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Journal of Pharmaceutical and Biomedical Analysis 39 (2005) 670–676
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Short communication

Quantification of propiverine by liquid chromatography/electrospray tandem mass spectrometry: Application to a bioequivalence study of two formulations in healthy subjects

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Received 10 January 2005; received in revised form 25 February 2005; accepted 1 March 2005
Available online 3 June 2005

Abstract

Here we report on the development and validation of a sensitive and rapid reversed-phase liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the quantitative determination of propiverine in human plasma. After adding an internal standard (oxybutynin chlroride) to human plasma, samples were extracted using *n*-hexane/ethylacetate (8:2, v/v). Compounds extracted were analyzed by reversed-phase high-performance liquid chromatography (HPLC) with multiple reaction monitoring (MRM) mode for analyte detection. This method for determination of propiverine proved accurate and reproducible, with a limit of quantitation of 0.5 ng/ml in human plasma. The standard calibration curve for propiverine was linear ($r^2 = 0.9988$) over the concentration range 0.5–1000.0 ng/ml in human plasma. The intra- and inter-day precision over this concentration range was lower than 8.66% (relative standard deviation, %R.S.D.), and accuracy was between 99.46 and 109.41%, respectively. This method was successfully applied to a bioequivalence study of two propiverine hydrochloride tablet formulations (20 mg) in 24 healthy subjects after a single administration.

Keywords: Propiverine; LC–MS/MS; Bioequivalence; Multiple reaction monitoring

1. Introduction

Propiverine hydrochloride (1-methyl-4-piperidinyl-diph enylpropoxyacetate hydrochloride) (Fig. 1) is an anticholinergic drug that is widely used for the symptomatic treatment of overactive bladder [1–3]. Different analytical methods, including gas chromatography–mass spectrometry (GC–MS) [4], high-performance liquid chromatography (HPLC) [5,6], and high-performance liquid chromatography–tandem mass spectrometry (LC–MS/MS) [7] have been developed for the simultaneous determination of propiverine hydrochloride and its metabolites. However, GC–MS-based methods require complicated derivatization, and HPLC-based methods are convenient but require a large volume of plasma due inherent sensitivity. Although a LC–MS/MS method has been reported [7], a more sensitive and cost-effective method is required for pharmacokinetic studies.

The aim of this study was to develop a more sensitive LC–MS/MS system with a 0.5 ng/ml lower limit of quantification (LLOQ) and a 2 min runtime. The developed method was used for a bioequivalence study of two tablet formulations of propiverine hydrochloride (20 mg) in healthy volunteers.

2. Experimental

2.1. Materials

Propiverine hydrochloride standard reference was obtained from Myungmoon Pharm. Co., Ltd. (Kyunggi-Do,

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doi:10.1016/j.jpba.2005.03.038

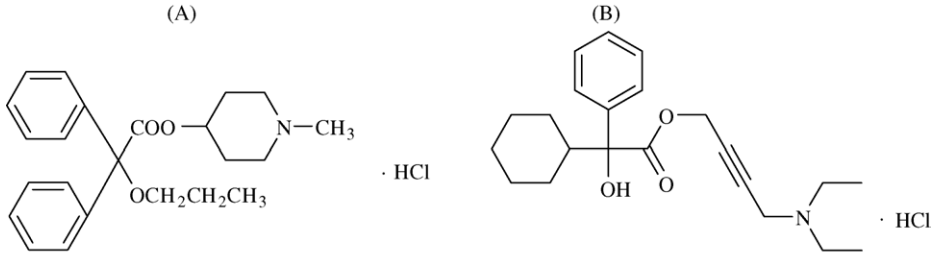

Fig. 1. Structures of (A) propiverine hydrochloride and (B) oxybutynin chloride.

