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Sensitive column-switching high-performance liquid chromatography method for determination of propiverine in human plasma

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Abstract

A sensitive column-switching high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection was developed for the determination of propiverine in human plasma. Propiverine and internal standard, oxybutynin, were extracted from human plasma that had been made basic with 5N sodium hydroxide into methyl *tert*-butyl ether. The extracted plasma sample was injected onto the HPLC system consisting of a pretreatment column, a concentrating column, and an analytical column, which were connected with a six-port switching valve. The assay was linear in concentration ranges of 2–200 ng/ml for propiverine in human plasma. This method showed excellent sensitivity (a limit of detection of 0.5 ng/ml), good precision and accuracy. This method is suitable for bioequivalence studies following single dose in healthy volunteers.

Keywords: Propiverine; Column switching; High-performance liquid chromatography (HPLC)

1. Introduction

Propiverine hydrochloride (1-methyl-4-piperidyl diphenylpropoxyacetate hydrochloride, PPV) has been developed for the treatment of hypertonic functional states in the region of the bladder, such as pollakisuria, nocturia and nocturial enuresis [1]. The urospasmolytic action of drug is due to the inhibition of calcium influx into smooth muscle cells and competitive antagonism with acetylcholine at muscarinic receptor sites [2]. Thin-layer chromatography (TLC) [3], gas chromatography (GC)–mass spectrometry (MS) [4], high-performance liquid chromatography (HPLC) [5] and LC–tandem mass spectrometry (MS/MS) methods [6–8] have been reported for the quantification of PPV in biological fluids. Among these methods, TLC method has been limited by the lack of sensitivity and selectivity and GC–MS method is complicated because of time-consuming derivatization and extraction procedures. LC/MS/MS system is not readily available in most clinical research laboratory because of cost although LC/MS/MS method has been revealed to be a powerful technique for the analysis of PPV in biological fluids with high sensitivity. HPLC/UV is a common method used for the determination of drugs in biofluids and has been developed for the determination of PPV. However, this was inadequate in our hand for bioequivalence study of PPV because of low sensitivity and specificity. More sensitive and specific methods are therefore required for the analysis of PPV in plasma. Recently, a number of papers have been published on the subject of HPLC column-switching [9–13]. Some of these papers showed that HPLC column-switching method can increase the sensitivity and specificity [9,10]. Therefore, in the present study, we describe the development and validation of a sensitive and specific column-switching HPLC method for the detection of PPV in human plasma using liquid–liquid extraction to accomplish the bioequivalence study of PPV. In this study, PPV was only analyzed because the analysis of metabolites is not required in bioequivalence study. This analytical method was applied to determine the level of PPV following a single dose in human healthy volunteers.

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2. Experimental

2.1. Chemical and materials

Propiverine (PPV) was obtained from Hanmi Pharm Co. Ltd (Seoul, Korea). As an internal standard (IS), oxybutynin chloride was purchased from Sigma (St. Louis, MO, USA). The chemical structures are showed in Fig. 1. The purity of PPV and IS was more than 95%. Acetonitrile and methyl-*tert*-butyl ether (MTBE) were obtained from Burdick & Jackson (Muskegon, MI, USA). Water was purified by a Milli-Q system (Millipore Corp., Bedford, MA, USA). All other reagents were of analytical reagent grade and used without further purification.

2.2. Standard solutions

Stock solutions of PPV and IS were prepared by dissolving the drug in methanol to a final concentration of 50 μg/ml and further diluted into 2–200 ng/ml for the preparation of plasma calibration standards. All solutions were stored at −20 °C. Using these standard solutions, seven calibration standard (CS) solutions containing 2.0, 5.0, 10.0, 25.0, 50.0, 100 and 200 ng/ml and quality controls (QC) solutions at concentrations of 2.0, 25.0 and 200 ng/ml were prepared in human plasma.

