A novel gradient LC-MS/MS method for simultaneous determination of trimebutine maleate and its two metabolites in human plasma

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ABSTRACT: A simple and rapid chromatography-tandem mass (LC-MS/MS) method has been developed for simultaneous determination of trimebutine maleate (TM) and its two major metabolites *N*-didemethyltrimebutine (APB) and 3, 4, 5-trimethoxybenzoic acid (TMBA) in human plasma. Trimebutine maleate and its metabolites were analyzed by protein precipitation followed by reverse-phase HPLC separation on a Sun Fire C_{18} column. An 5 min gradient elution of mobile phase was used to obtain quality chromatography. The API4000 mass spectrometer was operated in positive-negative switching ionization mode. Positive ionization was applied to detect TM, APB and BP (IS-1, positive internal standard) in the first 3.4 min. After an interval of 0.1 min, the instrument was automatically converted to negative ionization mode to detect TMBA and TOBA (IS-2, negative internal standard). The method was validated over the concentration range of 0.5–500 ng/mL for trimebutine maleate and APB, 50–50000 ng/mL for TMBA. Inter- and intra-day precision (RSD%) for trimebutine maleate and its metabolites were all within 15% and the accuracy was within 85–115%. The mean recoveries were 102.4% for TM, 100.9% for APB and 92.7% for TMBA. The method was applied to a pharmacokinetic study of trimebutine maleate and its metabolites in healthy Chinese volunteers.

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Introduction

Trimebutine maleate (TM), 2-dimethylamino-2-phenylbutyl-3, 4, 5-trimethoxybenzoate hydrogen maleate, is a prokinetic agent which confers a motility-modulating function by acting directly on the smooth muscle in the gastrointestinal tract [1]. It is clinically used in the treatment of various gastrointestinal disorders including irritable bowel syndrome and postoperative ileus [2-6]. Trimebutine maleate undergoes in vivo metabolism to produce mainly metabolites APB and TMBA [7]. The literature survey reveals several HPLC methods for the quantitation of trimebutine maleate and its metabolites [8-10]. But, these methods are not sensitive enough to evaluate the phamacokinetics of TM and its metabolisms. Wang HY *et al* [11]described a LC-MS/MS method for simultaneously determining trimebutine maleate and its three metabolites in human plasma. We improved the method in mobile phase and sample preparation procedure. The gradient elution and direct protein precipitation technique were used for the frist time. This method had shorten the total run time and improved the extraction recoveries significantly. The method was successfully applied for the single and multiple-dose pharmacokinetic study of trimebutine maleate tablets in healthy Chinese volunteers. No pharmacokinetic data have been published after multiple administration of trimebutine maleate tablets. Our study have compared the pharmacokinetic parameters of trimebutine maleate and its three metabolites after a first oral dose and repeated doses of 200 mg to healthy volunteers.

Experimental

2.1. Chemicals and reagents

The reference standard of Trimebutine maleate (purity 99.6%) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products of China (Beijing, PR China). APB (purity 99.1%) and TMBA (purity 97.0%) were provided by Kaikai Yuansheng Medicine Co., Ltd. (Henan, China) and TCI (Shanghai) Development Co., Ltd., respectively. The internal standards of BP (IS-1, purity 98.2%) and TOBA (IS-2, purity 98%) were provided by Adamas Reagent Co., Ltd. (Shanghai, China) and sigma-aldrich (USA), respectively. Structures of trimebutine maleate, its metabolites and internal standards are depicted in Fig. 1. Methanol (HPLC grade), acetonitrile (HPLC grade) and formic acid (analytical grade) were all purchased from Fisher Scientific (Massachusetts, USA). Deionized water was purified using PL5242 Purelab Classic UV (PALL Co. Ltd., USA) before use. Drug-free human plasma used in the research was supplied by Henan Provincial People's Hospital blood bank.

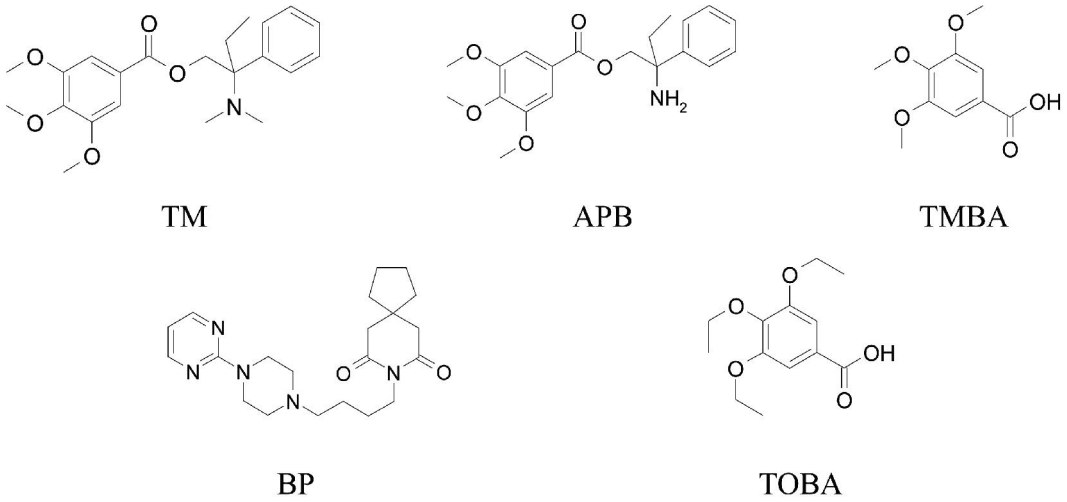

Figure 1. The chemical structures of trimebutine maleate, its metabolites and internal standards.

