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An HPLC–MS/MS method for the quantitative determination of 4-hydroxy-anethole trithione in human plasma and its application to a pharmacokinetic study

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ABSTRACT

A selective, rapid and sensitive method for the quantitation of 4-hydroxy-anethole trithione (ATX) in human plasma based on high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) was developed and validated. Paracetamol was used as the internal standard (I.S.). After liquid–liquid extraction of 500 μL plasma with ethyl acetate, ATX and the I.S. were chromatographed on an Inertsil® ODS-3 column. The mobile phase was consisted of methanol–water (75:25, v/v) with a flow rate of 0.25 mL/min. The detection was performed on a triple quadrupole tandem mass spectrometer by multiple reaction monitoring (MRM) mode via electrospray ionization (ESI) source. The calibration curve was linear over the range of 0.452–603 ng/mL ($r^2 \geq 0.99$) with a lower limit of quantitation (LLOQ) of 0.452 ng/mL. The intra- and inter-day precision (relative standard deviation, R.S.D.) values were below 13% and the accuracy (relative error, R.E.) was from −2.7% to −7.5% at three quality control levels. The assay herein described was successfully applied to a pharmacokinetic study of anethole trithione (ATT) tablet in healthy volunteers after oral administration.

1. Introduction

Anethole trithione (ATT, 5-(p-methoxyphenyl)-3H-1,2-dithiole-3-thione) which is a sulfur heterocyclic compound is used as a relatively new chologogue and marketed in many countries. ATT administration prevented the up-regulation in muscarinic acetylcholine receptor density and increased cholinergic and adrenergic responsiveness [1]. It was also reported to have a beneficial effect on xerostomia in patients treated with psychotropic drugs (tricyclic antidepressants or neuroleptics) [2]. As an adjunctive drug, it has been shown to be effective in the treatment of cholecystitis, gallstone, indigestion and acute/chronic hepatitis.

To our knowledge, few methods have been published for the quantitation of ATT in biological matrixes because of its low plasma concentration after an oral therapeutic dose. An HPLC–UV method was established to determine ATT in rabbit plasma with a lower limit of quantitation (LLOQ) of 0.5 ng/mL [3]. Recently, Li et al. [4] carried out the determination of ATT in human plasma by HPLC–MS/MS with a linear range of 0.02–5 ng/mL. Previous study [5] described that most ATT was metabolized into 4-hydroxy-anethole trithione (ATX) via O-demethylation and almost no ATT could be detected in human urine 24 h after administration, which probably corresponded to its concentration in plasma. Because of its pharmacological activity and/or possible toxicity, determining the concentration of the metabolite in clinical samples, especially for supporting pharmacokinetic studies, has become increasingly important. Therefore, it is essential to develop a specific, sensitive and rapid method for the determination of ATX in human plasma to characterize the pharmacokinetic profile of ATT. Li et al. [6] developed an HPLC–UV method to determine ATX in human plasma after a 16 h incubation with β-glucuronidase. However, the method was time-consuming and could not satisfy the need for high throughput in pharmacokinetic studies. Several other methods for the determination of ATX in biological samples have been reported, including HPLC–UV [7,8] and HPLC–MS/MS [9,10]. But the low sensitivity (LLOQ higher than 2.5 ng/mL) and long analysis time (longer than 8 min) may not meet the requirements for sensitivity and high throughput in biosample analysis.

This paper describes a selective, rapid and sensitive HPLC–MS/MS method for the quantitative determination of ATX in human plasma. An LLOQ of 0.452 ng/mL lower than those reported in the literatures [6–10] was achieved. The sample preparation was simple involving a single liquid–liquid extraction with ethyl acetate. Besides, the total run time of 3.5 min and the simple composition of the mobile phase made the method attractive

