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Short communication

HPLC determination of 4-hydroxy-anethole trithione in plasma via enzymatic hydrolysis and its application to bioequivalence study

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Abstract

A simple, selective and reproducible high-performance liquid chromatographic (HPLC) method via enzymatic hydrolysis of glucuronide conjugates of 4-hydroxy-anethole trithione (ATX) was established for simultaneous determination of ATX. Human plasma samples were hydrolyzed by β-glucuronidase and followed by subsequent extraction with cyclohexane–isopropanol (95:5, v/v) using mifepristone as the internal standard. Chromatography was carried out on a reverse phase C_{18} column (250 mm × 4.6 mm, 5 μm) and kept at 30 °C, with UV detection set at 346 nm. The mobile phase consisted of a mixture of methanol and water (75:25, v/v), at a flow rate of 1 ml/min. It was validated and proved to be linear in the range of 20–1500 ng/ml, with the regression equation $Y = 0.0016C - 0.0069$, $r = 0.9992$. And the limit of quantification (LOQ) concentration in plasma was 20 ng/ml. The absolute recoveries of ATX at three concentrations were 32.04, 38.95 and 44.06% and the relative recoveries were 104.80, 102.53 and 107.04%, which showed that the analytical method was sensible, accurate and reproducible. The method was utilized on a double-blind, randomized, single dose, two period, and crossover bioequivalence study of ATT tablets produced by different companies in 20 healthy male Chinese subjects, with a washout between every two periods. Blood samples were collected over each period of 10 h and various pharmacokinetic parameters were determined. Natural log-transformed values were compared by analysis of variance followed by classical 90% confidence interval for C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ and was found to be within the range, which indicated that the two products were bioequivalence.

Keywords: Anethole trithione; 4-Hydroxy-anethole trithione; β-Glucuronidase; HPLC; Bioequivalence

1. Introduction

Anethole trithione (ATT, 5-(*p*-methoxyphenyl)-3H-1,2-dithiole-3-thione), which is a sulfur heterocyclic compound first discovered in cruciferous vegetables [1], is a relatively new chologogue.

ATT increases salivary secretion for drug-induced xerostomia [2–5], stimulates post-receptor effect on the secretory response, increases cholinergic and adrenergic responsiveness, and prevent the up-regulation in muscarinic acethylcholine receptor density. It significantly inhibits carcinogenesis by increasing the activity of electrophile detoxification enzymes [6,7]. It also improves the level of glutathione and glutamyl cysteine syntetase, the activity of glutathione reductase and glutathione peroxidase, resulting in the enhancement of hepatic cell activity and bilifaction. Futhermore, it is prescribed as an adjunctive therapy for cholecystitis, gallstone, indigestion, acute/chronic hepatitis, and marketed in many countries.

Previous studies [8] described that most of ATT was metabolized into 4-hydroxy-anethole trithione (ATX) via *O*-demethylation in both animals (rats and rabbits) and humans, and then ATX formed the conjugates with glycuronic acid or sulfuric acid in vivo, such as $ATXK_2$, $ATXK_1$, $ATXK_2$ for rats, rabbits and human, respectively, as shown in Fig. 1. As reported, ATX was released from its glucuronide conjugate $ATXK_2$ through enzymolysis of β-glucuronidase in vitro [9,10]. No ATT, about 1.5–3% ATX and 12% $ATXK_2$ were detected in human urine 24 h later after administration, these probably corresponds to their concentrations in plasma [8].

There have been several articles reported about determination of ATT in animals or human plasma. Yu et al. carried out the determination of ATT with the mixture of standard ATT solution and blank plasma by high-performance liquid chromatography

