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Quantitative determination of trimebutine maleate and its three metabolites in human plasma by liquid chromatography-tandem mass spectrometry

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Abstract

A sensitive and selective HPLC–MS–MS method was developed for the determination of trimebutine maleate (TM) and its major metabolites *N*-monodemethyltrimebutine (TM-MPB), *N*-didemethyltrimebutine (APB) and 3,4,5-trimethoxybenzoic acid (TMBA) in human plasma. The analytes were extracted from plasma samples by liquid–liquid extraction and chromatographed on a YMC J'sphere C_{18} column. The mobile phase consisted of 2 m*M* ammonium acetate buffer (pH 6.5)–methanol (20:80, v/v), and at a flow-rate of 0.2 ml/min. Detection was carried out on a triple quadrupole tandem mass spectrometer in multiple reactions monitoring (MRM) mode using positive–negative switching electrospray ionization (ESI). The method was validated over the concentration range of 1–100 ng/ml for trimebutine maleate and APB, 1–500 ng/ml for MPB, and 50–10 000 ng/ml for TMBA. Inter- and intra-day precision (RSD%) for trimebutine maleate and its three metabolites were all within ±15% and the accuracy was within 85–115%. The limit of quantitation was 1 ng/ml for trimebutine maleate, TM-MPB and APB, and 50 ng/ml for TMBA. The extraction recovery was on average 58.2% for trimebutine maleate, 69.6% for MPB, 51.2% for APB and 62.5% for TMBA. The method was applied to the pharmacokinetic study of trimebutine maleate and its metabolites in healthy Chinese volunteers.
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Keywords: Trimebutine maleate; *N*-Monodemethyltrimebutine; *N*-Didemethyltrimebutine; 3,4,5-Trimethoxybenzoic acid

1. Introduction

Trimebutine maleate (TM) is a well-known prokinetic agent, which acts directly on the smooth muscle and possesses motility-regulating function on the gastrointestinal tract. It is clinically used in the treatment of various gastrointestinal disorders [1–4].

Trimebutine maleate undergoes in vivo metabolism to produce mainly metabolites TM-MPB, APB and TMBA [5]. Analytical methods based on HPLC have been described in literature for the quantitation of trimebutine maleate and metabolites MPB and APB [6–8]. However, these methods are inadequate for present pharmacokinetic study of trimebutine maleate and its metabolites because of low sensitivi-

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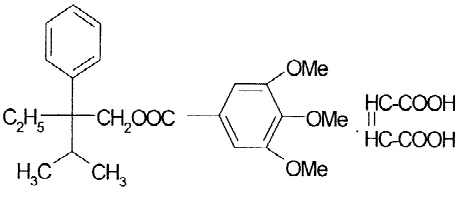
ty and no methods have been published for the quantitation of metabolite TMBA so far.

Therefore, we established a rapid, sensitive and selective LC–MS–MS method to determine trimebutine maleate and its three major metabolites in human plasma simultaneously. According to the structures of the analytes, trimebutine maleate and metabolites TM-MPB, APB are easily ionized in the positive mode because of ester bonds, but TMBA, a carboxylic acid, prefers the negative ionization mode to form $[M\text{-}H]^-$. Therefore, a timesaving HPLC–MS–MS method using positive–negative switching ionization was developed to detect all the compounds in one analytical run. It is the first time positive–negative switching ionization has been applied to the quantitative research of trimebutine maleate and its metabolites. The method proved to be sensitive and selective enough for the pharmacokinetic studies of trimebutine maleate in healthy Chinese volunteers.

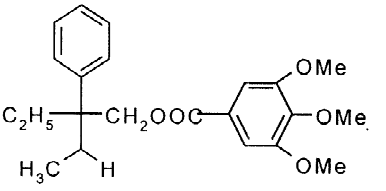
2. Experimental

2.1. Chemicals and reagents

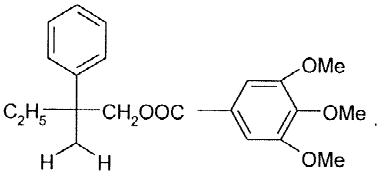
Trimebutine maleate (purity 99.6%), TM-MPB (purity 99.5%), APB (purity 99.1%), TMBA (purity 98.9%) and internal standards TM-PDP (IS-1, purity 99.2%), TOBA (IS-2, purity 99.7%) (Fig. 1) were provided by Tanabe Selyaku Co. Ltd. (Japan).



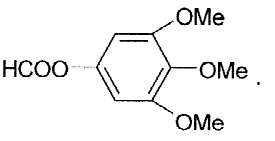
Trimebutine maleate (TM)



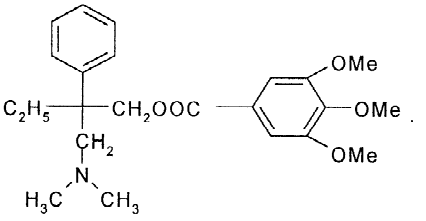
TM-MPB



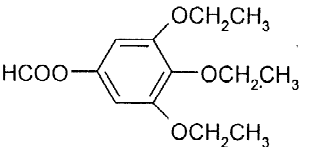
APB



TMBA



TM-PDP (IS-1)



TOBA(IS-2)

Fig. 1. Structures of trimebutine maleate, its metabolites and internal standards.

Methanol (HPLC grade) was purchased from Fisher (Fair Lawn, NJ, USA). Acetic acid, potassium phosphate and ammonium acetate were all of analytical grade and purchased from Peking Chemical Plant (Beijing, China). Drug-free human plasma used in the research was supplied by Peking Union Medical College Hospital blood bank. Distilled water was prepared with a Milli-Q water purifying system (Millipore, Bedford, USA).