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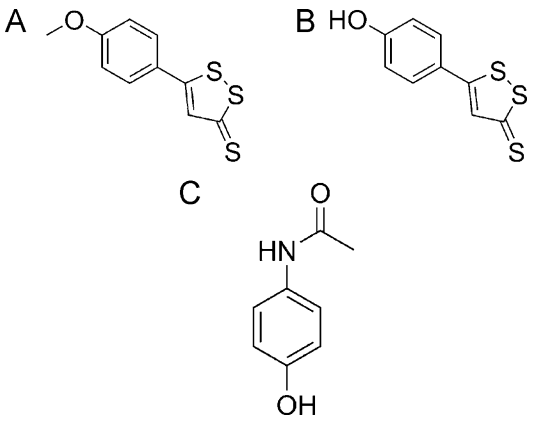

Fig. 1. Chemical structures of ATT (A), ATX (B) and paracetamol (I.S.) (C).

particularly in analyzing large number of plasma samples for pharmacokinetic studies. This method was fully validated and successfully applied to a pharmacokinetic study in 20 healthy volunteers after oral administration of 75 mg ATT in tablet.

2. Experimental

2.1. Reagents and chemicals

ATX (98.4% purity, Fig. 1B) was kindly provided by Changzhou Siyao Pharmaceutical Corporation (Jiangsu, PR China). Reference standard of paracetamol (internal standard, I.S., Batch No. 100018-200408, 99.5% purity, Fig. 1C) was purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, PR China). Methanol of HPLC grade was purchased from Tetia (Fairfield, OH, USA). Ethyl acetate of analytical grade was obtained from Yuwang Chemical Reagent Plant (Shandong, PR China). Water was purified by redistillation and filtered through 0.22 μm membrane filter before use.

2.2. Apparatus and operation conditions

2.2.1. Liquid chromatography

An ACQUITY Ultra Performance LC™ system (Waters Corp., Milford, MA, USA) was employed for the liquid chromatography. The separation was carried out on an Inertsil® ODS-3 column (50 mm × 2.1 mm i.d., 3 μm) at ambient temperature. The mobile phase was composed of methanol–water (75:25, v/v) and delivered at a flow rate of 0.25 mL/min. The autosampler temperature was kept at 4 °C and 3 μL of sample solution was injected with partial loop mode.

2.2.2. Mass spectrometry

The detection was performed on a triple quadrupole tandem mass spectrometer (Micromass® Quattro micro™ API mass spectrometer, Waters Corp., Milford, MA, USA) with an electrospray ionization (ESI) interface. The ESI source was set in negative ionization mode. The quantitation was conducted using multiple reaction monitoring (MRM) of the transitions of m/z 224.8 → 159.8 for ATX and m/z 149.7 → 106.5 for the I.S., with a scan time of 0.20 s per transition. The following MS parameters were carefully optimized to obtain maximum response of ATX: capillary voltage 3.0 kV, cone voltage 35 V, source temperature 110 °C and desolvation temperature 450 °C. Nitrogen was used as the desolvation and cone gas with flow rates of 600 L/h and 30 L/h, respectively. Argon was used as the collision gas at a pressure of approximately 2.7×10^{-3} mbar. The optimized collision energy for both ATX and I.S. was 25 eV. All data collected in the centroid mode were acquired and processed using MassLynx™ NT 4.1 software with the QuanLynx™ program (Waters Corp., Milford, MA, USA).

2.3. Preparation of standard and quality control samples

Stock standard solutions of ATX and I.S. were both prepared by dissolving the accurately weighed standard compounds in methanol to yield concentrations of 377 μg/mL and 100 μg/mL, respectively. The stock solution of ATX was then serially diluted with methanol–water (50:50, v/v) to obtain working standard solutions at desired concentrations for preparing calibration standard samples. For the preparation of quality control (QC) samples, appropriate amount of ATX was dissolved in methanol to give a concentration of 399 μg/mL. An I.S. working solution of 20.0 μg/mL was obtained by diluting the stock solution of I.S. with methanol–water (50:50, v/v). The working solutions of ATX and the I.S. were freshly prepared daily from the stock solutions. All stock and working solutions were stored at 4 °C and brought to room temperature before use.

The calibration standard samples were prepared by spiking 500 μL of blank plasma with working standard solutions (20 μL) of ATX giving concentrations of 0.452, 1.51, 4.52, 15.1, 45.2, 151 and 603 ng/mL. The QC samples were prepared in bulk at the beginning of the experiment with blank plasma at low, medium and high concentrations of 0.958, 16.0 and 478 ng/mL, and stored at −20 °C in aliquots. The standard and QC samples were extracted on each analysis day along with the unknown samples using the same procedure described below.