2.2Instrumentation

An API 4000 triple-quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada) via a Turbo Ion Spray ionization (ESI) interface, a LC-20 AD pump and a SIL-HT_A autosampler were used for LC–MS/MS analyses. Analyst 1.5 software (Applied Biosystems/MDS Sciex) was used for the control of equipment, acquisition and data analysis.

2.3. Chromatographic and Mass Spectrometric Conditions

Chromatographic separation was achieved on a Sun Fire C_{18} column (100 mm × 4.6 mm i.d., 5 μm; Waters, Milford, MA) with a 4.0 mm × 3.0 mm i.d. C_{18} (5 μm) security guard column (Phenomenex, Torrance, CA, USA). Gradient elution was performed mixing solvent A (acetonitrile containing 0.1% aqueous formic acid) and solvent B (water containing 0.1% aqueous formic acid) at a flow-rate of 0.8 mL/min. The final gradient program was: from 0 to 0.4 min isocratic at 5% A, from 0.4 to 1 min a linear gradient 5% A to 30% A, from 1 to 1.5 min a linear gradient 30% A to 50% A, from 1.5 to 3 min a linear gradient 50% A to 90% A, and from 3 to 5 min isocratic at 5% A. The injection volume was 10 μL. The column temperature was kept constant at 25 °C.

The API 4000 mass spectrometer was operated in positive-negative switching ionization mode. The ionspray voltage (IS) was set at 5 kV for positive ionization (0-3.4 min) and - 4.5 kV for negative mode (3.5-5 min). Positive ionization was applied to detect TM, APB and BP (IS-1, positive internal standard) in the first 3.4 min. After an interval of 0.1 min, which was specified for the instrument to prepare for switching, the instrument was automatically converted to negative ionization mode to detect TMBA and TOBA (IS-2, negative internal standard). Under both positive and negative ionization mode, the heater gas temperature was set at 500 °C with backpressures for collision gas of 6 psi, curtain gas of 30 psi, nebulizer gas of 50 psi and heater gas of 55 psi. Ultrapure nitrogen was used as nebulizer, heater, curtain, and collision-activated dissociation (CAD) gas. The fragmentation transitions for the multiple reaction monitoring (MRM) were m/z 388.0→343.3 for TM, m/z 360.0→195.0 for APB, m/z 386.3→122.1 for BP (IS-1), m/z 210.7→152.1 for TMBA and m/z 252.9→179.9 for TOBA (IS-2) (Fig. 2) with a dwell time of 150 ms per transition.

2.4. Preparation of Calibration Standards and Quality Control Samples

Stock solutions for TM, APB and TMBA were separately prepared by dissolving accurately weighed standard compounds with methanol to give a final concentration of 1.0 mg/mL. A series standard working solutions were obtained by further dilution of the stock solution with water–methanol (50:50, v/v).

Stock solutions for BP (IS-1) and TOBA (IS-2) were separately prepared by dissolving accurately weighed standard compounds with acetonitrile to give a final concentration of 1.0 mg/mL. The solutions were then mixed and diluted with acetonitrile to achieve a mixture containing BP (7.5 ng/mL) and TOBA (50 μg/mL) and used as working solutions.

Calibration standard and quality control samples (QC) in plasma were prepared by diluting corresponding working solutions with drug-free human plasma. Calibration curves were prepared in the following concentration ranges: 0.5, 2, 5, 20, 50, 100, 200, 500 ng/mL for TM and APB; 50, 200, 500, 2000, 5000, 10000, 20000, 50000 ng/mL for TMBA. The QC samples were prepared: 1.0, 10, 400 ng/mL for TM and APB; 100, 1000, 40000 ng/mL for TMBA. All the plasma samples were stored at −30°C.

