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A sensitive and specific liquid chromatography/tandem mass spectrometry method for determination of pinaverium bromide in human plasma: application to a pharmacokinetic study in healthy volunteers

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ABSTRACT: A sensitive and specific method using liquid chromatography–electrospray tandem mass spectrometry (LC-MS/MS) for the determination of pinaverium bromide in human plasma was developed and validated. Pinaverium bromide and an internal standard (paclitaxel) were isolated from plasma samples by precipitating plasma, and determined by LC-MS/MS in multiple-reaction monitoring mode. The main metabolite of pinaverium bromide and endogenous substances in plasma did not show any interference. The calibration curve was linear over the plasma concentration range of 10.0–10000.0 pg/mL with a correlation coefficient of 0.9979. The relative standard derivations intra- and inter-day at 30.0, 300.0 and 8000.0 pg/mL in plasma were less than 15%. The absolute recoveries of pinaverium bromide and the internal standard were 99.7–111.7 and 106.2%, respectively. The lower limit of quantitation was 10 pg/mL. The analytical method was successfully applied to study the pharmacokinetics of pinaverium bromide tablets in healthy Chinese volunteers.

Keywords: LC-MS/MS; pinaverium bromide; human plasma; pharmacokinetics

Introduction

Pinaverium bromide, a quaternary ammonium compound, is an L-type calcium channel blocker with selectivity for the gastrointestinal tract, which can effectively relieve pain, diarrhea and intestinal discomfort, and provide safe and effective treatment of irritable bowel syndrome (Awad *et al.*, 1995; Scarpignato and Pelosini, 1999; Lu *et al.*, 2000; Bouchoucha *et al.*, 2000). Pinaverium has a low absorption (8–10%), and maximum blood levels are reached 1 h after oral administration. Some 97% of the drug is bounded to protein in the plasma. With 1.5 h mean half-life, it undergoes a first-pass metabolism that reduces the bioavailability at therapeutic doses. Pinaverium bromide is almost eliminated after transformation in the liver (Evangelista, 2004). Thus, one of the major problems in the measurement of pinaverium bromide is the small amount present. Specific and sensitive analytical methodologies need to be developed in order to measure these small quantities without drawing large amounts of blood from patients.

In the literature, the gas chromatographic mass spectrometry (GC/MS) method was reported for the quantitation of pinaverium bromide in human serum (de Weerdt *et al.*, 1983). The method was complicated and time-consuming. However, there have been no reports on the quantitation of pinaverium bromide in human plasma by LC-MS/MS. This paper describes a validated method combining precipitation of plasma protein, reversed-phase LC and MS/MS detection to perform the selective determination of pinaverium bromide. Tandem mass spectrometry was selected in order to improve the selectivity and sensitivity of the method of determination, in which the lower limit of quantitation (LLOQ) was 10 pg/mL. The method was successfully applied for a pharmacokinetic study in 50 healthy Chinese volunteers.

Experimental

Materials and reagents

Pinaverium bromide was provided by Beijing Wansheng Pharmaceutical Ltd (Beijing, China). Paclitaxel was purchased from the National Institute for Control of Pharmaceutical and Biological Products (Beijing, China). The pinaverium bromide tablets, containing 0.05 g pinaverium bromide per tablet, were obtained from Solvay Pharma (France) and Beijing

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Abbreviations used: MRM, multiple-reaction monitoring.

Wansheng Pharmaceutical Ltd (Beijing, China). Acetonitrile was of HPLC grade (Fisher, USA). Ammomium acetate was of HPLC grade (Tedia, USA). Analytical-grade formic acid was purchased from The Research Institution of Guangfu Fine Chemicals (Tianjin, China). Distilled water was used throught the study.

LC-MS/MS and conditions

HPLC analysis was performed on a LC-20A system (Shimadzu, Japan) using an Allure C_{18} reversed-phase column (50 × 4.6 mm, 5 μm, Restek, USA). The mobile phase consisted of an acetonitrile–2 mmol/L ammonium acetate buffer solution containing 0.5% formic acid (70:30, v/v) with a flow rate of 0.5 mL/min. The sample volume injected was 30 μL. The column temperture was 20°C. The autosampler cooler was maintained at room temperature (20°C). The API 4000 triple quadrupole mass spectrometer (Applied Biosystems, USA) was used for the analysis in the multiple reaction monitoring (MRM) mode. Sample introduction was through ESI in the positive-ion mode. The ionspray voltage was 5 kV, and the source temperature was 600°C. Ions were collisionally activated at a collision energy of 35 V. Ion source gas 1 (GS 1), ion source gas 2 (GS 2), curtain gas (CUR) and collision gas (CAD) were set with a gas (N_2) flow rate of 40, 50, 30 and 8 L/min, respectively. The tandem mass spectrometer was tuned to monitor *m/z* 512.5 → *m/z* 131.3 for pinaverium bromide and *m/z* 854.3 → *m/z* 286.3 for paclitaxel (IS), using positive electrospray ionization with a dwell time of 100 ms. MRM data were acquired and analyzed by Analyst software (version 1.4.1, Applied Biosystems, USA).

