy atorvastatin (o-AT) (2). These three active compounds were subsequently equilibrated with their corresponding lactone forms at the ratio of approximately 1:1 (3). The chemical structures of the three active compounds and the three interconvertible products are shown in Figure 1.

There are two methods reported to determine the level of atorvastatin and its metabolites in human serum (4) or plasma using a UV detector (5). But their lower limit of quantitation (LLOQ) was too high (higher than 4 ng/mL) to delineate the pharmacokinetic profiles of atorvastatin in subjects who had taken 40 mg of atorvastatin, which is the medium dose in clinical application.

Because tandem mass spectrometry (MS) detection is more sensitive and does not need full chromatographic separation of the detected compounds, most published methods for the determination of AT and its metabolites in human plasma were high-performance liquid chromatography (HPLC)–MS–MS (6,7,8). Nevertheless, although the sensitivity and analysis speed were improved by these methods, it still requres more than 0.5 mL plasma sample from a subject for each time point. This will be very likely to decrease the compliance of the subjects. Besides, according to these methods, too much volume (at least 40 µL) of extracted solution was injected into HPLC–MS–MS system after condensation. This may also bear a possibility of HPLC–MS–MS system contamination.

Therefore, a fast, more sensitive, and much cleaner HPLC–MS–MS method with less sample volume was investigated and validated in this study, in order to fulfill the requirement of clinical phase I pharmacokinetic investigation.

Experimental

Chemicals

AT (purity 91.62%), *p*-AT (purity 80.89%), *o*-AT, AT lactone, *p*-AT lactone, AT-d5 (internal standard for AT), *p*-AT-d5 (internal standard for *p*-AT) were all provided by Pfizer. Except for AT and

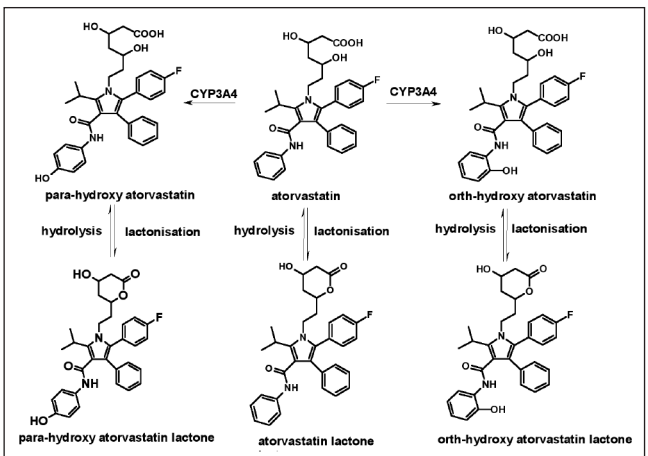

Figure 1. Chemical structures of atorvastatin and its metabolites *o*- and *p*-hydroxy atorvastatin and the interconcersion products atorvastatin lactone, *o*- and *p*-hydroxy atorvastatin lactone.

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p-AT, purities of other compounds were used as 100%. Methanol and acetonitrile (HPLC grade) were purchased from Burdick & Jackson (Muskegon, MI, USA). Glacial acetic acid and ammonium acetate were both of analytical grades and purchased from Peking Chemical Plant (Beijing, China). Drug-free human plasma (anticoagulant: heparin lithium) used in the study was supplied by Peking Union Medical College Hospital Blood Bank. Distilled water was prepared by a Milli-Q water purifying system (Millipore, Bedford, MA).