South Korea). Oxybutynin chloride (alpha-phenylcyclohexaneglycolic acid-4-[diethylamino]-2-butynyl ester hydrochloride, internal standard), and formic acid were purchased from Sigma (St. Louis, MO, USA). The solvents, i.e., methanol, *n*-hexane, and ethylacetate (all HPLC-grade) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). High purity nitrogen (99.9999%) was purchased from Shin Yang Gas Co. (Seoul, Korea), and all other chemicals and solvents were of the highest analytical grade available.

2.2. Preparation of standard solutions

Standard stock solutions of propiverine and oxybutynin were made up at 1.0 mg/ml in dimethyl sulfoxide as their free forms. The propiverine standard solution was serially diluted with 100% methanol and added to drug free plasma to obtain concentrations of 0.5, 1.0, 5.0, 10.0, 50.0, 100.0, 500.0, and 1000.0 ng/ml. Internal standard solution was prepared by diluting internal standard stock solution with 100% methanol.

2.3. Sample extraction

Frozen human plasma samples were thawed at ambient temperature and 200 μl aliquots of samples were placed in screw capped glass tubes. Ten microliters of internal standard working solution (2 μg/ml oxybutynin) and 50 μl of 10 mM potassium phosphate buffer (pH 7.0) were then added. After a thorough vortex mixing for 30 s, mixtures were extracted with 6 ml of *n*-hexane/ethylacetate (8:2, v/v), vortex-mixed for 5 min, and centrifuged at 3000 × *g* for 10 min. The organic layer was removed and evaporated under nitrogen gas in a Turbo Vap evaporator (Zymark, Hopkinton, MA, USA), and the dried residue obtained was dissolved in 100 μl of methanol:H_2O (50:50, v/v). Five microliters of this solution was then injected into the LC–MS/MS system.

2.4. Liquid chromatography–mass spectrometry

An Agilent 1100 HPLC system (Agilent Technologies, Inc., USA) was used, and separation was achieved on a Capcell Pak MG II C18 column (50 mm × 2.0 mm i.d., 3 μm, Shiseido, Tokyo, Japan) at a column temperature of 35 °C. The combination of the mobile phase, prepared by mixing methanol:0.01% formic acid in the ratio of 70:30 (v/v), the injection volume 5 μl, and the flow rate 200 μl/min. Separation was conducted under isocratic conditions and the total running time was 2 min. The system autosampler was controlled at 4 °C.

The HPLC system was coupled to an API 2000 triple quadrupole mass spectrometer (Applied Biosystems-SCIEX, Concord, Canada) equipped with a turbo ion spray source. Electrospray ionization (ESI) was performed in the positive mode and the optimum conditions for nebulizing gas (GS1) of nitrogen, turbo spray gas (GS2), and curtain gas (CUR) were 40, 50, and 20, respectively. The source temperature of GS2 was set at 320 °C, and the ion spray (IS) voltage used was 5400 V. Unit resolution was set for both Q1 and Q3 mass detection, and the collision energy (CE) was set at 65 and 35 V for propiverine and oxybutynin, respectively. MRM detection was employed using nitrogen as the collision gas (seven arbitrary value) with a dwell time of 150 ms for each transition. Analytical data were processed using Analyst software (Version 3.1.).

2.5. Calibration and validation

The calibration curves for propiverine in human plasma were obtained using eight calibration standards and each standard was prepared in triplicate. In order to assess the intra-day coefficient of variation (C.V.) and accuracy for plasma samples, samples of propiverine and oxybutynin were spiked into human plasma at final concentrations of 0.5, 1.0, 5.0, 10.0, 50.0, 100.0, 500.0, and 1000.0 ng/ml. C.V. and accuracy for inter-day assay were assessed at the same concentrations and repeated for 5 different days. Calibration curves were generated using least-squares linear regression without applying a weighting by plotting propiverine integrated peak area versus internal standard (I.S.) (oxybutynin) peak area ratios against the concentration ratios of analyte and internal standard in spiked plasma. The absolute recoveries of the analyte were calculated in triplicate in normal plasma by extracting drug-free plasma samples spiked with propiverine. Recoveries were calculated by comparison of the analyte peak areas of the extracted samples with those of the unextracted response

standard mixtures representing 100% recovery. This procedure was repeated for the three different concentrations of propiverine added, namely 0.5, 50.0, and 500.0 ng/ml.

