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# A rapid determination of propiverine and its N-oxide metabolite in human plasma by high performance liquid chromatography–electrospray ionization tandem mass spectrometry

Kyung-Hwan Yoon [a], So-Young Lee [a], Mi Jang [a], Sook-Hyun Ko [a], Won Kim [a], Jong-sei Park [a], Insook Park [b], Hie-Joon Kim [b,*]

[a] *LabFrontier, KSBC Bldg, Iui-dong, Youngtong-ku, Kyunggi-do 442-270, Republic of Korea*
[b] *Department of Chemistry, Seoul National University, Seoul 151-747, Republic of Korea*

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**Abstract**

A simple, fast and sensitive high-performance liquid chromatography (HPLC)–electrospray ionization (ESI) tandem mass spectrometric method (LC–MS/MS) has been developed for determination of propiverine and propiverine N-oxide metabolite in human plasma using oxybutynin as internal standard. Instead of extracting propiverine from plasma using organic solvents, which should be separated from the aqueous phase and evaporated before injecting the sample into the chromatograph, plasma sample containing propiverine and N-oxide was directly injected after precipitating proteins with acetonitrile. Numerous compounds in the plasma did not interfere with the highly specific multiple reaction monitoring in tandem mass spectrometric detection following $C_8$ reversed-phase chromatographic separation under conditions that eluted propiverine, N-oxide and oxybutynin within 2 min (0.1% formic acid in water/acetonitrile, 25:75, v/v). The LC–MS/MS method and an alternative LC–MS method, using methyl-*t*-butyl ether extraction and selected ion monitoring, were validated over 1–250 ng ml$^{-1}$ of propiverine and 2 to 500 ng ml$^{-1}$ of N-oxide, and successfully applied in a pharmacokinetic study. The lower limit of quantitation was 1 ng ml$^{-1}$ for propiverine and 2 ng ml$^{-1}$ for N-oxide in both methods.


*Keywords:* Propiverine; Electrospray ionization; LC–MS; LC–MS/MS

## 1. Introduction

Propiverine hydrochloride (1-methyl-4-piperidyl diphenylpropoxyacetate hydrochloride, MW = 403.0, Fig. 1 ) is an anticholinergic drug for treating hypertonic functional states in the bladder, such as pollakisuria, nouria and noturnal enuresis. Such anticholinergic effects are due to the direct effect of propiverine on contraction of the smooth muscle of the urinary bladder [1,2]. Propiverine is also known to block voltage-dependent $Ca^{2+}$ channels by recording inward $Ca^{2+}$ currents in single muscle cells from the urinary bladder [3]. Recently, it was found that calmodulin is the intracellular target molecule for propiverine. Inhibition of the action of calmodulin causes inhibition of actomyosin ATPase activity resulting in relaxation of the smooth muscle of the urinary bladder [4]. Its efficacy is as high as that of oxybutynin, a typical anticholinergic drug, with a lower frequency of side effects such as dry mouth [5]. Parkinsonism, however, has been reported in elderly patients taking propiverine [6].

Various methods for determining propiverine and its major metabolite N-oxide (propiverine N-oxide, Fig. 1) in human plasma or urine have been developed including gas chromatography–mass spectrometry (GC–MS) [7], high performance liquid chromatography (HPLC) [8,9], and liquid

* Corresponding author. Tel.: + 82 2 889 3415; fax: +82 2 889 1568.
*E-mail address:* hjkim1@snu.ac.kr (H.-J. Kim).

