Determination of Pinaverium Bromide in Human Plasma by a Sensitive and Robust UPLC–MS-MS Method and Application to a Pharmacokinetic Study in Mexican Subjects

Omar Patiño-Rodríguez1,3, Juan Ramón Zapata-Morales1,4, Abraham Escobedo-Moratilla1,5, Manuel Díaz de León-Cabrero1, Irma Torres-Roque2 and José Pérez-Urizar2*

1Dixpertia, Investigación Biofarmacéutica y Farmacológica S.C., San Luis Potosí, SLP, México, and 2Laboratorio de Farmacología y Fisiología, Facultad de Ciencias Químicas, UASLP, San Luis Potosí, SLP, México

Received 19 June 2014; revised 29 December 2014

A high-throughput ultra-performance liquid chromatography coupled to tandem mass spectrometry (LC–ESI–MS–MS) method was developed for the determination of pinaverium bromide in human plasma. Protein precipitation with acetonitrile–5 mM ammonium formate (80:20, v/v) was used as mobile phase. Isocratic elution at 0.3 mL/min was used. Detection was performed by positive ion electrospray tandem mass spectrometry on a XEVO TQ-S by multiple reaction monitoring mode. The mass transitions monitorized were as follows: m/z 511.2 → 230 for pinaverium bromide, and m/z 705.29 → 392.18 for the itraconazole. The method was validated over a concentration range of 12–12,000 pg/mL. A high-throughput ultra-performance liquid chromatography (UPLC) coupled to tandem mass spectrometry (MS/MS) (6). The UPLC–MS-MS method showed to be quicker and more accurate than the GC–MS method (6); nevertheless, the sample preparation for the HPLC method is based on a protein precipitation process, which can lead to a shortened useful life column and consequently increase the cost of the analyses. On the other hand, ultra-performance liquid chromatography (UPLC) coupled to MS–MS is a more sensitive, faster and more accurate technique than HPLC–MS–MS; this is due to the higher operating pressure and the smaller particle size in the stationary phase, leading to a higher resolution in chromatographic analysis (8, 9). The purpose of this study is to develop and validate an UPLC–MS–MS method with a simple sample preparation for the determination of PNV in human plasma. The data attained from analyzed samples were used to sustain a pharmacokinetic study of the oral administration of PNV in healthy Mexican subjects.

Introduction

Irritable bowel syndrome is a frequent disease in Mexican population (1) and the antispasmodics agents such as pinaverium bromide (PNV), mebeverine, ondansetron, hyoscine and trimetamitine (2) are among the most prescribed drug treatments in Mexico. PNV is a quaternary ammonium compound, which relaxes gastrointestinal structures primarily by inhibiting Ca2+ influx through potential-dependent channels in surface membranes of smooth muscle cells (3). PNV is effective in the treatment of other abnormal intestinal conditions including abdominal pain, diarrhea, constipation (4), higher antispasmodic activity, and to increase patients’ tolerability to otilonium, prifinium bromide and other antispasmodic agents (4). To date, little information of PNV pharmacokinetics has been reported, probably due to its low absorption through oral administration (5, 6). Since only a small amount of PNV can be found in patient’s plasma, due to its low bioavailability a linear, sensitive, selective analytical method for PNV in human plasma is a challenge. Few methods for PNV quantitation in plasma have been reported (6, 7). One of these methods is a multistep extraction followed by gas chromatography (GC) analysis coupled to mass spectrometry (MS) detection system (7); another method reported for the determination of PNV has fewer extraction steps and is followed by high-performance liquid chromatography analysis (HPLC) coupled to mass tandem spectrometry (MS/MS) (6). The HPLC–MS–MS method showed to be quicker and more accurate than the GC–MS method (6); nevertheless, the sample preparation for the HPLC method is based on a protein precipitation process, which can lead to a shortened useful life column and consequently increase the cost of the analyses. On the other hand, ultra-performance liquid chromatography (UPLC) coupled to MS–MS is a more sensitive, faster and more accurate technique than HPLC–MS–MS; this is due to the higher operating pressure and the smaller particle size in the stationary phase, leading to a higher resolution in chromatographic analysis (8, 9). The purpose of this study is to develop and validate an UPLC–MS–MS method with a simple sample preparation for the determination of PNV in human plasma. The data attained from analyzed samples were used to sustain a pharmacokinetic study of the oral administration of PNV in healthy Mexican subjects.