To test the short- and long-term stability of extracted propiverine, three samples, containing low (0.5 ng/ml), medium (50.0 ng/ml), and high (500.0 ng/ml) concentrations in plasma, were determined after several freeze and thaw cycles. The long-term storage stability at −70 °C was determined after 2 months. Moreover, the short-term stability of the extracted samples during storage for 24 h at 4 °C and room temperature was also determined.

2.6. Matrix effects

The matrix effect (undetected matrix components, which co-elute with analytes, may adversely affect the reproducibility of analyte ionization in a mass spectrometer' electrospray source) [8,9] was investigated by extracting 'blank' biological fluids from five different sources, reconstituted the final extract in 50% methanol containing a known amount of the analyte, analyzing the reconstituted extracts and then comparing the peak areas of the analyte.

2.7. Bioequivalence study

The developed method was used to evaluate the bioequivalence of two tablet formulations of propiverine hydrochloride in healthy volunteers: Urona® (test formulation from Myungmoon Pharm. Co., Ltd.; lot no. 401, expiration date July 2007) and BUP-4® (standard reference formulation from Jeil Pharm. Co., Ltd.; lot no. BPDB01, expiration date November 2006).

2.7.1. Subjects

The bioequivalence protocol used was approved by the Korean Food and Drug Administration. Twenty-four healthy male volunteers, aged between 19 and 55 years, were selected for this study after clinically assessing their health statuses evaluation (physical examination, electrocardiograph) and hematology, biochemistry, electrolytes, and urinalysis testing. No subject had a history or evidence of a renal, gastrointestinal, hepatic, or hematologic abnormality or any acute or chronic disease, or an allergy to any drugs. Subjects who had used drugs of any kind within the 2 weeks prior to the start of or during the study were also excluded. The volunteers had the following clinical characteristics (expressed as means ± S.D. [range]): age, 23.7 ± 1.9 years [20–29]; height, 175.0 ± 4.5 cm [164.2–181.1]; body weight, 68.5 ± 7.3 kg [54.2–83.3].

2.7.2. Drug administration

The study was based on a single dose, randomized, two-treatment, two-period crossover design. During phase 1 period, volunteers were hospitalized at 18:00 h and had a normal evening meal, and then after an overnight fast they were administered (at 08:00 h) a single dose of propiverine hydrochloride (20 mg of either tablet formulation). Water (240 ml) was given immediately after drug administration and the volunteers were then fasted for 4 h. A standard lunch was served at 4 h, and an evening meal was provided 12 h after administration. No other food was permitted during the 'in-house' period but liquid consumption was allowed ad libitum after lunch (with the exception of xanthine-containing drinks, such as tea, coffee, and cola). Systolic and diastolic arterial pressure (measured non-invasively with a sphygmomanometer), heart rate, and temperature were recorded just before and hourly after the administration of drug. After a wash period of 14 days, the study was repeated in the same manner (phase 2) to complete the crossover design.

2.7.3. Blood sampling

Heparinized blood samples (5 ml) were collected from a suitable forearm vein using an indwelling catheter into heparin containing tubes before (0 h) and 0.5, 1, 2, 3, 4, 5, 6, 12, 24, 48, and 72 h after dosing. The blood samples were centrifuged at 3000 rpm for 10 min, and plasma samples were separated and stored at −70 ± 5 °C until required for analysis.

2.7.4. Pharmacokinetics and statistical analysis

Pharmacokinetic analysis was performed using a non-compartmental method. Plasma area under the plasma concentration–time curve from time zero to the last measurable concentration (AUC_{0-t}) and area under the plasma concentration–time curve from time zero to infinity ($AUC_{0-\infty}$) were calculated using WinNonlin (Version 3.1; Scientific Consulting, KY, USA) with trapezoidal method. The peak propiverine concentrations (C_{max}) and the time to C_{max} (T_{max}) were determined by inspection of the individual plasma concentration–time profiles of the drug. The elimination rate (k_e) was obtained as the slope of the linear regression of the log-transformed concentration–time curve data in the terminal phase. The half-life ($t_{1/2}$) was calculated from ln 2 divided by k_e.