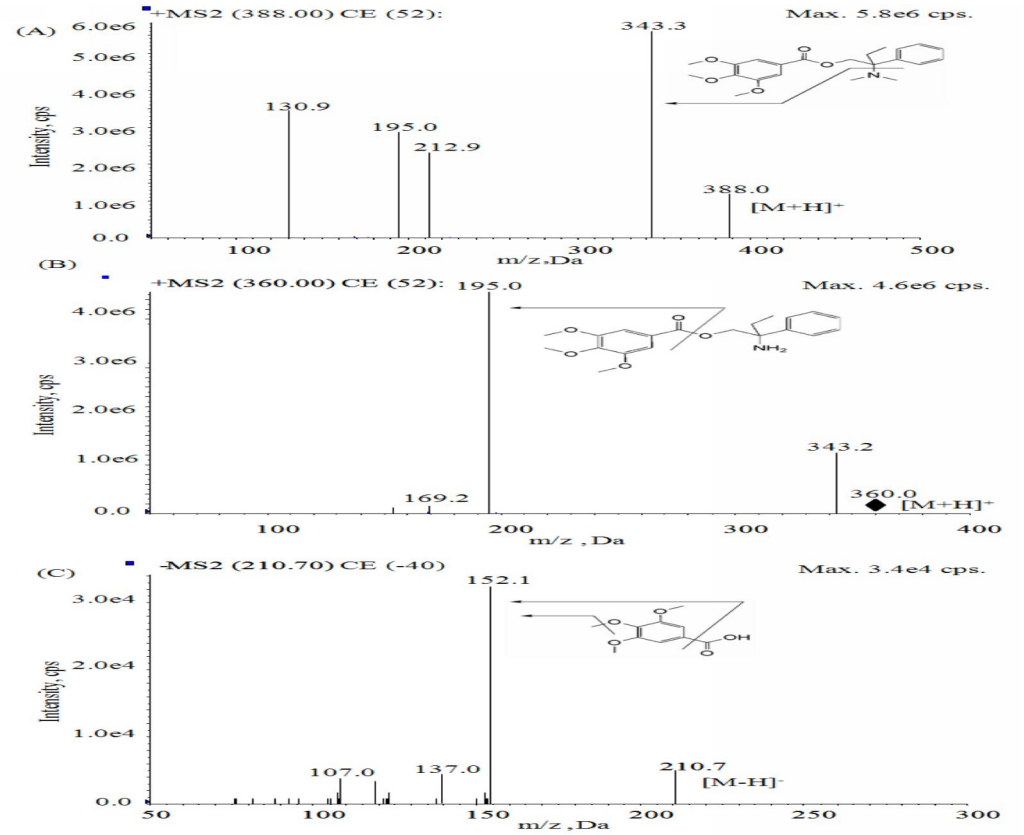

Figure 2. Product ion spectra of: (A) trimebutine maleate, (B) APB, (C) TMBA.

2.5. Sample preparation

Aliquot of 45 µL plasma sample was added with 5 µL water–methanol (50:50, v/v) and 100 µL IS(7.5 ng/mL of BP and 50 µg/mL of TOBA). After a thorough vortex mixing for 30 s, the mixture was centrifuged at 10000 r/min for 5 min. Aliquot of 50 µL of the supernatant liquid was added with 150 µL water containing 0.1% aqueous formic acid. The mixture was vortex mixing for 10 s and 10 µL was injected into the LC-MS/MS system for analysis.

2.6. Method Validation

The method validation assays were carried out according to the currently accepted US Food and Drug Administration (FDA) bioanalytical method validation guidance (US Department of Health and Human Services *et al*., 2001) [12]. The following parameters were considered.

The selectivity was assessed by comparing the chromatograms of six different donor's human blank plasma with the corresponding spiked plasma.

Calibration curves were constructed by analyzing spiked calibration samples. Samples were quantified using the peak area ratios of trimebutine maleate and its metabolites to the IS. The peak area ratios were plotted against nominal concentration of trimebutine maleate and its metabolites, and standard curves were constructed using linear regression analysis with a $1/x^2$ weighting factor. The limit of quantitation (LOQ) for trimebutine maleate and its metabolites were set at the concentration of the lowest non-zero calibration standard.

Intra- and inter-day precision and accuracy (relative recovery) were determined by assessing measured results of QC samples at low, medium and high concentrations.

The extraction recovery of the analytes was determined by comparing measured results of extracted QC samples at low, medium and high concentrations to unextracted calibration standards at the same concentration.

The matrix effect on the ionization of analytes was evaluated by comparing the peak area of analytes resolved in blank sample (the final solution of blank plasma after extraction and reconstitution) with that resolved in mobile phase. QC samples at low, medium and high concentrations were evaluated by analyzing five samples at each level. If the ratio <85 or >115%, an exogenous matrix effect was implied.

The short-term stability of trimebutine maleate and its metabolites in human plasma was investigated by assessing QC samples at low, medium and high concentrations after 6 h at room temperature (25°C). Freeze–thaw stability was checked after three cycles and long-term stability was acquired by

assessing QC samples stored at −30°C for 2 months. The stability of the analytes extracts was checked as follows: QC samples were prepared and injected with an autosampler, and after the samples of this batch were maintained in an auto-sampler at room temperature for 24 h, they were re-injected. The stability of the analytes in extracts was checked by comparing the measured results from the two runs.

2.7. Clinical study design and pharmacokinetic analysis

The pharmacokinetics of trimebutine maleate and its metabolites were investigated in 12 healthy subjects after single and multiple administration, and the situation of drug accumulation after multiple administration was evaluated. The clinical study protocol was approved by the Ethics Committee of Henan Provincial People's Hospital. All volunteers were given written informed consent to participate in the study according to the principles of the Declaration of Helsinki. Each volunteer participated in two phases: phases A and B, and there was a 7-day washout period between phases. In phrase A, 12 volunteers received 200 mg of trimebutine maleate tablet at 8:00 am following an overnight fast. In phrase B, 12 volunteers received 200 mg of trimebutine maleate tablet at 8:00 am after a standard breakfast on day 1, and were administered trimebutine maleate tablets 200 mg three times a day for 7 days. Water intake was allowed 2 hours after administration. The standard meals were provided after 4 h post-dose.

In phase A, venous blood samples were drawn pre-dose and at 0.25、0.5、0.75、1、1.5、2、4、6、9、12、24 and 36 hours post-dose. In phase B, venous blood samples were drawn before drug administration on days 4, 5 and 6 to determine the Cssmin. On day 7, venous blood samples were drawn at 0.25、0.5、0.75、1、1.5、2、4、6、9、12、24 and 36 hours post-dose.

Model-independent pharmacokinetic parameters were calculated for trimetazidine. The maximum plasma concentration (C_{max}) and the time to it (t_{max}) were noted directly. The elimination rate constant (kel) was calculated by linear regression of the terminal points of the semi-log plot of plasma concentration against time. Elimination half-life ($t_{1/2}$) was calculated using the formula $t_{1/2} = 0.693/kel$. The area under the plasma concentration–time curve $AUC_{0–36}$ to the last measurable plasma concentration was calculated by the linear trapezoidal rule.