2.3. Instruments

PPV was determined by column-switching HPLC with UV detector. All experiments were performed using an automated semi-microbore HPLC Nanospace SI-1 series (Shiseido, Tokyo, Japan) equipped with two 2001 pumps, a 2002 UV–vis detector, a 2003 autosampler, a 2004 column oven, a 2012 high-pressure switching valve and a 2009 degassing unit as schematically described in Fig. 2.

A Capcell Pak MF Ph-1 cartridge (20 mm × 4.0 mm i.d., 5 μm particles, Shiseido, Japan) was used for selective adsorption of PPV in plasma. A Capcell Pak C_{18} UG 120 V column

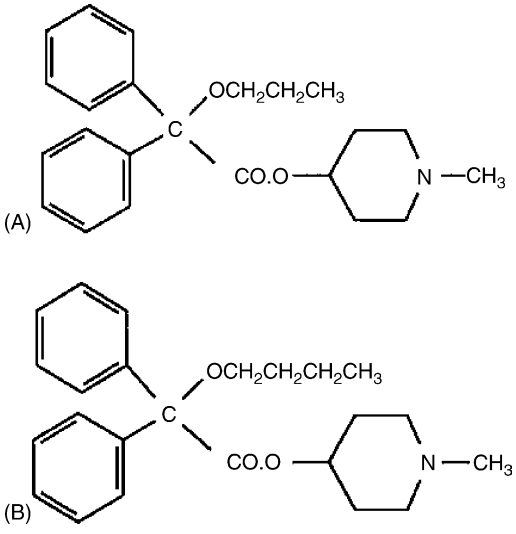

Fig. 1. Chemical structures of (A) propiverine (PPV) and (B) oxybutynin as an internal standard (IS).

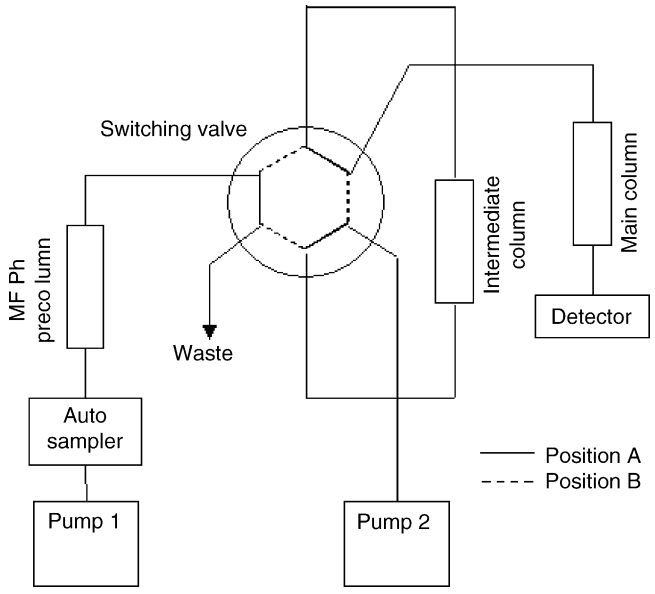

Fig. 2. Schematic diagram of a column-switching system.

(35 mm × 2.0 mm i.d., 5 μm particles, Shiseido) was used for concentrating PPV as an intermediate column. A Capcell Pak C_{18} MG II column (250 mm × 1.5 mm i.d., 5 μm particles, Shiseido) was used as a separation column. The pretreatment and separation column temperature was maintained at 30 °C during analysis. However, the concentration column was operated at room temperature.

2.4. Semi-microbore column-switching chromatographic conditions

The operation of this column-switching semi-micro HPLC consists of three main steps: sample loading and primary separation, enrichment of the analyte fraction and chromatographic separation.

