Research Article

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# Development and validation of a sensitive LC-MS/MS method with electrospray ionization for quantitation of pramipexole in human plasma: application to a clinical pharmacokinetic study

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**ABSTRACT: A highly sensitive and specific LC-MS/MS method has been developed and validated for the estimation of pramipexole (PPX) with 500 μL human plasma using memantine as an internal standard (IS). The API-4000 was operated under multiple-reaction monitoring mode (MRM) using the electrospray ionization technique. Solid-phase extraction was used to extract PPX and IS from human plasma. The resolution of peaks was achieved with 0.01 M ammonium acetate buffer (pH 4.4):acetonitrile (30:70, v/v) on a Discovery CN column. The total chromatographic run time was 3.0 min and the elution of PPX and IS occurred at approximately 2.32 and 2.52, respectively. The MS/MS ion transitions monitored were 212.10 → 153.10 for PPX and 180.20 → 107.30 for IS. The method was proved to be accurate and precise at linearity range of 20–3540 pg/mL with a correlation coefficient (*r*) of ≥0.999. The intra- and inter-day precision and accuracy values found to be within the assay variability limits as per the FDA guidelines. The developed assay method was applied to a pharmacokinetic study in human volunteers following oral administration of 0.25 mg PPX tablet. Copyright © 2008 John Wiley & Sons, Ltd.**

**Keywords:** pramipexole; human plasma; validation; LC-MS/ MS; pharmacokinetics

## Introduction

Pramipexole [PPX, (6*S*)-$N^6$-propyl-4,5,6,7-tetrahydro-1,3-benzothiazole-2,6-diamine, CAS No: 104632-26-0, Fig. 1] is an orally active, non-ergoline, dopamine agonist used for the treatment of Parkinson's disease and restless legs syndrome. PPX is a novel aminobenzothiazole compound having highest affinity towards $D_3$-subtype dopamine receptors (Mierau *et al*., 1995). Therapy with PPX tablets is initiated at a low dose (0.125 mg) and gradually titrated upwards according to clinical tolerability to obtain the optimum therapeutic effect.

Development of validated methods in preclinical species is essential at various stages of drug discovery and development. Only two LC-MS/MS methods (Lau *et al*., 1996; Nirogi *et al*., 2007) have been reported for quantification of PPX in human plasma. The first LC-MS/MS method, reported by Lau *et al*. (1996), has several disadvantages, viz. greater volume of plasma (1 mL) for analysis, utilization of non-commercial IS, time-consuming sample preparation and interference in the chromatography and longer run time (~4.5 min). The run time and LLOQ of this method were 3.5 min and 50 pg/mL, respectively (Lau *et al*., 1996). Subsequently, Nirogi *et al*., (2007) developed an LC-MS/MS using 500 μL plasma volume using a commercially available IS. However, this method's main drawback is higher LLOQ (200 pg/mL) compared with the earlier method reported by Lau *et al*. (1996), and also long run time (3.5 min), when compared with our method. By looking into the drawbacks/disadvantages of the two earlier reported LC-MS/MS methods and knowing the importance of and need for a highly sensitive method for quantification of PPX, which is used for treatment of Parkinson's disease (with a very low recommended starting dose of 0.125 mg), we felt that there is a need for a highly sensitive and high-throughput LC-MS/MS for the quantification of PPX in human plasma. In this manuscript, we are presenting a validated highly sensitive LC-MS/MS method (LLOQ: 20 pg/mL) with a high throughput (run time of 3.0 min) for quantification of PPX in human plasma and application of this method to derive pharmacokinetic parameters for PPX in humans.

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**Abbreviations used:** CE, collision energy; CXP, collision exit potential; DP, declustering potential; EP, entrance potential; MRM, multiple reaction-monitoring mode; PPX, pramipexole.

