Short communication

HPLC determination of anethole trithione and its application to pharmacokinetics in rabbits

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Abstract

To evaluate the relative bioavailability of anethole trithione (ATT) from self-microemulsifying drug delivery system (SMEDDS) and tablet, a sensitive, accurate and reliable liquid chromatography method was developed and validated to determine ATT in rabbit plasma. Chromatographic separation was performed on a Diamonsil C_{18} column by using a mixture of methanol–water (90:10, v/v) delivered at a flow rate of 1.0 ml/min. The wavelength was set at 348 nm and mifepristone was used as the internal standard. A linear relationship for ATT was found in the range of 0.5–32 ng/ml. The mean extraction recoveries of ATT determined over three concentrations were 84.7 ± 5.8, 92.3 ± 3.4 and $89.9 \pm 5.1\%$. After administration of SMEDDS and tablets to rabbits, significant differences were found in main pharmacokinetic parameters of T_{max}, C_{max} and $AUC_{0-\infty}$ between these two formulations, and a 2.5-fold enhancement of relative bioavailability of ATT was observed from the SMEDDS compared with tablets.
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1. Introduction

Anethole trithione (ATT, structure shown in Fig. 1, chemical name is 5-(*p*-methoxyphenyl)-3H-1,2-dithiole-3-thione) is a sulfur heterocyclic compound naturally found in cruciferous vegetables [1], and ATT tablets have been marketed in many countries such as France, Germany and China.

ATT has choleretic properties and can increase salivary secretion for xerostomia in patients treated with psychotropic drugs (tricyclic antidepressants or neuroleptics) [2–4]. In the past years, ATT has been drawing particular interest for cancer chemoprevention in humans since it can significantly inhibit carcinogenesis by increasing the activity of electrophile detoxification enzymes (phase II enzymes conjugating with carcinogens favoring their elimination) as well as increasing intracellular glutathione levels (increasing protection against free radicals, oxidants) [5,6]. Since ATT has very low intrinsic water solubility (0.0016 mM) [7,8], which results in some biopharmaceutical problems, it is necessary to increase the aqueous solubility of ATT and thus improve its bioavailability. A lot of new techniques and formulations have been investigated to enhance its bioavailability, such as complexation with β-cyclodextrins [9], solid dispersion with PEG [10]. Recently, a self-microemulsifying drug delivery system (SMEDDS) has been studied to improve the solubility and absorption for poorly water-soluble drugs [11–14]. A new SMEDDS formulation of ATT, which contained some inactive ingredients such as medium chain triglyceride, Cremophor, Tween80 and Labrosol, was developed in our laboratory to improve the oral bioavailability of ATT.

To our knowledge, the oral bioavailability of ATT SMEDDS has not been reported in either animals or humans. So far, there have been some methods for determination of ATT analogue (anethole dithiolthione) in urine [15,16], and few studies have been published for determination of ATT in plasma. Yu et al carried out the determination of ATT in the mixture of standard ATT
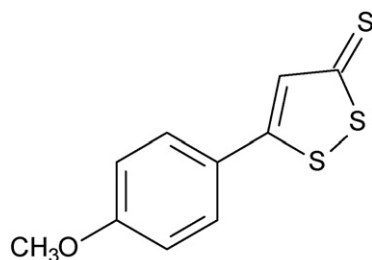
Fig. 1. Structure of ATT.

solution and blank plasma by HPLC [17]. However, the limit of quantitation (84 ng/ml) with the above method was insufficient to determine the low ATT concentration in plasma (about several ng/ml) after oral administration of ATT to animals. Moreover, the error would be very high in determination of the biologic samples without using internal standard. In this study, we developed and validated a sensitive, accurate and reliable liquid chromatography method by using liquid–liquid extraction and selecting appropriate internal standard to determine ATT concentration in rabbit plasma. The relative bioavailability of ATT SMEDDS compared with tablets was also investigated in rabbits.

2. Materials and methods

2.1. Chemicals and reagents

ATT reference standard was provided by National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). ATT tablet (containing 25 mg ATT per tablet) used as reference preparation was purchased from Purui Pharmaceutical Co. (Sichuan, China). ATT was provided by Shanghai Sixth Pharmaceutical Factory (Shanghai, China). ATT SMEDDS was prepared in our laboratory. Mifepristone was purchased from Hualian Pharmaceutical Co. (Shanghai, China). HPLC-grade methanol was obtained from Tedia Company Inc. (Fairfield, USA). All other chemicals used were of analytical grade unless otherwise indicated. Double-distilled water was used for all preparations.