2.7.5. Statistical analysis

Bioequivalence of the two propiverine products was assessed by calculating individual C_{max}, AUC_{0-t}, and $AUC_{0-\infty}$ values. Their ratios (test/reference) using log-transformed data, together with their means and 90.0% confidence intervals, were analyzed with analysis two-way ANOVA using the K-BE Test 2002 program [10] at a significant level of 0.05.

3. Results and discussion

3.1. MS optimization and quantification

In order to optimize ESI conditions for propiverine and oxybutynin, first quadrupole full-scans (Q1 scan) of

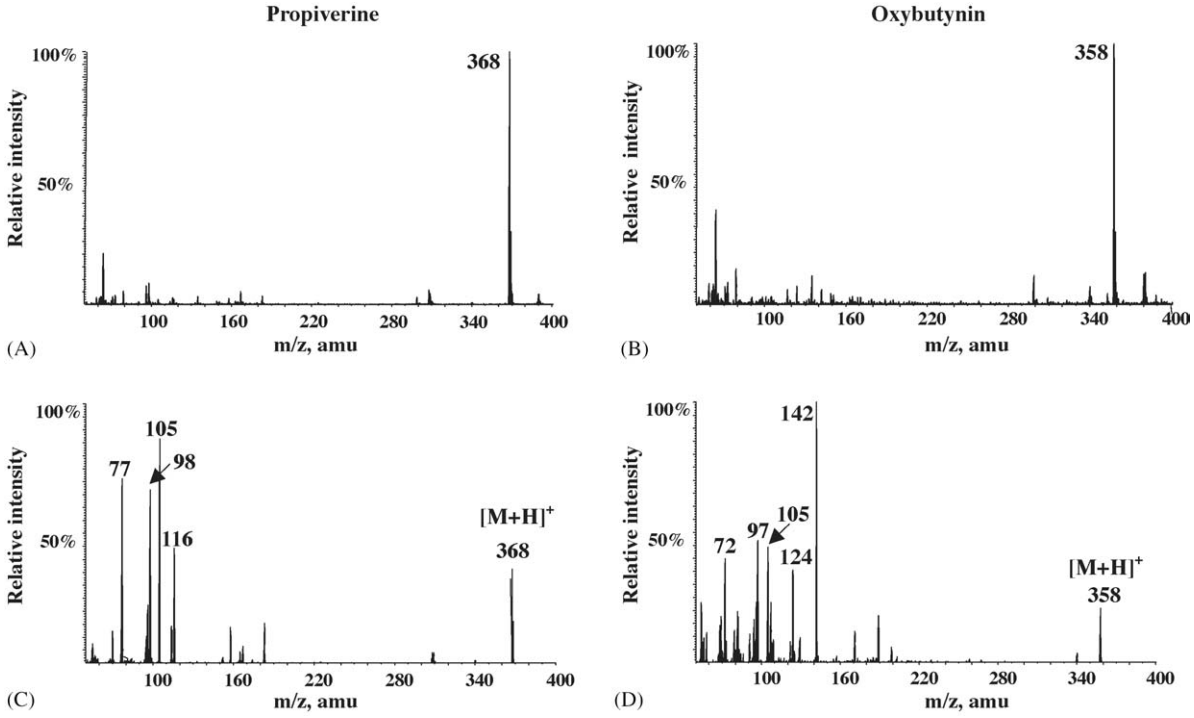

Fig. 2. Full-scan mass spectra (A) propiverine and (B) oxybutynin (I.S.), and product ion spectra of $[M + H]^+$ ions of (C) propiverine and (D) oxybutynin (I.S.).