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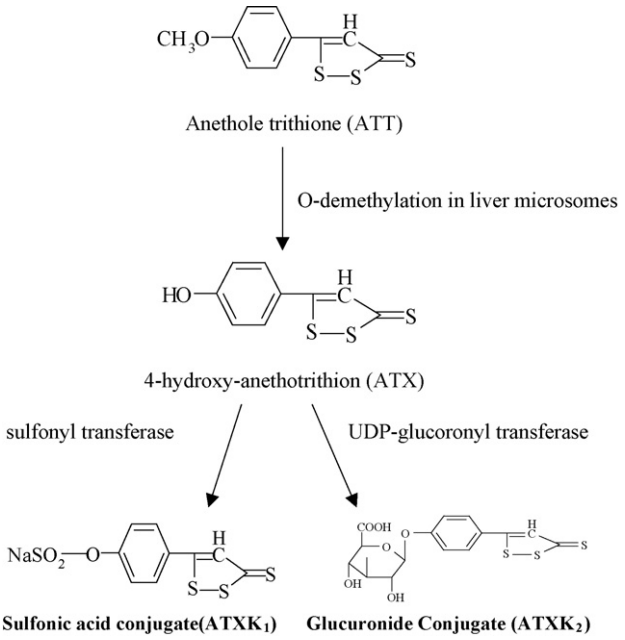

Fig. 1. Metabolic routine of ATT.

(HPLC), with the detection limit at 84 ng/ml [11]. Jing et al. reported the determination of ATT in rabbit plasma by HPLC with the detection limit at 0.5 ng/ml [12]. However, all of these HPLC-UV methods were not sensitive enough to determine the low-plasma concentration of ATT as the maximum concentration of ATT was no more than 0.8 ng/ml after administration of 75 mg of anethole trithione [13].

Although the HPLC–MS/MS method established by Li et al. [13] can detect the ATT concentration in plasma with a detection limit of 0.01 ng/ml, it is not practical for clinical monitoring of ATT. The introduction of enzymatic hydrolysis led to a significant increment of concentration of free ATX, the total concentration of ATX was more than 20 ng/ml in every subject 10 h after the administration of 75 mg of ATT. The method was validated and applied successfully to a pharmacokinetic study after single dose administration of 75 mg ATT tablets to healthy male Chinese volunteers. Some important pharmacokinetic parameters for ATX are also given for the first time.

2. Experiment

2.1. Chemicals and reagents

Anethole trithione tablets for test and reference were provided by Ji-Tai-An Pharmaceuticals Ltd. (Sichuan, PR China) and Sichuan Kinna United Pharmaceutical Company (Sichuan, PR China), respectively. ATT (not less than 99.0%) was supplied by Ji-Tai-An Pharmaceuticals Ltd. (Sichuan, PR China). ATX was synthesized and identified by School of Pharmacy, Tongji Medical College, Huazhong University of Science & Technology (Wuhan, PR China). β-Glucuronidase was purchased from Sigma–Aldrich Co. Mifepristone (I.S.) was provided by National Institute for the Control of Pharmaceutical and Biological Products (Beijing, PR China). Methanol (HPLC grade) obtained from Tianjin Kermel Chemical Reagent Co. Ltd. (PR China). Other chemicals were of analytical grade. HPLC-grade water was used throughout the study. Fresh frozen human plasma was collected from Union Hospital Affiliated to Tongji Medical College, Huazhong University of Science & Technology (Wuhan, PR China).

2.2. Synthesis and identification of ATX

ATX was synthesized as previously described [8]. Briefly, 1 g ATT was added to a flask containing 3.4 g pyridine hydrochloride, and then kept at 215 °C for 4 h with stirring. After reaction, the mixture was cooled to room temperature, dissolved with 20 ml hot water, filtrated and washed the deposition. The deposition was dissolved in 1 mol/L NaOH solution, and filtrated. Glacial acetic acid was added to the filtrate. The deposition was obtained, and recrystalized using alcohol. The structure was identified by H-NMR, MS and C-NMR. Its purity was more than 99.5% according to HPLC normalization.

2.3. Instrumentation and chromatography

The Waters liquid chromatographic system (Waters, USA), consisted of two 510 pumps, one Waters 486 detector, one manual injector port with 20 μl loop and one authorized chromatography work station. The analysis was carried out on a Venusil XBP-C_{18} column (250 mm × 4.6 mm, 5 μm, Agela Technologies, USA) thermostated at 30 °C, with a precolumn packed with C_{18} (5 μm, Turner Science Instrument Co. Ltd., PR China). Methanol:water (75:25 (v/v), filtered with a 0.45-μm film) was used as the mobile phase at a flow rate of 1.0 ml/min. The detection was performed at a wavelength of 346 nm after a injection volume of 20 μl.