Preparation of stock solutions

Stock solutions (1.00 mg/mL) of pinaverium bromide was prepared by dissolving in acetonitrile. The IS (paclitaxel) was also prepared as a stock solution (1.00 mg/mL) in acetonitrile and was further diluted with acetonitrile to give a concentration of 500 ng/mL, then used for all analyses.

Preparation of calibration standards and quality control samples

The calibration curve samples at concentrations of 10.0, 30.0, 100.0, 300.0, 1000.0, 3000.0 and 10000.0 pg/mL of pinaverium bromide were freshly prepared by serially diluting a stock solution with drug-free plasma. The quality control samples were prepared in the same way at concentrations of 10.0 (LLOQ), 30.0 (low), 300.0 (medium) and 8000.0 pg/mL (high) of pinaverium bromide. All plasma samples were stored at −70°C. Two hundred microliters of IS (500 ng/mL) was added to 0.1 mL of calibration curve samples and quality control samples. The further processing of both the calibration curve samples and the quality control samples was the same as described in the following section for collection and preparation of the samples. All standard stock solutions were prepared once a month and stored at −70°C.

Sample preparation

All frozen human plasma samples were thawed at room temperature. A 0.1 mL aliquot of human plasma was mixed with 0.2 mL of the IS working solution (500 ng/mL). Then, 0.1 mL acetonitrile was added to precipitate plasma protein. The mixture was vortexed for 0.5 min and centrifuged for 5 min at 14,836 ***g*** with a centrifugal separator (model TG16-WS, Xiangyi Centrifugal Separator Ltd, Hunan, China). The supernatant was the transferred to a glass autosampler vial and a 30 μL aliquot of the solution was injected into the LC-MS/MS system for analysis.

Pharmacokinetic study

Subjects. The developed LC-MS-MS method was applied to determine the plasma concentrations of pinaverium bromide from a clinical trial study in which 50 healthy male Chinese subjects were enrolled. The subjects were 21.2 ± 1.4 years old, body weight 64.4 ± 6.2 kg and height 173.1 ± 5.7 cm. All subjects were assessed as healthy based on medical history, clinical examination, blood pressure, electrocardiogram and laboratory investigation. No individuals had either a history or evidence of hepatic, renal, gastrointestinal or hematological abnormalities, or any acute/chronic disease or drug allergy. Subjects were advised not to take any medication at least 2 weeks before and during the study. The pharmacokinetic study was approved by the Medical Ethics Committee of the Second Hospital of Hebei Medical University. Informed consent was obtained from all subjects after explaining the aims and risks of the study.

Clinical protocol. Subjects were requested to fast for at least 10 h overnight the day before each treament. Subjects took one test (T) or reference (R) tablet according to cross-administration, containing 50 mg pinaverium bromide, with 250 mL water, and continued fasting for 4 h. The randomization plan was given to each subject in a fasting state for each treament peroid. The wash-out peroid between treament peroids was 1 week, which is longer than 7 times the elimination half-life of the drug. Safety assessments included vital signs (body temperature, respiration, blood pressure and pulse), a physical examination and adverse events.

Blood samples obtained from an antecubital vein prior to treatment and at 0.12, 0.23, 0.35, 0.50, 0.75,1.0, 1.5, 2.0, 3.0, 4.0, 5.0, 6.0, 8.0 and 10.0 h after treatment were placed in heparinized tubes. The blood samples were immediately centrifuged at 3000 ***g*** (4°C) for 10 min, then the plasma samples were transferred to suitable labeled tubes and frozen at −70°C until analysis.

