Journal of Chromatographic Science, Vol. 48, November/December 2010

Validated Ultra-Performance Liquid Chromatography Tandem Mass Spectrometry Method for the Determination of Pramipexole in Human Plasma

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Abstract

A sensitive and high throughput ultra-performance liquid chromatography tandem mass spectrometry (UPLC–MS–MS) method has been developed for the determination of pramipexole, a dopamine agonist, in human plasma. Sample preparation involved liquid–liquid extraction of pramipexole and ranitidine as the internal standard (IS) in ethyl acetate from 100 µL human plasma. The chromatographic separation is achieved on a Waters Acquity UPLC BEH C18 (100 mm × 2.1 mm, 1.7 µm) analytical column using an isocratic mobile phase, consisting of 10 mM ammonium formate (pH 7.50)–acetonitrile (15:85, v/v), at a flow-rate of 0.5 mL/min. The precursor → product ion transition for pramipexole (*m/z* 212.1 → 153.0) and IS (*m/z* 315.0 → 176.1) were monitored on a triple quadrupole mass spectrometer, operating in the multiple reaction monitoring (MRM) and positive ion mode. The method was validated over a wide dynamic concentration range of 20–4020 pg/mL. Matrix effect is assessed by post-column infusion experiment and the process efficiency were 91.9% and 85.7% for pramipexole and IS, respectively. The method is rugged and rapid with a total run time of 1.5 min and is applied to a bioequivalence study of 0.25 mg PPX tablet formulation in 30 healthy Indian male subjects under fasting condition.

Introduction

Pramipexole [PPX, (6S)-N^6-propyl-4,5,6,7-tetrahydro-1,3-benzothiazole-2,6-diamine] is a non-ergoline dopamine agonist used for the treatment of Parkinson's disease and idiopathic restless legs syndrome in adults (1,2). It acts by binding selectively to dopamine D_2-like receptors; in particular it shows high affinity for the D3 receptor subtype (3). It is effective both as monotherapy in the early stages and in the advanced phases in association with levodopa, which improves the motor fluctuations and dyskinesias. PPX has proved to exert neuroprotector effects and its use in clinical practice from the early stages of Parkinson's disease due to delay in the appearance of motor complications. PPX is free of severe side effects (fibrosis and valve disease) linked with ergotic dopaminergic agonists and causes fewer digestive and dysautonomic alterations (4). PPX is commercially available as the pure (S)-enantiomer, while its (R)-enantiomer is reported to show a 100-fold lower affinity for dopamine receptors (5). PPX is ~ 15% protein bound and is well absorbed after oral administration with bioavailability greater than 90%. It is generally administered three times daily, with dose ranging from 0.375–1.5 mg/day for Parkinson's disease (6), while a single daily administration (0.125–0.50 mg/day) is recommended for restless legs syndrome (2). Because elimination is primarily by renal clearance, accumulation of the drug can occur in patients with renal dysfunction and dosage reduction must be envisaged (7).

Due to very low PPX therapeutic levels, it is essential to develop sensitive, rugged, and rapid bioanalytical methods for its determination in biological fluids to minimize the risk of drug accumulation, for the optimization of therapy, and to reduce the frequency of adverse effects. The analysis of PPX in bulk drug and dosage form has been described by high-performance liquid chromatography (HPLC)–UV (8,9) methods. Pathare et al. (10) developed a chiral liquid chromatography method for the enantiomeric separation of PPX in bulk drugs. Few methods are presented to date for the determination of PPX in biological matrices like urine (11,12) and human plasma (11,13–15).