2.4. Preparation of stock solutions

Stock solutions of ATX (1 mg/ml) and mifepristone (I.S., 15 μg/ml) were prepared, respectively in methanol and stored at 4 °C. And the β-glucuronidase solution (3000 U/ml) was made by dissolving into sodium citrate tribasic dehydrate aqueous solution (0.03 g/ml, adjusted with citric acid to pH 5). All the solutions were diluted to the acquired concentration of working solutions if necessary.

2.5. Preparation of calibration standards and quality control samples

Calibration standards of ATX (20, 50, 100, 200, 500, 1000 and 1500 ng/ml) were prepared by adding appropriate amount of the standard solution into blank plasma.

Quality control (QC) samples were prepared by adding proper ATX into blank plasma to give the concentrations 50 ng/ml (low level), 500 ng/ml (middle level) and 1000 ng/ml (high level).

2.6. Sample preparation

150 μl of β-glucuronidase (3000 U/ml) was added to 0.5 ml of human plasma and incubated for 16 h under 37 °C. 100 μl of internal standard solution of mifepristone (15 μg/ml in ethanol) was added and vortexed for 1 min, extracted with 4 ml cyclohexane–isopropanol (95:5, v/v) by vortexing for 10 min, and then centrifuged at 4000 rpm for 10 min. The supernatant was separated and evaporated to dry at 40 °C under a gentle stream of nitrogen.

The solutions were reconstituted with 100 μl of mobile phase, vortex-mixed for 5 min and centrifuged at 10,000 rpm for 5 min. 20 μl aliquots of the supernatant were injected into the HPLC for the assay.

3. Method validations

The selectivity of the method was checked for interference from plasma. The standard curve consisting of seven points ranging from 20 to 1500 ng/ml was developed. Quality control samples as LQC, MQC, and HQC were used to determine the intra- and inter-day precision and accuracy of the assay. Peak area ratios of ATX to internal standard were fit to linear equation ($Y = 0.0016C - 0.0069$) using a weighted ($1/x^2$) linear regression, and drug concentration in control samples along with the same day standard curve samples were calculated using this equation. For all the curves the correlation coefficients were not less than 0.9992. The stability of ATX in human plasma kept at 20 °C for 8 h and at −80 °C for 2 weeks, the stability of the extracted ATX from plasma in the reconstituted solution kept at 20 °C for 24 h after processed as in Section 2.6 were assessed, respectively. The limit of quantification (LOQ) was estimated by analyzing ATX at low concentration of the calibration curves and defined as a concentration level in which accuracy and precision were still better than 10%. To determine the limit of detection (LOD), lower plasma concentrations than the lowest end of the calibration curves were used. The LOD was determined as the concentration which produces three times of signal/noise ratio (S/N = 3).

In order to calculate the absolute and relative recoveries of ATX, the plasma samples containing ATX at three QC levels (50, 500 and 1000 ng/ml, $n = 5$ at each concentration) and internal standard ($n = 5$) were prepared by adding 100 μl of ATX and I.S. standard solutions into 0.5 ml blank plasma samples. The peak areas for plasma extracts were compared to the peak areas of ATX and internal standard that were added to extracted blank plasma samples at the same concentration to calculate the absolute recoveries of ATX. Also, the concentrations of ATX calculated from the standard calibration were compared to the concentrations added to the blank plasma samples to calculate relative recoveries of ATX.

The plasma samples with only traces of ATX at 1 h after drug administration were selected for the study of the stability of $ATXK_2$ at 37 °C. 150 μl of β-glucuronidase (3000 U/ml) was added to the plasma samples under 37 °C at 0, 8 and 16 h, respectively, incubated for 16 h at 37 °C and then processed following "2.6" steps.