Pharmacokinetic data analysis

Calculation of the pharmacokinetic parameters was performed by noncompartmental assessment of data using the computer program Drug and Statistics (DAS version 2.0; Huang and Li, 2005). The maximum plasma concentrations (C_{max}) and their time of occurrence (T_{max}) were both obtained directly from the measured data. The area under the plasma concentration vs time curve (AUC_{0-t}) was estimated using the linear trapezoidal rule up to the last measurable time. $AUC_{0-\infty}$ was obtained by adding the part of the area extrapolated to infinity (last measurable concentration/k_e) to AUC_{0-t}, where k_e was estimated by log–linear regression of concentrations observed during the terminal phase of elimination. The elimination half-life ($t_{1/2}$) was estimated from the plasma data by using the equation $t_{1/2} = 0.693/k_e$. Relative bioavailability of pinaverium bromide T vs R was determined using the formula $F\% = [(AUC_{0-t})_T/(AUC_{0-t})_R] \times 100$. The values were expressed as mean ± SD.

Results and discussion

The reported GC-MS method for pinaverium bromide employed chloroform extraction, reduction of the residue with Raney–Nickel and toluene re-extraction. The method was costly, inconvenient and time-consuming. We developed an LC-MS/MS method with a common RP-C_{18} column. Only 3 min was needed for analysis of one sample, and precipitation of plasma protein was used for sample preparation. The method is simple and convenient.

The use of LC-MS/MS provides extremely high sensitivity and selectivity when compared with earlier methods such as gas chromatography mass spectrometry. The current assay required only 0.1 mL plasma samples and had a LLOQ of 10 pg/mL, far less than the limit of detection reported (1 ng/mL). In addition, we found that the LC-MS/MS method as described in this paper is robust, such that different laboratory personnel can repeat the assay with very similar results, as judged by the small inter-assay variability (<10% over 3 months).

The use of appropriate concentrations of ammonium acetate in the mobile phase may improve the chromatographic peak shapes. Several different concentrations of ammonium acetate buffer solution were tested in the mobile phsae. The results showed that 2 mmol/L ammonium acetate buffer was suitable. Different concentrations of formic acid at levels 0.1, 0.2 and