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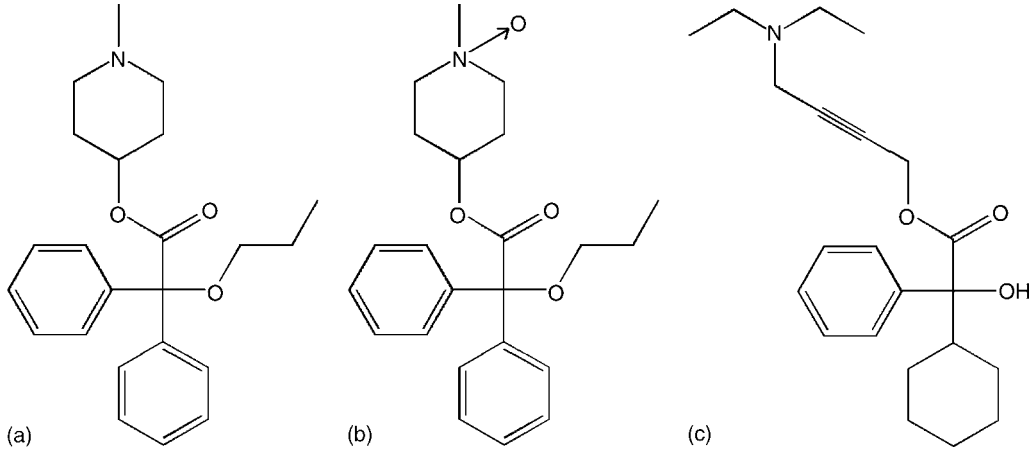


Fig. 1. Molecular structure of (a) propiverine, (b) propiverine N-oxide and (c) oxybutynin.

chromatography–tandem mass spectrometry (LC–MS/MS) [10,11]. Although the lower limit of quantitation (LLOQ) of propiverine in the GC–MS method is as low as 2 ng ml$^{-1}$, tedious sample preparation is required and the chromatographic run time is as long as 17 min [7]. Richter et al. used methyl-*t*-butyl ether (MTBE) extraction followed by reversed-phase (RP) HPLC with UV detection and reported LLOQ of 10 ng ml$^{-1}$ [9].

Recently, LC–MS/MS with its extremely high specificity for the fragmentation selected for monitoring has become a powerful alternative to HPLC with UV detection. Oertel et al. [10], used solid phase extraction and detected propiverine separated by RP HPLC by monitoring *m/z* 368 → 105 fragmentation. Komoto et al. used dichloromethane extraction, which required 7 ml dichloromethane for 200 μl plasma, 10 min shaking, 5 min centrifugation, and evaporating the solvent. Propiverine labeled with deuterium was used as internal standard. They monitored *m/z* 368 → 183 fragmentation for propiverine and *m/z* 368 → 183 fragmentation for propiverine N-oxide and reported LLOQ of 2 ng ml$^{-1}$ for propiverine and 4 ng ml$^{-1}$ for propiverine N-oxide [11].

Dealing with a large number of samples for pharmacokinetic studies, we were interested in simplifying the sample preparation step and significantly reducing the total analysis time. Substitution of solvent extraction with simple deproteination of the plasma was expected to reduce the sample preparation time by half. If the deproteination step could replace solvent extraction, it could be broadly applied to LC–MS/MS analysis of drugs and their metabolites in biological samples. Use of an internal standard without isotopic labeling was also an important consideration and structurally related oxybutynin was tried. In this paper, we report a fast and sensitive LC–MS/MS method for propiverine and its N-oxide in human plasma based on deproteination using acetonitrile and selected reaction monitoring of *m/z* 368 → 116 fragmentation for propiverine and *m/z* 384 → 183 fragmentation for propiverine N-oxide.

## 2. Experimental

### 2.1. Chemicals

Propiverine hydrochloride and propiverine N-oxide were kindly provided by Il-dong Pharmaceutical Co., Ltd. (Seoul, Korea). Oxybutynin chloride was obtained from Sigma (St. Louis, MO, USA). Propiverine, propiverine N-oxide and oxybutynin were 99.9% pure. HPLC grade methanol, isopropanol, acetonitrile, dichloromethane, *n*-hexane, *n*-heptane, ethyl ether, ethyl acetate and methyl-*t*-butyl ether were from Burdick & Jackson (Muskegon, MI, USA). Reagent grade formic acid, acetic acid and ammonium acetate were from Junsei Chemical (Tokyo, Japan). Deionized water was prepared using Aquamax Ultra system (Younglin Instrument, Anyang, Korea).