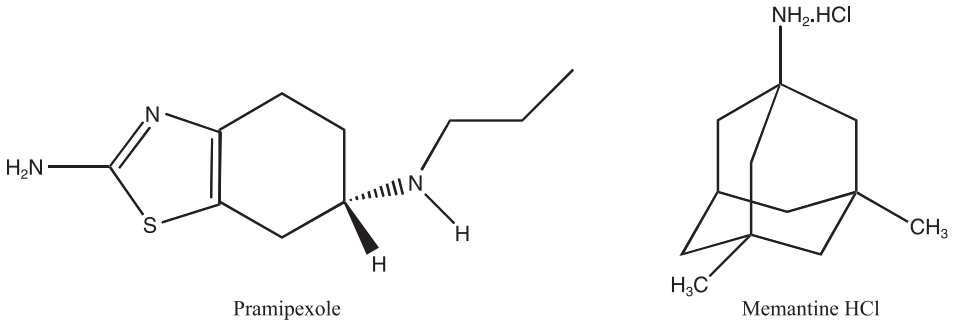


**Figure 1.** Structural representation of PPX and memantine (IS).

## Experimental

### Chemicals and Reagents

PPX was procured from Hetero Drugs, Hyderabad, India and memantine hydrochloride (IS, Fig. 1) was synthesized in API unit-II, Dr Reddy's Laboratories Ltd, Hyderabad, India and characterized using chromatographic (HPLC, LC-MS/MS) and spectral techniques (IR, UV, mass, $^{1}$H and $^{13}$C-NMR). Purity was found to be >98% for both the compounds. HPLC-grade acetonitrile and analytical-grade ammonium acetate, acetic acid and ethylenediaminetetraaceticacid (EDTA) disodium salt were purchased from Qualigens, Mumbai, India. All aqueous solutions, including the buffer for the mobile phase, were prepared with Milli Q (Millipore, Milford, MA, USA) grade water. The control $K_2$EDTA human plasma was purchased from Cauvery Diagnostics and Blood bank, Secunderabad, India.

### Instrumentation and Chromatographic Conditions

An Agilent (Agilent Technologies, Waldbronn, Germany) 1100 series LC system equipped with degasser (G1379A), binary pump (G1311A Quat pump), thermostat column oven (G1316A) along with an auto-sampler (G1367A WPALS) was used to inject 20 μL aliquots of the processed samples onto a Discovery column (4.6 × 50 mm, 5.0 μm, Thermo Electron Corporation, USA), which was maintained at ambient room temperature. The isocratic mobile phase, a mixture of 0.01 M ammonium acetate (pH 4.4):acetonitrile (30:70, v/v) was delivered at 0.5 mL/min into the mass spectrometer's electrospray ionization chamber.

Quantitation was achieved by MS/MS detection in positive ion mode using a PE Sciex (Foster City, CA, USA) API-4000 mass spectrometer, equipped with a Turboionspray™ interface at 450°C. The ion spray voltage was set at 5500 V. The common parameters, viz. curtain gas, GS1, GS2 and CAD gas, were set at 15, 30, 40 and 2 psi, respectively, whereas the compounds parameters, viz. declustering potential (DP), collision energy (CE), entrance potential (EP) and collision exit potential (CXP), were 45, 18, 10 and 15 V and 40, 35, 10 and 10 V for PPX and IS, respectively. Detection of the ions was performed in the multiple-reaction monitoring (MRM) mode, monitoring the transition pair of PPX at the *m/z* 212.10 precursor ion to the *m/z* 153.10 product ions for PPX and *m/z* 180.20 precursor ion to the *m/z* 107.30 product ion for IS. Quadrupoles Q1 and Q3 were set on unit resolution. The analytical data were processed using Analyst software (version 1.4.1).

### Preparation of Stock and Standard Solutions

Primary stock solutions of PPX for preparation of calibration standard and quality control (QC) samples were prepared from separate weighings. The primary stock solutions of the analytes and IS were prepared in methanol (1.01 mg/mL for analyte and 0.98 mg/mL for IS) and stored at −20 ± 2°C, and were found to be stable for 30 days (data not shown). Appropriate dilutions were made in methanol for PPX to produce working stock solution of 2.00, 4.00, 10.01, 17.56, 51.64, 172.1, 264.8, 311.6 and 354.1 ng/mL and on the day of analysis this set of stocks were used to prepare standards for the calibration curve (CC). Another set of working stock solutions of PPX was made in methanol (from second primary stock) at 2.00, 5.90, 155 and 293 ng/mL for preparation of QC samples. Working stock solutions were stored at approximately 5°C and were found to be stable for 15 days (data not shown). Individually QC and CC working stock solutions of PPX were made before spiking into QC and CC plasma samples accordingly. A working stock solution of IS (40 ng/mL) was prepared in methanol from primary stock solution of 1.00 mg/mL. Calibration standards were prepared by spiking in 490 μL of control human plasma with the appropriate amount of PPX (10 μL) and IS (50 μL) on the day of analysis. Samples for the determination of recovery, precision and accuracy were prepared by spiking control human plasma in bulk at appropriate concentrations [20.00 pg/mL (LLOQ), 59.08 pg/mL (QC low), 1554 pg/mL (QC medium) and 2933 pg/mL (QC high)] and 500 μL volumes were aliquoted into different tubes and were stored at −80 ± 10°C until analysis.