2.2. HPLC conditions

The HPLC system consisted of a Waters 510 HPLC pump, Waters automated gradient controller (Waters, USA) and a PE series 200 autosampler from Perkin-Elmer (Foster City, CA, USA). The analytical column was a YMC J'sphere C_{18} column (150×2.0 mm I.D., 4 μm) coupled to a C_{18} (Keystone) guard column. The mobile phase was composed of 2 m*M* ammonium acetate buffer (pH 6.5)–methanol (20:80, v/v). The flow-rate was 0.2 ml/min and the injection volume was 30 μl.

2.3. Mass spectrometer conditions

A Perkin-Elmer Sciex API 3000 triple-quadrupole mass spectrometer (Foster City, CA, USA) equipped with Turbo Ion Spray interface (ESI with auxiliary gas) was used for mass analysis and detection. Probe temperature was set at 350 °C with ultrahigh-purity nitrogen as curtain gas (10 l/min) and nebulizer gas (10 l/min). Auxiliary gas (nitrogen) was optimized daily. The detection was carried out in positive–negative switching ionization mode. The ionspray voltage (IS) was set at +4000 V for positive ionization and −3500 V for negative mode. 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 analyte were, respectively, determined in positive or negative mode using standard solutions.

Multiple reaction monitoring (MRM) mode was selected using nitrogen as collision gas (6 l/min), and with a dwell time of 100 ms for each transition. The specific precursor–product ions selected for monitoring are shown in Table 1.

The analytical time for each run was 7.5 min in total. Negative ionization was applied to detect TMBA and TOBA (IS-2, negative internal standard) in the first 2.5 min, and after an interval of 0.1 min, which was specified for the instrument to prepare for switching, the instrument was automatically converted to positive ionization mode to detect TM, TM-MPB, APB and TM-PDP (IS-1, positive internal standard).

2.4. Preparation of stock solutions and working solutions

Stock solutions for TM, TM-MPB, APB and TMBA, each in duplicate, one for calibration curve samples and the other for quality control (QC) samples, were separately prepared by dissolving accurately weighed standard compounds with methanol to give a final concentration of 100 μg/ml for each analyte.

Stock solutions for TM-PDP (IS-1) and TOBA (IS-2) were prepared as above to give the final concentration of 100 μg/ml for each internal standard. The solutions were then mixed and diluted with water–methanol (1:1, v/v) to achieve a mixture containing TOBA (1 μg/ml) and TM-PDP (20 ng/ml) and used as working solutions.

Table 1
The specific precursor–product ions of the analytes in MRM mode

Compound	Positive mode		Compound	Negative mode	
	Q1	Q3		Q1	Q3
TM	388	343	TMBA	211	167
TM-MPB	374	195			
APB	360	343	TOBA(IS)	253	180
TM-PDP(IS)	388	176			

Q1, the first quadrupole mass spectrometer; Q3, the third quadrupole mass spectrometer.

All the solutions were stored at −30 °C and were brought to room temperature before use.

2.5. Preparation of calibration curve and QC samples in human plasma

Calibration curves and QC samples in plasma were prepared by diluting corresponding stock solutions of each analyte with drug-free human plasma. Fresh human plasma from different individuals was pooled before use, and no significant interference was found in the determination. The final concentrations of calibration curves and QC samples are listed in Table 2. All the plasma samples were stored at −30 °C.

2.6. Extraction procedure of plasma samples

To each 0.2-ml volume of plasma sample, 100 μl of the internal standard working solution (20 ng/ml of TM-PDP and 1000 ng/ml of TOBA) was added and mixed, followed by the addition of 100 μl potassium phosphate (pH 9). Then 1.5 ml of cyclohexane was added using an Eppendorf repeater pipette, and the tubes containing samples were capped and shaken for 10 min on a shaker. The aqueous and the organic layers were separated by centrifugation at 1000 *g* for 10 min, and the organic layer was collected and transferred to a clean test-tube. The remaining aqueous layer was again extracted by adding 100 μl 1 N HCl and 1 ml cyclohexane–choloform (4:1, v/v). After centrifugation for 10 min (1000 *g*), the top layer was also collected and mixed with the organic layer acquired before. The mixture was then evaporated to dryness under a gentle stream of nitrogen at 50 °C. The residue was reconstituted with 200 μl of mobile phase and centrifuged in a micro-spin filter tube (0.2 μm nylon, Alltech) at 2500 *g* for 3 min. The filtrate was collected and stored at −30 °C until analysis.

Table 2
The concentration for calibration curve QC samples in human plasma

Compound	Calibration curve (ng/ml)	QC (ng/ml)
TM	1, 2.5, 5, 10, 20, 50, 100	2.5, 10, 50, 80
TM-MPB	1, 5, 10, 100, 250, 500	5, 50, 250, 400
APB	1, 2.5, 5, 10, 20, 50, 100	2.5, 10, 50, 80
TMBA	50, 100, 250, 500, 1000, 5000, 10 000	100, 500, 5000, 8000

2.7. Method validation

Calibration curve samples were prepared in duplicate every day for 5 days. The limit of quantitation (LOQ) for trimebutine maleate and its three metabolites were set at the concentration of the lowest non-zero calibration standard ($S/N \geq 10:1$). 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 short-term stability of trimebutine maleate and its three metabolites in human plasma was investigated by assessing QC samples at low, medium and high concentration after 24 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 3 months. QC samples at low, medium and high concentrations were prepared and injected with a PE series 200 autosampler, and after the samples of this batch were maintained in an autosampler at room temperature for 48 h, they were re-injected, and the stability of the analytes in extracts was checked by comparing the measured results from the two runs.