### 2.2. Sample preparation

Stock solutions containing 100 μg ml$^{-1}$ of propiverine, 100 μg ml$^{-1}$ of propiverine N-oxide and 100 μg ml$^{-1}$ of oxybutynin chloride were prepared in acetonitrile. Drug-free human plasma obtained from healthy male volunteers was stored at −30 °C and used as blank plasma. The calibration standard solutions of propiverine were prepared by serial dilution of the stock solution with blank plasma to 250, 100, 50, 25, 10, 5, 2.5 and 1 ng ml$^{-1}$. The quality control solutions of propiverine (150, 75, 15 and 7.5 ng ml$^{-1}$) were prepared similarly. For propiverine N-oxide, the calibration standard solutions were prepared at 500, 250, 100, 50, 25, 10, 5 and 2 ng ml$^{-1}$ and the quality control solutions were prepared at 400, 200, 40 and 20 ng ml$^{-1}$. The internal standard solution was prepared by dilution of the stock solution with acetonitrile to a final concentration of 1 μg ml$^{-1}$ for LC–MS and 50 ng ml$^{-1}$ for LC–MS/MS, respectively. All stock solutions and internal standard solution were stored at 4 °C and all plasma samples were stored at −30 °C.

For LC–MS, 20 μl of the internal standard solution and 1 ml of MTBE were added to 200 μl of plasma sample in a glass test tube, and the solution was vortex mixed for 5 min. After centrifugation at 3000 × *g* for 5 min, the supernatant organic layer was collected and evaporated to dryness under a stream of nitrogen at 40 °C. The residue was reconstituted with 200 μl of 50% acetonitrile and 2 μl aliquot was injected into the LC–MS system.

For LC–MS/MS, 200 μl of the internal standard solution was added to 100 μl of plasma sample in a 1.7 ml polypropylene tube, and the solution was vortex mixed for 10 s. After centrifugation at 17,000 × *g* for 10 min, a 5 μl aliquot of the supernatant was injected into the LC–MS/MS system.

### 2.3. Liquid chromatography and mass spectrometry

For LC–MS, Agilent (Wilmington, DE, USA) 1100 series HPLC system equipped with Agilent 1946D mass selective detector was used. Silica based $C_8$ column (Zorbax RX $C_8$, 5 μm, 150 mm × 2.1 mm) was also from Agilent. HPLC eluent was prepared by mixing 1 ml of formic acid with 500 ml deionized water and 500 ml acetonitrile. Flow rate was 0.4 ml $min^{-1}$. Agilent Chemstation was used for data management. Electrospray ionization was performed using nitrogen as nebulizing gas at 12 × l $min^{-1}$ flow rate, 40 psig nebulizing pressure, and 350 °C drying gas temperature. Capillary voltage and fragment voltage were set at 3000 and 150 V, respectively. Three channels of positive ion selective ion monitoring (SIM) mode were used to detect *m*/*z* 368 (propiverine), *m*/*z* 384 (propiverine N-oxide) and *m*/*z* 358 (oxybutynin), respectively.

For LC–MS/MS, Waters (Milford, MA, USA) Alliance system equipped with Waters Micromass Quattro Premier triple quadrupole mass spectrometer was used. Initially same HPLC conditions as for LC–MS were used. Later the acetonitrile content was increased to decrease the chromatographic run time and we found that eluent prepared by mixing 1 ml of formic acid with 250 ml deionized water and 750 ml acetonitrile was appropriate for LC–MS/MS.

Micromass Masslinx 4.0 and Quanlinx were used for data management. Electrospray ionization was performed using nitrogen as desolvation gas at 600 × l $h^{-1}$ flow rate, at 50 × l $h^{-1}$ cone gas flow rate and 350 °C desolvation temperature. Collision cell gas pressure was $3.3 \times 10^{-3}$ mbar. Other ion source parameters were as follows: capillary voltage, 3 kV; cone voltage, 30 V; extractor, 3 V; RF lens, 0.2 V; source temperature, 120 °C. MS analyzer parameters were as follows: resolution 1, 10(LM) and 5(HM); ion energy 1, 1.0 V; collision cell entrance potential, 1.0 V; collision energy, 30 V; collision cell exit potential, 20 V; resolution 2, 10(LM) and 10(HM); ion energy 2, 1.0 V; multiplier, 650 V; dwell time, 0.1 s. Three channels of positive ion multiple reaction monitoring (MRM) mode were used to detect *m*/*z* 368 → 116 (propiverine), *m*/*z* 384 → 183 (propiverine N-oxide) and *m*/*z* 358 → 142 (oxybutynin), respectively.

