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Validation of a simple liquid chromatography–tandem mass spectrometric method for the determination of propiverine hydrochloride and its *N*-oxide metabolite in human plasma

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Abstract

A simple high-performance liquid chromatography (HPLC)–tandem mass spectrometric method has been developed for determination of propiverine hydrochloride and its metabolite, propiverine *N*-oxide (M-1) in human plasma using stable isotopes, propiverine hydrochloride-d_{10} and M-1-d_{10}, as internal standards. The analytes were extracted with dichloromethane from 0.2 ml of plasma in neutral condition (pH 7.0) and separated by HPLC on a C18 reversed-phase column using methanol–1% acetic acid (50:50) as a mobile phase, and detected using positive electrospray ionization in selected reaction monitoring (SRM) mode. The method was validated over a concentration range of 2–500 ng/ml for propiverine hydrochloride and 4–1000 ng/ml for M-1 using 0.2 ml of human plasma per assay. The method developed was successfully applied to analysis of propiverine hydrochloride and M-1 in clinical studies.
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1. Introduction

Propiverine hydrochloride (1-methyl-4-piperidyl diphenylpropoxyacetate hydrochloride) (Fig. 1) is an anticholinergic drug widely used in the treatment of overactive bladder symptoms. A gas chromatography–mass spectrometry (GC–MS) method [1] and a high-performance liquid chromatographic (HPLC) method [2] have been developed for the simultaneous determination of propiverine hydrochloride and its metabolites. However, the GC–MS method includes complicated pretreatments to determine numerous metabolites. The HPLC method is convenient but requires a large volume of plasma due to insufficient sensitivity. A convenient and more sensitive analytical method is required in clinical pharmacokinetic studies to ease the burden on patients. This can be achieved using electrospray ionization–tandem mass spectrometry (ESI–MS–MS) for detection of propiverine hydrochloride, which has a sensitive tertiary amine substructure in its molecule. This article describes the validation of a high-performance liquid chromatographic–tandem mass spectrometric (LC–MS–MS) method that has been developed for measurement of propiverine hydrochloride and its principal metabolite, propiverine *N*-oxide (M-1, see Fig. 1) in human plasma using stable isotopes, propiverine hydrochloride-d_{10} and M-1-d_{10}, as internal standards.

2. Experimental

2.1. Materials

Propiverine hydrochloride was obtained from F. Hoffman-La Roche & Co. and its metabolite, M-1 and the internal standards propiverine hydrochloride-d_{10} and M-1-d_{10} were synthesized by Taiho Pharmaceutical Co. Ltd. (Tokyo, Japan). HPLC-grade methanol (MeOH), dichloromethane (CH_2Cl_2) and special-grade acetic acid, disodium hydrogen

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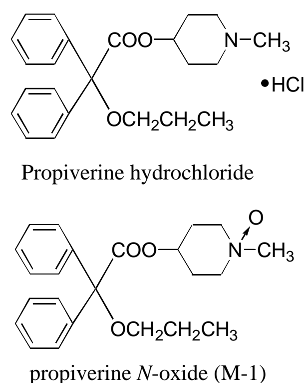

Fig. 1. Chemical structure of propiverine hydrochloride and its *N*-oxide.

phosphate dihydrate, potassium dihydrogen phosphate were obtained from Wako Pure Chemical (Osaka, Japan). Water was purified by Milli-Q SP-TOC (Nihon Millipore Kogyo, Japan). Pooled drug-free plasma samples from healthy volunteers were used as blank matrix. Blank plasma was stored at −80 °C until analysis.

2.2. Instruments and LC–MS–MS conditions

The HPLC system consisted of a Model 616 pump, a Model 717 plus autosampler and a Model 600S controller (Waters, Milford, MA, USA). Chromatographic separation was achieved on a Capcell Pak UG120 C18 (150 mm × 2.1 mm i.d., 5 μm) reversed-phase column from Shiseido (Tokyo, Japan) at a column temperature of 35 °C. The mobile phase was composed of methanol–1% acetic acid (50:50) with a flow-rate of 0.2 ml/min, and the cyclic time of assay was 10 min.