Lau et al. (11) have determined PPX in urine and human plasma by ion-pair chromatography on a Zorbax Rx C8 column with electrochemical and UV detection. The assay was linear in the range of 10–10000 ng/mL in urine and 0.050–15 ng/mL in human plasma. However, the method required very long (> 15 min) HPLC analysis run time. Recently, Musenga and coworkers (12) analyzed PPX in human urine by capillary electrophoresis with laser-induced fluorescence detection. The limit of detection and limit of quantitation were 10.0 and 25 ng/mL, respectively. The method involved a lengthy sample pre-treatment, which included LLE in ethyl acetate followed by derivatization and incubation for 4 h at 45°C. A sensitive method (50 pg/mL) for estimating PPX in human plasma by HPLC with atmospheric pressure chemical ionization tandem mass spectrometry (MS) has been presented by Lau et al. (13). The chromatographic analysis time was less than 5 min, but it employed a large plasma volume (1 mL) for processing. Also, the method was applied to study the pharmacokinetics of 0.25 mg oral dose of PPX administered to only one human volunteer. A less sensi-

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tive (200 pg/mL) but high throughput method has been proposed by Nirogi et al. (14) using 0.5 mL plasma for the quantification of PPX by LC–MS–MS. Very recently, Bharathi and co-workers (15) have developed and validated a highly sensitive and rapid LC–MS–MS method for PPX in 0.5 mL human plasma employing solid-phase extraction. The method was linear from 20–3540 pg/mL and the chromatographic run time was 3.0 min.

Ultra-high performance liquid chromatography (UHPLC) is emerging as a rapid device to separate complex mixtures in both isocratic and gradient modes. This technology is capable of achieving higher peak capacity, speed, and sensitivity than conventional HPLC by using sub-2 µm particles and optimized instrumentation. The frictional heating effect caused by sub-2 µm particles is minimized using 1.0–2.1-mm inner diameter (i.d.) columns and provides favorable sample loading capacity. In addition, solvent consumption can be significantly reduced compared to conventional 4.6 mm i.d. columns (16,17). Thus, in the present work an accurate, simple, and rapid UHPLC–MS–MS method has been developed and fully validated for reliable measurement of PPX in subject samples. The method requires only 100 µL human plasma for LLE and demonstrates excellent performance in terms of ruggedness and efficiency (1.5 min per sample). Interference due to matrix was ascertained by post column infusion technique. It was successfully applied to a bioequivalence study of 0.25 mg PPX tablet formulation in 30 healthy Indian male subjects under fasting condition.

Experimental

Chemicals and materials

Reference standards of pramipexole dihydrochloride (99.4%) and ranitidine hydrochloride (IS, 99.7%) were obtained from Samex Overseas (Ahmedabad, India). HPLC-grade methanol and acetonitrile were procured from Mallinckrodt Baker, S.A.de C.V. (Estado de Mexico, Mexico). HPLC-grade ethyl acetate, ammonia solution, and sodium hydroxide pellets were purchased from Merck Specialties Pvt. Ltd. (Mumbai, India), while ammonium formate was from Sigma-Aldrich (St. Louis, MO). Water used in the entire analysis was prepared from Milli-Q water purification system from Millipore (Bangalore, India). Blank human plasma was obtained from Supratech Micropath (Ahmedabad, India) and was stored at –20°C until use.

Liquid chromatographic conditions

A Waters Acquity UPLC system (MA) consisting of binary solvent manager, sample manager, and column manager was used for setting the reverse-phase liquid chromatographic conditions. The analysis of PPX and IS was performed on a Waters Acquity UPLC type BEH, C18 (100 × 2.1 mm, length × inner diameter) analytical column with 1.7-µm particle size and maintained at 40°C in a column oven. For isocratic separation, the mobile phase consisted of ammonium formate (10mM, pH 7.50 adjusted with ammonia)–acetonitrile (15:85, v/v). The flow rate of the mobile phase was maintained at 0.5 mL/min. The total chromatographic run time was 1.5 min. The sample manager temperature was maintained at 5°C and the pressure of the system was 4500 psi.