4. Clinical designs

The study protocol was formally accepted by State Food and Drug Administration and the Institution Ethics Committee. Twenty healthy male Chinese subjects with mean age 22.9 ± 1.5 years and average weight 64.3 ± 6.5 kg were enrolled into the study, under the criteria listed in our study protocol and declaration of Helsinki. The study was conducted as a 20 × 2 single dose, randomized, double-blind, and complete crossover design, with a 7-day wash-out period. Subjects were fastened overnight before and 2 h after either 75 mg test drug or reference drug was given with 200 ml water. Blood samples (5 ml) were collected at pre-dose (0 h) and at the following post-dose intervals: 0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 4, 5, 7 and 10 h through an indwelling cannula into heparinised vials. Standard diets were provided regularly. The blood samples were centrifuged immediately (4000 rpm, 10 min). Plasma was separated and stored at −80 °C until analyzed by the developed method.

5. Pharmacokinetic and statistical analysis

Pharmacokinetic analysis was performed as non-compartmental analysis. The parameters: C_{max} (maximum ATX concentrations) and T_{max} (the time of C_{max}) were determined by the individual plasma concentration–time profile. AUC_{0-t} curve (the total area under the plasma concentration–time) was calculated using the linear trapezoidal rule. $AUC_{0-\infty}$ (the AUC from 0 to infinity) was calculated according to the equation $AUC_{0-\infty} = AUC_{0-t} + C_t/K_e$, where C_t is the least plasma concentration that can be quantified. K_e (the elimination rate constant) was determined by linear regression analysis of the log-linear part of the plasma concentration–time curve, $t_{1/2}$ (the half-life) was calculated as $0.693/K_e$, the clearance (Cl/F) as $Cl/F = dose/AUC_{0-\infty}$, and the apparent volume of distribution (V_d/F) as $V_d/F = dose/(AUC \cdot K_e)$.

The main parameters AUC_{0-t}, $AUC_{0-\infty}$ and C_{max} were used to analyze the bioequivalence. The analysis of variance (ANOVA) for crossover design and the 90% confidence interval of the ratio of test/reference were determined using natural log-transformed data. The drugs were accepted as bioequivalence if the difference of parameters between them was not significant ($P > 0.05$) and if the confidence interval for these parameters was within 80–125%.

6. Results and discussion

6.1. Method development

ATT penetrates the gastro-intestinal mucosa easily, and is absorbed very fast [14,15]. However, ATT was not able to be detected in human plasma due to its rapid transformation to other metabolites. It has very low concentrations existed in human plasma because the maximum concentration of ATT was not more than 0.8 ng/ml [13] after 75 mg of anethole trithione administration. So, HPLC-UV method was not sensitive enough to detect the ATT in human plasma, and to develop the pharmacokinetic study.

ATT was metabolized into ATX conjugated mainly with glycuronic acid to form $ATXK_2$ in human body. The concentrations of ATX in human plasma without enzymatic hydrolysis were lower than 20 ng/ml (LOQ) in most volunteers 6 h after administration 75 mg of ATT tablet in our preliminary experiment. As shown on the chromatogram, only one peak was detected in the human plasma sample, whose retention time was the same as ATX. Due to the lack of reference substance of $ATXK_2$, it was unclear whether ATX has the same retention time with $ATXK_2$ or not. Moreover, the area of this weak peak on the chromatogram increased fourfolds after enzymatic hydrolysis as shown in Fig. 2 . These observations indicated that there was