2.2. Apparatus and chromatographic conditions

Chromatographic separation was performed on Waters liquid chromatographic system equipped with two 515 pumps, a 2487 absorbance detector, a 7725i manual injector. Empower chromatography workstation was applied for data collecting and processing (Waters, USA). An analytical column, Diamonsil C_{18} column (250 mm × 4.6 mm, 5 μm) from Dikma Technologies (Beijing, China) and a guard column packed with C_{18} (10 mm × 4.0 mm) from Turner Science Instrument Co., Ltd. (Tianjin, China) were used for chromatographic separation at a constant temperature (30 °C). Mobile phase consisted of a mixture of methanol–water (90:10, v/v) was delivered at a flow rate of 1.0 ml/min. The injection volume was 50 μl and the detection was performed at a wavelength of 348 nm.

2.3. Preparation of ATT SMEDDS

The components of SMEDDS (ATT, medium chain triglyceride, Cremophor, Tween80 and Labrosol) were accurately weighed into glass vials. They were mixed by gentle stirring and vortex mixing, and incubated at 40 °C in a water bath until ATT was completely dissolved. The formulation was stored at room temperature and was filled into hard capsules (contained 12.5 mg ATT per capsule) before bioavailability studies.

2.4. Sample preparation

A 50-μl of internal standard solution (mifepristone, 0.8 μg/ml in methanol) was added to 0.5 ml of rabbit plasma. The sample was extracted with 2.0 ml mixture of cyclohexane and isopropanol (95:5, v/v), then vortex mixed for 5 min, and centrifuged (1400 × g) for 10 min. The supernatant organic phase was separated and transferred into a clean conical tube for evaporation until dryness in a water bath at 40 °C under the protection of nitrogen stream. The residue was reconstituted with 100 μl of the mobile phase by vortex and a 50 μl volume was injected into the LC system.

2.5. Method validation

Plasma samples were quantified using the peak area ratio of ATT to mifepristone. To evaluate linearity, calibration standards in plasma at concentrations of 0.5, 1, 2, 4, 8, 16 and 32 ng/ml were prepared and assayed in triplicate on three consecutive days. The accuracy and precision were assessed by determining quality control (QC) samples at three concentration levels on three different days. The accuracy (R.E.) was expressed as (mean observed concentration − spiked concentration)/(spiked concentration) × 100% and the precision as relative standard deviation (R.S.D.). Concentrations of ATT in plasma samples were determined by back-calculation of the observed peak area ratios of the analyte to internal standard from the best-fit calibration curve using a weighted ($1/x^2$) linear regression. In order to calculate the extraction recoveries of ATT at three QC levels (0.5, 4 and 32 ng/ml, $n = 3$ at each concentration) and internal standard ($n = 3$), the peak areas for plasma extracts were compared to the peak areas of ATT and internal standard that were added to extracted blank plasma samples at the same concentration. The stability of ATT in the reconstituted solution under 20 °C for 24 h and at −20 °C for 1 month in dryness after processed as Section 2.4 was assessed by placing QC samples at three concentrations in triplicate.