Mass spectrometric conditions

Ionization and detection of PPX and IS was carried out on a triple quadrupole mass spectrometer, MDS SCIEX API-4000 (Toronto, Canada), equipped with turbo ion spray interface and operating in positive ion mode. Quantitation was performed using multiple reaction monitoring (MRM) mode to monitor precursor → product ion transitions for PPX *m/z* 212.1 → 153.0 and *m/z* 315.0 → 176.1 for IS (Figure 1). The source dependent parameters maintained for both the drugs were: Gas 1 (nebulizer gas), 50 psig; Gas 2 (heater gas), 60 psig; ion spray voltage (ISV), 5500 V; turbo heater temperature (TEM), 400°C; interface heater (Ihe), ON; entrance potential (EP), 10 V; collisional activation dissociation (CAD), 6 psig and curtain gas (CUR, nitrogen), 15 psig. The optimum values for compound dependent parameters like declustering potential (DP), collision energy (CE) and cell exit potential (CXP) set were 50, 21, and 10 eV for PPX and 14, 21, and 4.0 eV for IS, respectively. Quadrupole 1 and 3 were maintained at unit mass resolution and the dwell time was set at 200 ms for both the drugs. Data collection, peak integration, and calculations were performed using Analyst classic software version 1.4.1.

Standard stock, calibration standards, and quality control sample preparation

The standard stock solution of pramipexole (1 mg/mL) was prepared by dissolving requisite amount in methanol. Calibration standards and quality control (QC) samples were prepared by spiking (2% of total plasma volume) blank plasma with stock solution. Calibration curve standards were made at 20.0, 40.0, 125, 251, 502, 1005, 2010, and 4020 pg/mL concentrations, respectively, while quality control samples were prepared at four levels, viz. 3663 pg/mL (HQC, high quality control), 308 pg/mL (MQC, middle quality control) 55.0 pg/mL (LQC, low quality control), and 20.5 pg/mL (LLOQ QC, lower limit of quantification quality control). Stock solution (1 mg/mL) of the internal standard (IS) was prepared by dissolving 25.0 mg of ranitidine in 25.0 mL of methanol. Its working solution (50 ng/mL) was prepared by appropriate dilution of the stock solution in methanol. All the solutions (standard stock, calibration standards and quality control samples) were stored at 2–8°C until use.

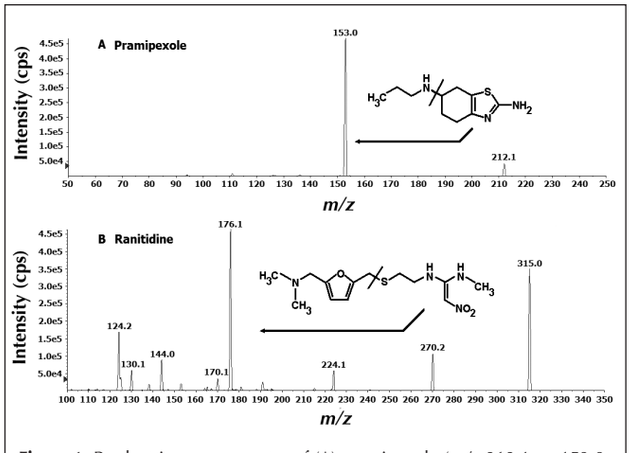

Figure 1. Product ion mass spectra of (A) pramipexole (*m/z* 212.1 → 153.0, scan range 50–250 amu) and (B) internal standard, ranitidine (*m/z* 315.0 → 176.1, scan range 100–350 amu) in positive ionization mode.