Calibration standard and quality control samples in human plasma

Stock solutions of AT, *p*-AT, *o*-AT, AT lactone, and *p*-AT lactone for calibration standards (CS) and quality control (QC) were prepared separately in acetonitrile–water (50:50, v/v). Stock solutions of AT-d5 and *p*-AT-d5 were also prepared in acetonitrile–water (50:50, v/v). In order to investigate whether these lactones and *o*-PT interfere the concentration of AT and *p*-AT in clinical samples during analysis procedure, *o*-AT, AT lactone and *p*-AT lactone were mixed with them and were stored together. The concentrations of stock solution were 0.200 mg/mL for *o*-AT, AT lactone, and *p*-AT lactone, 0.183 mg/mL for AT and 0.162 mg/mL for *p*-AT. The stock solutions of AT, *p*-AT, *o*-AT, AT lactone, and *p*-AT lactone were mixed and further diluted with acetonitrile–water (50:50, v/v) to obtain working solutions at several concentration levels waiting for the preparation of calibration standard and quality control. Different stock solutions of AT, *p*-AT, *o*-AT, AT lactone, and *p*-AT lactone were used for preparation of calibration standards and QC samples.

Calibration standard and QC samples in plasma were prepared by diluting corresponding mixed working solutions with drug-free human plasma. The final concentrations of calibration standard in plasma were 0.229, 0.458, 0.916, 2.29, 4.58, 9.16, 22.9, 45.8, and 91.6 ng/mL for AT, and 0.202, 0.404, 0.809, 2.02, 4.04, 8.09, 20.2, 40.4, and 80.9 ng/mL for *p*-AT. The final concentration of QC in plasma samples were 0.229, 0.733, 7.33, and 73.3 ng/mL for AT and 0.202, 0.647, 6.47, and 64.7 ng/mL for *p*-AT. The concentrations of *o*-AT, AT lactone, and *p*-AT lactone in calibration standard and QC samples were proportional to the corresponding concentrations of AT and *p*-AT, and their concentrations were not to be determined. Internal standard working solution was prepared in methanol.

All the plasma samples were stored at –70°C.

Extraction procedure

Calibration standard samples, QC samples, and clinical plasma samples were extracted using an SPE technique. 50 µL of 5 ng/mL internal standard working solution and 0.4 mL of 100mM ammonium acetate (pH 4.5) were added to 0.1 mL of plasma treated with heparin. Following vortex mixing, the mixtures were loaded onto Waters Oasis HLB SPE columns (30 mg), which were pretreated with 0.4 mL of methanol followed by 0.8 mL of 100 mmol/L ammonium acetate (pH 4.6). After the mixtures were loaded, the SPE columns were washed with 0.5 mL of 100 mmol/L ammonium acetate (pH 4.6) followed by 0.9 mL of methanol–water (20:80, v/v). Then, the columns were vacuumed to dryness and the analytes were eluted with 0.3 mL of methanol–water (95/5, v/v). The eluates were collected and evaporated to dryness under nitrogen stream at 35°C, then reconstituted with 0.2 mL of mobile phase.

Liquid chromatography–mass spectrometry

HPLC was performed on Waters Alliance 2790 HPLC system. Chromatographic separation was carried out on a Genesis C_{18} column (2.1 × 50 mm, 4 µm) at ambient temperature. The mobile phase was composed of 0.1% acetic acid in water–acetonitrile (4:6, v/v). The flow rate was 0.2 mL/min and the injection volume was 15 µL for each sample.

Mass chromatograms were recorded using an API 3000 triple-quadrupole mass spectrometer with Turbo IonSpray interface (Applied Biosystems/MDS Sciex, Foster City, CA). Analysis was performed with an ionizing voltage of 5000 V. Ion source temperature was set at 350°C with ultrahigh-purity nitrogen as curtain gas (8 L/h), nebulizer gas (13 L/h), and auxiliary gas (8 L/h). Other mass-dependent parameters such as orifice plate voltage (OR), focusing ring voltage (RNG), Q2 rod offset voltage (RO_2), RF-stubbies voltage (ST_3), and Q2 rod offset voltage (RO_3) for each compound were determined in positive mode using standard solutions. Multiple-reaction monitoring (MRM) was carried out using nitrogen as collision gas (8 L/h), and with a dwell time of 200 ms for each transition. The analytes were detected by monitoring the transitions *m/z* 559.3 → 440.1, 564.1 → 445.4, 575.0 → 440.0, and 580.4 → 445.4 with the collision energy 34 V for AT, AT-d5, *p*-AT, and *p*-AT-d5, respectively. The analytical time for each run was 2.5 min in total.