2.8. Data acquisition and analysis

Data acquisition was performed using Sample Control and MultiView software (PE-Sciex). Peak integration and calibration were processed with PE MacQuan software. Peak area ratios of the analytes to internal standards were used to construct calibration curves, and the linear regression between plasma concentration and peak area ratios was weighted by $1/x^2$. Concentrations of QC and unknown samples were calculated by interpolation from the calibration curves.

3. Results and discussion

3.1. LC–MS–MS optimization

An HPLC–MS–MS method for the detection of trimebutine maleate (TM) and its three metabolites in human plasma was investigated. The analytes were introduced into the mass spectrometer using the Turbo Ionspray interface, and parameters such as IS, OR and RNG were optimized to obtain protonated molecular ions $[M+H]^+$ or $[M\text{-}H]^-$. MRM scan mode was selected to ensure high specificity of this method and the most suitable collision energy was determined when observing the maximum response for fragment ions. The product ion mass spectra of the analytes are depicted in Fig. 2 where $[M+H]^+$ or $[M\text{-}H]^-$ of each analyte was selected as precursor ion, and the most abundant or specific fragment ion was chosen as the product ion in the MRM acquisition.

During the early stage of method development, we tried to apply one ionization mode to detect all the analytes considering the simplification of method development. However, the final results showed that TM, APB and TM-MPB had a strong response in positive ionization mode, but as for the carboxylic

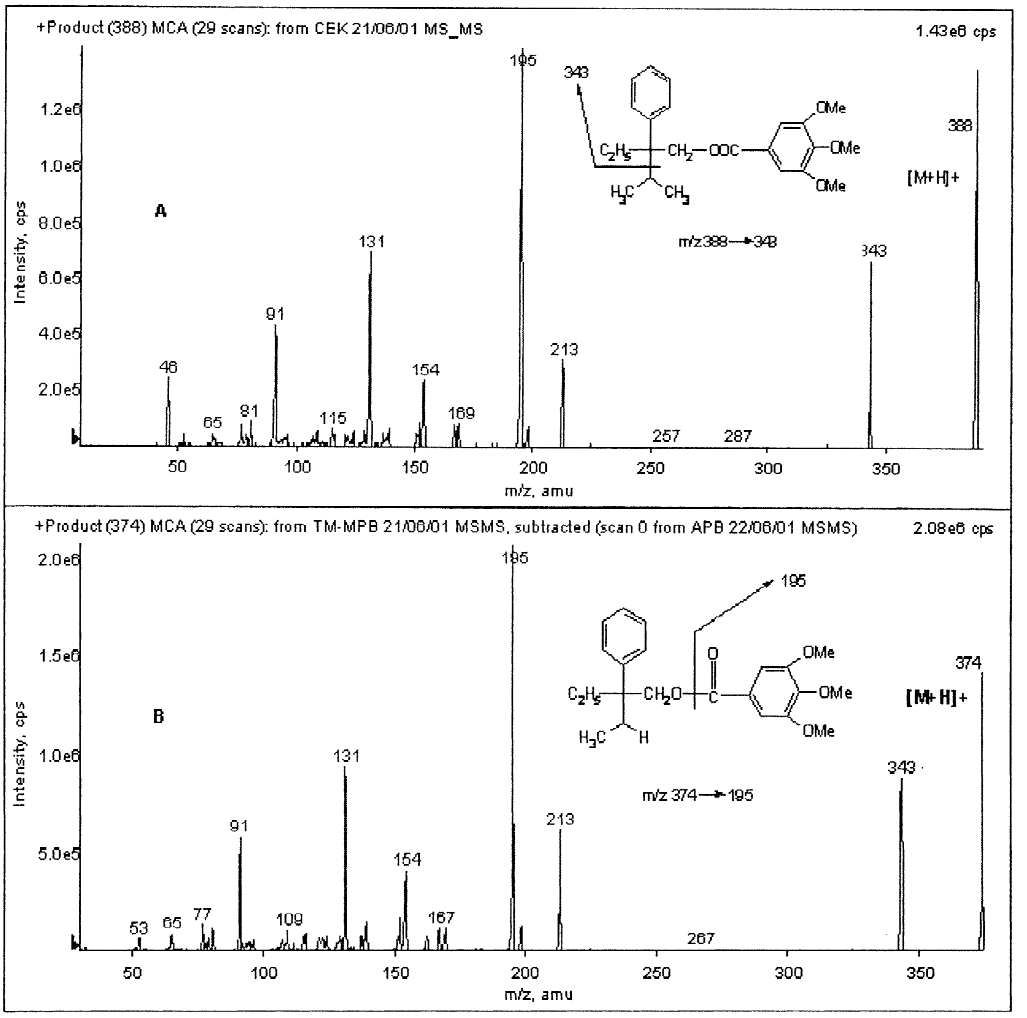


Fig. 2. Product ion spectra of: (A) trimebutine maleate, (B) TM-MPB, (C) APB, (D) TMBA.