A Finnigan TSQ 7000 triple-stage quadrupole mass spectrometer (Thermo Electron, Massachusetts, USA) was used with an electrospray interface at a spray voltage of 4.5 kV in the positive ion mode. Monitored quasi-molecular ion transitions $[M + H]^+$ for propiverine hydrochloride and M-1 in the selected reaction monitoring (SRM) mode were *m/z* 368 → 183 and *m/z* 384 → 183, respectively. The heated capillary was maintained at 250 °C. Argon was used as the collision gas at a pressure of 0.23–0.25 Pa and the collision energy was set at −35 eV. Nitrogen served both as the sheath gas at a pressure of 480 kPa and as the auxiliary gas at a flow-rate of 10 units.

2.3. Sample preparation

2.3.1. Standard, calibration curve and quality control samples

The standard stock solution of 100 μg/ml of propiverine hydrochloride and 200 μg/ml of M-1 were prepared in methanol. The standard solutions were prepared by diluting standard stock solution with methanol. The internal standard stock solutions of 20 μg/ml of propiverine hydrochloride-d_{10} and 20 μg/ml of M-1-d_{10} were prepared in methanol. Internal standard solution was prepared by diluting internal standard stock solution with methanol. All solutions were stored at 5 °C. The corresponding standard solutions were evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was reconstituted in 0.2 ml of blank plasma to prepare the calibration standards containing 500, 100, 25, 10, 5 and 2 ng/ml for propiverine hydrochloride and 1000, 200, 50, 20, 10 and 4 ng/ml for M-1.

Quality control (QC) samples were prepared in the same way as calibration standards with blank plasma and standard solutions to obtain the following concentrations; GQCH: F400 ng/ml of propiverine hydrochloride, 800 ng/ml of M-1, QCM: 40 ng/ml of propiverine hydrochloride, 80 ng/ml of M-1, and QCL: 3 ng/ml of propiverine hydrochloride, 6 ng/ml of M-1 in plasma. QC samples were stored at −80 °C until analysis.

2.3.2. Sample pretreatment

A 0.5 ml portion of 10 mM phosphate buffer (pH 7.0) and 0.05 ml of internal standard solution (2 μg/ml of propiverine hydrochloride-d_{10}, 4 μg/ml of M-1-d_{10}) were added to 0.2 ml of plasma samples. After adding 7 ml of CH_2Cl_2, the mixture was shaken for 10 min, and then centrifuged at 3000 rpm, for 5 min at 5 °C. The organic layer was separated and evaporated to dryness at 40 °C under a gentle stream of nitrogen. The residue was redissolved in 100 μl of 50% methanol and a 20 μl aliquot was injected into the LC–MS–MS system.

2.4. Validation

2.4.1. Linearity

Linearity of calibration was tested by extraction and assayed ($n = 5$). The peak area of propiverine hydrochloride or M-1 to the internal standard (propiverine hydrochloride/propiverine hydrochloride-d_{10}, M-1/M-1-d_{10}) was used as the assay parameter. The peak area ratios were plotted against nominal concentrations. Calibration curves were obtained from weighted ($1/x^2$) least-squares linear regression analysis of the data ($y = ax + b$), where y is the peak area ratio and x the nominal concentrations.

2.4.2. Accuracy and precision

Intra-day accuracy and precision (each, $n = 5$) were evaluated by analysis of QC samples and lower limit of quantitation (LLOQ) on the same day. Inter-assay accuracy and precision were determined by repeated analysis over five consecutive days ($n = 1$ series per day). The concentration of each sample was determined using calibration standards prepared on the same day. Accuracy was defined as the deviation of the mean from nominal concentration, and precision as the coefficient of variation (CV).

2.4.3. Stability

The effect of three freeze-thaw cycles, the compound stability for at 4 h at room temperature in plasma were evaluated

by repeated analysis ($n = 3$) of QCH and QCM samples. Long-term stability in plasma was also tested by assaying frozen QCH and QCM samples after storage at −20 °C for 1, 3 and 6 months. Stability was expressed as a percentage of nominal concentration.