3. Results and discussion

3.1. Conditions for Chromatography

When selecting the mobile phase for HPLC-MS system, attention was paid to the influence of mobile phase on the chromatographic retention and the MS sensitivity. During the early stage of method development, we tried to apply isocratic elution to detect all the analytes considering the simplification of method development. The final results showed that TM，APB had similar retention time, but as for different chemical properties, the retention time of TMAB was quite different with TM and APB. Considering the time and expense of the experiment, a method with gradient elution which can simultaneously determine TM and its two metabolites within 5 min was developed. Formic acid in mobile phase could improve peak symmetry and ionization efficiency under positive ionization mode while inhibition of ionization efficiency under negative ionization mode. Different amount of formic acid (0.05%, 0.1, 0.2 %) were tested based on peak shape and ionization efficiency. It was found that peak shape did not show any significant changes when the formic acid percentage changed from 0.1% to 0.2%, but ionization efficiency under negative ionization mode slightly decreased when the percentage was further increased up to 0.2%. The 0.1% formic acid was consequently selected.

3.2. Conditions for ESI-MS

Due to amino groups in its chemical structure, TM and APB had mass spectrometric response in the positive ion mode. But as for the carboxylic compound TMBA, the response appeared much stronger using negative ionization. A LC–MS/MS method using positive–negative switching ionization was choosed.

3.3. Sample preparation

During the early stage of method development, liquid–liquid extraction (LLE) was utilized for clean-up and extraction from the plasma. Different extraction solvents such as ethyl acetate, cyclohexane and ethyl acetate–acetone (3:1, v/v) were tested. However, the experiment results presented that the liquid-liquid extraction recovery rate was low (less than 60%), which can be due to the polar group in chemical sturctures of the analytes. Finally, we developed the direct precipitation method which could improve the extraction recoveries and simplify the sample preparation procedure significantly.

3.4. Selectivity

Selectivity was assessed by comparing the chromatograms of six different batches of blank human plasma with the corresponding spiked plasma. Fig. 3-5 show chromatograms of extracted blank plasma, plasma samples spiked with drugs. There was no interference observed at the retention times of the analytes in human plasma samples.

3.5. Linearity and lower limits of quantification

The calibration curves were validated over the concentration range of 0.5–500 ng/mL for trimebutine maleate and APB, and 50–50000 ng/mL for TMBA in human plasma. Typical equations of

calibration curves are as follows:
Trimebutine maleate: $Y = 4.98 \times 10^{-3} C - 3.20 \times 10^{-4}$, r = 0.9981
APB: $Y = 1.53 \times 10^{-3} C + 4.06 \times 10^{-4}$, r = 0.9960
TMBA: $Y = 1.37 \times 10^{-5} C + 1.91 \times 10^{-4}$, r = 0.9972

Here, Y represents peak area ratio, and C describes plasma concentration. The lowest concentration of determination was taken as the LLOQ which was found to be 0.5 ng/mL for TM and APB, and 50 ng/mL for TMBA. In all the cases, the calculated concentrations in the calibration curves were within ±15% bias from the nominal value except at the LLOQ, which was set at ±20%.