When the column-switching valve was at the A position, an aliquot of extracted plasma sample was loaded to precolumn and primary separation of PPV and IS from plasma sample were performed using mobile phase 1 at a flow rate of 0.4 ml/min. Then, the valve was switched to the B position, and PPV and IS fraction were eluted from precolumn and concentrated in enrichment column by mobile phase 1 [28% (v/v) acetonitrile in 30 mM phosphate buffer (pH 7.5)] at a flow rate of 0.4 ml/min. Then, the valve position was returned to A, and PPV and IS concentrated in the enrichment column were separated on an analytical column using mobile phase 2 [40% (v/v) acetonitrile in 30 mM phosphate buffer (pH 2.5) and 0.02% TEA] at a flow rate of 0.15 ml/min. UV detection was performed at 220 nm using a chromatogram integration software dSChrom (Donam Instrument, Korea).

2.5. Extraction procedure

Extraction of PPV in plasma was conducted by the reported method [5] with slight modification. Briefly, a 20 μl of 5N NaOH solution and 5 μl of internal standard solution (50 μg/ml) were

added to 1 ml of plasma sample in a glass test tube, respectively. The tube was vortex-mixed for 10 s and then 5 ml of MTBE was added as extraction solvent. After 30 s of shaking, the samples were centrifuged at 3000 × *g* at 4 °C for 10 min and the organic phase was evaporated under a steam of nitrogen gas at 40 °C. The dried analytes were reconstituted using 100 μl of mobile phase 1, and the solutions were vortex-mixed for 10 s. A 100 μl aliquot was transferred to autosampler vial and a 70 μl aliquot was injected into the HPLC system.

2.6. Method validation

2.6.1. Recovery

The extraction recovery was calculated by comparing the peak area of the extracted quality control sample to that of the unextracted standard solution containing the same concentration. Also, the transfer recovery by column-switching system was evaluated at the concentrations of 2, 25 and 200 ng/ml in mobile phase.

2.6.2. Linearity and limit of quantification

Linearity was demonstrated by running plasma standards at seven concentrations over the range of 2–200 ng/ml for five consecutive days. Peak area ratios of each PPV to IS were measured and the calibration curve was obtained from least-square linear regression (no weighing factor). The suitability of the standard curve was confirmed by back-calculating the concentrations of the standard curve. The lower limit of quantification (LLOQ) was defined as the concentration of the sample that can be quantified within 20% deviation. The limit of detection (LOD) for PPV was defined as the drug concentration in the plasma after the sample clean-up method that corresponds to three times the signal-to-noise ratio for PPV.

2.6.3. Precision and accuracy

Precision and accuracy were assessed in conjunction with the linearity studies using PPV-spiked plasma samples at each of four concentrations (nominally, 2, 10, 50 and 200 ng/ml) on five different days. The coefficient of variation (CV) was calculated from the ratio of the standard deviation (S.D.) to the mean. Accuracy was comparing the differences between the spiked value and the real concentrations, and determined from the bias calculations.

2.6.4. Stability

The short-term stability of PPV was examined by keeping replicates of PPV-spiked samples at room temperature for 24 h. Freeze–thaw stability of the sample was obtained over three freeze–thaw cycles, by thawing at room temperature for 2–3 h, refrozen for 12–24 h. Autosampler stability of PPV in the autosampler tray was evaluated by the analysis of processed and reconstituted low and high concentrations of PPV obtained from PPV-spiked plasma test samples for 24 h. Long-term stability of PPV in human plasma was tested after storage at −70 °C for 2 months. For each concentration and each storage condition, six replicates were analyzed in one analytical batch.

2.6.5. Ruggedness

Ruggedness test was conducted by different analysts and columns.

2.7. Application in one volunteer's plasma

Informed consent was obtained from three volunteers in this study. One tablet containing 20 mg of PPV from Hanmi Pharm Co. was administered to the healthy male volunteers. Venous blood samples were collected in heparinized tubes during the 0–72 h time interval after administration. The plasma collected before the dose was employed as a blank. All blood samples were centrifuged immediately, the plasma separated and stored at −70 °C until analysis.