Stability on the autosampler was studied. A prepared sample was placed on the autosampler at 10 °C for 48 h, and then the sample solution was injected into LC–MS–MS. The peak area ratio was compared with the immediate sample peak area ratio.

The standard solutions and the internal solution had been stored in refrigerator at 5 °C for 6 months, and then the stability was evaluated. The standard solutions were injected into LC–MS–MS after spiking with freshly prepared internal standard solution. The peak area ratio of propiverine hydrochloride or M-1 to the corresponding internal standard (propiverine hydrochloride/propiverine hydrochloride-d_{10}, M-1/M-1-d_{10}) was compared between freshly prepared solution and that after 6 months storage. The stability of internal standard solution was evaluated by the same procedure using the standard solutions as the internal reference.

3. Results and discussion

3.1. Mass spectrum analysis

Protonated molecules $[M+\mathrm{H}]^+$ were detected at *m/z* 368 for propiverine hydrochloride and at *m/z* 384 for M-1. The precursor ion selected $[M+\mathrm{H}]^+$, and product ion mass spectra were recorded at collision energies of −25, −30, −35 and −40 eV (Fig. 2). SRM using precursor → product ion combination at *m/z* 368 → 183 for propiverine hydrochloride, and *m/z* 384 → 183 for M-1 permitted highly sensitive detection of the analyte. Thus, the collision energy of −35 eV was chosen for MS–MS quantitation.

3.2. Chromatography and matrix effect

Typical chromatograms are shown in Fig. 3. The retention times for propiverine hydrochloride and M-1 were 4.63 and 5.46 min, respectively, and no endogenous peaks that would interfere with the detection of propiverine hydrochloride, propiverine hydrochloride-d_{10}, M-1, and M-1-d_{10} was observed.

The matrix effect was evaluated by referring the peak area of extracted sample to that of standard solution without matrix. The peak area were enhanced in the ratio of 1.26 for propiverine hydrochloride and 1.62 for M-1, respectively. Although the sample matrix may enhanced the ionization of analytes [3,4], the matrix effect for the corresponding internal standard was equivalent to that of target analyte. Thus, the ratio of peak area was not influenced by the endogenous components via sample matrix.

3.3. Linearity of calibration curve

The calibration curve were liner within range of 2–500 ng/ml for propiverine hydrochloride and 4–1000 ng/ml for M-1. The correlation coefficients were ranged in 0.99802–0.99959 for propiverine hydrochloride, 0.99820–0.99969 for M-1. The calibration parameters are shown in Table 1. The accuracy of back-calculated concentrations ranged in 90.9–107.2% for propiverine hydrochloride, and in 90.4–106.1% for M-1.

3.4. Precision and accuracy

The intra- and inter-day ($n = 5$) precision and accuracy are shown in Table 2. The precision at LLOQ (propiverine hydrochloride: 2 ng/ml, M-1:4 ng/ml) was 15.2% for propiverine hydrochloride, and 8.7% for M-1, and the accuracy was 88.9% for propiverine hydrochloride and 88.5% for M-1. The precision for the QC samples for propiverine hydrochloride was 2.8–12.4%, while that for M-1 was 5.4–9.8%. The accuracy for the QC samples for propiverine hydrochloride was 97.5–110.5%, while that for M-1 was 100.4–110.2%. These results satisfied validation criteria [5], since the accuracy and precision were within 85–115 and 15%, except for the LLOQ, at which the accuracy and precision were within 80–120 and 20%, respectively.