Sample extraction protocol

Prior to analysis, all frozen subject samples, calibration standards, and quality control samples were thawed and allowed to equilibrate at room temperature. To an aliquot of 100 µL of spiked plasma sample, 50 µL of IS was added and vortexed for 10 s. Further, 50 µL of 0.5 N sodium hydroxide solution was added and vortexed for another 10 s. LLE was carried out with 2.0 mL of ethyl acetate on rotary mixer (rotospin) for 5 min at 32 × g. Samples were then centrifuged at 3204 × g for 5 min at 10°C. After centrifugation, 1.5 mL of the supernatant organic layer was transferred and evaporated to dryness in a thermostatically controlled water-bath maintained at 40°C under a gentle stream of nitrogen. The dried samples were reconstituted in 100 µL of mobile phase and 2 µL was used for injection in the chromatographic system.

Method Validation

The bioanalytical method was thoroughly validated following the USFDA guidelines (18). System suitability experiments were performed by injecting six consecutive injections using aqueous standard mixture of PPX (308 pg/mL) and ranitidine (50 ng/mL) at the start of each batch during method validation. System performance was studied by injecting one extracted LLOQ sample with IS at the beginning of each analytical batch and before re-injecting any sample during method validation. The carryover effect of the autosampler was evaluated by sequentially injecting solutions of aqueous standard of PPX, reconstitution solution (mobile phase), standard blank, and extracted standard of PPX equivalent to highest standard in the calibration range.

The selectivity of the method towards endogenous plasma matrix components was assessed in 12 different batches (6 normal of K3 EDTA, 2 haemolysed, 2 lipemic, and 2 heparinised) of blank plasma. A check for interference due to commonly used medications in human volunteers was done for paracetamol, chlorpheniramine maleate, caffeine, acetylsalicylic acid and ibuprofen. Their stock solutions (100 µg/mL) were prepared by dissolving requisite amount in methanol. Further, working solutions (100 ng/mL) were prepared in the mobile phase, and 2 µL was injected to check any possible interference at the retention time of PPX and IS.

The linearity of the method was determined by analysis of five linearity curves containing eight non-zero concentrations. The area ratio response for PPX/IS obtained from multiple reaction monitoring was used for regression analysis. Each calibration curve was analyzed individually by using least square weighted ($1/x^2$) linear regression, which was finalized during pre-method validation. A correlation coefficient (r^2) value > 0.99 was desirable for all the calibration curves. The lowest standard on the calibration curve was accepted as the lower limit of quantitation (LLOQ), if the analyte response was at least five times more than that of drug free (blank) extracted plasma. In addition, the analyte peak of LLOQ sample should be identifiable, discrete, and reproducible with a precision (%CV) no greater than 20% and accuracy within 80–120%. The deviation of standards other than LLOQ from the nominal concentration should not be more than ± 15%.

For determining the intra-batch accuracy and precision, replicate analysis of plasma samples of PPX was performed on the same day. The run consisted of a calibration curve and five replicates of LLOQ QC, LQC, MQC, and HQC samples. The inter-assay accuracy and precision were assessed by analyzing five precision and accuracy batches on three consecutive validation days. The deviation at each concentration level from the nominal concentration was expected to be within ± 15% except LLOQ, for which it should be within ± 20%. Similarly, the mean accuracy should not deviate by ± 15% except for the LLOQ where it can be ± 20% of the nominal concentration.

Ion suppression/enhancement effects on the MRM LC–MS–MS sensitivity were evaluated by the post column analyte infusion experiment. A standard solution containing PPX (at MQC level) and ranitidine (IS) was infused post column via a "T" connector into the mobile phase at 10 µL/min employing in-built infusion pump. Aliquots of 2 µL of extracted control plasma were then injected into the column by the auto-sampler and MRM LC–MS–MS chromatogram was acquired for PPX. Any dip in the baseline upon injection of double blank plasma (without IS) would indicate ion suppression, while a peak at the retention time of PPX or IS indicates ion enhancement.