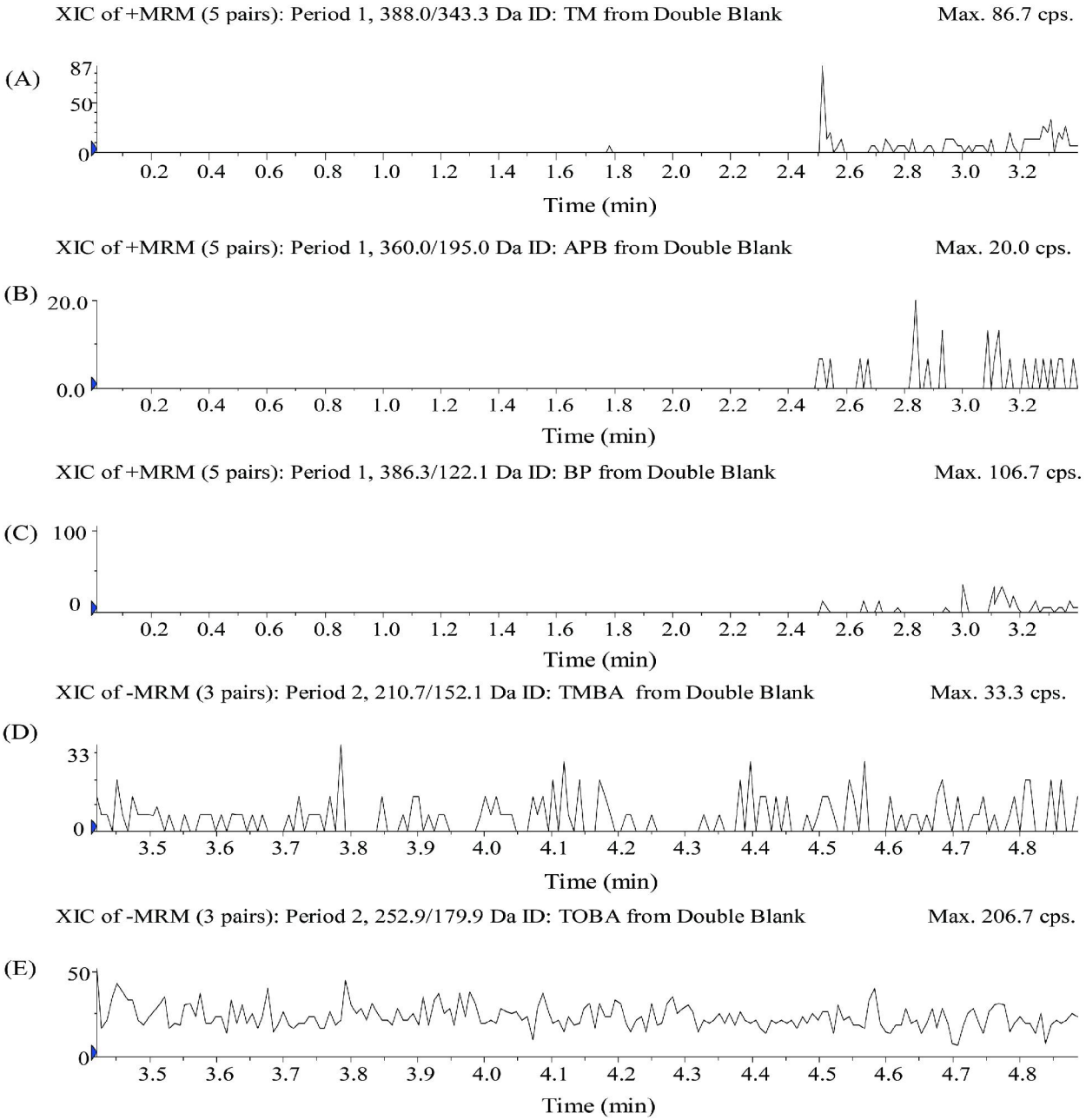

Figure 3. Typical MRM chromatograms obtained from blank plasma samples without any drugs and internal standards added (A): TM, (B): APB, (C): BP (IS-1), (D): TMBA, (E): TOBA(IS-2).

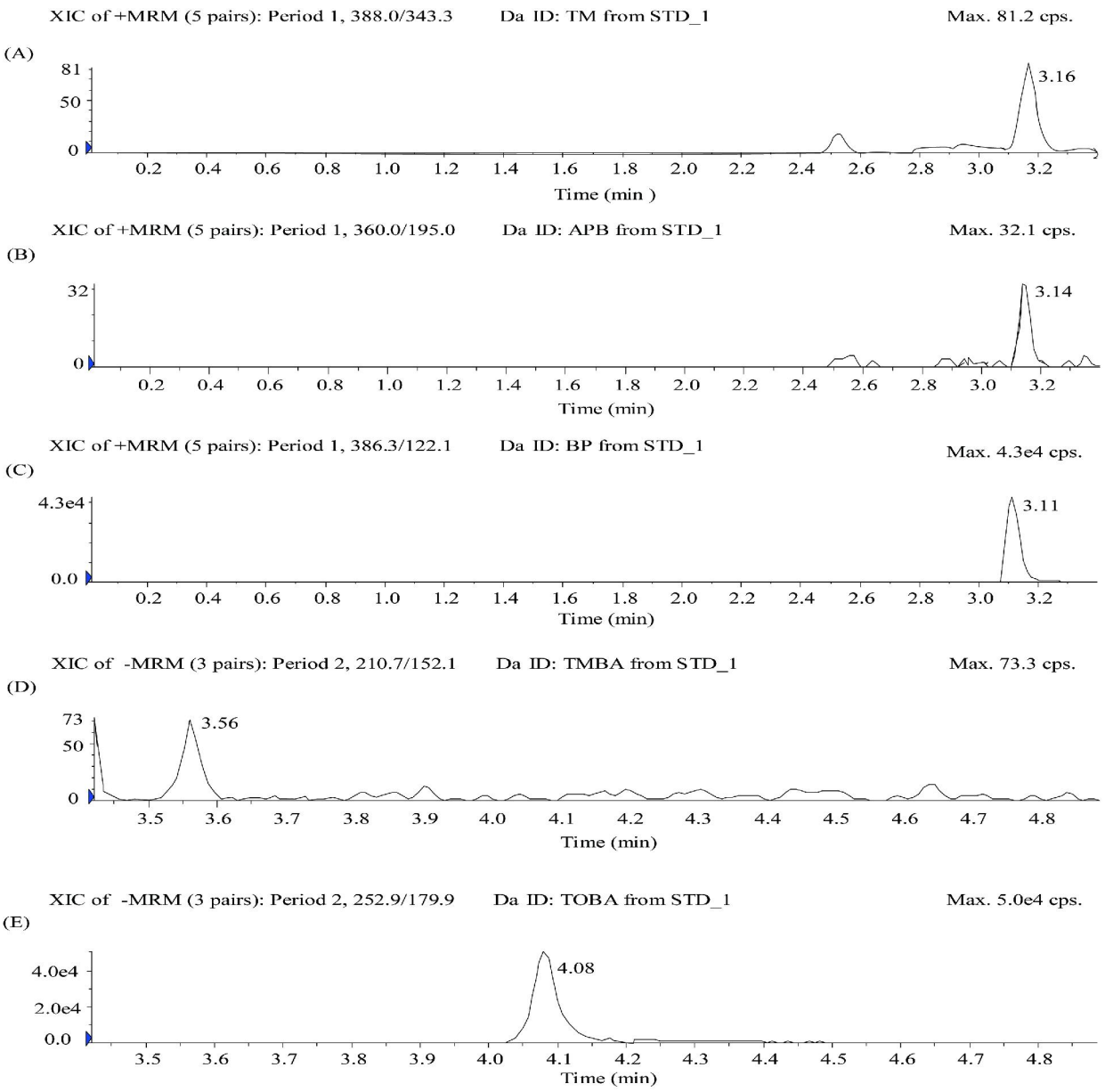

Figure 4. Typical MRM chromatograms obtained from plasma samples spiked with analytes and the IS (A): TM (0.5 ng/mL), (B): APB(0.5 ng/mL), (C): BP (IS-1), (D): TMBA (50 ng/mL), (E): TOBA(IS-2).