### Recovery

The recovery of PPX and IS, through solid-phase extraction procedure, was determined by comparing the responses of the analytes extracted from replicate QC samples ($n = 6$) with the response of analytes from aqueous standard samples at equivalent concentrations (Dams *et al*., 2003). Recoveries of PPX were determined at QC low, QC medium and QC high concentrations, viz. 59.08, 1554 and 2933 pg/mL, whereas the recovery of the IS was determined at a single concentration of 40 ng/mL.

### Sample Preparation

To an aliquot of 500 μL human plasma sample, IS solution (50 μL) was added, diluted with 500 μL of Milli Q water and vortex mixed for 30 s on a cyclomixer (Remi Instruments, Mumbai, India). This sample mixture was loaded on pre-conditioned (1 mL acetonitrile followed by 1 mL water) Oasis HLB cartridges (1 cm$^3$, 30 mg) and



  

washed with 1 mL water followed by 1 mL 10% acetonitrile in water and finally eluted with 500 μL of mobile phase. From the eluate 20 μL was directly injected onto LC-MS/MS system.

### Validation Procedures

A full validation according to the FDA guidelines (US DHHS *et al*., 2001) was performed for the assay in human plasma.

**Specificity and selectivity.** The specificity of the method was evaluated by analyzing human plasma samples from at least six different sources to investigate the potential interferences at the LC peak region for analyte and IS.

**Matrix effect.** The effect of human plasma constituents over the ionization of PPX and IS was determined by comparing the responses of the post extracted plasma standard QC samples ($n = 6$) with the response of analytes from neat standard samples (10 μL of required working stock sample spiked into 490 μL of methanol instead of blank plasma) at equivalent concentrations (Hubert *et al*., 1999; Dams *et al*., 2003). The matrix effect was determined at LLOQ concentrations viz., 20.00 pg/mL, whereas the matrix effect over the IS was determined at working concentration of 40 ng/mL.

**Calibration curve.** The calibration curve was acquired by plotting the ratio of sum of peak area of PPX to that of IS against the nominal concentration of calibration standards. The final concentrations of calibration standards obtained for plotting the calibration curve were 20.01, 40.03, 100.08, 175.58, 516.42, 1721.4, 2648.3, 3115.7 and 3540.6 pg/mL. The results were fitted to linear regression analysis using $1/X^2$ as weighting factor. The calibration curve had to have a correlation coefficient ($r$) of 0.999 or better. The acceptance criteria for each back-calculated standard concentration were ±15% deviation from the nominal value except at LLOQ, which was set at ±20% (US DHHS *et al*., 2001).

**Precision and accuracy.** The intra-day assay precision and accuracy were estimated by analyzing six replicates at four different QC levels, i.e. 20.08, 59.08, 1554.8 and 2933.6 pg/mL. The inter-assay precision was determined by analyzing the four levels QC samples on three different runs. The criteria for acceptability of the data included accuracy within ±15% standard deviation (SD) from the nominal values and a precision of within ± 15% relative standard deviation (RSD), except for LLOQ, where it should not exceed ±20% of accuracy as well as precision (US DHHS *et al*., 2001).

**Stability experiments.** The stability of PPX and IS in the injection solvent was determined periodically by injecting replicate preparations of processed samples up to 26 h (auto-sampler) after the initial injection. The peak areas of the PPX and IS obtained at 0 h on day 1 were used as the reference to determine the relative stability of the analyte at subsequent points. In all stability studies two QC concentrations were used, viz. QC low and QC high. Stability of PPX in the biomatrix during 8 h exposure at room temperature in human plasma (bench-top) was determined at ambient temperature (25 ± 1°C) in six replicates at each concentration. Freezer stability of PPX in human plasma was assessed by analyzing the QC samples stored at −80 ± 10°C for at least 15 days. The stability of PPX in human plasma following repeated freeze–thaw cycles was assessed using QC samples spiked with PPX. The samples were stored at −80 ± 10 °C between freeze–thaw cycles. The stability of PPX was assessed after the third freeze–thaw cycle. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (i.e. ±15% SD) and precision (i.e. ±15% RSD).