### 2.4. Method validation and clinical test

Quantitative analyses were performed using internal standard. Standard calibration curves were obtained by the chromatographic area ratios of propiverine and propiverine N-oxide against oxybutynin. Concentrations of both propiverine and propiverine N-oxide were calculated from their area ratio and the calibration curve.

The intra- and inter-day accuracy and precision were studied by performing five separate analyses per day for 5 days at eight propiverine and propiverine N-oxide concentrations. Lower limit of detection (LOD) was obtained by means of signal to noise ratio (S/N) = 3 and lower limit of quantitation (LLOQ) was obtained by means of S/N = 10. Precision and accuracy of LLOQ was required to be within 20% by the Korean Food and Drug Administration.

For clinical test, 24 healthy male volunteers were given a single oral dose of propiverine hydrochloride (20 mg). Plasma samples were obtained by centrifuging blood samples collected before dosing and at 0.5, 1, 2, 3, 4, 5, 6, 8, 12, 24, 48 and 72 h after intake. Plasma samples were frozen at −30 °C until analysis. Three hundred and twelve clinical samples were divided into four batches. Each batch included an eight-point calibration samples before clinical samples and two replicates of three-point quality control samples after clinical samples. The precision and accuracy of the intermediate samples were evaluated using quality controls.

## 3. Results and discussion

### 3.1. Optimization of the LC–MS method

As acetonitrile and methanol are the organic modifiers of choice in LC–MS, both were tested. Among acetic acid, ammonium acetate, and formic acid tested as an additive to the mobile phase, formic acid gave the shortest retention time and the highest peak. Five concentrations of formic acid (0.05, 0.1, 0.2, 0.5 and 1%) were tested and 0.1% was selected, because ionization of propiverine and propiverine N-oxide appeared most efficient at this concentration. Thus, 1 ml of formic acid was added to the mixture of 500 ml of deionized water and 500 ml of organic modifier. Propiverine was eluted at 1.5 min using acetonitrile. Retention time was 9.1 min and peak area was only 53% using methanol instead of acetonitrile, which seems to have caused increased retention time and LLOQ in Komoto et al. [11]; thus acetonitrile was selected. An adequate chromatographic separation was achieved with a total run time of 2.5 min using 0.1% formic acid in water/acetonitrile (50:50, v/v). Propiverine was eluted at 1.5 min, propiverine N-oxide at 2.1 min and oxybutynin at 1.2 min as shown in a representative chromatogram in Fig. 2. No interfering peaks appeared around these analyte peaks.

For sample preparation, a simple deproteination with two volumes of acetonitrile was tried. LLOQ determined from S/N = 10 was 5 ng $ml^{-1}$ with injection volume of 5 μl and