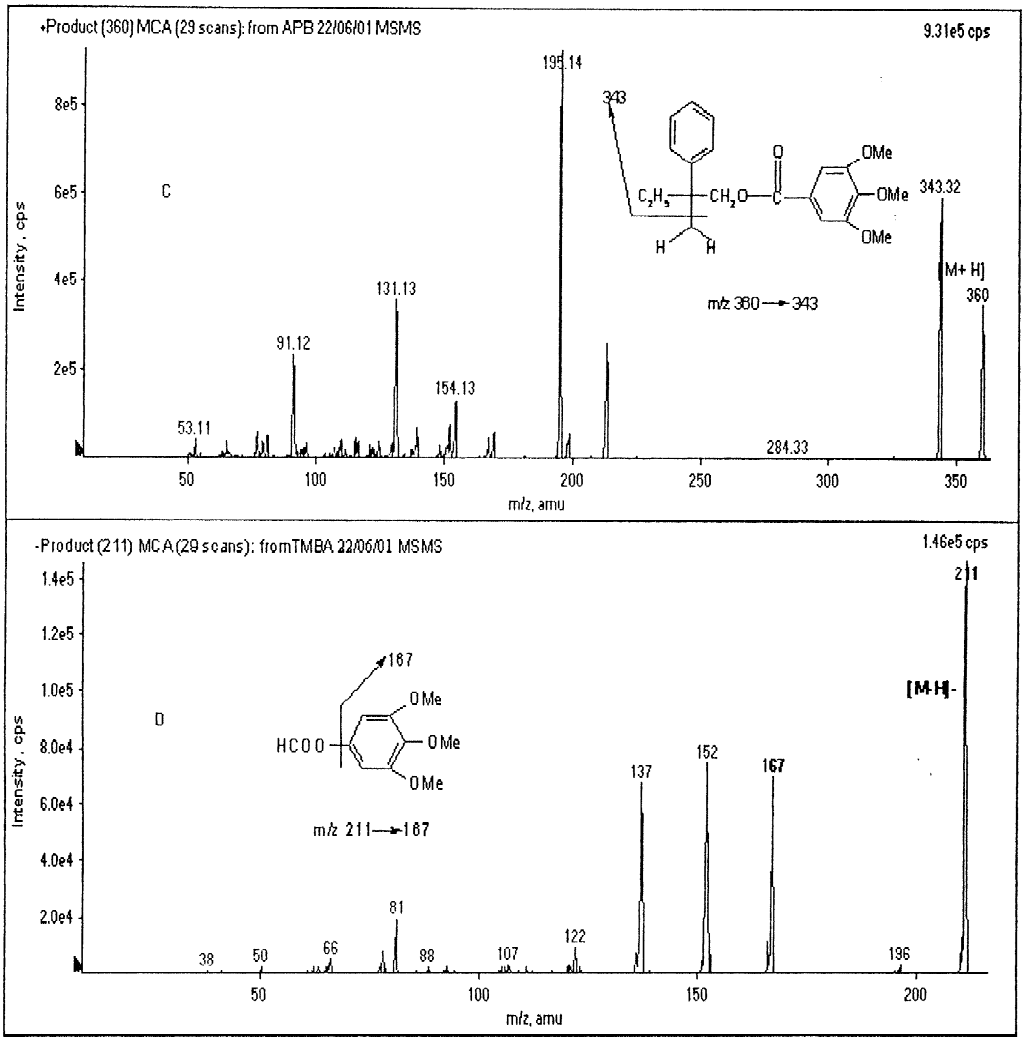


Fig. 2. (*continued*)

compound TMBA, the response appeared much stronger using negative ionization. So a method with only one ionization mode could not provide enough sensitivity for all the analytes simultaneously, which meant the plasma samples needed to be measured twice, i.e. one time for TM, APB and TM-MPB in positive mode and the other time for TMBA in negative mode. Considering the time and expense of the experiment, a LC–MS–MS method using positive–negative switching ionization was developed, adjusting the ion source and mass analyzer of API 3000 between the different states. The method could successively detect positive and negative compounds in one analytical run. Firstly, TMBA and TOBA (IS-2, negative internal standard) were detected in negative mode in the first 2.5 min of each analytical run. Secondly, the instrument was automatically turned to the opposite state after an interval of 0.1 min, and then TM, TM-MPB, APB and TM-PDP (IS-1, positive internal standard) were ionized and detected in positive mode. A timesaving and specific method including two ionization modes was established in this way. Representative MRM chromatograms of an unknown plasma sample from a vol-

unteer, at 3 h after an oral dose of 100 mg, performed on positive–negative switching ionization modes, are depicted in Fig. 3. Although metabolite TMBA and its internal standard TOBA were not completely separated, ion suppression caused by the co-elute was not found during the process of analysis.

The key to combine two ionization modes into one method was to keep the mass spectrometer in a stable state during the process of switching. It was found that a long delay between switching would increase analytical time, but too short an interval (<0.7 s) would not provide enough time for the instrument to adapt to the switching, and the stability of detection would be poor. So the interval between the positive–negative switching should be optimized in the method development. In addition, parameters of the ion source and mass analyzer, for examples, the ionspray voltage, RO_2, ST_3 and RO_3 were also crucial. With the automatic switching of ionization