Method validation

The method was validated for selectivity, intra- and inter-day precision and accuracy, linearity, sensitivity, recovery, and various stabilities according to the China State Food and Drug Administration (SFDA) guidelines (9) for the validation of bioanalytical methods.

The specificity of this method was investigated by analyzing six different individual human blank blood samples. Each blank sample was tested for interference using the proposed extraction procedure and HPLC–MS–MS conditions and compared with a spiked sample at a concentration of the LLOQ for each analyte in plasma.

Calibration standard samples in human plasma were prepared for five separate days. Intra- and inter-day precision and accuracy were measured by determining the concentrations of AT and p-AT in plasma in five replicates of QC samples at four different concentrations for five separate days.

The extraction recoveries of AT and *p*-AT were determined by comparing quantitative results of extracted QC samples at three concentrations (LQC, MQC, and HQC) to unextracted QC samples at the same concentration.

The stabilities of AT and *p*-AT in biological matrix and in working solution at different storage conditions were evaluated as follows, and the results were expressed as percentage recoveries. The stabilities of AT and *p*-AT working solutions were tested for 6 h at room temperature. The stabilities of AT and *p*-AT in plasma sample at three concentrations were examined under different study conditions (i.e., standing at 4°C for 12 h and storing at –70°C for 2 weeks). The stabilities of AT and *p*-AT in plasma extracts were also tested by storing samples at 4°C for 24 h.

Freeze/thaw stability was determined after freezing (–70°C) and thawing QC samples for three cycles.

Result and Discussion

HPLC–MS–MS optimization

First, an HPLC–MS–MS method for the detection of AT and p-AT in plasma was investigated. The analytes were introduced into the mass spectrometer using an electrospary interface, and the parameters such as IS, OR and RNG were optimized to obtain protonated molecular ion $[M+H]^+$. In order to ensure the high specificity of the method, MRM scan mode was selected to assay the analytes and the most suitable collision energy was determined when observing the maximum response for fragment ions. The product ion mass spectra of the compounds are depicted in Figure 2 where $[M+H]^+$ of each compound was selected as precursor ion, and the most abundant fragment ion was chosen as the product ion in the MRM acquisition with the optimized collision energy for each compound.

Then, Genesis C18 column was selected to retain the analytes. The sensitivity and retention time of AT and p-AT was increased as the concentration level of acetic acid in water was increased.