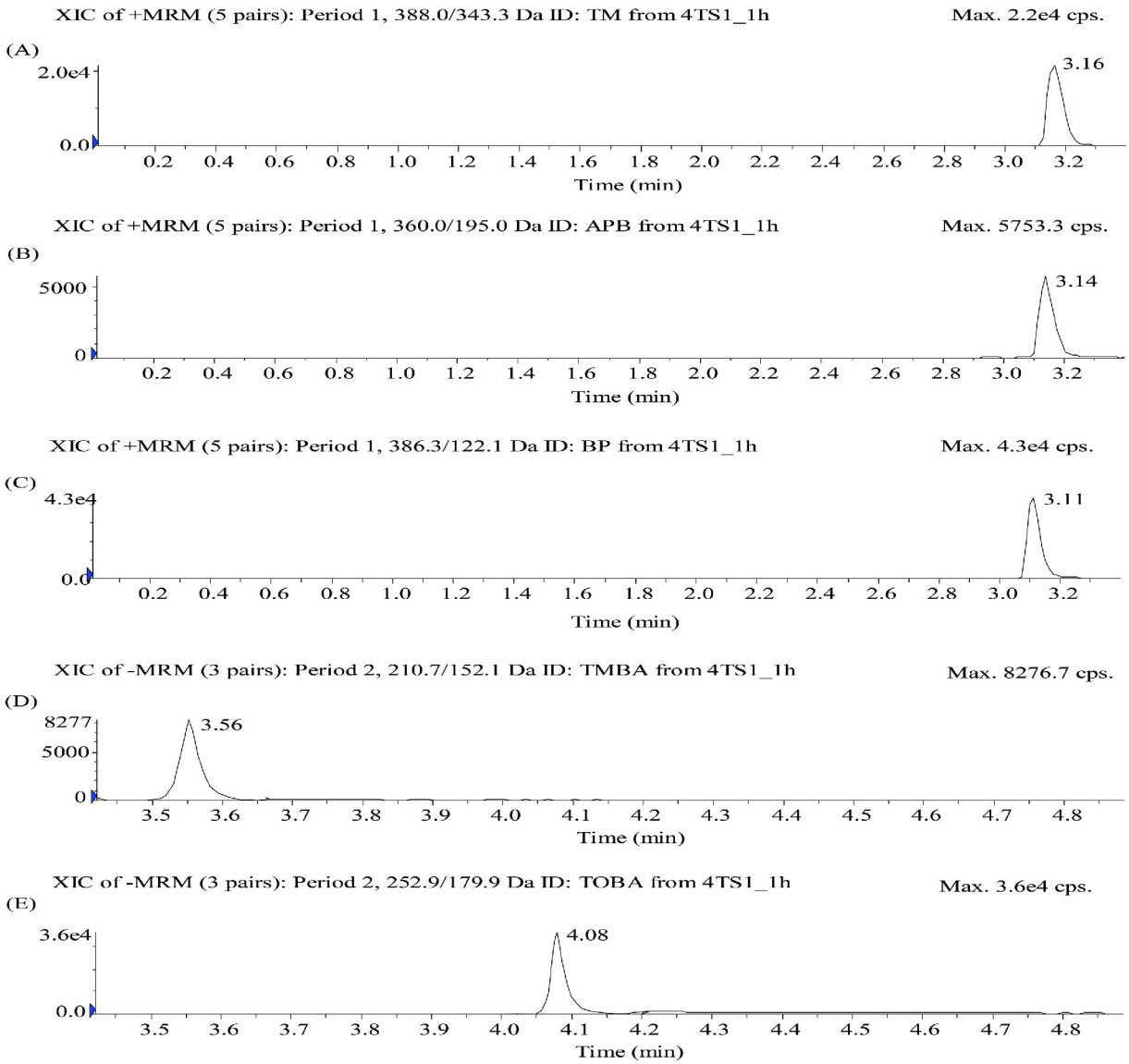

Figure 5. Typical MRM chromatograms obtained from a Chinese volunteer, 1 h after oral administration of 200 mg trimebutine maleate tablet.

3.6. Precision and accuracy

The QC samples at low, medium and high concentrations were prepared and analyzed on three separate analytical batches to evaluate the accuracy and precision of the analytical method. The accuracy and precision of the method were determined by analyzing five replicates of the QC samples along with one standard curve on each of three batches. Assay precision was calculated as the relative standard deviation (RSD, %) by use of one-way analysis of variance. The accuracy is the degree of closeness of the determined value to the nominal true value under the prescribed conditions. Accuracy is defined as the relative deviation of the value (E) of a standard from that of its true value (T) expressed as a percentage (RE%). It was calculated using the formula RE%= (E − T)/T × 100. Intra- and inter-day precisions were required to be less than 15%, and the accuracy to be within ±15%.

Table 1 summarizes the intra- and inter-day precision and accuracy for TM, APB and TMBP evaluated by assaying the QC samples.

3.7. Recovery and matrix effect

The observed value of extraction recovery of the method is shown in Table 2. The extraction recoveries were on average 102.4% for TMB, 100.9% for APB and 92.7% for TMBA. No significant ion suppression or enhancement was observed at the expected retention time of the targeted ions.