2.6. Application of the developed LC method

Six male rabbits (weighed 2.5 ± 0.2 kg) were provided by the Center of Laboratory Animals of Second Military Medical University. The study was based on a single-dose, randomized, two-period crossover design at an interval of 1 week. All rabbits were dosed following an overnight fast. They were given single dose of either two ATT SMEDDS capsules (equivalent to 25 mg ATT) or four ATT tablets (equivalent to 100 mg ATT) with 20 ml water. No other food was allowed until after collection of the 10 h
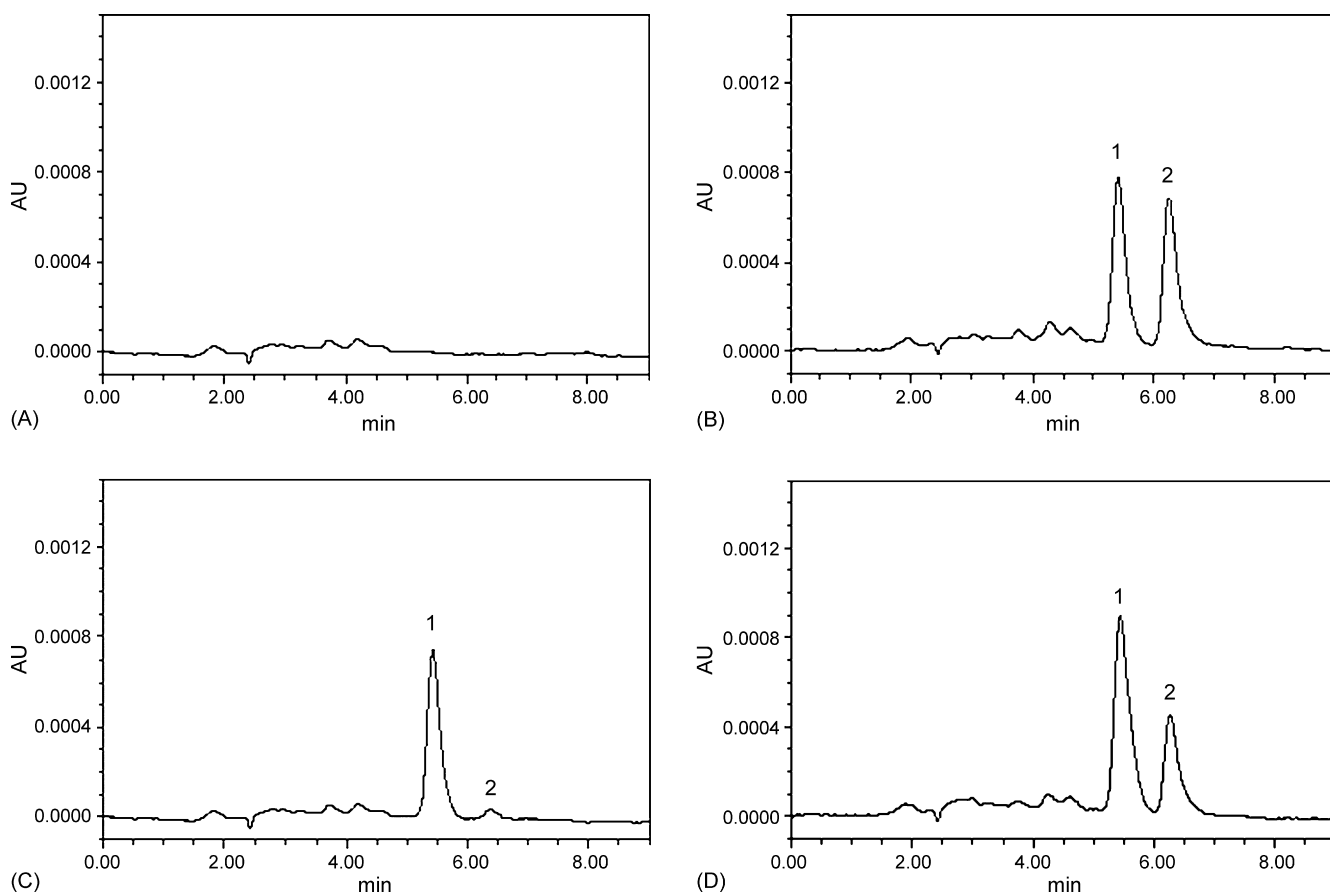
Fig. 2. Chromatograms of ATT and mifepristone in plasma samples. (A) Blank plasma sample; (B) blank plasma sample spiked with ATT (8 ng/ml) and mifepristone; (C) blank plasma sample spiked with ATT (0.5 ng/ml) and mifepristone; (D) 2-h rabbit plasma sample after oral administration of SMEDDS. Peak 1, mifepristone ($t_R = 5.4$); peak 2, ATT ($t_R = 6.3$).

sample while water intake was free. About 1 ml of blood sample was collected from the ear vein into heparinized test tube at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 8 and 10 h following oral administration of ATT formulations. Plasma was separated by centrifugation and processed as Section 2.4 until dryness. These dried samples were kept frozen at −20 °C until analysis. The study was conducted one more time after a washout period of 7 days.