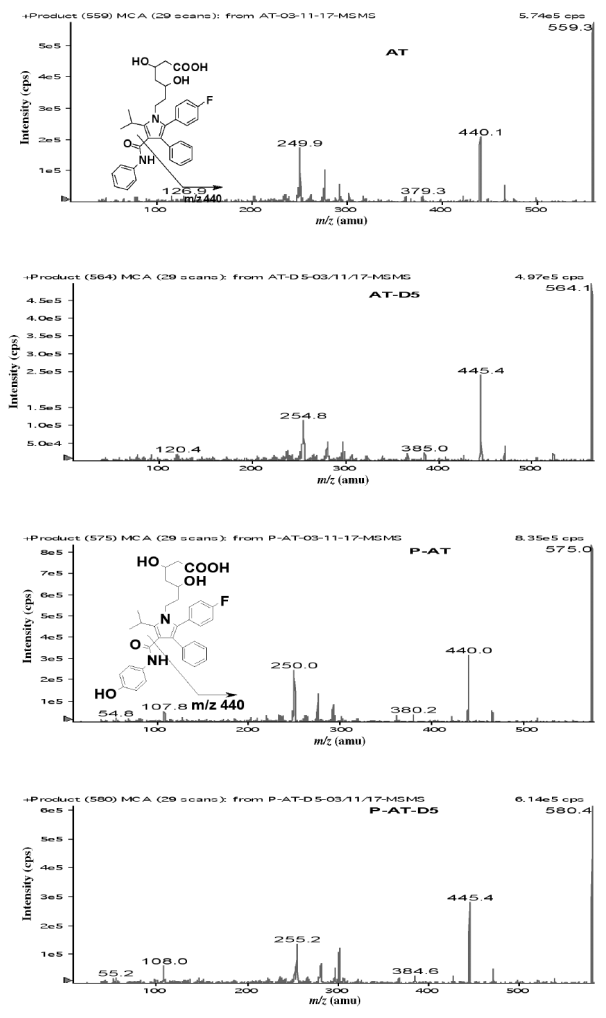

Figure 2. Electrospray product ion mass spectra of the precursor ions of AT, AT-d5, *p*-AT, and *p*-AT-d5.

Finally, the mobile phase contained 0.1% acetic acid in water and acetonitrile was chosen because of their acceptable response and appropriate retention time.

Extraction procedure optimization

The methods of sample preparation were developed using solid-phase extraction (SPE) method. In order to avoid the transformation of AT and *p*-AT into their lactone form at room temperature, the plasma samples were thawed in 4°C until loaded onto SPE cartridges.

Validation step

Specificity and sensitivity

The chromatograms of blank sample and LLOQ were used to estimate the specificity and sensitivity of the method. From the chromatograms, we found no endogenous source of interference at the retention times of the analytes, which were approximately 1.70 min for AT and AT-d5 and 0.88 min for *p*-AT and *p*-AT-d5. Typical chromatograms obtained from blank plasma, plasma sample at LLOQ level for both of AT and *p*-AT are presented in Figures 3 and 4, respectively.

Linearity

A calibration curve was established on each validation day. The calibration curve was linear over the concentration range of 0.229–91.6 ng/mL for AT and 0.202–80.9 for *p*-AT with coefficient of correlation (r) > 0.996, respectively. A weighting factor of $1/x$ for both of AT and *p*-AT was chosen. Linearity was found to be quite satisfactory.

Recovery

The recoveries of the extraction method from plasma observed (value and CV %, n = 5) for both of AT and *p*-AT are shown in Table I. Recoveries were more than 42.8% at different concentrations for both AT and *p*-AT with acceptable variance.

Matrix effect

Matrix effects and inter-subject variability data from plasma of individual subjects were not investigated in the present method validation because of the use of isotope-labeled compound as

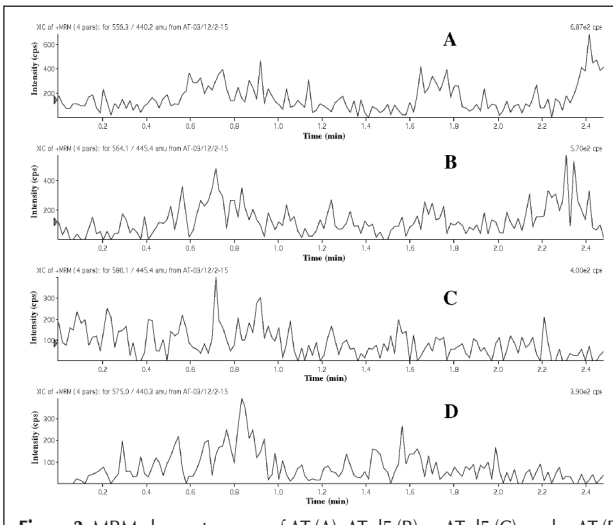

Figure 3. MRM chromatograms of AT (A), AT-d5 (B), *p*-AT-d5 (C), and *p*-AT (D) in blank plasma (free of drugs and IS).

internal standard. The use of isotope analog as internal standard is expected to experience the same exact matrix effect as analyte in any batch of plasma and will ensure that the accuracy/precision of the method is unaffected by presence of the matrix effect (10,11).