3. Results

3.1. Optimization of chromatographic conditions

In this study, the optimum column-switching HPLC condition for the detection of PPV in human plasma was investigated. In column switching, the choice of washing solvent and column-switching time is crucial in order to obtain complete recovery and clean chromatograms [14]. It was therefore, tested various combinations of the mobile phase composition and pH using acetonitrile and 30 mM sodium phosphate in order to decide the optimum column-switching time.

Mobile phase pH at 6.5, 7.5 and 8.5 and acetonitrile percentages of 25, 28 and 30% were investigated in precolumn. The decrease in pH resulted in a reduction in resolution between PPV and IS due to the decrease of retention time. The result was an overlapping between the analyte peaks at pH 7.5, which consequently led to a clean chromatogram within short time (Fig. 3). Although an overlapping of peaks was shown at lower pH than pH 7.5, it was not possible to obtain adequate separation between analyte and endogenous compounds because the retention time under these conditions was very short. The retention time for PPV and IS was longer as the percentage of acetonitrile was decreased. The mobile phases containing acetonitrile below 28% (v/v) showed very long retention time for PPV and that containing acetonitrile higher than 28% (v/v) gave more interference plasma peaks in column switching. Therefore, the optimum condition of mobile phase in precolumn was acetonitrile–30 mM phosphate buffer (pH 7.5) (28:72, v/v). To determine the appropriate time for column-switching, the separation profile of PPV and IS in plasma on MF Ph-1 precolumn was evaluated using this mobile phase. According to these results, time program and switching valve position are shown in Table 1.

The analytes isolated from precolumn by valve switching step were focused on the top of intermediate C18 column to obtain sharp peaks in final separation. And then, the analytes were separated in analytical column.

To achieve better separation and peak shapes, the mobile phase containing varying pH and acetonitrile content and additive were tested in analytical column. Mobile phases containing phosphate buffer of various pH values ranging from 2.5 to 10 and 40% acetonitrile were investigated for elution from the

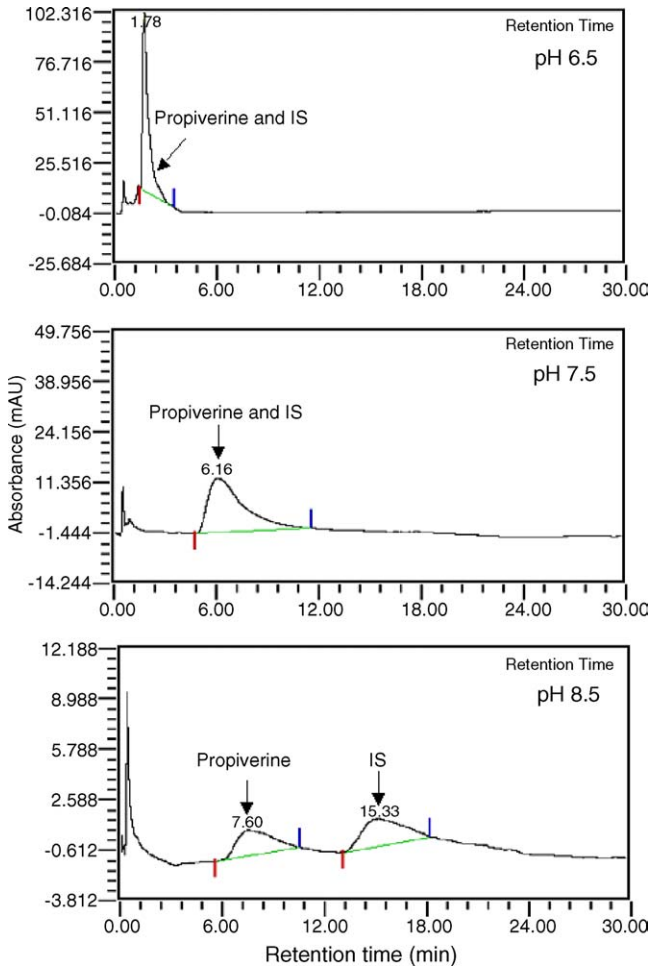

Fig. 3. Effect of the mobile phase pH on retention of propiverine in precolumn (acetonitrile content was kept constant at 28%).