### Pharmacokinetic Study in Humans

A pharmacokinetic study was performed in 14 healthy male subjects. The ethics committee approved the protocol and the volunteers provided written informed consent. Blood samples were obtained following oral administration of 0.25 mg PPX tablet into polypropylene tubes containing $K_2$EDTA solution as an anticoagulant at pre-dose, 0.33, 0.67, 1.00, 1.33, 1.67, 2.00, 2.33, 2.67, 3.00, 3.50, 4.00, 5.00, 6.00, 8.00, 10.0, 12.0, 24.0, 36.0 and 48.0 h. Plasma was harvested by centrifuging the blood using Multifuge (Hereaus, Germany) at 1760***g*** for 5 min and stored frozen at −80 ± 10°C until analysis.

An aliquot of 500 μL of thawed plasma samples were spiked with IS and processed as mentioned in the Sample Preparation section. Along with study samples, QC samples at low, medium and high concentration were assayed in duplicate and were distributed among unknown samples in the analytical run. The criteria for acceptance of the analytical runs encompassed the following: (i) not more than 33% of the QC samples were greater than ±15% of the nominal concentration; (ii) not less than 50% at each QC concentration level met the acceptance criteria. Plasma concentration–time data of PPX was analyzed by non-compartmental method using WinNonlin Version 5.1 (Pharsight Corporation, Mountain View, CA, USA).

## Results

### Method Development, Liquid Chromatography and Mass Spectroscopy

In order to remove the interfering ballasts from the biomatrix and to increase the selectivity and sensitivity of the analytical method, different methods of sample pre-treatment were investigated. Protein precipitation and liquid–liquid extraction with various organic solvents and their mixtures resulted in non-reproducible recoveries and interferences from the sample matrix with the chromatography of the analyte and/or IS (data not shown). Subsequently, SPE was investigated as samples pre-treatment technique. Following optimization of various types of SPEs and several dilution, conditioning, washing and elution reagents, we have finally selected Oasis HLB cartridges (1 $cm^3$, 30 mg) in this assay.

In pursuit of symmetric peak shape and shorter run time, feasibility of various mixture(s) of solvents such as acetonitrile and methanol using different buffers such as ammonium acetate, ammonium formate and formic acid with variable pH range of 4.0–7.0, along with altered flow-rates (in the range of 0.3–1.1 mL/min) were tested for complete chromatographic resolution of PPX and IS (data not shown). The resolution of peaks was achieved with 0.01 M ammonium acetate (pH 4.4):acetonitrile (30:70, v/v) with a flow rate of 0.5 mL/min, on a Discovery CN column and was found to be suitable for the determination of electrospray response for PPX and IS.

In order to optimize ESI conditions for PPX and IS, quadrupole full scans were carried out in positive ion detection mode. During a direct infusion experiment, the mass spectra for PPX and IS revealed peaks at *m/z* 212.10 and 180.20, respectively, as protonated molecular ions, $[M + H]^+$. Following detailed optimization



  

of mass spectrometry conditions (provided in the Instrumentation and Chromatographic Conditions section), the *m/z* 212.10 precursor ion to the *m/z* 153.10 was used for quantification for PPX. Similarly, for IS the *m/z* 180.20 precursor ion to the *m/z* 107.30 was used for quantification purpose. As the earlier publications (Lau *et al*., 1996; Nirogi *et al*., 2007) have discussed extensively the fragmentation pattern of PPX, we are not presenting the data pertaining to this.

### Recovery

The results of the comparison of pre-extracted standards vs post-extracted plasma standards were estimated for PPX at 59.08, 1554 and 2933 pg/mL and the mean recovery was 60.05 ± 2.69, 61.12 ± 1.60 and 56.22 ± 11.47%. The SPE process yielded a clean chromatogram when the blank plasma sample was processed and, further, no interference was observed at the retention time of PPX and IS. The recovery of IS at 40 ng/mL was 87.07 ± 2.45%.