Precision and accuracy

Five quality control samples at each concentration level (0.229, 0.733, 7.33, 73.3 ng/mL for AT and 0.202, 0.647, 6.47, and 64.7 ng/mL for *p*-AT) were processed and calculated each batch of five for five batches to provide precision (RSD %) and accuracy of this method. The intra- and inter-day precision and accuracy data for AT and *p*-AT in plasma are summarized in Table II and III, respectively. For both of AT and *p*-AT over the five batches, intra-day precision was less than 8.0%, and the accuracy ranged from 92.9% to 109.0%; inter-day precision was less than 7.9% and the accuracy ranged from 92.6% to 100.1%, where intra- and inter-day precisions of LLOQ were less than 11.3% and intra- and inter-day accuracies of LLOQ ranged from 96.9% to 116.3%. The result verified that the method had a good precision and accuracy for AT and *p*-AT in plasma samples.

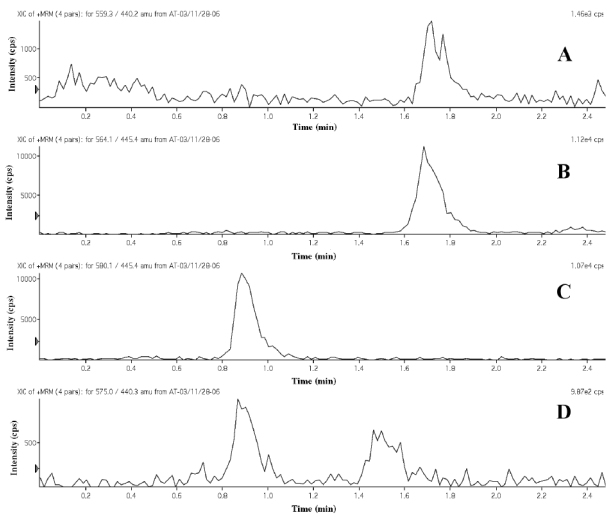

Figure 4. MRM chromatograms of AT (A), AT-d5 (B), *p*-AT-d5 (C), and *p*-AT (D) in plasma sample at LLOQ level.

Table I. Results of Extraction Recovery of ATV and *p*-ATV in Plasma (*n* = 5)

Analyte	ATV			p-ATV		
Nominal conc. (ng/mL)	0.733	7.33	73.3	0.647	6.47	64.
Overall recovery (%)	44.2	56.7	58.3	42.5	67.5	64.2
RSD (%)	2.3	15.0	12.3	1.6	11.9	6.2

Table II. Accuracy and Inter-, Intra-Batch Precision for the Detection of ATV in Plasma

	LLOQ	Q1	Q2	Q3
Intra-batch*	0.229	0.733	7.33	73.3
Mean	0.228	0.706	7.30	72.8
SD (*n* = 5)	0.01	0.05	0.18	0.73
Overall accuracy (%)	99.6	96.3	99.6	99.3
Precision (%)	5.9	6.7	2.5	1.0
Inter-batch*	0.229	0.733	7.33	73.3
Mean	0.230	0.734	7.20	71.5
SD (*n* = 5)	0.02	0.05	0.28	2.42
Overall accuracy (%)	100.3	100.1	98.2	97.5
Precision (%)	7.9	6.7	3.9	3.4

* Nominal concentration (ng/mL).

Table III. Accuracy and Inter-, Intra-Precision for the Detection of *p*-ATV in Plasma

	LLOQ	Q1	Q2	Q3
Intra-batch*	0.202	0.647	6.47	64.7
Mean	0.220	0.609	6.31	62.6
SD (*n* = 5)	0.01	0.02	0.21	2.09
Accuracy (%)	108.9	94.1	97.5	96.8
Precision (%)	4.9	3.1	3.3	3.3
Inter-batch	0.202	0.647	6.47	64.7
Mean	0.218	0.663	6.23	63.2
SD (*n* = 5)	0.02	0.05	0.27	3.06
Accuracy (%)	107.8	102.4	96.3	97.6
Precision (%)	7.8	7.6	4.3	4.8

* Nominal concentration (ng/mL).