Table 1. Summary of precision and accuracy of QC samples at different concentration levels (in three analysis batch, five replicates for each batch)

	Concentration (ng/mL)	Precision (RSD%)		Accuracy (%)
		Intra-batch	Inter-batch	
TM	1	9.4	6.5	91.7
	10	6.9	5.4	102.0
	400	3.1	8.4	92.2
APB	1	8.8	12.2	101.0
	10	8.6	14.8	99.5
	400	3.7	13.0	103.1
TMBA	100	7.9	8.3	101.0
	1000	6.8	9.1	106.5
	40000	6.1	10.8	92.6

Table 2. Summary of extraction recoveries for trimebutine maleate (TM) and its metabolites in human plasma (n=5)

	Concentration (ng/mL)		Recovery (%)	R.S.D (%)
	Nominal	Found		
TM	1.0	0.958	95.8	9.7
	10	10.6	106.0	7.3
	400	421	105.3	1.3
APB	1.0	1.01	101.1	8.5
	10	10.2	101.6	7.2
	400	400.4	100.1	1.4
TMBA	100	96.2	96.2	9.0
	1000	901	90.1	1.7
	40000	36720	91.8	6.2

3.8. Stability

The results obtained from determination of stability (Table 3) showed no significant degradation occurred under the conditions tested. The plasma samples were stable at ambient temperature for 6 h, during three freeze-thaw cycles, and at -80°C for two months. The analytes in extracts were found to be stable for 24 h in the autosampler at room temperature.

Table 3. Summary of stability for trimebutine maleate (TM) and its metabolites in human plasma under different storage conditions (n = 3)

Compoud	Spiked conc. (ng/mL)	6 h at room temperature (R.E. %)	2 months at -30°C (R.E. %)	Stability of freeze-thaw (R.E. %)	Stability of extracts (R.E. %)
TM	1.0	1.4	3.1	0.8	6.8
	10	2.6	-0.9	1.6	-3.3
	400	-4.4	5.7	-2.0	-2.5
APB	1.0	-5.0	2.6	2.4	2.1
	10	3.9	-3.2	5.7	1.6
	400	10.3	-1.8	10.5	9.7
TMBA	100	-4.6	3.8	4.0	-1.9
	1000	5.1	4.1	-1.9	5.7
	40000	5.8	2.9	-0.2	7.8

3.9. Application of the method in pharmacokinetic studies

The method described above was successfully applied to study pharmacokinetics of trimebutine maleate tablets in healthy Chinese volunteers. After single and multiple oral administration of 200 mg trimebutine maleate tablets to 12 volunteers, plasma concentrations of trimebutine maleate and its metabolites APB and TMBA were simultaneously determined by the described LC–MS/MS method. Means of plasma concentration–time curves are shown in Fig. 6 and Fig. 7 after data were log transformed. The PK parameters are summarized in Table 4 and Table 5. Steady state was achieved after administration of trimebutine maleate tablet for 7 consecutive days. Compared with single-dose administration, the $AUC_{0\text{-}36}$ of multiple-dose administration are increased significantly. At the same time, the $t_{1/2}$ and *MRT* are prolonged, which suggested that accumulation in plasma occurred.

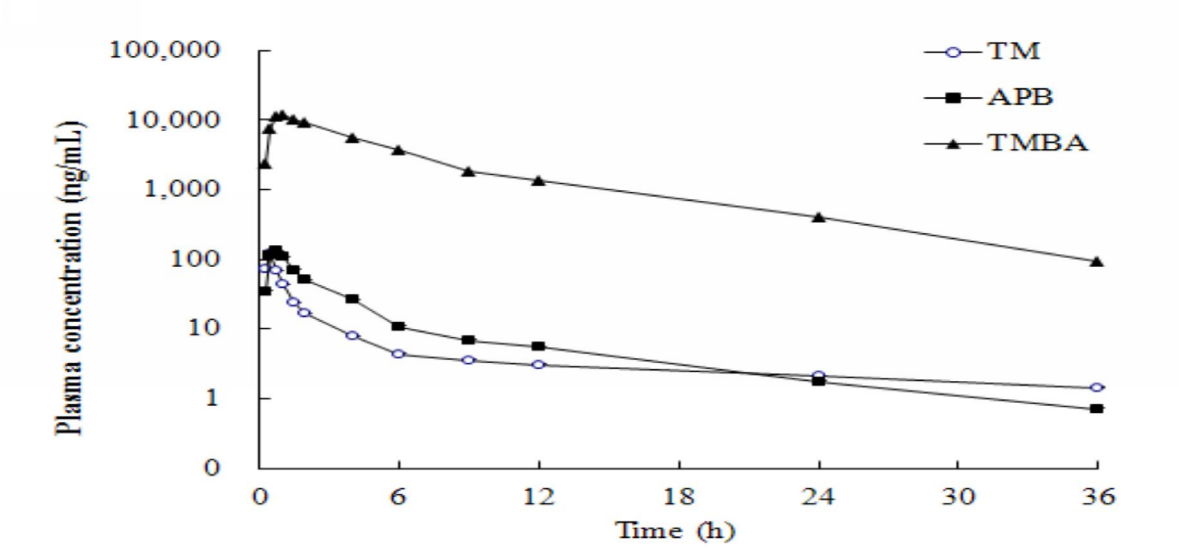

Figure 6. The mean plasma concentration–time curves of trimebutine maleate and its two metabolites in healthy Chinese volunteers (n=12) after single oral administration of 200 mg trimebutine maleate tablet.