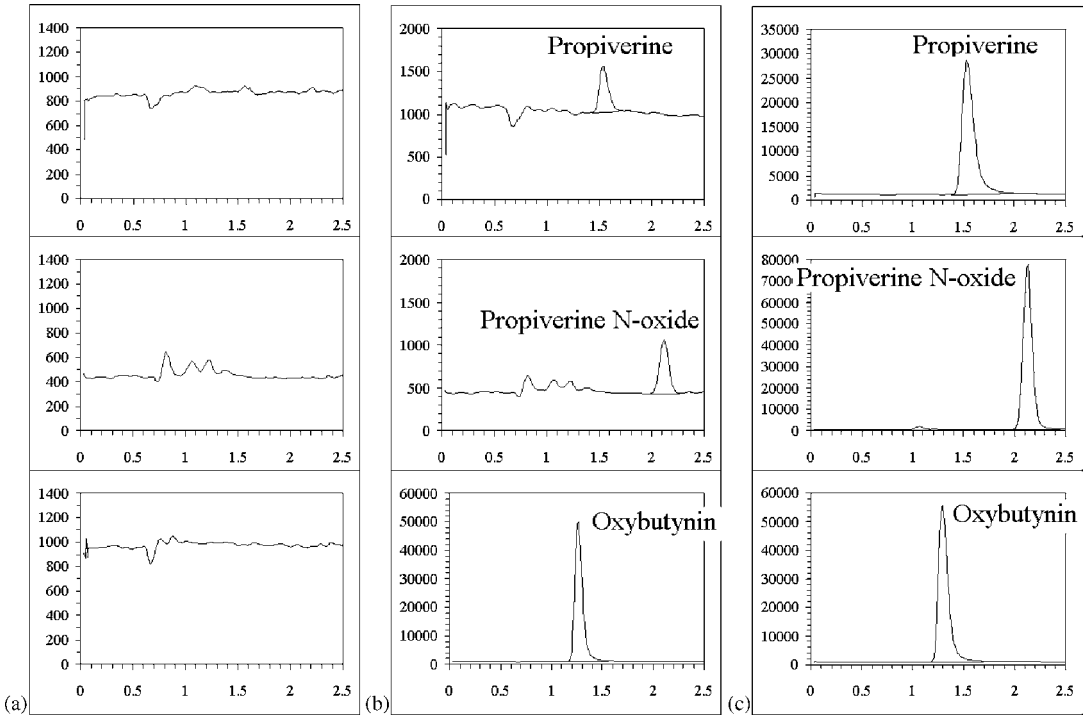


Fig. 2. LC–MS chromatograms from (a) blank plasma, (b) plasma spiked with 1 ng ml$^{-1}$ of propiverine and 2 ng ml$^{-1}$ propiverine N-oxide, and (c) sample plasma (measured propiverine concentration: 62.73 ng ml$^{-1}$ and propiverine N-oxide: 207.34 ng ml$^{-1}$).

good chromatographic separation within 7 min was achieved with 0.1% formic acid in water/acetonitrile (30:70, v/v). To decrease LLOQ and analysis time liquid–liquid extraction was tried. Recovery of propiverine, propiverine N-oxide and oxybutynin was examined using seven different extraction solvents (Fig. 3, Table 1). MTBE was chosen for high recovery of analytes and its low boiling point saved evaporation time. To minimize the waste, volume of MTBE was reduced to 1 ml maintaining satisfactory recovery. LLOQ of propiverine was reduced to 1 ng ml$^{-1}$ with 2 μl injection volume.

In the ESI MS detection, change of capillary voltage did not significantly change the ionization efficiency. Fragment voltage, however, had two important effects. Although 100 V is the default voltage recommended by Agilent, peak intensity of propiverine, propiverine N-oxide and oxybutynin was enhanced by as much as 52% at 150 V compared to 100 V. The baseline ion count was reduced by 22%. Therefore, fragment voltage was set at 150 V. At either 100 or 150 V, all of the propiverine, propiverine N-oxide and oxybutynin gave a sharp signal at *m*/*z* 368, 384 and 358, respectively.

### 3.2. Optimization of the LC–MS/MS method

To optimize the LC–MS/MS method capillary voltage, cone voltage and collision energy were varied. Using MS scan mode at optimal voltages, propiverine, propiverine N-oxide and oxybutynin gave sharp signals at *m*/*z* 368, 384 and 358, respectively. Tandem mass spectra for all the analytes are shown in Fig. 2. The most abundant product ions at the selected voltages were *m*/*z* 116 from the parent *m*/*z* 368 ion, 183 from 384 and 142 from 358. Therefore, multiple reaction monitoring (MRM) mode was used at *m*/*z* 368 → 116 for propiverine, *m*/*z* 384 → 183 for propiverine N-oxide and *m*/*z* 358 → 142 for oxybutynin. Optimum tandem MS parameters including resolution, ion energy, entrance and exit potential and collision energy were set to maximize the product ions and the optimum values mentioned in the experimental section were obtained.