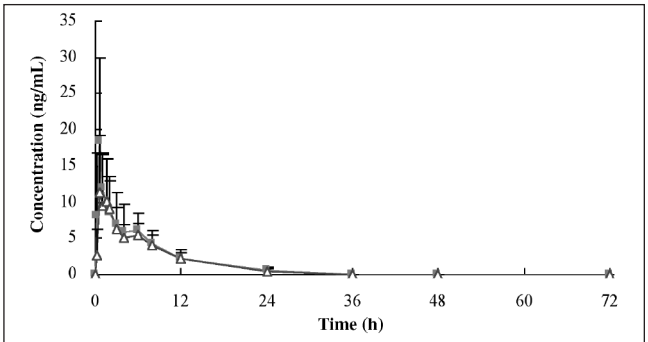

Figure 5. The average concentration-time curves of AT in plasma from healthy Chinese subject after administration of test drug (Δ) and reference drug (❑) (Mean ± SD, *n* = 30).

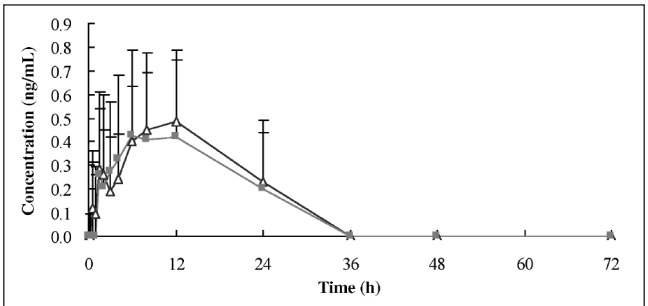

Figure 6. The average concentration-time curves of *p*-AT in plasma from healthy Chinese subjects after administration of test drug (Δ) and reference drug (❑) (Mean ± SD, *n* = 30).

Stability

The stability tests of the analytes were designed to cover expected conditions of handling of clinical samples. The stabilities of the analytes in human plasma were investigated under a variety of storage and processing conditions. Briefly, three freeze/thaw cycles and 4°C storage of the QC samples up to 12 h appeared to have no effect on results of quantification of AT and *p*-AT in plasma. QC samples stored in a freezer at or below –70°C remained stable for at least 2 weeks. Processed samples were allowed to stand at 4°C in reconstituted solution for 24 h prior to analysis, with no observed effect on results of quantification. After working solution of AT and *p*-AT in methanol was stored at room temperature for 6 h, the analytes were found to be stable.

Carryover test

The MRM chromatogram of double blank (free of AT, *p*-AT, and its internal standard) analyzed by following the samples with the highest concentration of ULQ (91.6 ng/mL for AT and 80.9 ng/mL for *p*-AT) had showed that there was no carryover in the condition of the present method.

Inter-conversion test

Due to the presence of lactone form in clinical plasma samples, the inter-conversion between the two forms should be assessed before the determination of AT and *p*-AT in these clinical plasma samples. From the results of linearity, precision and accuracy, and various stabilities, the inter-conversion between the two forms during the analysis procedure were verified to have no effect on the determination of AT and *p*-AT with the present method and could be used to determine AT and *p*-AT in clinical samples.

Application of the method in pharmacokinetic studies

The HPLC–MS–MS method described in this paper was used to investigate the plasma pharmacokinetic profiles of AT and *p*-AT in healthy Chinese subjects after administering with 40 mg atorvastatin tablet in two different formulations (n = 30). The mean plasma concentration–time curve of AT and *p*-AT in subjects after dosing atorvastatin are shown in Figures 5 and 6, respectively. The terminal phase of *p*-AT was not collected completely, which had little effect on the assessment of bioequivalence (BE) between the two different formulations of atorvastatin tablet. This was because the BE study asked for more attention to the parent drug rather than its metabolites (12).

Conclusion

A more sensitive and cleaner HPLC–MS–MS method with SPE procedure has been developed and validated for the determination of AT and *p*-AT in plasma of healthy Chinese subjects. The extraction procedure and HPLC–MS–MS conditions were optimized in order to improve the sensitivity and robustness of the method. The procedure was fully validated to meet the requirements of China State Food and Drug Administration and GLP Guidelines for Industry. This procedure was successfully applied to the determination of atorvastatin in human plasma.

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