3. Results and discussion

3.1. Optimization of analytical condition

A trial test indicated that the solvents mixture of cyclohexane and isopropanol (95:5, v/v) for extraction could obtain a relative high recovery of ATT and less endogenous substances from the rabbit plasma. A symmetric peak was observed for ATT in chromatograph and a good separation between ATT and the interfering peaks was achieved.

3.2. Selectivity

The method selectivity was assessed by comparing the chromatograms of blank rabbits plasma with the corresponding spiked plasma. Fig. 2 showed the typical chromatograms of a blank plasma sample, a blank plasma sample spiked with ATT

(8 ng/ml) and mifepristone (0.8 μg/ml), a blank plasma sample spiked with ATT (0.5 ng/ml) and mifepristone (0.8 μg/ml), and a 2-h rabbit plasma sample after oral administration of ATT SMEDDS. No significant interferences of endogenous substances from the blank rabbit plasma with ATT or mifepristone were detected. Typical retention time for ATT and mifepristone were 6.3 and 5.4 min, respectively. Therefore, the described LC method was selective for the determination of ATT in rabbit plasma.

3.3. Linearity

Calibration standards were prepared in blank rabbit plasma to give plasma concentrations 0.5, 1, 2, 4, 8, 16 and 32 ng/ml for ATT. The linear regression of the peak area ratio versus concentration was fitted over the concentration range of 0.5–32 ng/ml in rabbit plasma. A typical equation of the calibration curve was as follows: $R = 0.1249C - 0.0207$ ($r = 0.9982$), where R was the peak area ratio of ATT to mifepristone, and C was the concentration of ATT.

3.4. Accuracy and precision

The limit of quantification (LOQ) for determination of ATT in rabbit plasma, defined as the smallest sample concentration

Table 1
Accuracy and precision of the developed LC method for the determination of ATT in rabbit plasma

	Concentration (ng/ml)		
	0.5	4	32
Intra-day ($n = 6$)			
Mean ± S.D. (ng/ml)	0.51 ± 0.04	4.06 ± 0.26	31.46 ± 0.77
R.S.D. (%)	8.4	6.3	2.5
R.E. (%)	2.7	1.5	−1.7
Inter-day ($n = 6$)			
Mean ± S.D. (ng/ml)	0.52 ± 0.06	3.91 ± 0.34	31.70 ± 1.56
R.S.D. (%)	11.8	8.6	4.9
R.E. (%)	3.7	−2.2	−0.9

S.D., standard deviation; R.S.D., relative standard deviation; R.E., relative error. R.E. (%)=100 × ((mean concentration − nominal concentration)/nominal concentration).

above which quantitation could be carried out with adequate accuracy and precision (Table 1), was found to be 0.5 ng/ml. Compared with literature method [17], the LOQ was appropriate for pharmacokinetic studies of ATT preparations in rabbits. Intra-day accuracy and precision ranged from −1.7 to 2.7% and from 2.5 to 8.4%, respectively; inter-day accuracy and precision ranged from −2.2 to 3.7% and from 4.9 to 11.8%, respectively.

3.5. Extraction recovery and stability

The extraction recoveries of ATT, determined at three concentrations (0.5, 4.0 and 32.0 ng/ml), were 84.7 ± 5.8, 92.3 ± 3.4 and 89.9 ± 5.1% ($n = 3$). The extraction recovery of mifepristone was 86.4 ± 3.7% ($n = 3$).

The relative error of ATT between the initial concentrations and the concentrations stored at −20 °C for 1 month was less than 7.1%, which indicated ATT sample in dryness was stable for at least 1 month under storage condition after rabbit plasma was processed. ATT samples were also stable (the relative error of ATT less than 6.2%) in the reconstituted solution of methanol–water (90:10, v/v) for at least 24 h at room temperature (20 °C).

3.6. Bioavailability study and pharmacokinetic analysis

Pharmacokinetic analysis was performed by non-compartmental analysis. The maximum ATT concentration (C_{max}) and corresponding peak time (T_{max}) were obtained directly from concentration–time profiles. The slope of least-square fitted terminal log-linear portion of the plasma concentration-time profile gave the elimination rate constant (K_e) and the elimination half-life ($t_{1/2}$) was calculated by $0.693/K_e$. The area under the plasma concentration–time curve of ATT from time zero to infinity ($AUC_{0-\infty}$) was calculated by the trapezoidal rule which included area from time 0 to the last measurable concentration (C_t) point t and those from time t to infinity, calculated as C_t/K_e.