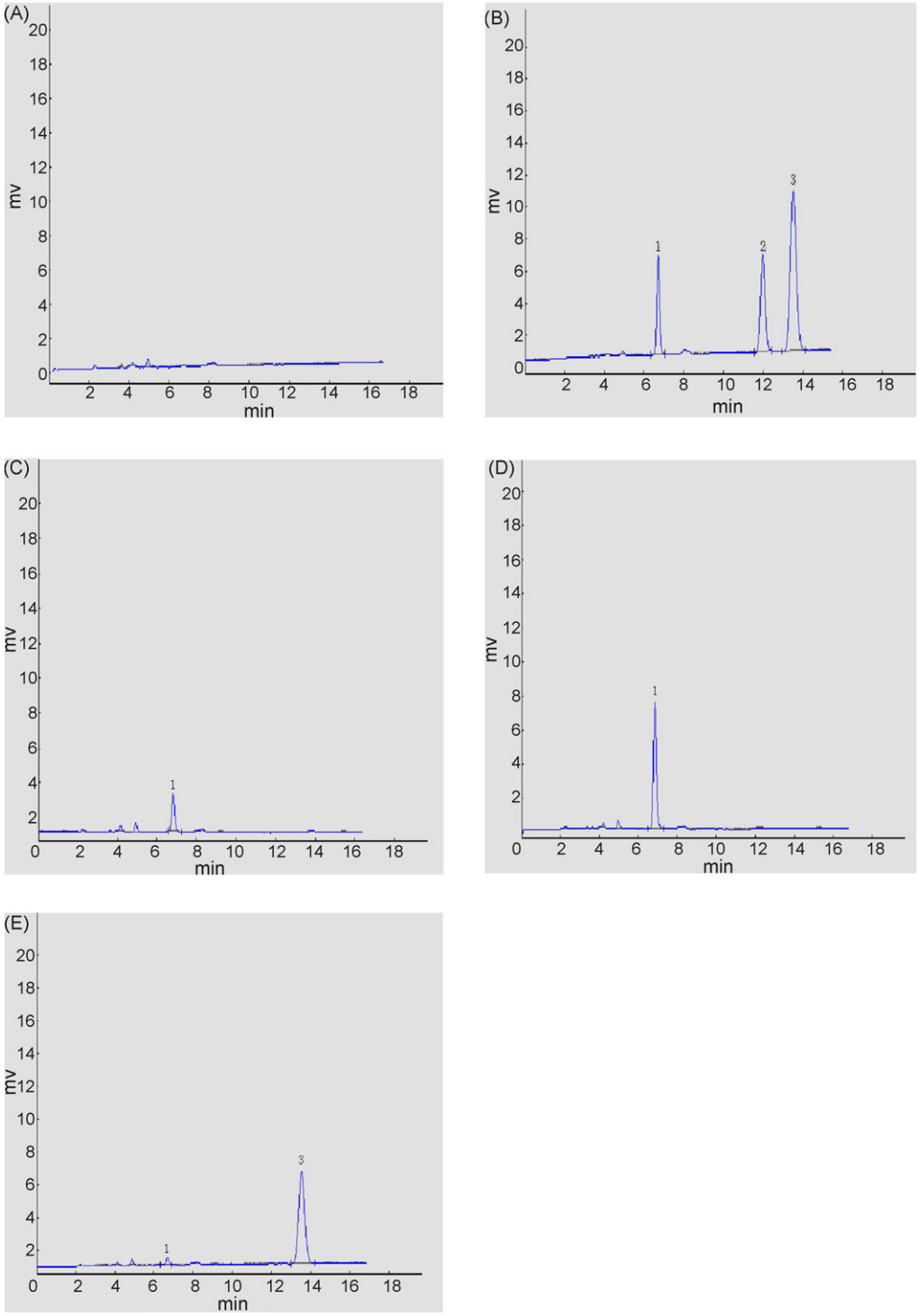

Fig. 2. Representative chromatograms of HPLC selectivity-comparation. (A) Drug-free plasma; (B) blank plasma spiked with ATT (500 ng/ml), ATX (500 ng/ml) and internal standard (3 μg/ml); (C) human plasma sample 2 h after administration of ATT; (D) human plasma sample via enzymatic hydrolysis 2 h after administration of ATT; (E) blank plasma spiked with ATX (20 ng/ml) and internal standard (3 μg/ml). Peak 1, ATX (t_R = 6.677); peak 2, ATT (t_R = 11.950); peak 3, mifepristone (t_R = 13.487).