2.4. Plasma sample preparation

To a 500 μL aliquot of plasma in 10 mL clean glass tube, 20 μL of I.S. solution (20.0 μg/mL) was added. The mixture was vortexed for 30 s and extracted with 3 mL of ethyl acetate by vortex mixing for 1 min and centrifugation at 3500 rpm for 10 min. The upper organic layer was transferred into another set of clean glass tubes and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 100 μL of methanol–water (75:25, v/v), followed by vortexing and centrifugation at 13000 rpm for 10 min. The supernatant was transferred into 700 μL glass vials, and an aliquot of 3 μL was injected into the HPLC–MS/MS system.

2.5. Method validation

The method validation experiment was designed according to the FDA guideline [11] for validation of bioanalytical methods, including selectivity, linearity, accuracy, precision, extraction recovery, matrix effect and stability. The validation runs were conducted on three consecutive days. The peak area ratios of ATX to the I.S. of QC samples were interpolated from the calibration curve on the same day to give the concentrations of ATX. The results from QC samples for three runs were used to evaluate the precision and accuracy of the method.

2.5.1. Selectivity

The selectivity was evaluated by comparing the chromatograms of six different batches of blank plasma obtained from six subjects with those of corresponding blank plasma spiked with ATX and I.S., and those of plasma samples from volunteers after oral administration of ATT tablets.

2.5.2. Linearity and LLOQ

Calibration curves were constructed by assaying standard plasma samples at seven concentrations in the range of 0.452–603 ng/mL with weighted ($1/x^2$) least squares linear regression. The linearity was determined by plotting the peak area ratio

(y) of ATX to I.S. versus the nominal concentration (x) of ATX. The LLOQ is defined as the lowest concentration on the calibration curve at which an acceptable accuracy (relative error, R.E.) within ±20% and a precision (relative standard deviation, R.S.D.) below 20% can be obtained.

2.5.3. Precision and accuracy

The intra-day precision and accuracy were evaluated by determining replicate QC samples of ATX on the same day. The run included two sets of calibration standards and six replicates of LLOQ, low, medium and high concentrations of QC samples. For determining the inter-day accuracy and precision, analysis of three batches of QC samples was performed on three consecutive days. The precision was expressed as the R.S.D. and the accuracy as the R.E.

2.5.4. Extraction recovery and matrix effect

An amount of mixed blank plasma was obtained from 20 individuals to investigate the extraction recovery. The extraction recovery was evaluated by analyzing six replicates of plasma samples at three QC concentration levels of 0.958, 16.0 and 478 ng/mL. The recovery was calculated by comparing the peak areas obtained from mixed blank plasma samples spiked with the analyte before extraction with those from blank plasma samples to which the analyte was added after extraction. To evaluate the matrix effect, standard solutions at three QC concentration levels were added to the dried extracts of 500 μL of the blank plasma samples obtained from six different subjects, dried and reconstituted with 100 μL solution of methanol–water (75:25, v/v). The corresponding peak areas were compared with those obtained from pure ATX standard solutions at equivalent concentrations dried directly and reconstituted with the same solution. The extraction recovery and matrix effect of the I.S. were evaluated in the similar way.

2.5.5. Stability

To evaluate the stock solution stability of ATX and the I.S., five aliquots of the stock solutions of ATX (377 μg/mL) and the I.S. (100 μg/mL) were kept at 4 °C for 30 days. Thereafter, the mean peak areas of ATX and the I.S. from five replicate chromatographic runs were compared with those from freshly prepared solutions at the same concentration.

The stability of ATX in plasma was investigated by analyzing three replicates of low, medium and high QC samples during the sample storage and processing procedures. The freeze–thaw stability was performed by allowing QC samples to undergo three freeze (−20 °C)–thaw (room temperature) cycles before extraction. The long-term and short-term stability were estimated by processing QC samples after the storage at −20 °C for 30 days and at room temperature for 4 h, respectively. The post-preparation stability was carried out by keeping the extracted QC samples in the autosampler at 4 °C for 12 h before analysis. The concentrations of QC samples were calculated by using a freshly prepared calibration curve and compared with nominal values.