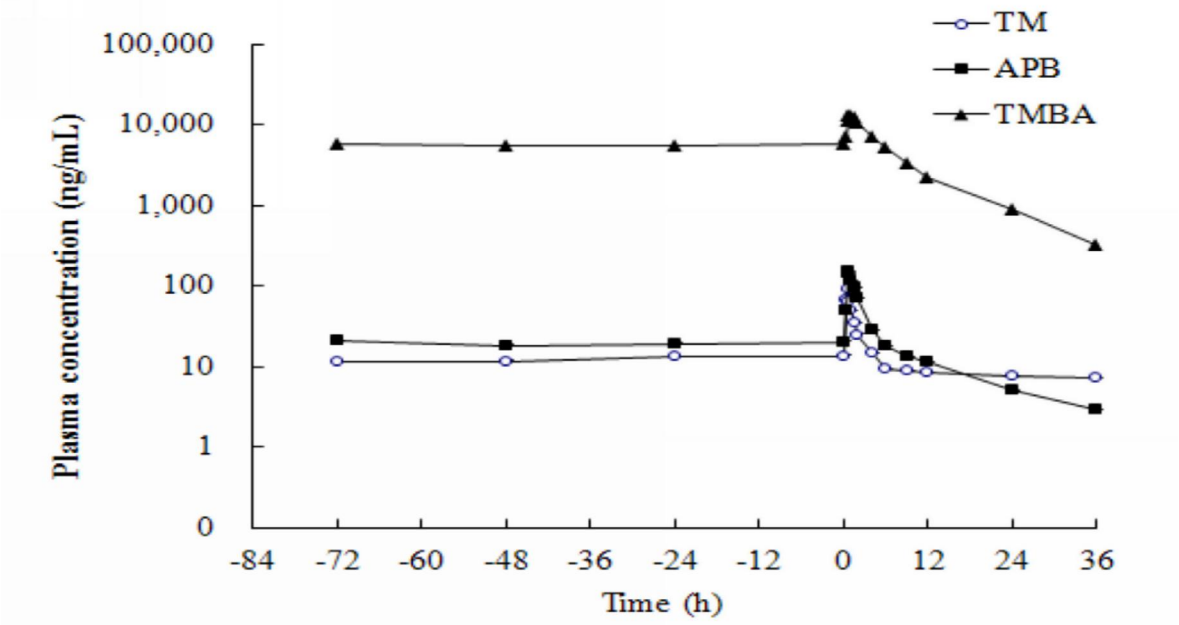

Figure 7. The mean plasma concentration–time curves of trimebutine maleate and its two metabolites in healthy Chinese volunteers (n=12) after multiple oral administration of 200 mg trimebutine maleate tablets.

Table 4. The main pharmacokinetic parameters for trimebutine maleate (TM) and its metabolites after single oral administration trimebutine maleate tablet in healthy Chinese volunteers. (mean ± SD, n = 12)

	TM	APB	TMBA
C_{max} (ng/mL)	131.3 ± 215.3	138.3 ± 62.8	12176.7 ± 2380.8
T_{max} (h)	0.6 ± 0.2	0.8 ± 0.3	1.0 ± 0.5
$t_{1/2}$ (h)	8.6 ± 2.3	8.3 ± 3.8	6.6 ± 1.3
$AUC_{0\text{-}36}$ (ng·h/mL)	219.9 ± 228.2	365.2 ±199.6	69343 ± 15478
$AUC_{0\text{-}\infty}$ (ng·h/mL)	243.6 ± 241.5	377.5 ± 201.9	70842 ± 15817
MRT (h)	8.0 ± 1.9	4.7 ± 1.0	6.7 ± 1.1

Table 5. The main pharmacokinetic parameters for trimebutine maleate (TM) and its metabolites after multiple oral administration trimebutine maleate tablets in healthy Chinese volunteers. (mean ± SD, n = 12)

	TM	APB	TMBA
C_{ss_max} (ng/mL)	94.16 ± 72.82	168.3 ± 82.8	13991.7 ± 3098.8
C_{ss_min}	13.45 ± 10.71	19.78 ± 5.43	5680.0 ± 2053.9
C_{av}	20.32 ± 15.09	42.99 ± 15.20	6568.6 ± 2043.4
T_{max} (h)	0.6 ± 0.3	0.7 ± 0.3	1.0 ± 0.3
$t_{1/2}$ (h)	14.7 ± 3.4	10.61 ± 1.71	8.3 ± 0.6
$AUC_{0\text{-}36}$ (ng·h/mL)	403.4 ± 326.8	572.5 ±188.4	99401 ± 36196
$AUC_{0\text{-}\infty}$ (ng·h/mL)	571.6 ± 483.9	617.2 ± 204.6	103389 ± 38590
MRT (h)	12.6 ± 1.5	7.5 ± 0.9	7.8 ± 0.8
DF	3.9 ± 1.3	3.4 ± 0.9	1.3 ± 0.3

4. Conclusion

A simple, sensitive and specific HPLC-MS/MS method has been developed and validated for the determination of trimebutine maleate and its two metabolites in human plasma. Considering the different chemical structures and properties of the analytes, a gradient elution was used for the first time to improve peak shape and shorten the total run time. Besides, the direct protein precipitation technique had improve the extraction recoveries and simplify the sample preparation procedure significantly. No significant interference and matrix effect caused by endogenous compounds was observed. The method proved to be timesaving, sensitive and specific, and it has been applied to pharmacokinetic studies of trimebutine maleate and its major metabolites in healthy Chinese volunteers.

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