Table 1
Results of intra- and inter-day variability for validation of the proposed method

Theoretical concentration (ng/ml)	Intra-day ($n=5$)			Inter-day ($n=5$)		
	Concentration found (ng/ml)	R.S.D. (%)	Accuracy (%)	Concentration found (ng/ml)	R.S.D. (%)	Accuracy (%)
50	50.55 ± 2.48	4.9	101.0	52.99 ± 4.30	8.1	106.0
500	475.92 ± 20.74	4.4	95.2	483.10 ± 13.01	2.7	96.6
1000	984.05 ± 37.48	3.8	98.4	994.66 ± 47.72	4.8	99.5

none or trace amount $ATXK_2$ in the organic extracted solvent probably due to its water-soluble properties. Thus, it is essential to release ATX from its glycuronic acid conjugate via enzymatic hydrolysis to carry out accurate bioequivalent studies.

The amount of ATX in human plasma increased four times after 150 μl of β-glucuronidase (3000 U/ml) was added to 0.5 ml of human plasma sample. It increased no more even if 150 μl of β-glucuronidase (6000 U/ml) was added into 0.5 ml of human plasma. It showed that 150 μl of β-glucuronidase (3000 U/ml) was the optimum for the complete transformation of ATX conjugates into free ATX in 0.5 ml of plasma. The concentrations of ATX in human plasma samples are more than 20 ng/ml (LOQ) 10 h after administration of 75 mg of ATT via enzymatic hydrolysis, and all of concentrations fall into the linear range of ATX.

ATX is the metabolite of ATT via *O*-demethylation, and its physicochemical properties are similar to ATT, which is not easily dissolved with water. ATX was extracted from plasma using organic solvents based on the paper published by Jing et al. [12]. Liquid–liquid extraction procedure was tried using different ratios of cyclohexane and isopropanol as extracting solvent as well. It was found that there were no interference with plasma samples and cleaner chromatograms could be obtained when extracted with cyclohexane:isopropanol (95:5, v/v). These data prove that the selectivity and recovery of the method is satisfactory with cyclohexane:isopropanol (95:5, v/v) as the extracting solvent.

To achieve the best peak resolution and retention time, various mobile phases in different proportion were evaluated in this study. The composition of the mobile phase (methanol:water in the ratio of 75:25 at a flow rate of 1 ml/min) chosen here provide concomitantly the best resolution from both analyte and internal standard.

6.2. Selectivity

In blend, ATX, ATT and internal standard were well dissolved and separated completely, with a retention time 6.677, 11.950 and 13.487 min, respectively. At these retention times, no drug free plasma samples yield endogenous interference with these materials (Fig. 2A–E).

6.3. The Stability of ATXK2 in human plasma

The peak area of ATX in human plasma after incubated at 37 °C at 0, 8 and 16 h were 35,721.67 ± 410.44, 36,382.67 ± 662.83 and 35,612.67 ± 1367.65 ($n=5$), respectively, no significant difference in peak areas between them showed that $ATXK_2$ was stable in human plasma at 37 °C for 16 h.

6.4. Accuracy and precision

The coefficient of variation calculated for the intra-day at three points was within 4.9%, while assay accuracy ranged from 95.2 to 101.0%. For inter-day calculation, the coefficient was within 8.1% and accuracy ranged from 96.6 to 106.0% (Table 1).

6.5. Linearity and quantification limit

The calibration curve was drawn by plotting the peak area ratio of ATX to internal standard versus nominal concentration of ATX. The peak area response was linear over the range 20–1500 ng/ml with a regression equation, $Y = 0.0016C - 0.0069$. The correlation coefficient was 0.9992. The detection limit for ATX (LOD) was 8 ng/ml with a signal-to-noise = 3. The limit of quantification was 20 ng/ml, with a coefficient of variation of 9.3%.

6.6. Recovery and stability

As shown in Table 2, the absolute extraction recoveries of ATX at three levels (50, 500 and 1000 ng/ml) were 32.04 ± 1.26, 38.95 ± 3.5 and 44.06 ± 1.07%, respectively ($n=5$), and it was 84.77 ± 2.6% ($n=5$) for mifepristone. And the relative extraction recoveries of ATX were 104.80 ± 1.25, 102.53 ± 4.38 and 107.04 ± 1.72% ($n=5$), respectively.