Experimental

Analytical standards of PNV (purity of 99.1%) and itraconazole (IS) (purity of 22.7%) were donated by a pharmaceutical company (RIMSA, Guadalajara, Mexico). Commercial formulations PNV in 100 mg capsules (Dicetel®; Abbott Laboratoríes de México, S. A. de C. V.) were used for the pharmacokinetic study. Acetonitrile LC–MS grade (EMD-Chemicals, NY, USA) and Ammonium formate HPLC grade (Fluka, VA, USA) were used. All working solutions were prepared with deionized water.

Chromatographic analysis was performed on a Waters XEVO TQ-S (MA, USA), an UPLC–MS–MS commercial system consisting of an Acquity UPLC coupled to tandem mass spectrometry detection system. The XEVO TQ-S system was equipped with an Waters Acquity UPLC BEH C18 column (1.7 μm, 2.1 × 100 mm, MA, USA). The mobile phase consisted of acetonitrile–5 mM ammonium formate buffer solution (80:20, v/v) at a flow rate of 0.3 mL/min. The run time was 2.5 min and the sample volume injected was 1.0 μL. The column temperature was set

© The Author 2015. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com
to 40°C. The autosampler cooler was set to 8°C. The tandem mass spectrometer was set in multiple reaction monitoring (MRM) mode and in ESI positive ionization mode. Collision energy and cone voltage were 18 and 15 V, respectively. Cone and desolvation gas flow rates were set to 150 and 200 L/h, respectively, using Argon as collision gas at a flow rate of 0.17 mL/min. Mass spectrometry tandem was used based on the conditions previously reported by Ren et al. (6). The system was tuned to monitoring m/z 511.2 → 230 m/z transition for PNV and m/z 705.29 → m/z 392.18 transition for the IS, with a dwell time of 0.3 s. MRM data were acquired and analyzed through Waters MassLynx software (MA, USA).

A stock solution (1.0 mg/mL) of PNV was prepared by dissolving it in methanol, additional working dilutions of 100 and 1,000 ng/mL were prepared from the 1.0 mg/mL stock. An IS stock solution was prepared (1.0 mg/mL) in methanol and added to PNV samples.

The concentration of calibration standards and quality control samples were chosen based on the expected concentration in human samples according to the literature (6), with an increase in the upper limit of the range to cover possible variability as there are no reports of bioavailability of PNV in Mexican subjects. The PNV calibration curve (12, 720, 3,000, 4,800, 7,800, 9,600 and 12,000 pg/mL) was prepared by serial dilution of the stock solution. Quality control (QC) samples were prepared in the same way for PNV concentrations of 1,440 (low, QCL), 6,000 (medium, QCM) and 10,800 pg/mL was prepared by serial dilution of the stock solution. Quality control (QC) samples were prepared in the same way for PNV concentrations of 1,440 (low, QCL), 6,000 (medium, QCM) and 10,800 pg/mL (high, QCJ). The calibration curve and quality control concentrations were proposed according to Mexican regulations (NOM-177-SSA1). All standard stock solutions were prepared once a month and stored at −20°C.

Frozen 0.5 mL plasma samples were thawed at room temperature, then 10 μL of calibration curve or quality control solutions each and 10 μL of IS solution were added to each sample. To promote protein precipitation, 1.0 mL of acetonitrile was added to each sample, and the mixture was vortexed for 0.5 min and centrifuged for 7 min at 14,000 g on a bench top centrifuge Eppendorf 5418 (Hamburg, Germany). The supernatant was transferred to a fresh microtube and frozen for 5 min at −80°C. Latter, a new centrifugation cycle was performed (5 min at 14,000 g) and 150 μL of supernatant was transferred to a glass total recovery autosampler vial. The sample was injected into the LC–MS–MS system for analysis.