propiverine and oxybutynin were carried out in positive ion detection mode. The mass spectra of propiverine and oxybutynin revealed base peaks at *m/z* 368 and *m/z* 358, respectively, and protonated molecular ions $[M + H]^+$ (Fig. 2A and B). Major fragment ions of propiverine and oxybutynin were observed at *m/z* 105 [6] (Fig. 2C) and *m/z* 142 [12] (Fig. 2D), respectively. Full-scan mass spectra and product ion mass were collected during direct infusion experiment, and the collision activated dissociation (CAD) of each protonated $[M + H]^+$ was conducted at different collision energies to optimize the output signal. The product ions of *m/z* 105 and *m/z* 142 provided high sensitivity for quantification in MRM mode. Instrumental parameters are summarized in Table 1.

The specificity and selectivity of the method were investigated by preparing and analyzing human plasma blanks from five different batches of pooled human plasma. The product ion chromatograms extracted from plasma are depicted in Fig. 3. As shown, the chromatogram retention times for propiverine and oxybutynin (I.S.) were 1.1 and 0.9 min, respectively, which are lower than those reported by Ikumi et al. [7]. Total HPLC–MS/MS analysis time was 2 min per sample. Fig. 3A shows an HPLC chromatogram for a blank plasma sample, indicating no endogenous peaks at the retention times of propiverine or internal standard (oxybutynin). Fig. 3B shows the MRM chromatograms obtained by the analysis of plasma spiked with 100 ng/ml propiverine and 2 μg/ml oxybutynin.

Table 1
LC–MS/MS instruments parameters

Parameters	Analyte	I.S.
Curtain gas (arbitrary unit)	20	20
Nebulizing gas (arbitrary unit)	40	40
Turbo spray gas (arbitrary unit)	50	50
Protonated molecule (*m/z*)	368	358
Product ion (*m/z*)	105	142
Dwell time (ms)	150	150
Declustering potential (V)	26	21
Focusing potential (V)	370	370
Entrance potential (V)	10	9
Collision cell entrance potential (V)	22	22
Collision energy (V)	65	35
Collision cell exit potential (V)	2	2

The purpose of these investigations was to develop a specific and sensitive assay for the determination of propiverine in human plasma using LC–MS/MS for pharmacokinetic studies.

3.2. Method validation

A calibration curve was constructed using eight different propiverine concentrations and processed by least-squares linear regression analysis (no weighting was applied). The standard calibrations curve for spiked human plasma containing propiverine were linear over in the concentration range 0.5–1000.0 ng/ml. Least-squares equations for propiverine calibration showed a correlation coefficient greater than 0.9988. When we compared our method to the method developed by Ikumi et al. [7], even the methods are similar, the sensitivity of our method was 10-fold higher, which was probably due to the different LC–MS/MS systems used.