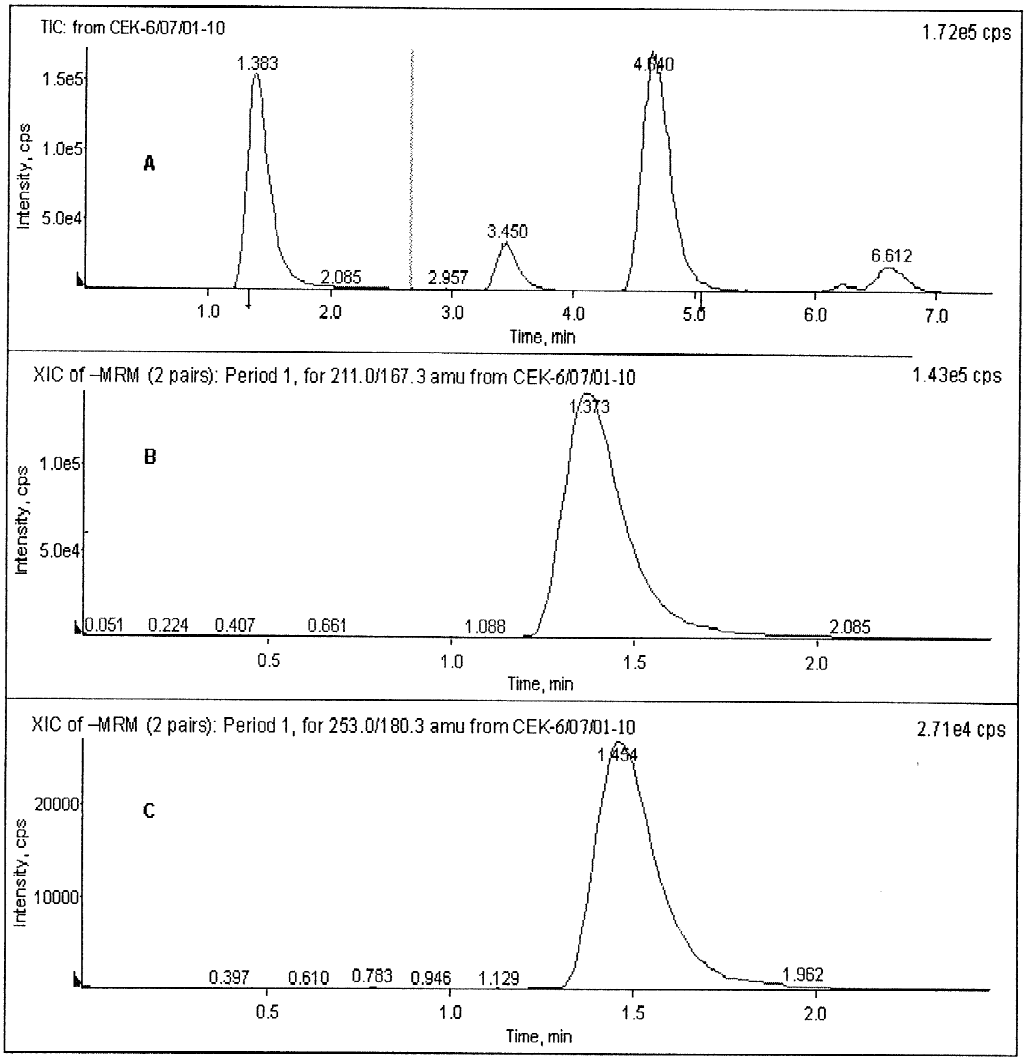


Fig. 3. Representative MRM chromatograms of unknown plasma sample from a volunteer, at 3 h after an oral dose of 100 mg, carried out on positive–negative switching ionization mode: (A) total ion chromatogram, (B) TMBA, (C) TOBA (IS-2, negative internal standard), (D) APB, (E) TM-MPB, (F) trimebutine maleate (TM), (G) TM-PDP (IS-1, positive internal standard).