Table 1
Parameters of calibration curves

	Propiverine hydrochloride			M-1		
	Slope (*a*)	Intercept (*b*)	Correlation coefficient (*r*)	Slope (*a*)	Intercept (*b*)	Correlation coefficient (*r*)
Day 1	0.00202	0.00042	0.99813	0.00135	0.00119	0.99952
Day 2	0.00215	−0.00048	0.99869	0.00146	0.00095	0.99886
Day 3	0.00224	−0.00085	0.99959	0.00137	0.00021	0.99919
Day 4	0.00206	0.00202	0.99802	0.00154	0.00035	0.99820
Day 5	0.00221	0.00016	0.99926	0.00135	0.00054	0.99969
Mean	0.00214	0.00025	0.99874	0.00141	0.00065	0.99909
S.D.	0.00009	0.00111	0.00069	0.00008	0.00041	0.00059

$y = a \times x + b$, where y is the peak area ratio (propiverine hydrochloride/propiverine hydrochloride-d_{10}, M-1/M-1-d_{10}) and x the nominal concentration; weighting: $1/x^2$.

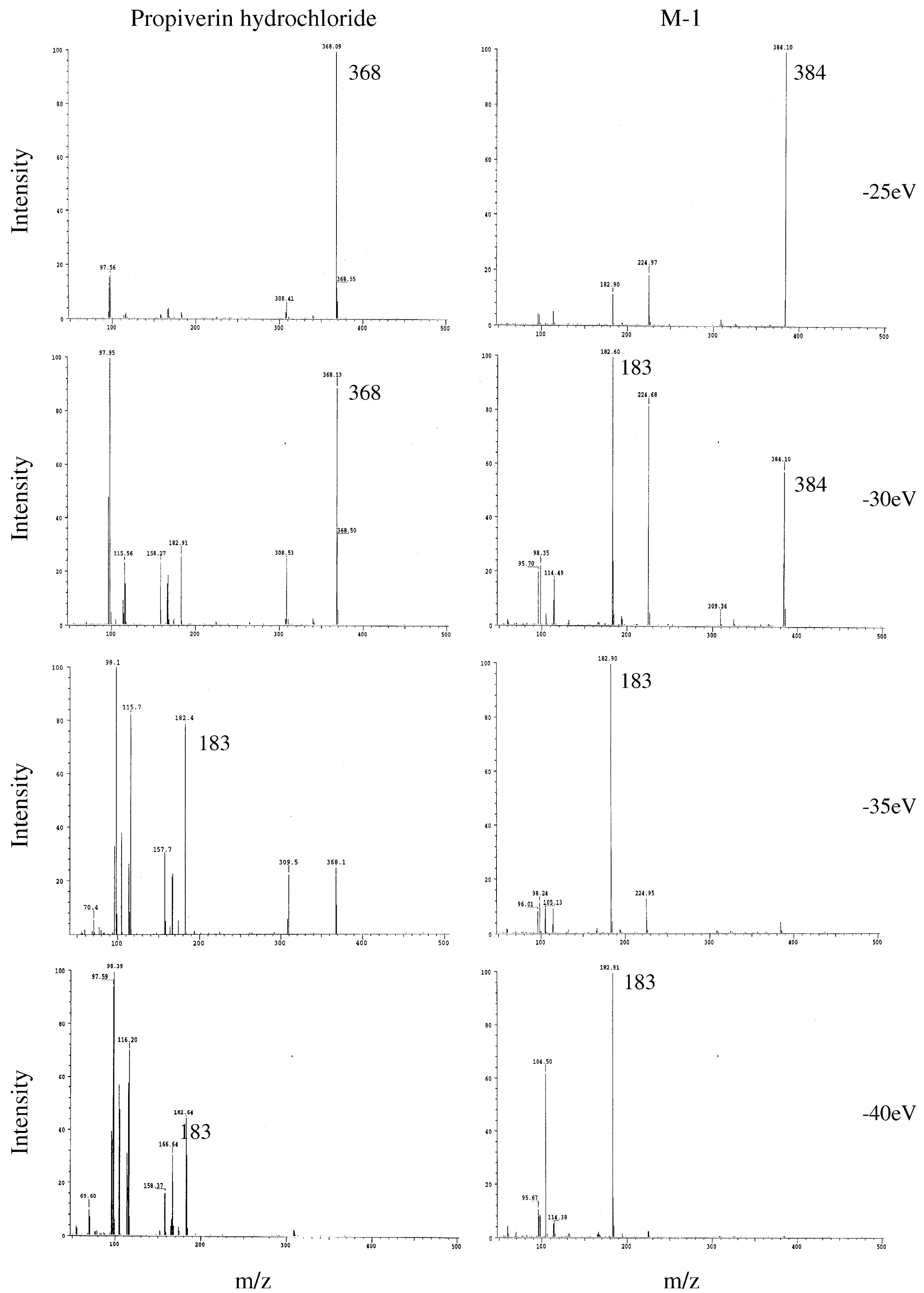

Fig. 2. Product ion mass spectra of propiverine hydrochloride and M-1 obtained from different collision energies of −25, −30, −35 and −40 eV.