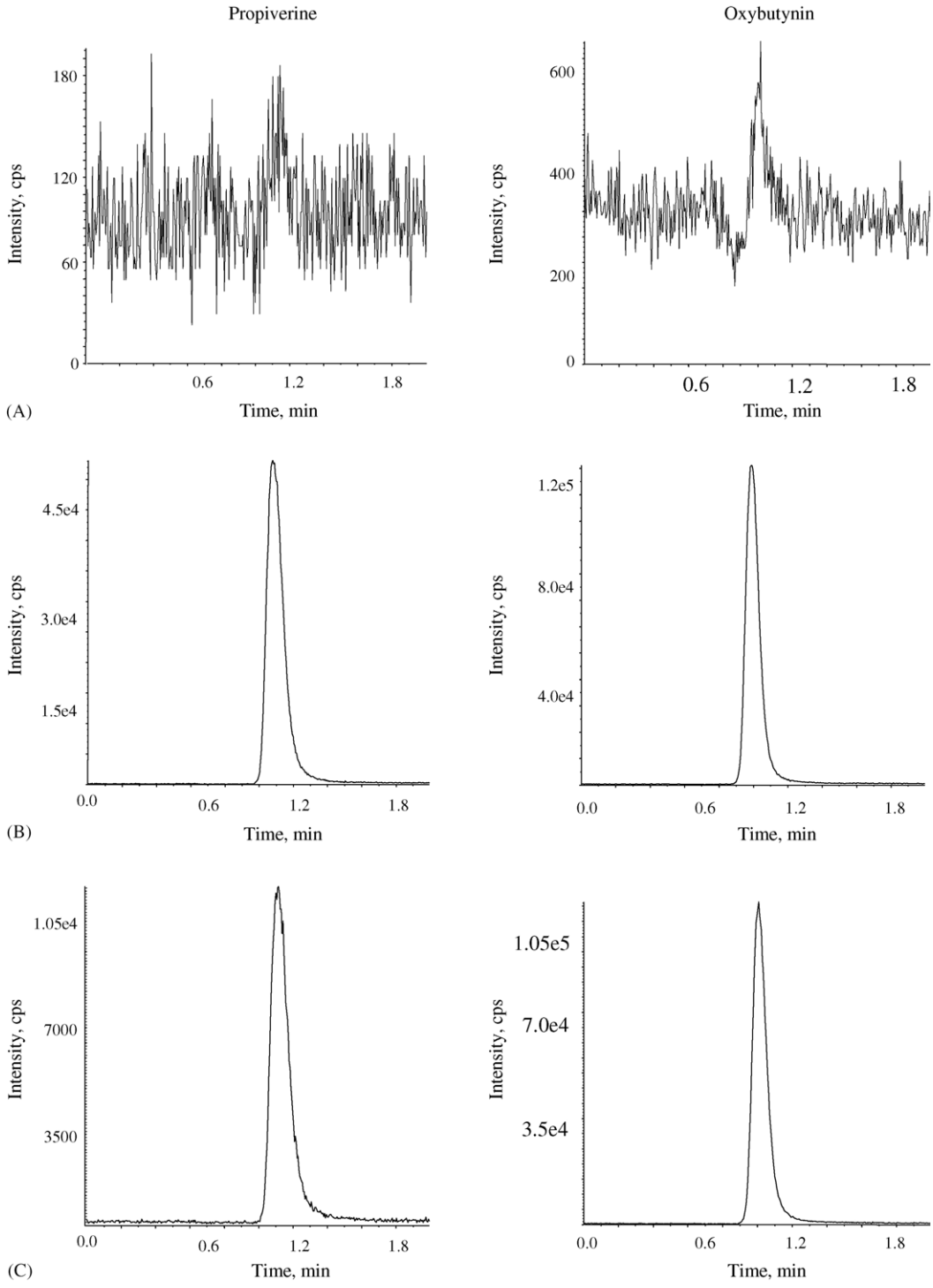

Fig. 3. LC–MS/MS chromatogram of (A) blank human plasma, (B) plasma spiked with 100 ng/ml propiverine and oxybutynin (2 μg/ml), and (C) plasma collected 1.0 h after single oral administration of propiverine hydrochloride tablet (20 mg).

The intra- and inter-day variations of propiverine determination in human plasma are summarized in Table 2. The intra-day coefficients of variation were between 1.58 and 6.88% and accuracies ranged from 99.46 to 106.88%. The inter-day coefficients of variation were between 3.94 and 8.66% and accuracies were between 100.15 and 109.41%. Under these conditions, a lower limit of quantification (LLOQ) of 0.5 ng/ml was achieved for propiverine using a 0.2 ml plasma sample volume. This was the lowest concentration of analyte that can be measured with both a coefficient of variation and accuracy of <15%. Moreover, this LLOQ is sufficient for pharmacokinetic studies. The extraction recov-

Table 2
Intra- and inter-day coefficient of variation and accuracy for determination of propiverine in human plasma ($n = 5$)