The results from our earlier experiments showed that the bioavailability and the plasma concentration of ATT after oral administration of ATT formulation to rabbits increased with the increasing of dose. Based on the assumption that
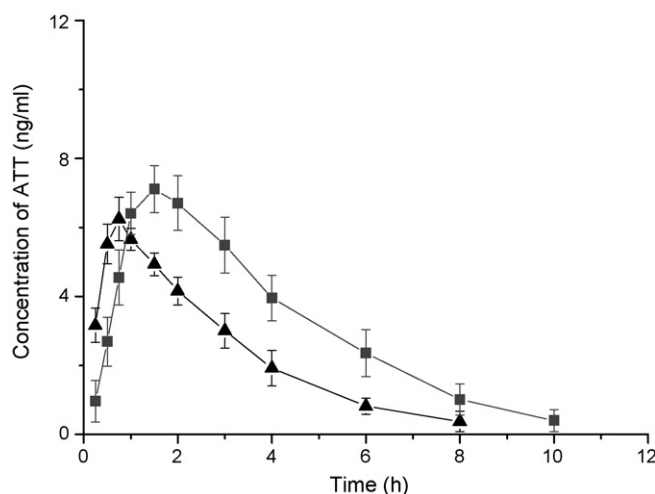

Fig. 3. Mean plasma concentration–time curves of ATT after oral administration of SMEDDS capsules (equivalent to 25 mg ATT) and tablets (equivalent to 100 mg ATT). Each point represented the mean and standard deviation of six rabbits. Formulation type: (▲) SMEDDS capsules; (■) tablets.

AUC might increase proportionally with the dose administered, the relative bioavailability of ATT from SMEDDS against the tablets was calculated by $(AUC_{0-\infty})_{SMEDDS}/(AUC_{0-\infty})_{tablet} \times dose_{tablet}/dose_{SMEDDS}$ to find the extent of absorption of ATT. The mean plasma concentration–time profiles for these two formulations were presented in Fig. 3 and the pharmacokinetic parameters were given in Table 2.

There were significant difference in pharmacokinetics parameters such as $AUC_{0-\infty}$, T_{max} and C_{max} between ATT SMEDDS and tablets ($P < 0.01$, the statistic calculation of $AUC_{0-\infty}$ and C_{max} values were carried out by $AUC_{0-\infty}/dose$ and $C_{max}/dose$). The $AUC_{0-\infty}$ for SMEDDS (dose 25 mg) and tablets (dose 100 mg) of ATT were 20.38 and 32.92 ng h/ml, respectively. A 2.5-fold enhancement of bioavailability and shorter T_{max} was observed from SMEDDS compared with tablet. It seemed that the solubilization process played a dominant role in the absorption. The reason might be that the spontaneous formation of an microemulsion upon drug release in the GI tract advantageously presented the drug in a dissolved form, the small droplet size provided a large interfacial surface area for drug absorption and ATT was likely to penetrate the gastro-intestinal mucosa easily, thus absorption might occur very quickly [12,18].

Table 2
Pharmacokinetic parameters for ATT in rabbits following oral administration of SMEDDS and tablets formulations ($n = 6$)

	SMEDDS	Tablets
T_{max} (h)	0.71 ± 0.10[*]	1.50 ± 0.32
C_{max} (ng/ml)[a]	6.26 ± 0.61[*]	7.11 ± 0.68
$AUC_{0-\infty}$ (ng h/ml)[b]	20.38 ± 3.24[*]	32.92 ± 6.16
K_e (h^{-1})	0.3988 ± 0.0663	0.3496 ± 0.0504
$t_{1/2}$ (h)	1.78 ± 0.31	2.02 ± 0.38

[a] The statistic calculation of C_{max} values was carried out by $C_{max}/dose$.
[b] The statistic calculation of $AUC_{0-\infty}$ values was carried out by $AUC_{0-\infty}/dose$.
[*] $p < 0.01$.

4. Conclusions

In summary, a sensitive, accurate and reliable HPLC method was developed and validated to determine ATT in rabbit plasma. A 2.5-fold enhancement of bioavailability in rabbits was observed from ATT SMEDDS compared with tablets. This study could be very useful for pharmacokinetics studies of ATT preparations in human.

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