separation column. In this study, the pK_a values of PPV and IS are close to 8.5 and 8.0, respectively, and thus, the retention time for PPV and IS was reduced as the pH was decreased. As a result, it was not possible to obtain reproducible results within pH range of 6.0–9.0 because even minor pH changes affected the resolution and retention time of peaks. A stable retention time for PPV and IS was achieved at the pH values higher than 9.0. However, the complete separation of two peaks was not achieved. Based on these results, a mobile phase pH of 2.5 was chosen for the analytical separation. Different percentages of acetonitrile as organic modifier was also tested. The suitable condition was obtained with an analytical mobile phase composed of 40% acetonitrile in 30 mM phosphate buffer pH 2.5.

Table 2
Assessment of recovery ($n = 3$)

Propiverine concentrations (ng/ml)	Recovery (%) ± S.D.
2	91.4 ± 7. 4
25	101.6 ± 1.9
200	96.6 ± 1.4

However, severe tailing of PPV peak was observed in this condition. Thus, triethylamine (TEA) was added to mobile phase because with an addition of TEA to mobile phase sharp and symmetric peaks can be obtained without any effect on UV signal. In order to determine appropriated TEA concentration, various concentrations of TEA were tested and successful improvement in peak symmetry was observed at 0.02%. As a result, the optimum separation PPV and IS in plasma was achieved using a mobile phase consisting of mixture of acetonitrile–30 mM phosphate buffer pH 2.5 (40:60, v/v) containing 0.02% TEA. Fig. 4 shows typical chromatograms of blank plasma, plasma sample spiked with PPV at LOD (0.5 ng/ml), and plasma sample obtained from a healthy subject following an oral 20 mg dose of PPV. The retention time of IS and PPV was approximately 14.9 and 17.4 min, respectively, with complete baseline separation between peaks of interest. No interfering peaks from the endogenous plasma components were observed at the retention time of PPV. There were minor peaks near the IS region but these minor peaks were less than 5% of IS peak height near the IS region and did not affect quantification.

3.2. Validation

3.2.1. Recovery

The extraction and transfer recovery were excellent for PPV at concentrations of 2, 25 and 200 ng/ml. The mean extraction recovery of PPV for human plasma was higher than 91.4% (Table 2) and the transfer recovery by column-switching system was higher than 99.3%.

3.2.2. Linearity and sensitivity

The calibration curves were linear over the concentration range from 2 to 200 ng/ml. Typical equation for the calibration curve for PPV was $y = 0.0075(\pm 0.0003)x + 0.0058(\pm 0.0030)$. The correlation coefficient for PPV was greater than 0.9996. The limit of detection (LOD) for PPV was 0.5 ng/ml as shown in Fig. 4C and this sensitivity was showed similar result when compared to the other LC/MS/MS method [8]. The lower limit of quantification (LLOQ) for PPV was estimated as 2 ng/ml.

Table 1
Time schedule of column-switching HPLC for the analysis of propiverine (PPV) and oxybutynin (IS)

Time after injection (min)	Switching valve position	Comments
0.0–7.7	Loading (A)	Sample loading (70 μl) by mobile phase 1 at 0.4 ml/min
7.7–8.7	Concentration (B)	Transfer of PPV and IS from pre-column to intermediate column by mobile phase 1 at 0.4 ml/min
8.7–20	Separation (A)	Intermediate column back-flush onto the analytical column followed by mobile phase 2 at 0.15 ml/min

Mobile phase 1: 28% (v/v) acetonitrile in 30 mM phosphate buffer (pH 7.5); mobile phase 2: 40% (v/v) acetonitrile in 30 mM phosphate buffer (pH 2.5) containing 0.02% TEA.