Validation procedure
The validation was performed according to the current international guidelines (10, 11). The limit of quantitation (LOQ) was determined by injecting decreasing concentrations of PNV into the analytical system to determine the minimal concentration providing a signal-to-noise ratio of > 10 with adequate precision and accuracy (LOQ of 80–120%). Calibration standards and blanks were prepared and analyzed in duplicate to establish the calibration range with acceptable accuracy and precision (calibration standards except LOQ, 85–115%). The acceptance criteria for the calibration curve were a linearity of r ≤ 0.99 and r² ≤ 0.98, with at least five calibration points. The analyte–IS ratio (response) was calculated for each sample by dividing the area of the PNV peak by the area of the IS peak. Standard curves of PNV were constructed using linear regression analysis by plotting the response against the PNV (theoretical) concentration in each sample. The accuracy and precision of the assay were determined by analyzing five replicates of PNV samples at the LOQ, QCL, QCM and QCH, and this set was prepared and analyzed five times each in an independent analytical run, with the freshly prepared calibration curves as described above. Back-calculated concentrations of calibration and quality control samples were estimated using the equation generated via linear regression analysis. Accuracy was calculated as the relative difference (% Diff) between the back-calculated concentration and the theoretical concentration using 1/x weighting equation (12). Precision was calculated as the relative standard deviation (% CV) of the calculated concentrations of each standard solution. Intra- and inter-assay precision were calculated for each set of quality control samples.

Recovery
The absolute recovery, or extraction efficiency, was determined by comparing in three separate occasions, the peak areas of the three QC samples (QCL, QCM and QCH) first added then processed, against the peak areas from equivalent samples first processed then spiked to the same final concentration of PNV and IS. As the extraction efficiency of PNV and IS was determined simultaneously, the recovery was calculated as the PNV–IS extraction ratio.

Determination of matrix effect
To investigate whether endogenous matrix constituents interfered with the assay for free and total PNV, samples of 4,800 pg/mL in the ACN–water mixture were prepared. Non-analyte-added plasma samples were processed and then spiked with PNV and IS to a final concentration equivalent to 4,800 pg/mL. Matrix effect was calculated by dividing the calculated concentration of the post-extraction spiked plasma samples by the calculated concentration of PNV in ACN–water mixture.

Carry-over effect
To determine the presence of carry-over contamination in the assay, ACN–water mixture injections were performed within the analytical run, specifically after the calibration point of higher concentration was injected. Carry-over was determined by calculating the concentration (if any) of PNV in each ACN–water mixture injection.

Stability
All stability studies were conducted at three concentration levels (QCL, QCM and QCH) by using duplicate. The stability of PNV in human plasma was determined after three freeze–thaw cycles (−74 to −86°C), after storage at −80°C for 2 weeks, over 69 h in the autosampler (8°C) and after 4 h refrigeration (2–6°C). Stability was expressed as the percentage recovery of the assayed solution relative to a freshly processed analyte, added plasma sample (t = 0).

Pharmacokinetic study
Clinical study design
Twenty-five healthy Mexicans volunteers of both gender who were between the ages of 18 and 45 (mean ± SEM: 24.71 ± 0.03 years), had heights between 140.0 and 190.0 cm (163.0 ± 0.005 cm) and weighed between 43.50 and 79.50 kg (62.15 ± 1.9 kg) were enrolled in the study. The study protocol
was approved by an independent ethics committee as well as by the regulatory authority in Mexico (COFEPRIS), and it was conducted following the ethical principles described in the Declaration of Helsinki.

The study was an open label exploratory bioavailability trial in healthy subjects under fasting conditions. The participants received one dose of 100 mg of PNV, and blood samples were obtained prior to dosing (time 0) and at 0.17, 0.33, 0.5, 0.75, 1, 1.33, 1.67, 2, 2.5, 3, 4, 8, 12 and 24 h after administration. After blood withdrawal, the blood samples were immediately centrifuged at 3,000 rpm for 10 min, and the plasma obtained was stored at −20°C until analysis.

Pharmacokinetic data analysis
Pharmacokinetic parameters for PNV were calculated using non-compartmental and compartmental models with WinNonlin 6.2.1 software (Pharsight, Mountain View, CA, USA, 2011). From the individual data, it was estimated the pharmacokinetic parameters of PNV. The maximum plasma concentration ($C_{\text{max}}$), time to reach $C_{\text{max}}$ ($T_{\text{max}}$), area under the plasma concentration time curve from time zero to the time of the last measurable concentration (AUC$_{0-t}$) and AUC extrapolation to infinity (AUC$_{0-\infty}$) were calculated according to the non-compartmental method.