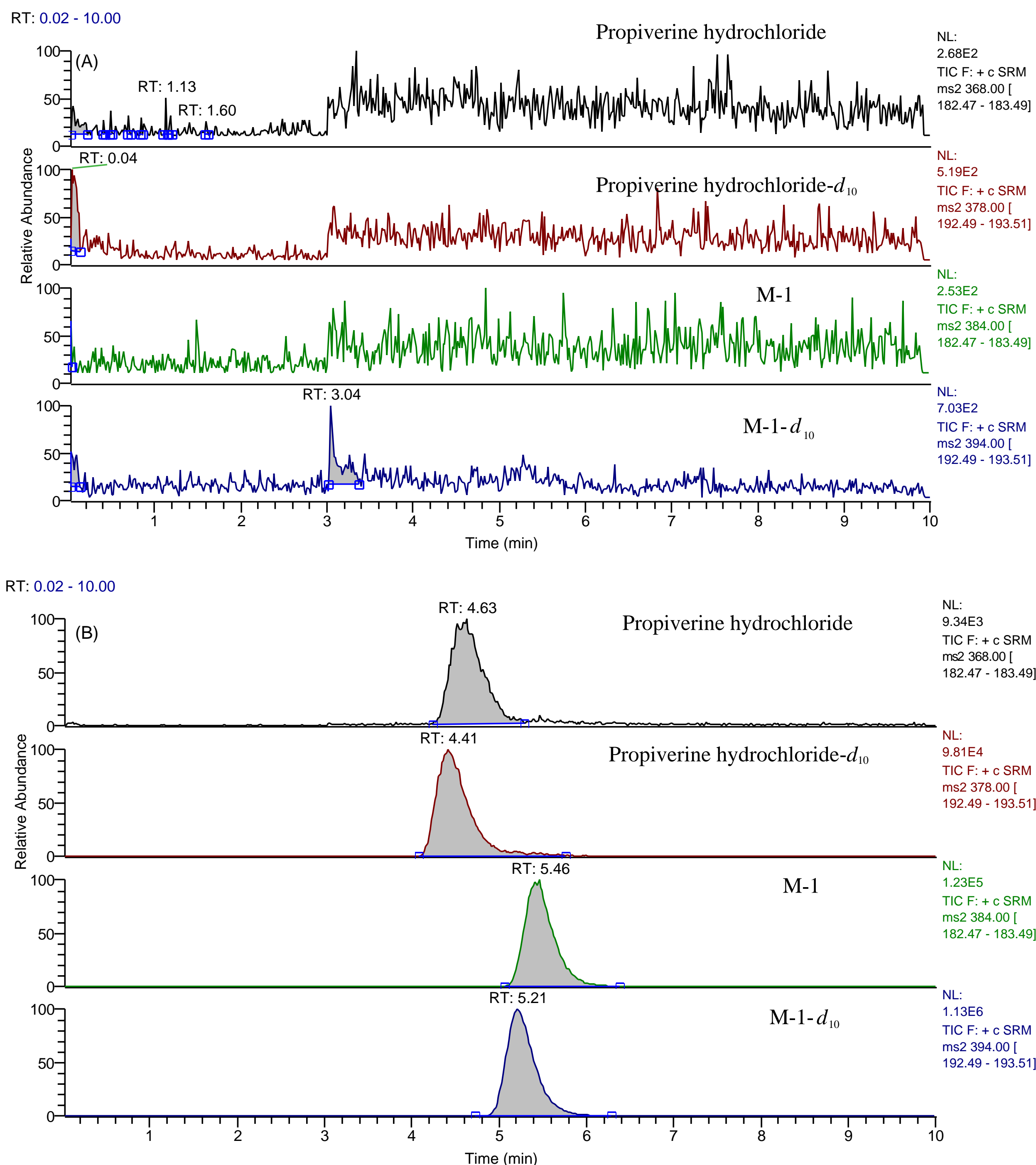

Fig. 3. Typical chromatograms of human blank plasma (A), and human plasma spiked with propiverine hydrochloride (40 ng/ml), M-1 (80 ng/ml), propiverine hydrochloride-d_{10} (500 ng/ml) and M-1-d_{10} (1000 ng/ml) (B).

3.5. Stability

All stability results are shown in Table 3. Propiverine hydrochloride and M-1 were stable for at least 4 h at room temperature in plasma samples; the mean recoveries from the nominal concentration were 114.3 and 97.8%, respectively, at 400 and 800 ng/ml, and 94.1 and 101.0%, respectively, at 40 and 80 ng/ml. Propiverine hydrochloride and M-1 were stable in plasma samples when stored at −20 °C for a 6-month period, and following three freeze-thaw cycles.

Stability on the autosampler results are shown in Table 4. The area ratio did not change at 10 °C for 48 h. Prepared samples were stable for the period of analysis.

The standard solutions and the internal standard solution stored at 5 °C were stable for a 6-month period, the stability

Table 2
Precision and accuracy of assay for determination of propiverine hydrochloride and M-1 in plasma ($n = 5$)

	Nominal concentration (ng/ml)	Intra-assay			Inter-assay		