2.6. Application to pharmacokinetic study

The validated method was applied to a clinical trail in which 20 healthy male volunteers received an ATT tablet (containing 75 mg ATT) each after a 12 h fast. The pharmacokinetic study was approved by the local Ethics Committee and carried out in the hospital. All volunteers gave their signed informed consent to participate in the study according to the principles of the Declaration of Helsinki. No other prescribed or OTC drugs should be taken from 2 weeks before the administration of ATT to the end of the trial. The blood samples were collected before and at 0.13, 0.33, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0, 4.0, 6.0, 8.0, 14.0 and 16.0 h post-dosing. The plasma was rapidly separated by centrifugation and stored at −20 °C until analyzed.

The maximum plasma concentration (C_{max}) and the time of the maximum plasma concentration (T_{max}) were noted directly from the measured data. The elimination rate constant (k_e) was calculated by linear regression of the terminal points for a semi-log plot of plasma concentration against time. The elimination half-life ($t_{1/2}$) was calculated using the formula $t_{1/2} = 0.693/k_e$. The area under the plasma concentration–time curve (AUC_{0-t}) to the last measurable plasma concentration (C_t) was calculated by the linear trapezoidal rule. The area under the plasma concentration–time curve to time infinity ($AUC_{0-\infty}$) was calculated as: $AUC_{0-\infty} = AUC_{0-t} + C_t/k_e$.

3. Results and discussion

3.1. Selection of internal standard

A good I.S. should be capable of tracking extraction, on-column retention, in-source ionization and matrix effect, etc., for a target analyte. An ideal I.S. in LC/MS analysis is a deuterated form of the analyte, however, it is not commercially available in this study. Therefore, candidates with abundance response in negative ionization mode, such as indomethacin, paracetamol and ibuprofen were considered. Paracetamol was finally chosen as the I.S. due to its similarity to ATX in retention, ionization and extraction efficiency. Indomethacin and ibuprofen were not adopted because of the poor peak shape and long retention time (4.0 min and 8.5 min, respectively).

3.2. HPLC–MS/MS condition optimization

The HPLC–MS/MS operation parameters were carefully optimized for the determination of ATX. Both the positive and negative modes with ESI were investigated and the signal intensity of ATX obtained in negative ionization mode was much greater than that in positive ionization mode. In the precursor ion full-scan spectra, the most abundant ions were $[M-H]^-$ m/z 224.8 and 149.7 for ATX and the I.S., respectively. Parameters such as desolvation temperature, ESI source temperature, capillary and cone voltage, flow rate of desolvation gas and cone gas were optimized to obtain the highest intensity of $[M-H]^-$ of ATX. The product ion scan spectra showed high abundance fragment ions at m/z 159.8 and 106.5 for ATX and the I.S., respectively. The product-ion spectra of the two compounds are shown in Fig. 2. The collision gas pressure and collision energy of collision-induced decomposition (CID) were optimized for maximum response of the fragmentation of ATX. The precursor → product ion transitions of m/z 224.8 → 159.8 for ATX and m/z 149.7 → 106.5 for the I.S. were chosen for MRM.

The chromatographic conditions were optimized to obtain high sensitivity, good peak shape and short retention. The separation and ionization of ATX and I.S. were affected by the composition of mobile phase. Methanol and acetonitrile were tested as the organic modifier of mobile phase. Methanol was finally adopted for it produced symmetric peak shape and higher detection response of analyte than acetonitrile. Then, the organic solvent percentage in the mobile phase was investigated over the range of 65–80% and 75% was chosen due to the high response of ATX and suitable retention times provided. Formic acid and ammonium acetate were considered as additives in the mobile phase to improve the response of analyte. However, no significant improvement was observed and the peak shape was not as good as that without additive. Finally, a mobile phase of methanol–water (75:25, v/v) was used, which was simpler than the methods reported [9,10]. Although the chromatography was performed on an HPLC column,