The higher specificity of the LC–MS/MS method was expected to permit less rigorous sample preparation and higher acetonitrile content in the eluent. In fact, the total chromatographic run time could be reduced to 2 min using 75% acetonitrile in the mobile phase with propiverine at 1.3 min, propiverine N-oxide at 1.6 min and oxybutynin at 1.2 min, even though simple deproteination with two volume of acetonitrile was used instead of solvent extraction. This result illustrated clearly demonstrated the advantage of MS/MS detection. LLOQ was 1 ng ml$^{-1}$ for propiverine with injection volume of 5 μl. Recovery of propiverine and propiverine N-oxide from plasma evaluated using three replicate samples at 100 ng ml$^{-1}$ was better than 97.5%.

### 3.3. Stability

Komoto et al. [11] have studied the stability of propiverine and propiverine N-oxide. Both propiverine and propiverine N-oxide are known to be stable at −20 °C in plasma for 6 months and their standard solutions were stable at 5 °C for 6 months. Standard solution of oxybutynin is also stable according to Kim et al. [12]. Therefore, we skipped stability

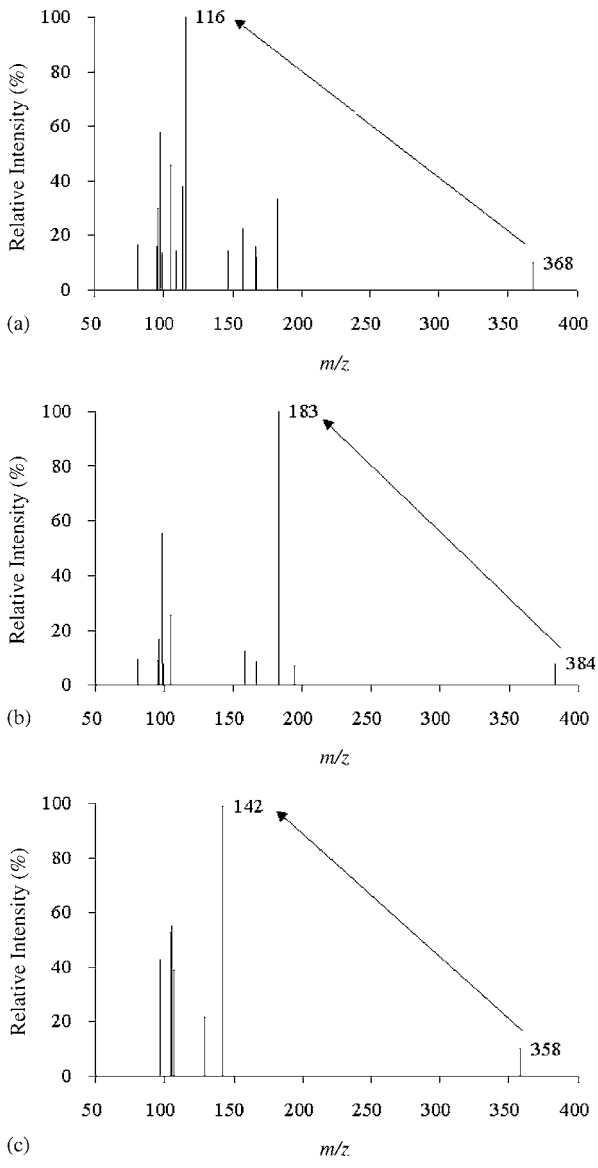


Fig. 3. MS/MS spectra of (a) propiverine, (b) propiverine N-oxide and (c) oxybutynin at collision energy of 30 eV and collision cell gas pressure of $3.3 \times 10^{-3}$ mbar.