Theoretical concentration (ng/ml)	Intra-day			Inter-day		
	Mean concentration found (ng/ml)	C.V. (%)	Accuracy (%)	Mean concentration found (ng/ml)	C.V. (%)	Accuracy (%)
0.5	0.48	6.88	103.48	0.50	4.27	100.15
1.0	0.97	3.41	103.54	0.91	5.80	109.41
5.0	4.68	5.74	106.88	4.75	6.40	105.32
10.0	9.64	4.08	103.71	9.64	8.31	103.73
50.0	49.40	3.13	101.21	47.20	8.61	105.93
100.0	100.54	1.75	99.46	95.27	3.94	104.97
500.0	487.80	2.99	102.50	458.36	6.89	109.08
1000.0	987.40	1.58	101.28	927.48	8.66	107.82

ery determined for propiverine was shown to be consistent and reproducible. The mean recoveries of the 0.5, 50.0, and 500.0 ng/ml levels were 94.3, 92.4, and 95.6%, respectively. To evaluate propiverine stability in human plasma, drug-free plasma samples were spiked at 0.5, 50.0, and 500.0 ng/ml. After extraction, samples were arranged in the autosampler and were analyzed. In the short-term stability study, propiverine was found to be stable for 24 h at 4 °C and room temperature (Table 3). In the long-term stability study, the plasma samples spiked with propiverine also showed no loss of analytes when they were stored for 2 months at −70 °C. The final stability test was demonstrated after three freeze-thaw cycles. No significant deterioration of the analytes was observed under any of these conditions (Table 3).

3.3. Matrix effects

The C.V. (%) of the analyte peak areas for these five determinations was calculated. From the calculated C.V. of 3.34% for the analyte that if the analyte did co-elute with endogenous matrix components, this did not adversely affect the reproducibility of ionization.

3.4. Bioequivalence of propiverine

This method was applied to a bioequivalence study of two propiverine hydrochloride tablet formulations. The mean (±S.D.) plasma concentrations–time profiles of propiverine after a single oral dose of 20 mg of either formulation in tablet form are shown in Fig. 4. The pharmacokinetic parameters of the two propiverine formulations are shown in

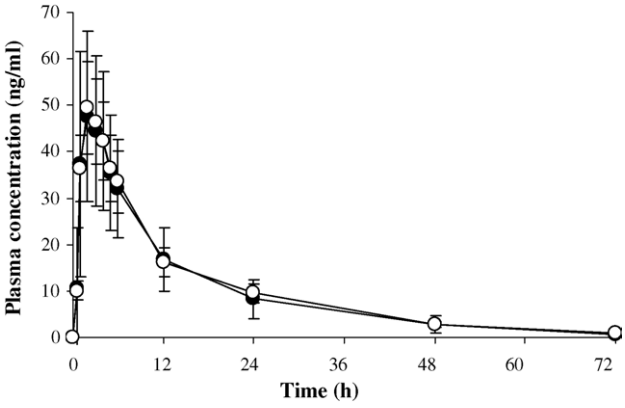

Fig. 4. Mean plasma concentration vs. time graph of propiverine after administration of test (URONA®; ○) and reference (BUP-4®; ●) formulations to healthy, adult, male human subjects under fasting condition.

Table 4. All pharmacokinetic parameters values obtained are in good agreement with previously reported values [3], and no statistically significant difference was found between the two formulations. For the bioequivalence test, AUC_{0-t}, $AUC_{0-\infty}$, and C_{max} were evaluated as primary parameters. The means and standard deviations of these parameters for the two brands were similar, indicating that the pharmacokinetics of propiverine in the two brands are similar. The 90.0% confidence intervals for the ratios of test drug to reference drug in terms of AUC_{0-t}, $AUC_{0-\infty}$, and C_{max} were within the range 80.0–125.0%, which is the range accepted by the Korean and US Food and Drug Administration [11,13].