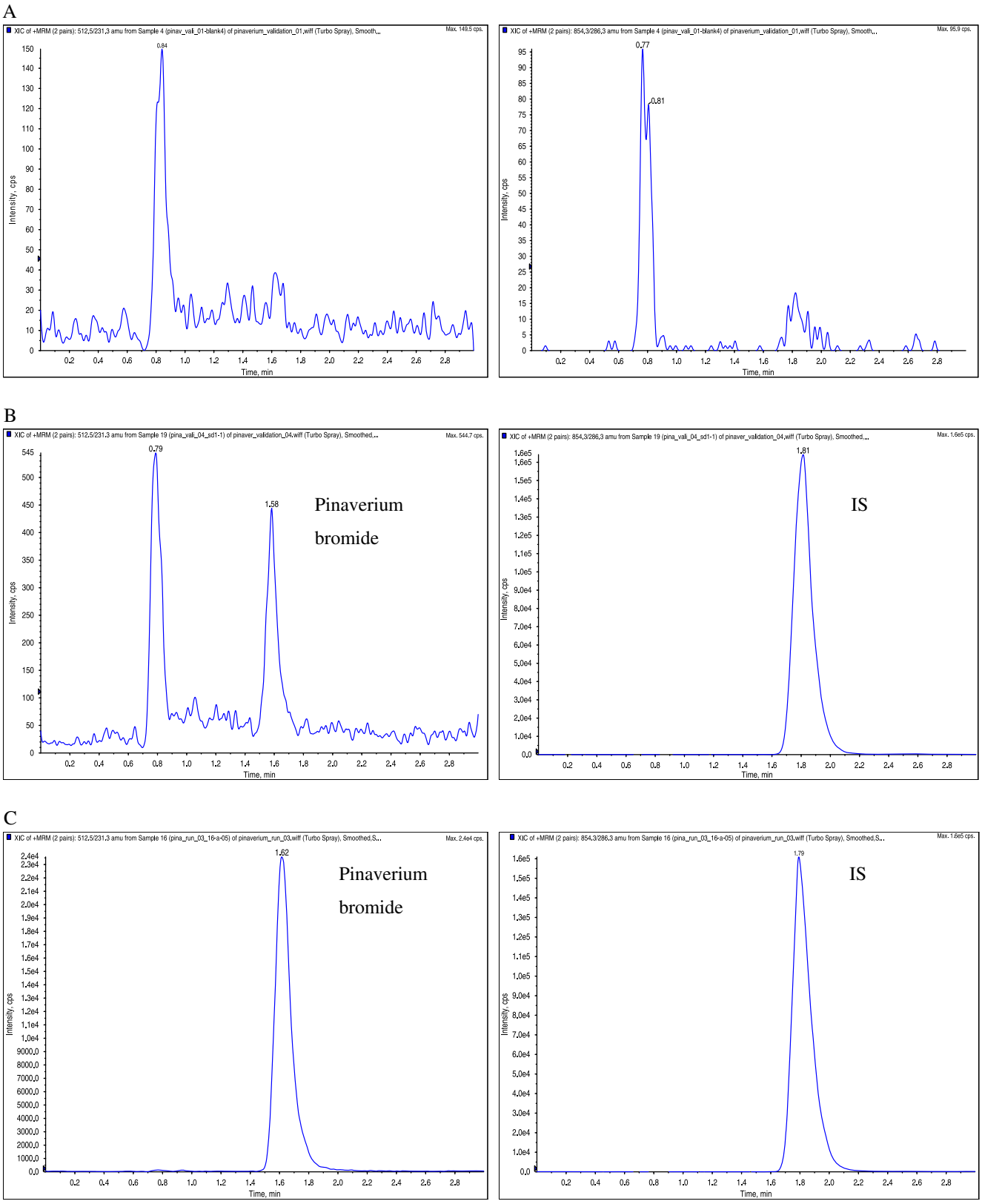

Figure 1. Representative chromatograms. (A) Blank plasma (endogenous background) without IS. (B) LLOQ for pinaverium bromide in plasma(10 pg/mL) and the IS. (C) plasma sample equivalent to 1440.0 pg/mL for pinaverium bromide from a volunteer 0.5 h after an oral dose of 50 mg pinaverium bromide tablets.

0.5% were tested in the mobile phase. The test result showed that lower pH buffer enhanced the ionization of pinaverium-bromide and IS, improving the chromatographic peak shapes.

Using the LC-MS/MS assay described in this paper, we were able to measure plasma concentrations of pinaverium bromide for pharmacokinetic study in volunteers following a single oral dose (50 mg/person). To our knowledge, this is the first report of a plasma concentration time profile of pinaverium bromide in Chinese people following a single oral administration. The method can be adapted for clinical pharmacokinetic studies with a small plasma sample size.

Table 1. Precision and accuracy of the intra- and inter-day assay ($n = 4$ runs, six replicates per run)

Concentrarion added (pg/mL)	Concentration measured (pg/mL) (mean ± SD)	Precision (%, RSD)		Accuracy (%, RE)
		Intra-day	Inter-day	
30.0	30.8 ± 1.48	4.4	9.1	2.8
300.0	324.0 ± 8.9	2.2	5.4	8.0
8000.0	8160.0 ± 430.5	2.4	12.7	2.0

Method validation

Specificity and matrix effect. Selectivity was assessed by comparing chromatograms of six different lots of blank human plasma with the corresponding spiked plasma. Individual blank plasma samples, LLOQ and quality control samples ($n = 6$) were prepared according to the sample preparation procedure described above and screened for interference. As can be seen in Fig. 1, no significant interference from endogenous substances with pinaverium bromide or IS was detected. Typical retention times for pinaverium bromide and IS were 1.7 and 1.8 min, respectively. The bioanalytical method was proved to be selective.

The matrix effects were evaluated by spiking blank plasma sample extracts with neat standards at 30.0, 300.0 and 8000.0 pg/mL of pinaverium bromide. Assaying as many as five different lots of plasma, the calculated matrix effects were within the range 96.8–101.5%. The matrix effect of the IS was also evaluated and the result was 99.8%. Thus the ion suppression and enhancement from plasma matrix was negligible for this method.

Linearity and range. The calibration curves in plasma provided a reliable response from 10.0 to 10000.0 pg/mL ($n = 7$) for pinaverium bromide. The weighted least-square linear regression was used for the equation, and the best weighting factor $1/C^2$ was seclected. The calibration curve of spiked plasma samples showed excellent linearity, with correlation coefficients (r) exceeding 0.997. The typical equation of the calibration curve obtained from four batches in method validation was $f = 0.0000595C + 0.000371$ ($r = 0.9979$), where the f represents the peak area ratio of pinaverium bromide to the IS, and the C represents the plasma concentration of pinaverium bromide. The LLOQ of the method was 10 pg/mL.