Table 1
Recovery of propiverine, propiverine N-oxide and oxybutynin using different solvents for extraction in the LC–MS method

| | Propiverine (%) | Propiverine N-oxide (%) | Oxybutynin (%) |
|---|---|---|---|
| Dichloromethane [11] | 93.3 (±5.3) | 92.8 (±4.5) | 97.4 (±4.6) |
| Chloroform [7] | 87.7 (±7.1) | 89.3 (±4.3) | 91.7 (±6.1) |
| Ethyl ether | 41.2 (±12.1) | 37.9 (±6.4) | 35.4 (±7.8) |
| Ethyl acetate | 38.9 (±9.8) | 35.4 (±11.2) | 35.2 (±7.2) |
| *n*-Heptane/isopropanol (95/5) [8] | 101.9 (±4.7) | 99.5 (±5.4) | 106.7 (±3.7) |
| *n*-Hexane/acetonitrile (80/10) [12] | 91.0 (±6.2) | 93.5 (±8.3) | 98.4 (±5.9) |
| Methyl-*t*-butyl ether [9] | 102.0 (±4.4) | 101.5 (±3.7) | 103.4 (±5.2) |

tests of propiverine and propiverine N-oxide except for the stability after sample preparation on the auto sampler: the area ratio of propiverine and propiverine N-oxide against oxybutynin after sample preparation did not change for at least 12 h (data not shown), which indicates that the sample preparation method is appropriate.

### 3.4. Precision, accuracy, linearity and limit of detection

Within-assay and between-assay precision and accuracy determined in human plasma for four levels of concentration of propiverine and propiverine N-oxide are summarized in Table 2. All results were within appropriate range of R.S.D. (%) and accuracy (%) and were acceptable for bio-analytical applications.

Linearity of the method was evaluated with calibration curves ranging from 1 to 250 ng ml$^{-1}$ of propiverine and 2–500 ng ml$^{-1}$ of propiverine N-oxide. Each point represents an average of five determinations. Following linear regression equations, a good linear relationship between peak area ratios and concentrations was established: for propiverine, $y = 0.01056x$ (±0.00015) + 0.00200 (±0.00175); $R^2 = 0.99996$ (±0.00004), where $y$ is the peak area ratio (propiverine/oxybutynin) and $x$ is the concentration (ng ml$^{-1}$) of propiverine for LC–MS, and $y = 0.01874x$ (±0.00039) + 0.00172 (±0.00139); $R^2 = 0.99991$ (±0.00011) for LC–MS/MS, and for propiverine N-oxide, $y = 0.05702x$ (±0.00078) + 0.00351 (±0.00132); $R^2 = 0.99998$ (±0.00003), where $y$ is the peak area ratio (propiverine N-oxide/oxybutynin) and $x$ is the concentration (ng ml$^{-1}$) of propiverine N-oxide for LC–MS, and $y = 0.09183x$ (±0.00192) + 0.00845 (± 0.00238); $R^2 = 0.99994$ (±0.00006) for LC–MS/MS.

The LOD, 0.3 ng ml$^{-1}$ for propiverine and 0.6 ng ml$^{-1}$ for propiverine N-oxide were determined as the concentration of analyte at signal to noise ratio of 3. And the LLOQ, 1 ng ml$^{-1}$ for propiverine and 2 ng ml$^{-1}$ for propiverine N-

Table 2
Intra- and inter-day precision and accuracy of the LC–MS and LC–MS/MS methods for propiverine and N-oxide in human plasma

| | R.S.D. (%) | Accuracy (%) |
|---|---|---|
| Propiverine | | |
| LC–MS | | |
| Intra-day | 0.52–4.71 | 97.9–105.0 |
| Inter-day | 0.66–12.6 | 95.8–107.1 |
| LC–MS/MS | | |
| Intra-day | 1.04–6.20 | 96.9–105.0 |
| Inter-day | 1.59–10.2 | 93.9–104.7 |
| Propiverine N-oxide | | |
| LC–MS | | |
| Intra-day | 0.68–3.68 | 97.9–106.7 |
| Inter-day | 1.07–4.02 | 97.1–108.2 |
| LC–MS/MS | | |
| Intra-day | 1.56–7.96 | 96.8–101.4 |
| Inter-day | 1.02–9.57 | 95.2–105.9 |