### Validation Procedures

**Matrix effect, specificity and selectivity.** In this study, the matrix effect was evaluated by analyzing the LLOQ sample. Average matrix factor values (matrix factor = response of post spiked concentrations/response of neat concentrations) obtained for PPX were + 1.06 (CV 9.06%, $n = 6$) at the LLOQ level, whereas on the IS it was found to be –0.94 (CV 1.29%, $n = 6$) at the tested concentration of 40 ng/mL. Hence, it is concluded that there is 6% ion enhancement in the case of PPX with a CV of 9.06, and 6% ion suppression in the case of IS with a CV of 1.29%, observed at PPX and IS retention times at LLOQ level concentration.

Figure 2 shows a typical overlaid chromatogram for the control human plasma (free of analyte and IS), human plasma spiked with IS, human plasma spiked with PPX at LLOQ and IS and an *in vivo* human plasma sample obtained at 1.67 h after oral administration of PPX tablet. No interfering peaks from endogenous compounds were observed at the retention times of analyte and IS. The retention time of PPX and IS was ~2.32 and 2.52 min, respectively. The total chromatographic run time was 3.00 min, which is shorter than that in earlier reported methods (Lau *et al*., 1996; Nirogi *et al*., 2007).

**Calibration curve.** The plasma calibration curve was constructed using nine calibration standards (viz., 20.01–3540.6 pg/mL). The calibration standard curve had a reliable reproducibility over the standard concentrations across the calibration range. The calibration curve was prepared by determining the best fit of peak-area ratios (peak area analyte/peak area IS) vs concentration, and fitted to the $y = mx + c$ using weighing factor ($1/X^2$). The average regression ($n = 4$) was found to be ≥0.999. The