For estimation of the absorption rate constant ($K_a$), half-life of the absorption process ($T_{1/2\text{ abs}}$) as well as the disposition and elimination parameters: apparent volume of distribution ($V/F$), clearance apparent ($CL/F$), elimination rate constant ($K_e$) and elimination half-life ($T_{1/2}$), the best model that described the individual pharmacokinetic data was fitted as an open model of one compartment with first-order absorption without lag-time.

Results
This study was undertaken to develop a clean, fast, sensitive and selective method to determine PNV in human plasma, designed for subject sample analysis. The data through the chromatographic method reported in this work are intended to determine pharmacokinetic parameters of healthy Mexican volunteers after an oral dose of PNV.

Chromatography
Fragmentation of PNV (Figure 1A) and IS (Figure 1B) is shown in Figure 1. Representative chromatograms of PNV processed samples are shown in Figure 2. A chromatogram of a non-analyte-added plasma sample is presented in Figure 2A. The calibration curve processed plasma samples for lower (12 pg/mL) and higher (12,000 pg/mL) PNV concentration limits are presented in Figure 2B and Figure 2C, respectively; PNV retention time is measured in 2.09 min. A chromatogram of a processed sample containing IS is presented in Figure 2C, and IS presented a retention time of 1.15 min. Chromatograms show little interference for both PNV and IS.
Validation of the LC–MS–MS assay

Calibration curve and LOQ

All five calibration curves were linear over the analyzed concentration range of 12–12,000 pg/mL. Three validation runs were conducted on separate days and the standard curves obtained for PNV were linear (Table I). The validated method for pinaverium showed a linear behavior in the range of concentrations analyzed (12–12,000 pg/mL), \( y = 0.00003 + 0.00029x \), with a 0.9991 correlation coefficient (\( r \)). For the LOQ five samples were analyzed and showed that the detection limit is reproducible (Table II) based on calculated concentrations of 14.23 ± 2.5 pg/mL. Signal-to-noise ratio for the LOQ presented and calculated on 21 (Figure 2B). The detection limit was evaluated by injecting duplicate 50:50 serial dilutions of the lowest concentration of the working range to the point where the PNV signal was more than three times the background noise, the detection limit was reached at the 1:8 dilution, equivalent to an average concentration of 1.5 pg/mL PNV.

Accuracy and precision

The intra- and inter-assay accuracy and precision for the calibration curve samples are reported in Table I and demonstrate that the accuracy and precision was <10% for each analysis. For the quality control samples, the intra-assay coefficient of variation was <15%, and accuracy ranged from −0.9 to 4.6% (Table II); these results exhibit a higher accuracy compared against the method reported by Ren et al. (6), which reported an accuracy in the range of 2 and 8%. The quality control sample values showed a better profile of precision in inter-day analysis than the method described by Ren et al. (5.4–12.7% versus 3.7–8.1%). However, the precision of the method reported in this work has an intra-day precision similar to the methods previously reported (6, 7).

Table I

<table>
<thead>
<tr>
<th>Concentration (pg/mL)</th>
<th>Mean observed concentration (pg/mL) ± SD</th>
<th>Accuracy (%)</th>
<th>Precision intra-assay (%)</th>
<th>Precision inter-assay (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>12 ± 0.1</td>
<td>0.13</td>
<td>2.81</td>
<td>1.37</td>
</tr>
<tr>
<td>720</td>
<td>723 ± 13</td>
<td>0.42</td>
<td>3.32</td>
<td>4.06</td>
</tr>
<tr>
<td>3,000</td>
<td>2,960 ± 121</td>
<td>−1.32</td>
<td>7.14</td>
<td>4.99</td>
</tr>
<tr>
<td>4,800</td>
<td>4,827 ± 245</td>
<td>0.56</td>
<td>8.82</td>
<td>2.40</td>
</tr>
<tr>
<td>7,800</td>
<td>7,896 ± 167</td>
<td>1.23</td>
<td>3.68</td>
<td>2.77</td>
</tr>
<tr>
<td>9,600</td>
<td>9,448 ± 265</td>
<td>−1.58</td>
<td>4.87</td>
<td>6.54</td>
</tr>
<tr>
<td>12,000</td>
<td>12,064 ± 182</td>
<td>0.54</td>
<td>2.63</td>
<td>1.61</td>
</tr>
</tbody>
</table>