In the stability study, the relative error was 3.62% for the 500 ng/ml ATX solution kept at 20 °C till 8 h, and it showed that ATX in human plasma was stable at 20 °C. Short-term and long-term stability of ATX in human plasma were evaluated, the results indicated ATX in plasma was stable when stored at

Table 2
Results of relative recovery and absolute recovery of ATX in human plasma ($n=5$)

Added ($\mu g\,l^{-1}$)	Found ($\mu g\,l^{-1}$)	Relative recovery (%)	R.S.D. (%)	Absolute recovery (%)	R.S.D. (%)
50	52.40 ± 0.63	104.80 ± 1.25	1.20	32.04 ± 1.26	4.32
500	539.47 ± 21.91	102.53 ± 4.38	4.27	38.95 ± 3.50	8.99
1000	1070.43 ± 17.24	107.04 ± 1.72	1.61	44.06 ± 1.07	2.42

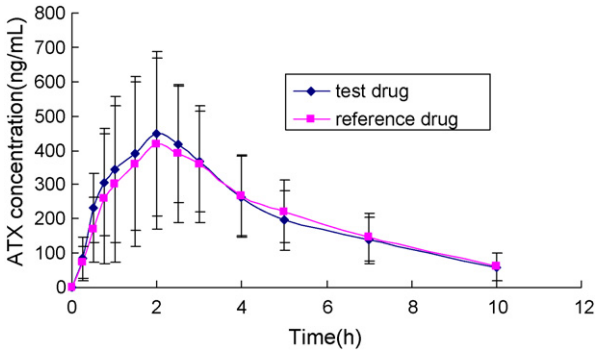

Fig. 3. Mean plasma concentration time profile of 75 mg ATT in 20 healthy male Chinese subjects.

Table 3
Pharmacokinetic parameters of test and reference ATT

Parameters	Mean ± S.D.	
	Reference drug	Test drug
T_{max} (h)	2.01 ± 0.58	1.88 ± 0.70
C_{max} (ng/ml)	464.10 ± 226.09	511.66 ± 219.95
$t_{1/2}$	3.11 ± 1.23	3.21 ± 1.64
K_e (h^{-1})	0.25 ± 0.10	0.27 ± 0.12
AUC_{0-t} (ng/(ml h))	2146.58 ± 1006.63	2179.72 ± 820.07
$AUC_{0-\infty}$ (ng/(ml h))	2483.67 ± 1221.64	2518.13 ± 1030.58
V_d/F (l)	0.164 ± 0.137	0.158 ± 0.116
Cl/F (1/h)	0.036 ± 0.014	0.034 ± 0.012

Table 4
90% confidence interval for AUC_{0-t}, $AUC_{0-\infty}$ and C_{max}

Pharmacokinetic parameters	90% CI
AUC_{0-t}	93.5–115.8%
$AUC_{0-\infty}$	95.1–113.9%
C_{max}	98.8–126.3%

−80 °C for 2 weeks due to the relative error less than 7.81%. ATX samples were also stable in the reconstituted solution of methanol–water (75:25, v/v) at 20 °C for at least 24 h with the relative error of ATX less than 7.6%.

6.7. *Bioavailability and pharmacokinetic analysis*

Fig. 3 shows plasma ATX concentration time profile after administration of the different tablets. All calculated pharmacokinetic parameter values were shown in Table 3.

For bioequivalence evaluation, the mean and standard deviation of the test and reference tablets were very close. And the analysis of variance (ANOVA), after natural log-transformation of the data, showed statistically insignificant ($P > 0.05$) (Table 4). All of these indicated that these two kinds of tablets were bioequivalence.

7. Conclusion

The HPLC-UV via enzymatic hydrolysis has been established to quantify ATX, the metabolite of ATT in plasma. Its simplicity and higher detection limit make it convenient, reproducible, reliable, and useful in the determination of ATX in human plasma and bioequivalence study. The statistical analysis of AUC and C_{max} indicated that there was no significance for the two kinds of tablets. 90% confidence interval for the mean (T/R) of AUC_{0-t}, $AUC_{0-\infty}$ and C_{max} suggested that the reported values were within the acceptance range (80–125%). Taken together, the two tablets produced by different manufacturers are bioequivalent, and can be interchanged in clinical therapy.

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