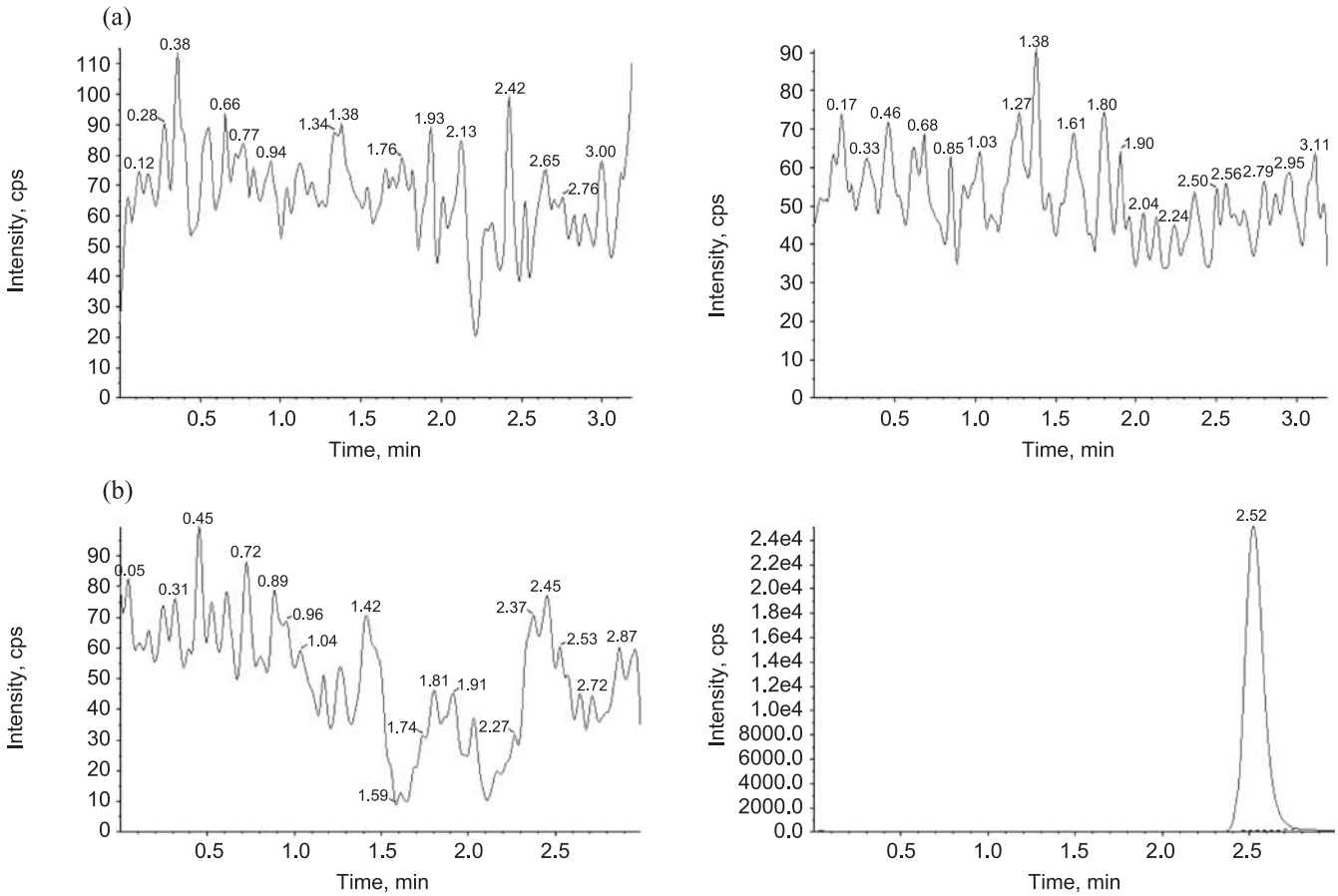


**Figure 2.** Typical MRM chromatograms of PPX (left panel) and IS (right panel) in (a) human blank plasma; (b) human plasma spiked with IS; (c) human plasma spiked with PPX at LLOQ (20 pg/mL) and IS; and (d) a 1.67 h plasma sample showing PPX peak (675.14 pg/mL) obtained following an oral dose of a PPX tablet to a healthy volunteer along with IS.