		Measured concentration (ng/ml) (mean ± S.D.)	Precision (%)	Accuracy (%)	Measured concentration (ng/ml) (mean ± S.D.)	Precision (%)	Accuracy (%)
Propiverine hydrochloride	2	1.78 ± 0.27	15.2	88.9			
	3	3.11 ± 0.22	7.1	103.7	2.93 ± 0.36	12.4	97.5
	40	42.18 ± 1.17	2.8	105.4	40.83 ± 3.44	8.4	102.1
	400	408.14 ± 33.17	8.1	102.0	441.95 ± 27.37	6.2	110.5
M-1	4	3.54 ± 0.31	8.7	88.5			
	6	6.33 ± 0.37	5.9	105.4	6.03 ± 0.42	6.9	100.4
	80	82.93 ± 4.46	5.4	103.7	82.17 ± 8.08	9.8	102.7
	800	881.61 ± 69.23	7.9	110.2	831.23 ± 50.60	6.1	103.9

Table 3
Stability of propiverine hydrochloride and M-1 in plasma

	Propiverine hydrochloride, nominal concentration (ng/ml)		M-1, nominal concentration (ng/ml)	
	400	40	800	80
Three freeze-thaw cycles				
Measured concentration (ng/ml) (mean ± S.D.)	449.74 ± 15.23	42.05 ± 6.86	788.36 ± 27.95	82.22 ± 8.13
Accuracy (%)	112.4	105.1	98.5	102.8
Room temperature (4 h)				
Measured concentration (ng/ml) (mean ± S.D.)	457.05 ± 13.88	37.62 ± 6.47	782.44 ± 11.09	80.79 ± 2.43
Accuracy (%)	114.3	94.1	97.8	101.0
Stored below −20 °C for 6 months				
Measured concentration (ng/ml) (mean ± S.D.)	403.95 ± 6.92	37.51 ± 1.06	791.50 ± 15.42	78.78 ± 0.81
Accuracy (%)	101.0	93.8	98.9	98.5

Accuracy (%): measured concentration/nominal concentration ×100 (%).