Table 3
Stability data for propiverine ($n = 3$ per test and each concentration)

Theoretical concentration (ng/ml)	5.0	50.0	500.0
Long-term			
2 Months, −70 °C (%)	101.24 ± 1.03	97.35 ± 1.05	98.26 ± 1.62
Short-term			
24 h, room temperature (%)	100.54 ± 2.01	102.78 ± 1.23	101.45 ± 0.45
24 h, 4 °C (%)	102.79 ± 0.47	102.94 ± 2.21	97.81 ± 1.98
Freeze/thaw stability (%)	97.47 ± 0.61	99.46 ± 0.84	102.98 ± 1.46

Table 4
Mean pharmacokinetic parameters and 90.0% confidence interval for propiverine, after the administration of an oral dose of 20 mg of test (URONA®) and reference (BUP-4®) formulations to healthy human volunteers

Pharmacokinetic parameters	BUP-4® (mean ± S.D.)	URONA® (mean ± S.D.)	Confidence limit 90.0%
T_{max} (h)	2.39 ± 1.03	2.48 ± 1.07	–
C_{max} (ng/ml)	52.26 ± 20.04	52.50 ± 24.27	90.67–108.03
AUC_{0-t} (ng h/ml)	648.87 ± 238.89	678.50 ± 324.79	92.24–110.64
$AUC_{0-\infty}$ (ng h/ml)	661.35 ± 246.63	702.70 ± 348.84	92.62–112.60
$t_{1/2}$ (h)	15.66 ± 4.71	14.06 ± 3.25	–
k_e (h^{-1})	5.09 ± 1.02	5.17 ± 1.58	–

4. Conclusions

The LC–MS/MS method developed have for the determination for propiverine using a simple liquid–liquid extraction procedure and isocratic chromatography, provided a fast and sensitive analytical method. The developed method has excellent sensitivity, reproducibility and specificity. The method has been successfully used to provide the bioequivalent study of propiverine in human plasma.

Acknowledgements

The authors wish to thanks Dr. Jin-Sung Kim of Applied Biosystems, Korea, for his insightful comments. This work was supported by a grant no. R13-2002-020-01002-0 from the Korea Science & Engineering Foundation.

References

[1] W.D. Steers, in: P.C. Walsh, AB. Retik, ED. Vaughan Jr., AJ. Wein (Eds.), Campbell's Urology, vol. 1, seventh ed., Saunders, Philadelphia, 1997, pp. 870–915 (Chapter 26).
[2] K. Miyamae, M. Yoshida, S. Murakami, H. Iwashita, M. Ohtani, K. Masunaga, S. Ueda, Pharmacology 69 (2003) 205–211.
[3] R.P.G. David, Clin. Pharmacokinet. 42 (2003) 1243–1285.
[4] T. Marunaka, Y. Umeno, Y. Minami, E. Matsushima, M. Maniwa, K. Yoshida, M. Nagamachi, J. Chromatogr. 420 (1987) 43–52.
[5] K. Richter, S. Scheithauer, D. Thümmler, J. Chromatogr. B 708 (1998) 325–329.
[6] R. Oertel, K. Richter, J. Fauler, W. Kirch, J. Chromatogr. A 948 (2002) 187–192.
[7] K. Ikumi, Y. Ken-ichiro, M. Eiji, Y. Kazumasa, A. Tsutomu, A. Shunji, J. Chromatogr. B 799 (2004) 141–147.
[8] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 70 (1998) 882–889.
[9] I. Fu, E.J. Woolf, B.K. Matuszewski, J. Pharm. Biomed. Anal. 18 (1998) 347–357.
[10] Y.J. Lee, S.J. Jung, C.K. Shim, K-BE Test 2002 for Window, Version 1.2.1, 2002.
[11] KFDA Guidance for Industry, Statistical Approaches to Establishing Bioequivalence, Bioequivalence Division, Pharmacology Department, National Institute of Toxicology Department, 2003 (website: http://www.kfda.go.kr/).
[12] H.H. Kim, S.B. Han, J. Pharm. Biomed. Anal. 31 (2003) 341–349.
[13] FDA Guidance for Industry, Statistical Approaches to Establishing Bioequivalence, US Department of health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), 2001 (website: http://www.fda.gov/cder/guidance/index.htm).