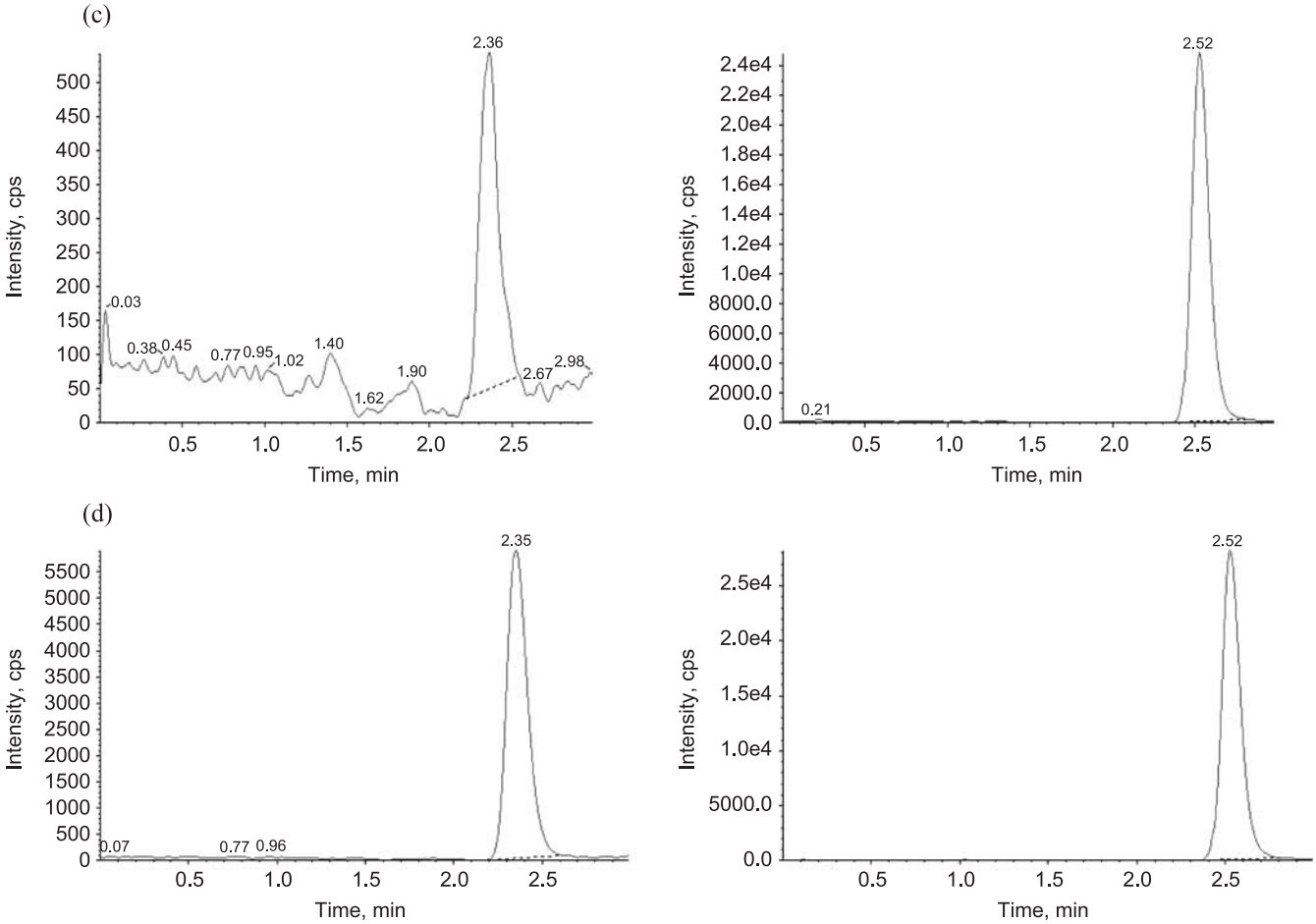


**Figure 2.** (*Continued*)

lowest concentration with the RSD <20% was taken as LLOQ and was found to be 20 pg/mL. The percentage accuracy observed for the mean of back-calculated concentrations for three calibration curves was within 93.1–103, while the precision (% CV) values ranged from 1.00 to 8.40.

**Accuracy and precision.** The accuracy and precision data for intra- and inter-day plasma samples are presented in Table 1. The assay values on both the occasions (intra- and inter-day) were found to be within the accepted variable limits.

**Stability.** The predicted concentrations for PPX at 59.08 and 2933 pg/mL samples deviated within ±15% of the nominal concentrations in a battery of stability tests, viz. in-injector (26 h), bench-top (8 h), repeated three freeze–thaw cycles and at −80 ± 10°C for at least for 15 days (Table 2). The results were found to be within the assay variability limits during the entire process.

### Pharmacokinetic Study in Humans

The sensitivity and specificity of the assay were found to be sufficient for accurately characterizing the pharmacokinetics of PPX in humans. Profiles of the mean plasma concentration versus time are shown in Fig. 3. Maximum concentration in plasma ($C_{max}$ 454.05 ± 75.85 pg/mL) was achieved at 2.47 ± 1.17 h. The half-life ($t_{1/2}$) of PPX was 7.78 ± 2.46 h, while $AUC_{(0-\infty)}$ was 5711.96 ± 1479.61 pg h/mL.

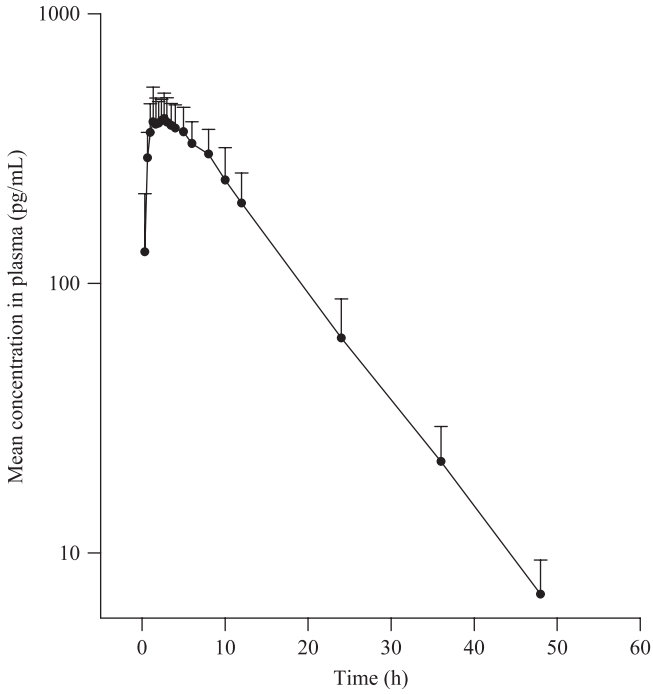


**Figure 3.** Mean ± SD plasma concentration–time profile of PPX in human plasma following oral dosing of PPX tablet to 14 subjects.