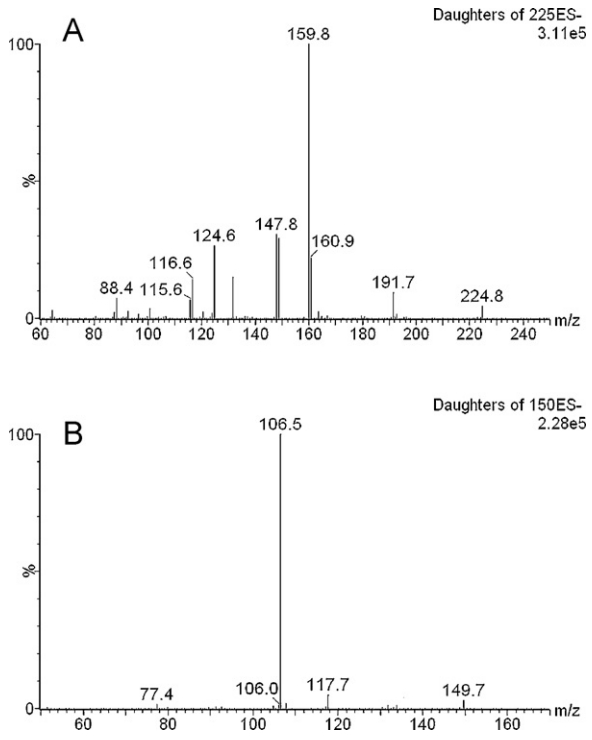

Fig. 2. Full-scan product-ion mass spectra of $[M–H]^-$ of ATX (A) and I.S. (B).

the small dead volume of the Acquity system benefited the separation efficiency and the run time. Under the optimal conditions, the total run time for each sample was only 3.5 min, with symmetric peak shape and high sensitivity. Two channels in the mass spectrometer were used for recording the response, channel 1 for ATX and channel 2 for the I.S. (Fig. 3).

3.3. Selection of extraction method

Liquid–liquid extraction (LLE) and protein precipitation (PPT) were investigated for sample preparation. Compared with PPT, LLE produced more purified as well as concentrated samples and improved the sensitivity and robustness of the assay. Therefore, LLE was adopted to extract ATX and the I.S. from plasma. Several extraction solvents such as ethyl acetate, diethyl ether, cyclohexane and n-hexane were tested. Ethyl acetate was found to be more efficient with extraction recoveries above 90%, while the recoveries of others was below 50%.

3.4. Method validation

3.4.1. Selectivity

The selectivity was determined by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. As shown in Fig. 3, no interference from endogenous substance at the retention times of ATX and the I.S. was observed.

3.4.2. Linearity and LLOQ

The peak area ratio of ATX to I.S. in human plasma varied linearly over the concentration range of 0.452–603 ng/mL ($r^2 \geq 0.99$), which was wide enough for the determination of unknown samples in pharmacokinetic study. A typical regression equation for the calibration curves was $y = 9.63 \times 10^{-3}\,x + 4.09 \times 10^{-4}$, $r = 0.9973$, where y is the peak area ratio of ATX to I.S., and x is the concentration of ATX in plasma. The LLOQ was 0.452 ng/mL in plasma, which was lower than that reported in the literatures [6–10]. A corresponding chromatogram is shown in Fig. 3B. The LLOQ ensured the determination of ATX in plasma samples until 16 h after a single oral dose of 75 mg ATT, which was sensitive enough to investigate the pharmacokinetic behavior of ATT in human plasma.

3.4.3. Precision and accuracy

The results for intra- and inter-day precision and accuracy for the determination of ATX from QC samples are summarized in Table 1. The precision and accuracy of the present method complied with the criteria for analysis of biological samples according to the guidance of FDA where the R.S.D. determined at each concentration level of QC samples was required not exceeding 15% (20% for the LLOQ) and R.E. within ±15% (±20% for the LLOQ) of the actual value [11].