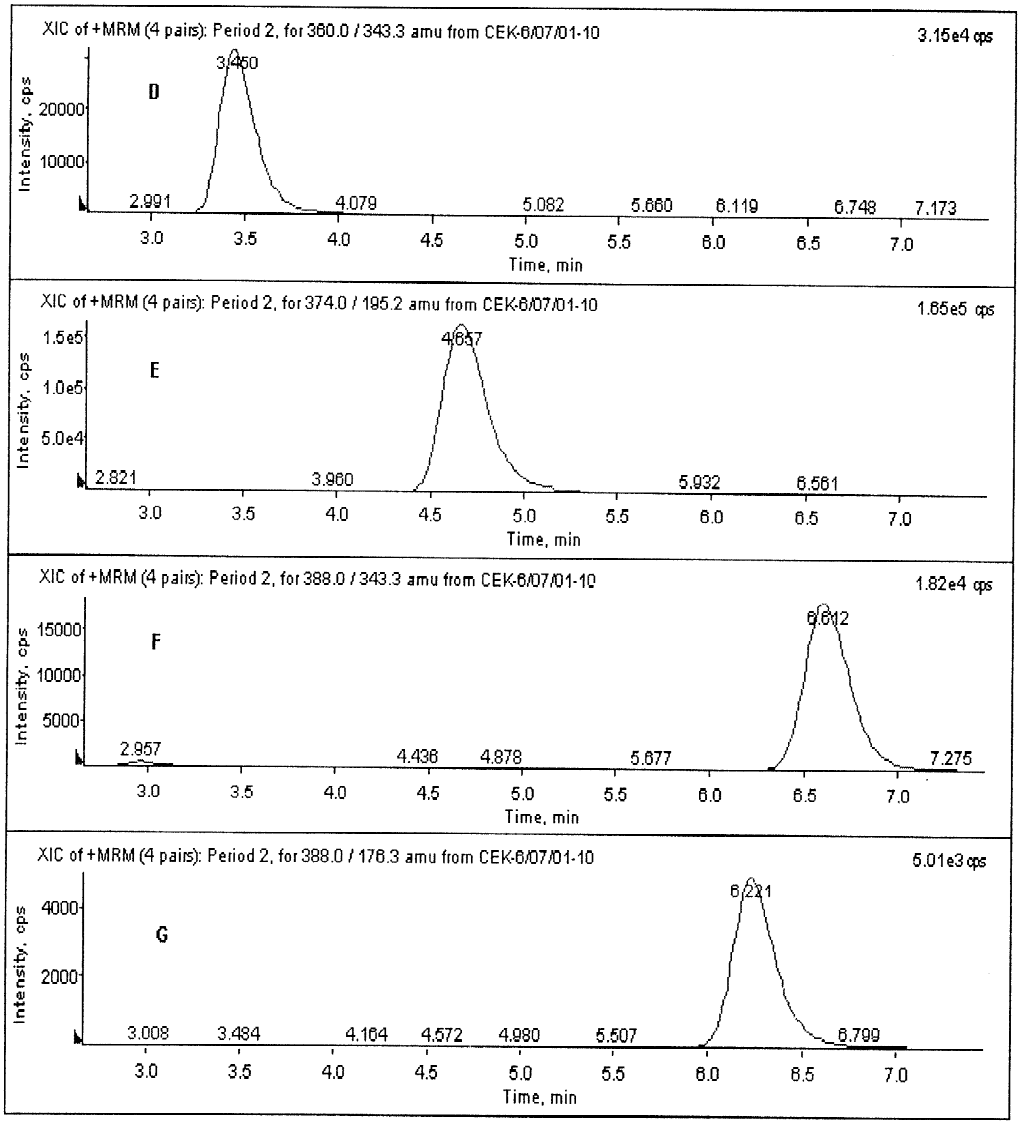


Fig. 3. (*continued*)

mode, these parameters would turn to the opposite polarity in a very short time, so more attention should be paid to them in order that the instrument could complete the switching in a stable state. Other parameters such as temperature of ion source, flow of curtain gas, nebulizer and auxiliary gas were mainly determined by the flow-rate and component of mobile phase, so these parameters should be the same for the two modes during the course of switching.

3.2. Extraction procedure optimization

Trimebutine maleate and metabolites APB, MPB were much more easily extracted from plasma

samples under relatively alkaline conditions compared with the carboxylic compound TMBA. The extraction pH conditions for the latter should be acidic to keep it non-ionized, so a two-part liquid–liquid extraction was needed to improve the extraction recovery of all the analytes. Firstly, potassium phosphate buffer (pH 9) was added to basify the samples, and TM, APB and TM-MPB were extracted into organic layer by cyclohexane in this way, but TMBA was still kept in the remaining aqueous layer. Secondly, TMBA was extracted from the aqueous layer by cyclohexane–choloform (4:1, v/v) after acidification with 1 N HCl. This extraction method improved the recovery of all the analytes.

3.3. Method validation

3.3.1. Specificity

Representative chromatograms of extracted blank plasma, plasma sample spiked with drugs were