Table 3
Summary of sample batch quality controls ($n = 8$) during analysis of samples from 24 healthy male volunteers using the LC–MS/MS method

| | Mean ± S.D. (ng ml$^{-1}$) | R.S.D. (%) | Accuracy (%) |
|---|---|---|---|
| Propiverine | | | |
| 15 | 15.3 ± 1.42 | 9.23 | 102.1 |
| 75 | 75.3 ± 1.65 | 2.19 | 100.4 |
| 150 | 153.6 ± 5.42 | 3.53 | 102.4 |
| Propiverine N-oxide | | | |
| 40 | 41.3 ± 4.22 | 10.22 | 103.2 |
| 200 | 198.5 ± 5.30 | 2.67 | 99.2 |
| 400 | 405.2 ± 9.43 | 2.33 | 101.3 |

oxide were determined at S/N = 10 and from precision and accuracy within 20% for both LC–MS and LC–MS/MS. For LC–MS, reduction of reconstitution volume after evaporation in sample preparation and increase of injection volume could enhance the sensitivity up to 10 times. However, the mean concentration of both propiverine and propiverine N-oxide at 72 h after intake was 1.69 and 2.95 ng ml$^{-1}$, respectively, which indicates 1 ng ml$^{-1}$ for propiverine and 2 ng ml$^{-1}$ for propiverine N-oxide are appropriate for LLOQ.

### 3.5. Application to the clinical test

Established methods were applied to the clinical test involving 24 subjects. Initially samples from six subjects were analyzed by both LC–MS and LC–MS/MS. The pharmacokinetic parameters obtained by both methods were almost identical. LC–MS/MS was used for samples from the remaining 18 subjects.

All 312 clinical samples were divided into four batches and each batch consisted of eight-point calibration curve, 78 samples from six subjects, and six quality controls. It took about half an hour for sample preparation and 5 h for sample analysis. All samples were analyzed in 2 days. Precision and accuracy of quality controls are summarized in Table 3. Variations in instrumental response observed within batches were minimal. Both results demonstrate the reproducibility and reliability of the analytical method. Average pharmacokinetic data for propiverine and propiverine N-oxide obtained from 24 healthy male volunteers are summarized in Table 4 and Fig. 4.

Table 4
Summary of pharmacokinetic parameters of propiverine and propiverine N-oxide from 24 healthy male volunteers given a single oral dose of propiverine hydrochloride (20 mg)

| | Propiverine | Propiverine N-oxide |
|---|---|---|
| $C_{max}$ (ng ml$^{-1}$) | 58.84 ± 28.59 | 282.52 ± 60.46 |
| $AUC_{72\,h}$ (ng ml$^{-1}$ h) | 765.4 ± 479.9 | 2410.7 ± 1148.8 |
| $AUC_{inf}$ (ng ml$^{-1}$ h) | 814.3 ± 573.4 | 2698.5 ± 1349.8 |
| $T_{max}$ (h) | 2.33 ± 0.76 | 1.33 ± 0.82 |
| $t_{1/2}$ (h) | 19.95 ± 4.72 | 16.65 ± 5.43 |

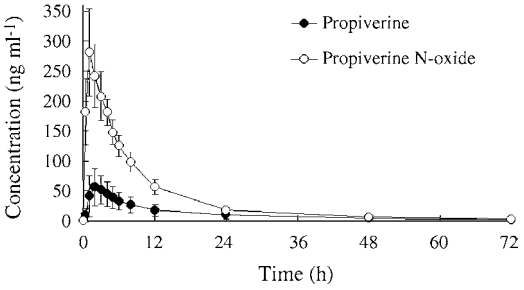


Fig. 4. Mean concentration-time pharmacokinetic profile of propiverine and propiverine N-oxide in plasma from 24 healthy male volunteers after single oral administration of propiverine hydrochloride tablet (20 mg).

## 4. Conclusion

The highly specific LC–MS/MS method could be used to determine propiverine and propiverine N-oxide in deproteinated human plasma. Solvent extraction is needed for the less specific LC–MS method. Both methods are reliable and can be used in pharmacokinetic studies with LLOQ of 1 ng ml$^{-1}$ for propiverine. The deproteination step has a potential to simplify sample preparation for LC–MS/MS analysis in many biological systems.

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