3.4.4. Extraction recovery and matrix effect

The extraction recoveries of ATX from QC samples were 95.1 ± 8.3%, 97.2 ± 7.4%, 93.2 ± 3.9% at low, medium and high concentrations, respectively. The mean extraction recovery of the I.S. was 80.4 ± 3.3%. Thus, the consistency in recoveries of ATX and the I.S. supported the extraction procedure for its application to routine sample analysis. The matrix effects for ATX at three concentration levels were 99.0 ± 5.5%, 99.9 ± 2.2%, 102.2 ± 3.3%, respectively, indicating that the ionization competition between the analyte and the endogenous co-elutions could be neglected.

3.4.5. Stability

The R.E. between the stocked and the freshly prepared solutions for ATX and the I.S. were −3.0% and 0.7%, respectively, which indicated the stock solutions of ATX and the I.S. were stable for at least 30 days.

The results of stability tests under certain storage conditions are listed in Table 2. No chemical or biological degradation or decomposition of ATX was observed during all of the sample storage, preparation and analysis periods. The method was therefore proved to be applicable for routine analysis.

3.5. Application to a pharmacokinetic study

The validated HPLC–MS/MS method was applied to a pharmacokinetic study of ATT tablet in 20 healthy male volunteers after oral administration of 75 mg ATT. The mean plasma concentration–time curve of ATX in single dose study is shown in Fig. 4. The maximum plasma concentration (C_{max}) was 279.6 ± 110.0 ng/mL, the time of maximum plasma concentration (T_{max}) was 1.55 ± 0.62 h, the area under the plasma concentration–time curve from 0 h to the time of last measurable concentration (AUC_{0-t}) was 951.1 ± 568.2 ng h/mL,

Table 1
Precision and accuracy for the determination of ATX in human plasma (intra-day: $n = 6$; inter-day: $n = 6$ series per day, 3 days).

Concentrations (ng/mL)		Intra-run R.S.D. (%)	Inter-run R.S.D. (%)	Accuracy R.E. (%)
Added	Found			
0.452	0.421 ± 0.039	8.9	12	−6.8
0.958	0.886 ± 0.048	5.8	1.2	−7.5
16.0	15.4 ± 1.0	5.8	12	−3.8
478	466 ± 32	6.4	9.5	−2.7