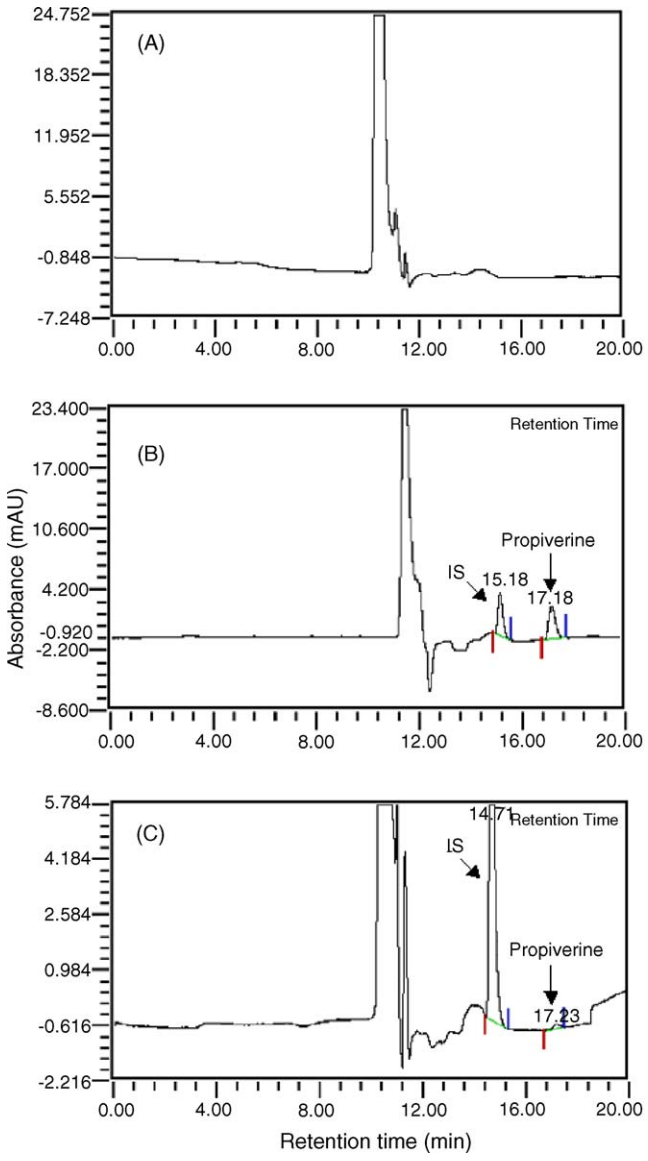

Fig. 4. Chromatograms of (A) blank plasma, plasma sample spiked at (B) plasma sample from volunteer plasma at 1.5 h after a single 20 mg oral dose of propiverine and (C) 0.5 ng/ml (LOD) of propiverine.

LLOQ value of PPV was lower than that obtained by previous HPLC (2 ng/ml versus 10 ng/ml) [5].

3.2.3. Precision and accuracy

The results shown in Table 3 indicate that the assay method is reproducible for the replicate analysis of PPV in human plasma within the same and different days. The precision values for intra- and inter-assay at the LLOQ and at 10, 50 and 200 ng/ml of PPV in plasma were reproducible with an average intra-day CV less than 8.0%, an average inter-day CV less than 7.7%. The accuracy values at the LLOQ and at 10, 50 and 200 ng/ml of PPV in plasma were within 102.8%.

Table 3
Reproducibility of Propiverine in human plasma ($n = 5$)

Propiverine concentrations (ng/ml)	Precision (CV, %)		Accuracy (%)
	Intra-day	Inter-day	
2 (LOQ)	8.1	6.9	102.8
10	7.7	7.7	96.2
50	7.4	5.0	100.8
200	1.6	4.8	100.0

Table 4
Stability of the samples (stability % ± S.D., $n = 3$)

	Propiverine concentrations (ng/ml)		
	2	25	200
Freeze–thaw	86.0 ± 9.9	88.5 ± 0.3	93.1 ± 2.4
Short-term (24 h)	96.7 ± 4.8	93.1 ± 0.4	96.2 ± 0.5
Long-term (2 months)	82.5 ± 12.0	92.4 ± 0.6	86.7 ± 0.2
Autosampler (24 h)	95.9 ± 8.7	100.2 ± 1.5	95.8 ± 0.2

3.2.4. Ruggedness

When the method was validated on other analytical columns by another analyst, the CV value was less than 5.0% at the LLOQ and at 10, 50 and 200 ng/ml of PPV in plasma at the same chromatographic condition.