Figure 2. MRM ion-chromatograms of (A) extracted blank plasma (without IS and analyte), (B) pinaverium bromide LOQ and (C) real sample, volunteer number 05, first period (I), sample 05 in the time of 1.0 h (V05-I-05) (m/z 511.2 → 230) and (D) itraconazole (m/z 705.29 → 392.18) as used for internal standard (IS).
Stability studies
The solutions used for the validation of the analytical method for the determination of plasma PNV proved to be stable during a 7-day period, meeting the acceptance criteria for the parameters of the line, slope (m), intercept (b) and correlation coefficient (r). In the identity test of the line to the point of lowest concentration of the calibration curve (CC1), coefficient of variation (CV %) was within ±20% and for the remaining points of the curve within ±15%. The criterion for precision and accuracy, as the average accuracy was 100 ± 15% of the theoretical concentration for all three levels evaluated. Finally, the value of precision measured, as coefficient of variation (%) was <15% for all levels.

In stability studies, two replicates of QCL, QCM and QCH levels were analyzed. The results are presented in Table III. The data demonstrated that PNV samples had adequate stability under refrigeration (2–6°C) for 4 h. Similarly, it is determined that PNV is stable in three freezing–thawing cycles, additionally in the autosampler at 8°C for 69 h. The long-term stability of PNV stored in a temperature range between −74 and −86°C was evaluated for 2 weeks resulted be stable this storage time.

Assay specificity, carry-over and matrix effect
Selectivity of the method was demonstrated to use of hemolyzed, lipemic plasma and plasma spiked with concomitant drugs, such as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciprofloxacin, paracetamol, difenidol, ranitidine and caffeine. An enhancement of signal exists; however, no matrix effect is observed, as ciproflo
the inter-assay was evaluated with the totality of data of calibration curve during all validation, and the average inter-assay precision was 3.3%. This result shows a reproducible analytical method. Moreover, because of the use of UPLC technique the total analysis time (extraction and chromatography) is the quickest for the determination of PNV compared with the methods reported previously (6, 7). In addition, the column loading of PNV at LOQ was only 12 pg/mL per sample injection volume (1 µL), which is a low concentration that may extend column usage and efficiency, allowing the analysis of a greater number of injections. The method presented in this work showed an improved performance and a reduced consumption of analysis reagents.

In this application, significant reduction in the time of analysis was achieved by using UPLC when compared with an HPLC analysis method (2.5 min run time and a flow rate of 0.2 mL/min versus 3.0 min run time and a flow rate of 0.5 mL/min) (6), allowing full utilization of the rapid scanning speeds made possible by the triple-quadrupole MS.

This method of analysis was successfully validated and was used as an essential tool to determine the pharmacokinetic parameters of PNV after the administration a single 100 mg oral dose of PNV. Figure 3 shows the plasma concentration of PNV versus time profile human subjects under fasting conditions. The method was sensitive enough to monitoring the PNV plasma concentration up to 20 h. In all ~800 samples including the calibration, QC and volunteer samples were run and analyzed for a period of 3 days and the precision and accuracy were found to be well within the acceptable limits.

Conclusion
The proposed validated method for the estimation of PNV in human plasma is accurate, precise and rapid compared with published reports. The method offers significant advantages over those previously reported in terms of sample amount requirements, fast extraction procedure against lower interferences in the chromatographic analysis and overall time of analysis due to the UPLC system. The efficiency of the protein precipitation method for extraction and a chromatographic run time of 2.5 min per sample make this method of analysis an attractive procedure in high-throughput bioanalysis of PNV.

Acknowledgments
The authors thank to RIMSA, Representaciones e Investigaciones Médicas, S. A. de C. V. (Guadalajara, Mexico) for the funding for this study. The authors appreciate the technical assistance provided to Maricela Martínez-Delgado and Israel Luna-Zavala.

References
3. Christen, M.O.; Action of pinaverium bromide, a calcium-antagonist, on gastrointestinal motility disorders; General Pharmacology, (1990); 21: 821–825.

Figure 3. The mean plasma concentration–time profile of pinaverium bromide after oral administration of (100 mg pinaverium bromide capsules) formulation to 25 healthy volunteers under fasting conditions.