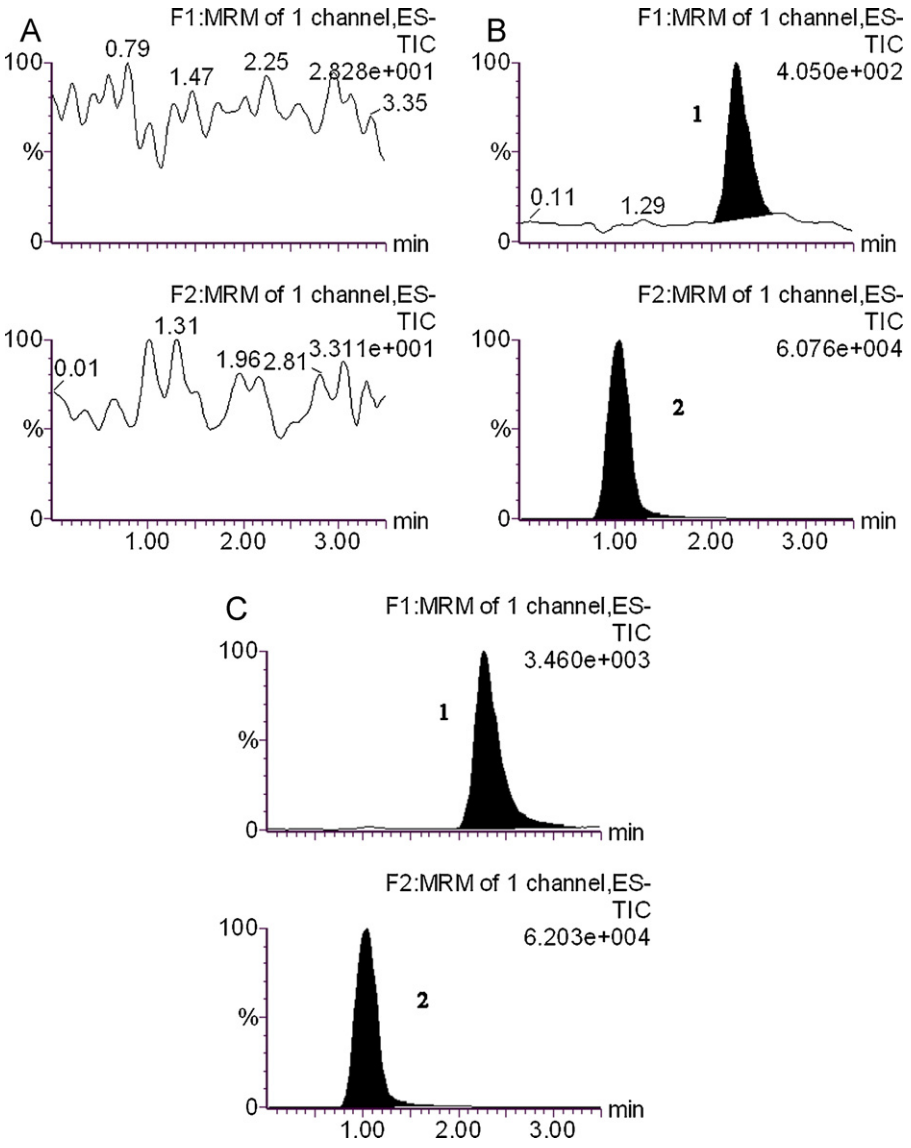

Fig. 3. Representative MRM chromatograms of ATX (peak 1, channel 1) and I.S. (peak 2, channel 2) in human plasma samples. (A) A blank plasma sample; (B) a blank plasma sample spiked with ATX at the LLOQ of 0.452 ng/mL and I.S. (20.0 μg/mL); (C) a plasma sample from a volunteer 0.33 h after oral administration of 75 mg ATT. The retention times of ATX and I.S. were 2.3 min and 1.0 min, respectively.

Table 2
Stability of ATX in human plasma at three QC levels ($n = 3$).

Nominal concentration (ng/mL)	Concentration found (ng/mL; mean ± S.D.)	R.S.D. (%)	R.E. (%)
Short term (room temperature for 4 h)			
0.958	0.911 ± 0.057	6.2	−4.9
16.0	16.0 ± 0.5	3.1	0.1
478	473 ± 15	3.1	−1.2
Long term (−20 °C for 30 days)			
0.958	0.935 ± 0.075	8.0	−2.4
16.0	14.2 ± 0.5	3.5	−11
478	437 ± 13	3.0	−8.8
Three freeze–thaw cycles			
0.958	0.882 ± 0.059	6.7	−8.0
16.0	16.1 ± 1.0	6.5	0.9
478	486 ± 29	6.0	1.4
Post-preparative (4 °C for 12 h)			
0.958	1.03 ± 0.02	1.5	7.3
16.0	16.9 ± 0.9	5.3	5.6
478	509 ± 28	5.6	6.3

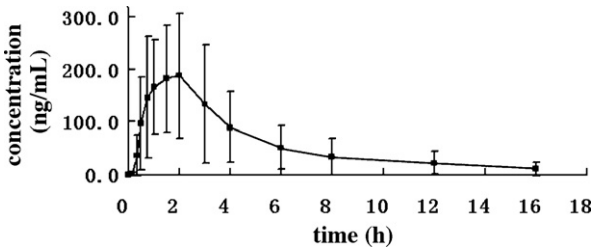

Fig. 4. Mean plasma concentration–time curve of ATX in male volunteers after a single oral dose of 75 mg ATT.

the area under the plasma concentration–time curve from 0 h to infinity ($AUC_{0-\infty}$) was 1035.2 ± 655.0 ng h/mL, the half-life of drug elimination at the terminal phase ($t_{1/2}$) was 4.50 ± 1.06 h.

4. Conclusion

A sensitive, selective and rapid HPLC–MS/MS method for the quantitation of ATX in human plasma was developed and validated.

Compared with the analytical methods reported before, this assay offered superior sensitivity with an LLOQ of 0.452 ng/mL, short run time of 3.5 min and simple composition of the mobile phase. The method has been successfully applied to the pharmacokinetic study of ATT tablet in healthy volunteers.

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