Table 4
Stability on autosampler

	Nominal concentration (ng/ml)	Mean area ratio for sample immediate after treatment ± S.D.	Mean area ratio for sample left for 48 h on autosampler ± S.D.
Propiverine hydrochloride	40	0.088553 ± 0.000170	0.090208 ± 0.001231
M-1	80	0.113264 ± 0.000284	0.112766 ± 0.000088

of standard solutions were 104.9 and 105.0% respectively, at 10 and 0.4 μg/ml for propiverine hydrochloride, and 105.6 and 105.9%, respectively, at 20 and 0.8 μg/ml for M-1. The stability of internal solution were 97.2% at 2 μg/ml for propiverine hydrochloride-d_{10}, and 102.0% at 4 μg/ml for M-1-d_{10}.

3.6. Application to pharmacokinetic study in a pediatric population

The present method was applied to determine the plasma concentrations of propiverine hydrochloride and its metabolite, M-1. Blood samples were collected once per subject from a total of 105 patients (6–14 years old) receiving propiverine hydrochloride therapy (10–40 mg/body per day) [6]. The time after dosing varied from 2 to 38.13 h. The concentrations of propiverine hydrochloride are normalized to 20 mg/63 kg by dose and body weight, which is a typical dosing condition in the adult populations, and the obtained data are plotted in Fig. 4. Although the propiverine hydrochloride levels in this pediatric population varied moderately compared with that for healthy adult volunteers (Tei et al., an in-house report, Taiho Pharmaceutical Co. Ltd.), the concentration–time profile was similar to that for adults. Furthermore, the ratios of M-1 to propiverine hydrochloride were slightly lower than those for adults (Fig. 5). Since this analytical method requires only 0.2 ml of plasma, the blood collection load on patients was minimal.

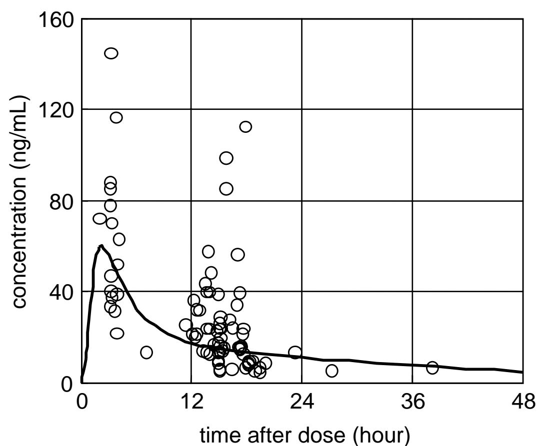

Fig. 4. Plasma concentration–time profile of propiverine(as free-base) in pediatric patients. (The line indicates the concentration in healthy adult volunteers.)

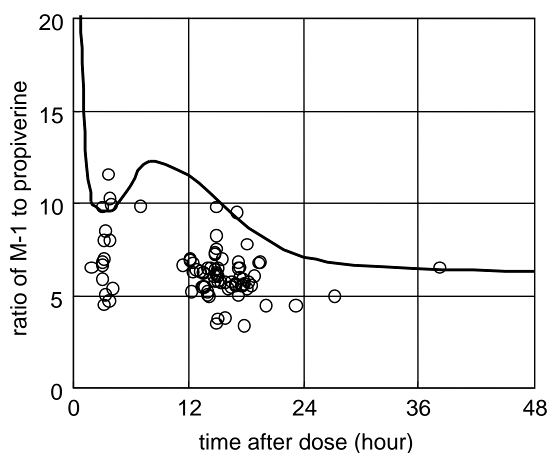

Fig. 5. The ratio of M-1 to propiverine(as free-base) in pediatric patients. (The line indicates the ratio in healthy adult volunteers.)

4. Conclusions

A simple and rapid LC–MS–MS method for the determination of propiverine hydrochloride and its metabolite M-1 in human plasma has been developed and validated. This method used a simple one-step liquid–liquid extraction and C18 column coupled with MS–MS. The results obtained exhibited good precision and accuracy. The validated assay used a 0.2 ml plasma sample, and the calibration curve range was 2–500 ng/ml for propiverine hydrochloride and 4–1000 ng/ml for M-1. This method can be applied to analysis of propiverine hydrochloride and its metabolite M-1 in clinical pharmacokinetics studies.

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