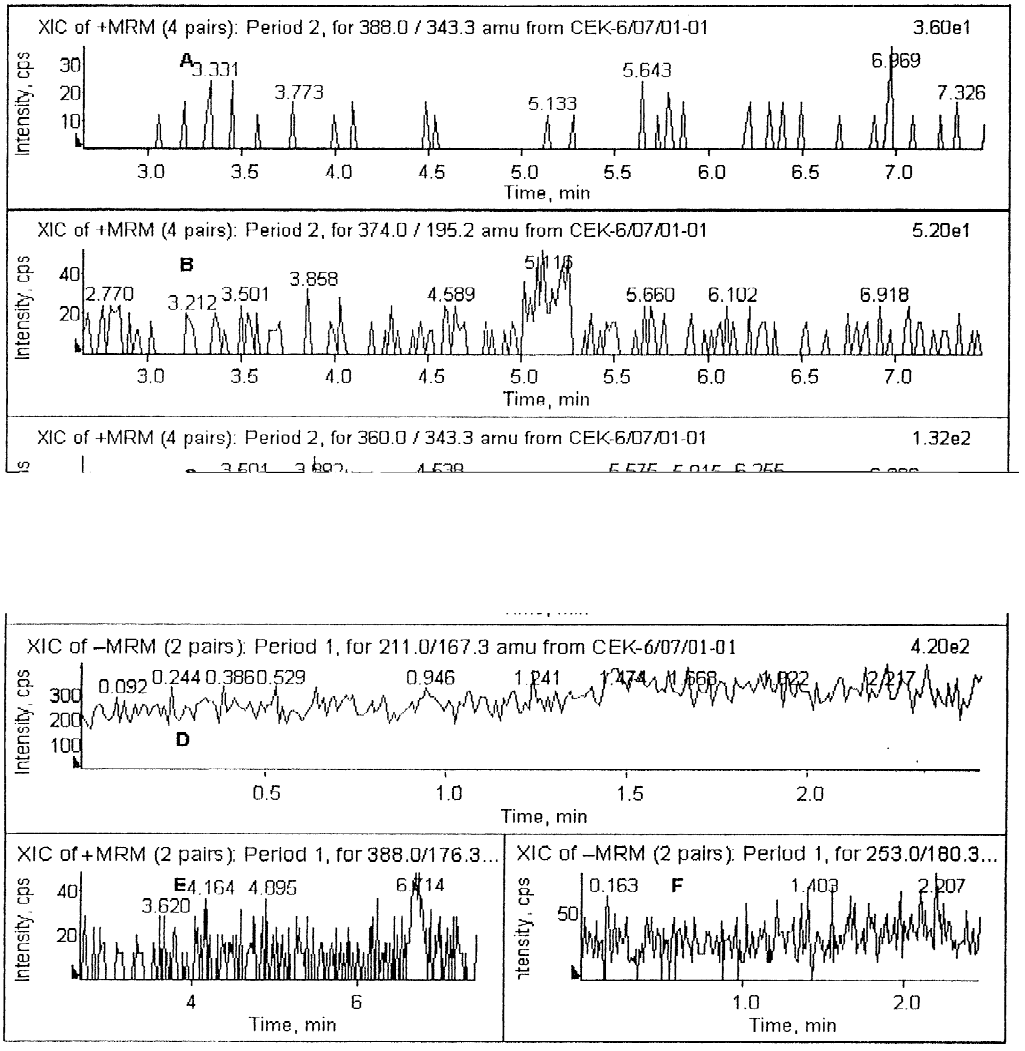


Fig. 4. An extracted blank plasma sample without any drugs and internal standards added: (A) trimebutine maleate (TM), (B) TM-MPB, (C) APB, (D) TMBA, (E) TM-PDP (IS-1), (F) TOBA (IS-2).

obtained under the selected analytical conditions described in Sections 2.2 and 2.3. It was demonstrated that extracted plasma components did not cause any effects of ion suppression on the determination of the analytes and their internal standards (Figs. 4–6).

3.3.2. Calibration curve

The calibration curves were validated over the concentration range of 1–100 ng/ml for trimebutine maleate and APB, 1–500 ng/ml for TM-MPB, and 50–10 000 ng/ml for TMBA in human plasma. Calibration curves were prepared in duplicate every day for 5 days and the deviations of the back-calculated concentrations from their nominal values were within ±15%. Typical equations of calibration curves are as follows ($n=10$):

Trimebutine maleate: $Y = 4.494 \times 10^{-3} + 1.909 \times 10^{-2}X$, $r = 0.9996$

TM-MPB: $Y = 3.160 \times 10^{-3} + 2.254 \times 10^{-2}X$, $r = 0.9973$

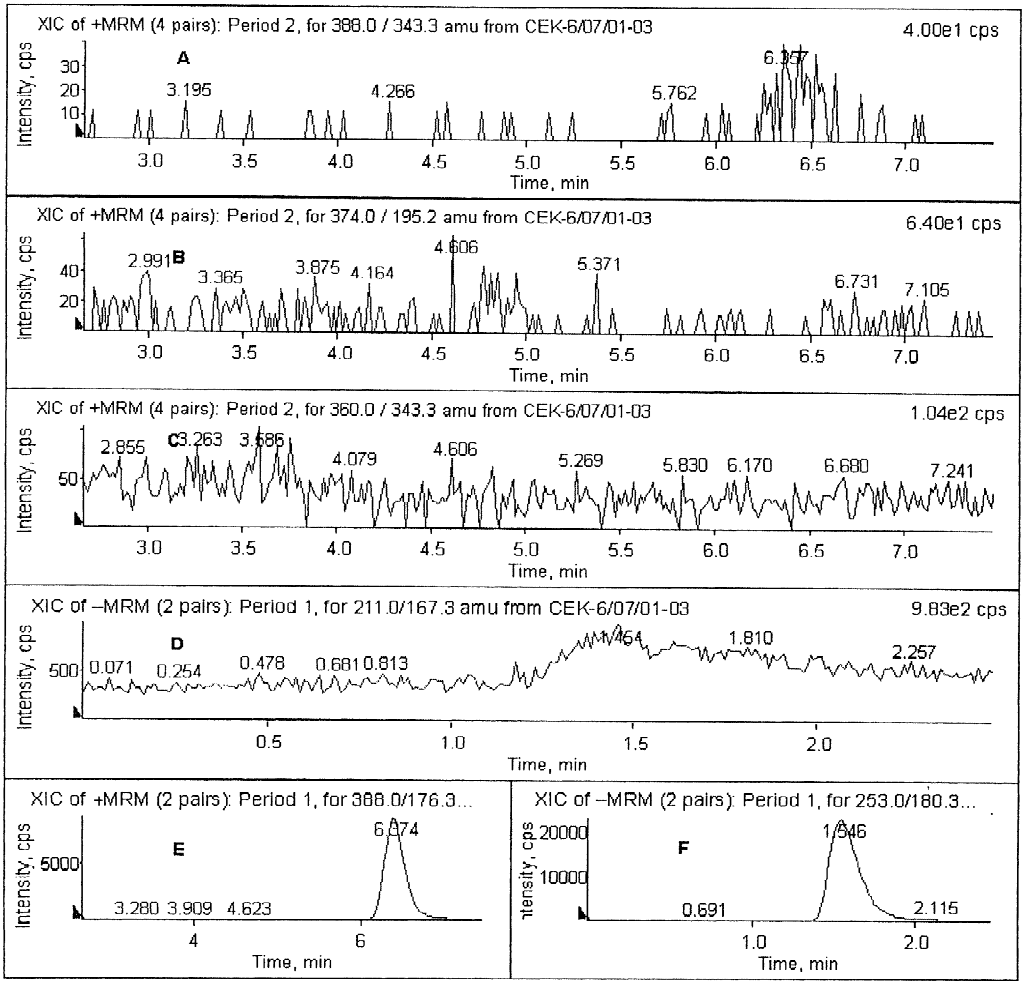


Fig. 5. An extracted blank plasma sample with internal standard 1 (TM-PDP) for positive ion detection and internal standard 2 (TOBA) for negative ion detection added: (A) trimebutine maleate (TM), (B) TM-MPB, (C) APB, (D) TMBA, (E) TM-PDP (IS-1, 20 ng/ml), (F) TOBA (IS-2, 1000 ng/ml).