  

**Table 1.** Intra- and inter-day precision of determination of PPX in human plasma

| Theoretical concentration (pg/mL) | Run | Measured concentration (pg/mL) Mean | SD | RSD | Accuracy (%) |
|---|---|---|---|---|---|
| *Intra-day variation (six replicates at each concentration)* | | | | | |
| 20.08 | 1 | 19.52 | 1.65 | 8.49 | 97.2 |
| | 2 | 22.17 | 2.14 | 9.67 | 110 |
| | 3 | 20.10 | 1.84 | 9.15 | 100 |
| 59.08 | 1 | 59.25 | 2.98 | 5.04 | 100 |
| | 2 | 54.97 | 2.73 | 4.97 | 93.0 |
| | 3 | 57.60 | 4.02 | 7.00 | 97.5 |
| 1554 | 1 | 1532 | 97.7 | 6.38 | 98.5 |
| | 2 | 1582 | 110 | 7.00 | 101 |
| | 3 | 1504 | 29.3 | 1.95 | 96.7 |
| 2933 | 1 | 2879 | 168 | 5.84 | 98.1 |
| | 2 | 2991 | 167 | 5.61 | 102 |
| | 3 | 2909 | 80.0 | 2.75 | 99.1 |
| *Inter-day variation (18 replicates at each concentration)* | | | | | |
| 20.08 | | 20.72 | 2.13 | 10.2 | 103 |
| 59.08 | | 57.45 | 3.58 | 6.24 | 97.2 |
| 1554 | | 1539 | 88.1 | 5.72 | 99.0 |
| 2933 | | 2926 | 144 | 4.93 | 99.7 |

RSD: relative standard deviation (SD × 100/mean).

**Table 2.** Stability data PPX quality controls in human plasma

| Nominal concentration (pg/mL) | Stability | Mean ± SD,[a] $n = 6$ (pg/mL) | Accuracy (%)[b] | Precision (% CV) |
|---|---|---|---|---|
| | 0 h (for all) | 57.60 ± 4.02 | 97.5 | 7.00 |
| | 3rd freeze–thaw | 63.336 ± 3.39 | 107 | 5.37 |
| 59.08 | 8 h (bench-top) | 55.95 ± 3.92 | 94.7 | 7.01 |
| | 26 h (in-injector) | 60.26 ± 1.65 | 102 | 2.74 |
| | 15 day at −80°C | 62.36 ± 3.27 | 105 | 5.26 |
| | 0 h (for all) | 2909 ± 80.0 | 99.1 | 2.75 |
| | 3rd freeze–thaw | 2871 ± 145 | 97.8 | 5.07 |
| 2933 | 8 h (bench-top) | 2848 ± 167 | 97.0 | 5.87 |
| | 26 h (in-injector) | 3041 ± 145 | 103 | 4.77 |
| | 15 day at −80°C | 2960 ± 169 | 100 | 5.74 |

[a] Back-calculated plasma concentrations; [b] (mean assayed concentration/mean assayed concentration at 0 h) × 100.

## Discussion

So far there have been only two LC-MS/MS methods (Lau *et al*., 1996; Nirogi *et al*., 2007) published for the determination of PPX in human plasma. Both the reported methods have disadvantages and drawbacks. The method reported by Lau *et al*. (1996) utilizes a non-commercial IS and has a longer run time, cumbersome sample preparation and a strong interference peak just before the retention time of the analyte. The method reported by Nirogi *et al*. (2007) has longer run time compared with ours and higher LLOQ (200 pg/mL, which is 10-fold higher than our LLOQ of 20 pg/mL). The effective amount of PPX loaded onto the HPLC column for analysis is also several fold lower than the earlier reported methods [0.4 vs 35 pg (Lau *et al*., 1996) and 0.4 vs 10 pg (Nirogi *et al*., 2007)]. In a nutshell, our method has overcome all the disadvantages/drawbacks of the earlier reported methods. The applicability of the method in clinical pharmacokinetic studies has been demonstrated in healthy humans.

## Conclusions

In summary, we have developed a method for the determination of PPX in human plasma, which offers the highest sensitivity (20 pg/mL) compared with other methods described in the literature using a simple SPE extraction procedure and commercially available IS. From the results of all the validation parameters and



  

applicability of the assay, we can conclude that the present method can be useful for clinical pharmacokinetic studies of PPX with desired precision and accuracy.

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