Precision and accuracy. The accuracy and precision of this method were calculated for three concentrations of pinaverium bromide in human plasma. Six replicate samples having pinaverium bromide theoretical concentrations of 30.0, 300.0 and 8000.0 pg/mL were injected into the system, and then the intra-day parameters obtained. The inter-day precision and accuracy were also evaluated using six aliquots for each quality control sample concentration, prepared and analysed on four different days. The intra- and inter-day precision and accuracy of this method are listed in Table 1. The intra- and inter-day precisions of analysis were less than 4.4 and 12.7%, respectively. The accuracy of the method ranged from 102.0 to 108.0%.

Recovery. The recovery was calculated in plasma samples ($n = 5$) spiked with pinaverium bromide standard at concentrations of 30.0, 300.0 and 8000.0 pg/mL, and determined by comparing the response (area) obtained from processed quality control samples and the response obtained from samples with equal concentration to that of quality control samples spiked in the supernatant of processed blank plasma samples. The recovery values of pinaverium bromide were 111.7 ± 3.9, 104.9 ± 2.1 and 99.7 ± 3.5%, respectively.

Stability. The stability of pinaverium bromide was studied in human plasma at room temperature(20°C) for 4 h. The bulk-spiked plasma samples stored at −70°C underwent three freeze–thaw cycles. Stability studies of the analyte after sample processing were performed at room temperature (20°C) for 24 h. In addition, a long-term (7 and 30 days) stability study was carried in human plasma stored at −70°C. In stability studies, six replicates of low and high quality control levels were analyzed. The results are presented in Table 2. The data demonstrated that pinaverium bromide was stable under the conditions evaluated, reflecting actual sample handling and analysis.

Pharmacokinetic analysis

The pharmacokinetic study was performed in 50 healthy Chinese volunteers after administration of pinaverium bromide at a single dose of 50 mg. Pharmacokinetic parameters were estimated using non-compartmental analysis of curves of pinaverium bromide plasma concentrations vs time. Figure 2 and Table 3 show the mean plasma concentration of pinaverium bromide–time curves and mean pharmacokinetic parameters of pinaverium bromide after oral test and reference tablets ($n = 50$), respectively. The last time point for which all subjects had measurable concentrations of pinaverium bromide was 10 h.

Table 2. Results of the stability tests of pinaverium bromide in human plasma

	Concentration added (pg/mL)	Concentration measured (pg/mL)	RE (%)
Short-term stability	30.0	30.9 ± 0.80	3.0
	8000.0	8385.0 ± 374.3	4.8
Stability after sample processing (24 h)	30.0	30.9 ± 1.48	2.9
	8000.0	7910.0 ± 183.1	−1.1
Freeze–thaw stability	30.0	31.0 ± 1.05	3.4
	8000.0	8375.0 ± 380.7	4.7
Long-term stability (30 days)	30.0	30.2 ± 1.51	0.6
	8000.0	8055.0 ± 161.7	0.7

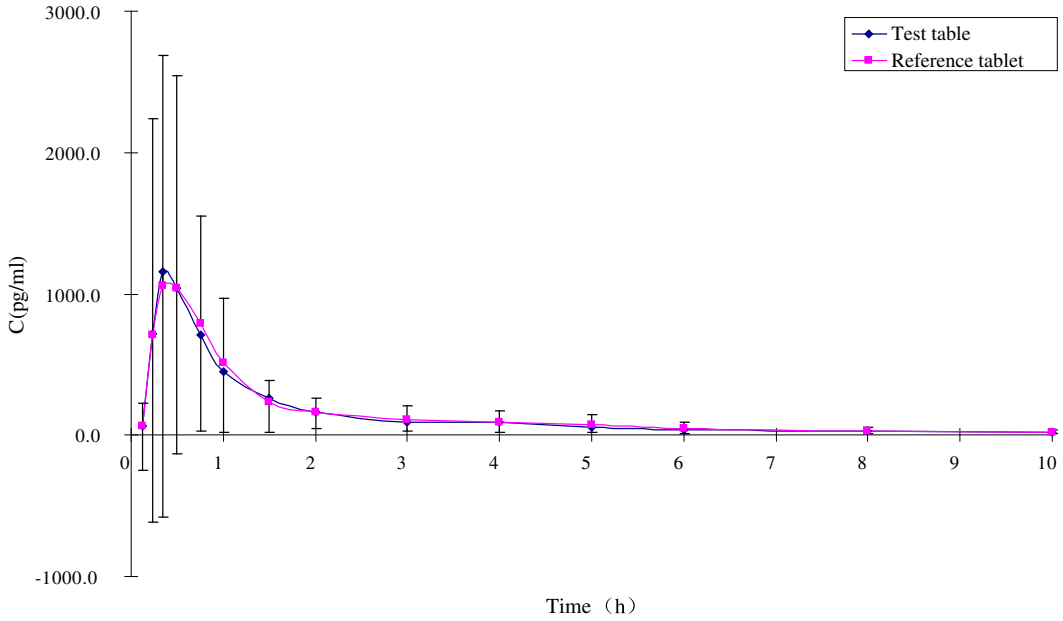

Figure 2. Mean plasma concentration–time profile of pinaverium bromide after oral test table and reference table ($n = 50$).