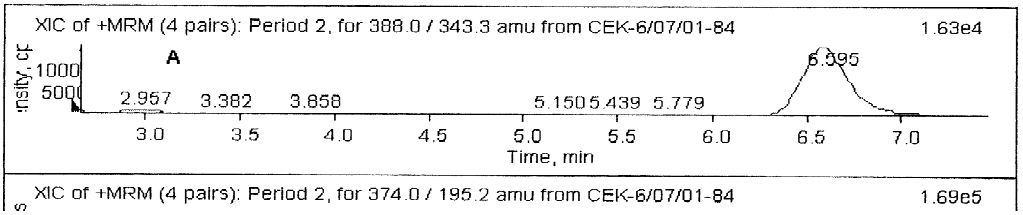


Fig. 6. An extracted plasma sample (medium QC) spiked with drugs and internal standards: (A) trimebutine maleate (50 ng/ml), (B) TM-MPB (250 ng/ml), (C) APB (50 ng/ml), (D) TMBA (5000 ng/ml), (E) TM-PDP (20 ng/ml), (F) TOBA (1000 ng/ml).

APB: $Y = 2.662 \times 10^{-3} + 2.182 \times 10^{-2}X$,
$r = 0.9982$

TMBA: $Y = 3.386 \times 10^{-3} + 3.226 \times 10^{-2}X$,
$r = 0.9983$

Here, Y represents peak area ratio, and X describes plasma concentration. Standard errors of slopes and intercepts for each equation were 4.526×10^{-4} and 8.131×10^{-3} for trimebutine maleate, 5.173×10^{-4} and 3.676×10^{-3} for TM-MPB, 1.280×10^{-4} and 1.792×10^{-3} for APB, and 1.921×10^{-4} and 1.937×10^{-3} for TMBA, respectively.

3.3.3. Precision and accuracy

Six quality control samples at each concentration level (2.5, 10, 50, 80 ng/ml for TM and APB; 5, 50, 250, 400 ng/ml for TM-MPB and 100, 500, 5000, 8000 ng/ml for TMBA) were processed and calculated every day for 5 days to provide precision

Table 5
Summary of the stability for trimebutine maleate (TM) and its three metabolites at different storage and process conditions, $n=6$

Compound	Nominal conc. (ng/ml)	Freeze–thaw stability (R.E.%)	Storage stability (R.E.%)		Stability of extracts (R.E.%)
			24 h at room temperature	3 months at −30 °C	
TM	2.5	0.6	−4.0	−14.0	−9.3
	10	8.8	14.3	−10.2	3.2
	80	−1.9	12.3	−9.3	−10.2
TM-MPB	5	10.4	−14.5	5.1	−6.2
	50	14.8	−7.0	−3.9	−12.4
	400	−14.0	−7.1	−4.6	−8.5
APB	2.5	7.4	−17.6	4.0	2.5
	10	−11.0	−7.1	4.5	1.6
	80	3.8	−14.7	8.9	−4.6
TMBA	100	−6.5	−12.8	−9.6	−7.3
	500	4.0	−6.1	−10.6	−12.1
	8000	5.2	−25.5	−13.5	−5.9

R.E.%, % relative error from nominal.

ing conditions. The analytes were found to be stable in human plasma after three cycles of freeze–thaw and for at least 24 h at room temperature (25 °C). The results of long-term stability showed all the analytes were still stable in human plasma for at least 3 months at −30 °C, and the analytes in extracts were found to be stable for 48 h in the autosampler at room temperature (Table 5).

3.4. Application of the method in pharmacokinetic studies

After a single oral administration of 100 mg trimebutine maleate to 30 healthy subjects, plasma concentrations of trimebutine maleate and its metabolites APB, TM-MPB and TMBA were simultaneously determined by the described LC–MS–MS

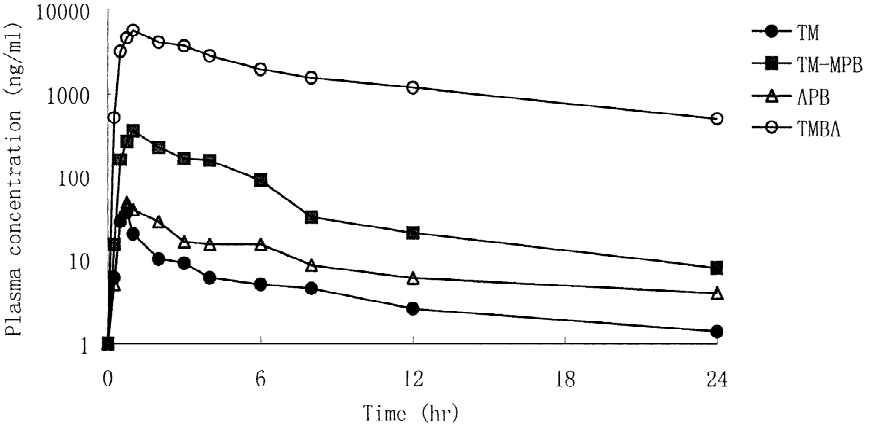


Fig. 8. Mean plasma concentration–time curves of trimebutine maleate and its three metabolites after oral administration of 100 mg trimebutine maleate to healthy subjects ($n=30$).

method. Means of (plasma concentration)–time curves are shown in Fig. 8 after data were log transformed.

4. Conclusion

A sensitive and specific HPLC–MS–MS method has been developed and validated for the determination of trimebutine maleate and its three metabolites in human plasma. Considering the different chemical structures and properties of the analytes, a technique with positive–negative switching ionization was for the first time used to complete the detection of all the compounds in one analytical run. 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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