3.2.5. Stability

The stability of PPV was investigated after storing PPV-spiked plasma samples at −70 °C for 2 months. During the storing period, three freeze–thaw cycles, short-term and autosampler stability were carried out. All stability results were presented in Table 4. The mean concentrations following this storage period were higher than 82.72 ± 11.95% ($n = 3$) of the normal value, indicating that plasma samples containing PPV were stable at −70 °C for at least 2 months.

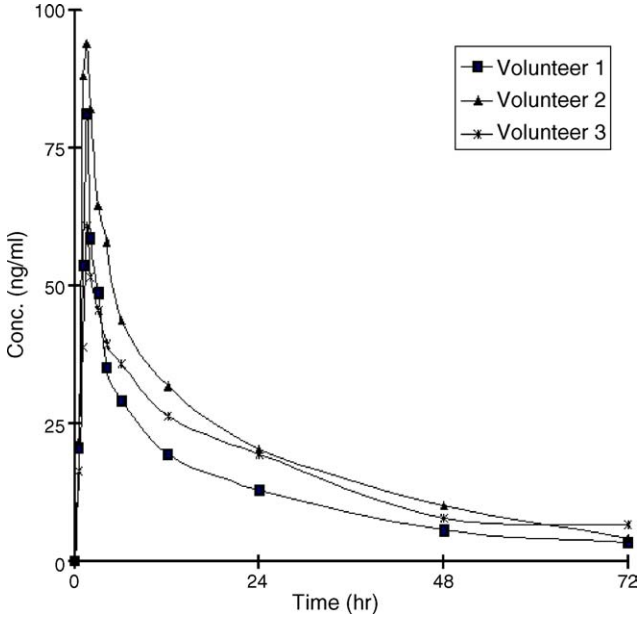

Fig. 5. Plasma concentration–time profiles of PPV in three healthy male volunteers following oral administration of 20 mg of propiverine tablet.

3.3. Application to human plasma

Three male volunteers were orally administrated with one 20 mg PPV tablet. The concentration–time profiles of PPV in plasma were monitored for 72 h as shown in Fig. 5. The mean maximum PPV plasma concentration (C_{max}) was 78.6 ng/ml and mean time to reach the C_{max} (T_{max}) was 1.5 h. The sensitivity in the present simple HPLC method showed enabling the monitoring of plasma concentrations of PPV up to 72 h after the dose.

4. Discussion

Previous studies using HPLC/UV, have shown simultaneous determination of PPV and its metabolites in biofluids. However, these methods were inadequate in our lab system because of low sensitivity and specificity between PPV and its metabolite. Thus, in order to develop a HPLC/UV method with sufficient sensitivity and specificity, a column-switching system was investigated. In this study, metabolites of PPV and other endogenous components in plasma were excluded using column-switching system. Only PPV was analyzed and detected because our investigation purpose was to develop an analytical method for the bioequivalence study of PPV, and this study is not necessarily an analysis of metabolites.

5. Conclusion

An HPLC method was developed using column-switching and liquid–liquid extraction for the determination of PPV in human plasma. This method showed excellent sensitivity (0.5 ng/ml), reproducibility, specificity and speed (analysis time 20 min). Especially, the LLOQ value was five-fold lower than that obtained by Richter et al. [5], and LOD was close to that obtained by LC/MS/MS method [7,8]. In conclusion, this paper describes a simple, specific and sensitive HPLC method for the determination of PPV in human plasma. Moreover, the limit of quantification obtained allows the measurement of bioequivalence studies of PPV using human volunteers.

Acknowledgement

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