Table 3. Mean pharmacokinetic parameters of pinaverium bromide after oral administration of 50 mg pinaverium bromide tablets in 50 volunteers (mean ± SD, $n = 50$)

Parameter	Test tablet	Reference tablet
C_{max} (pg/L)	1943.4 ± 2125.5	1714.7 ± 1974.0
T_{max} (h)	0.52 ± 0.29	0.62 ± 0.40
$t_{1/2}$ (h)	2.69 ± 1.20	2.50 ± 0.81
MRT (h)	1.85 ± 0.55	2.05 ± 0.62
AUC_{0-t} (pg h/mL)	1731.8 ± 1230.1	1664.7 ± 1046.9
$AUC_{0-\infty}$ (pg h/mL)	1808.8 ± 1264.4	1730.7 ± 1075.5
F (%)	105.9 ± 47.1	

All pinaverium bromide tablets were absorbted similarly, and there was no significant difference in T_{max} and C_{max} between test and reference tablet. After subjects were given test and reference tablets, pinaverium bromide reached peak plasma concentrations of 125.0–9480.0 pg/mL within 1.0 h. Some individual differences in pharmacokinetic parameters apparently existed among healthy subjects. The test and reference tablets showed basic conformity in absorbtion *in vivo*. Compared with the imported reference tablet, the relative bioavailability of the domestic test tablet was 105.9 ± 47.1%.

Analysis of variance for C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ were performed after logarithmic conversion. There were no significant differences between the test tablet and reference tablet ($p > 0.05$). Only individual differences in C_{max}, AUC_{0-t} and $AUC_{0-\infty}$ existed ($p < 0.05$). Two one-sided t-tests and analysis of the 90% confidence interval were carried out to evaluate the bioequivalence between preparation, which showed that the test tablet was bioequivalent to the reference tablet.

Conclusion

A sentive and reproductible LC-MS/MS method for determination of pinaverium bromide in human plasma was developed and validated for the first time. This method did not require extraction or reduction and provided a rapid analysis. Validation results demonstrated that the method was specific, reproducible and reliable. The method is suitable for pharmacokinetic study of pinaverium bromide in human subjects. The relative bioavailability test for domestic pinaverium bromide tablet was evaluated by comparison with an imported tablet.

References

Awad R, Dibildox M and Ortiz F. Irritable bowel syndrome treatment using pinaverium bromide as a calcium channel blocker. A randomized double-blind placebo-controlled trial. *Acta Gastroenterologica Latinoamerica* 1995; **25**: 137–144.

Bouchoucha M, Faye A, Devroede G and Arsac M. Effect of oral pinaverium bromide on colonic response to food in irritable bowel syndrome patients. *Biomedical Pharmacotherapy* 2000; **54**: 381–387.

de Weerdt GA, Beke RP, Verdievel HG, Barbier F, Jonckheere JA and de Leenheer AP. Quantitative gas chromatographic mass spectrometric determination of pinaverium-bromide in human serum. *Biomedical Mass Spectrometry* 1983; **10**: 162–167.

Evangelista S. Quaternary ammonium derivatives as spasmolytics for irritable bowel syndrome. *Current Pharmaceutical Design* 2004; **10**: 3561–3568.

Huang XH and Li J. Drug and Statistics Software (DAS), version 2.0. Mathematical Pharmacology Professional Committee of China. *American Journal of Pharmaceutical Education* 2005; **69**: 397–398.

Lu CL, Chen CY, Chang FY, Chang SS, Kang LJ, Lu RH and Lee SD. Effect of a calcium channcl blocker and antispasmldic in diarrhoea-predominant irritable bowel syndrome. *Journal of Gastrsenterology and Hepatology* 2000; **15**: 925–930.

Scarpignato C and Pelosini I. Management of irritable bowel syndrome: noverl approaches to the pharmacology of gut motility. *Canadian Journal of Gastroenterology* 1999; **13**: 50A–65A.