The relative recovery, matrix effect, and process efficiency were assessed as recommended by Matuszewski et al. (19). All three parameters were evaluated at HQC, MQC, and LQC levels in six replicates. Relative recovery (RE) was calculated by comparing the mean area response of pre-spiked samples (spiked before extraction) to that of extracts with post-spike samples (spiked after extraction) at each QC level. The recovery of IS was similarly estimated. Absolute matrix effect (ME) was assessed by comparing the mean area response of unextracted samples (spiked after extraction) with mean area of neat standard solutions. The overall "process efficiency" (%PE) was calculated as (ME × RE)/100. Further, the effect of plasma matrix (relative matrix effect) on analyte quantification was also checked in six different batches/lots of plasma. From each batch, six samples at LLOQ level were prepared (spiked before extraction) and checked for the % accuracy and precision (% CV). The deviation of the standards should not be more than ± 15% and at least 90% of the lots at each QC level should be within the aforementioned criteria.

All stability results were evaluated by measuring the area response (PPX/IS) of stability samples against freshly prepared comparison standards with identical concentration. Stock solutions of PPX and IS were checked for short-term stability at room temperature and long-term stability at 2–8°C. The solutions were considered stable if the deviation from nominal value was within ± 10.0%. Autosampler stability (wet extract), dry extract, bench top (at room temperature), and freeze-thaw stability were performed at LQC and HQC using six replicates at each level. Freeze-thaw stability was evaluated by successive cycles of freezing (at –20°C and –70°C) and thawing (without warming) at room temperature. Long-term stability of spiked plasma samples stored at –20°C and –70°C was also studied at both these levels. The samples were considered stable if the deviation from the mean calculated concentration of freshly thawed quality control samples was within ± 15.0%.

To authenticate the ruggedness of the proposed method, it was performed on two precision and accuracy batches. The first batch was analyzed by different analysts, while the second batch

was studied on two different columns. Dilution integrity experiment was evaluated by diluting the stock solution prepared as spiked standard at 8040 pg/mL PPX concentration in the screened plasma. The precision and accuracy for dilution integrity standards at 1/5th (1608 pg/mL) and 1/10th (804 pg/mL) dilution were determined by analyzing the samples against calibration curve standards.

Bioequivalence study design

The design of the study comprised of "An open label, balanced, randomized, two-treatment, two-period, two-sequence, single dose, crossover bioequivalence study of a test formulation of pramipexole hydrochloride (0.25 mg tablets of an Indian Company) and a reference formulation (Sifrol tablets containing 0.25 mg pramipexole hydrochloride) in 30 healthy adult Indian subjects under fasting conditions". Each subject was judged to be in good health through an evaluation of medical history, physical examination, and routine laboratory tests. Written consent was taken from all the subjects after informing them about the objectives and possible risks involved in the study. An independent ethics committee constituted as per Indian Council of Medical Research (ICMR) approved the study protocol. The study was conducted strictly in accordance with guidelines laid down by International Conference on Harmonization and USFDA (20). The subjects were orally administered a single dose of test and reference formulations after recommended wash out period of 7 days with 200 mL of water. Blood samples were collected at 0.0 (pre-dose), 0.33, 0.66, 1.0, 1.3, 1.6, 2.0, 2.3, 2.6, 3.0, 3.3, 3.6, 4.0, 5.0, 6.0, 8.0, 10.0, 12.0, 16.0, 24.0, 36.0, and 48.0 h after oral administration of the dose for test and reference formulation in labeled EDTA-vacuettes. The maximum volume of blood withdrawn during the entire study was 287 mL, which included (other than for measurement) up to 10 mL for screening, ~ 10 mL for post study safety assessment (hematology and biochemical tests) while 0.5 mL of heparinised blood was discarded prior to each sampling through venous cannula. Plasma was separated by centrifugation and kept frozen at –20°C until the completion of period and then at –50°C until analysis. During study, subjects had a standard diet while water intake was free. An incurred sample re-analysis (assay reproducibility test) was also conducted by computerized random selection of 20 subject samples. The results obtained were compared with the data obtained earlier for the same sample using the same procedure. The percent change in the value should not be more than ± 20% (21).

Results and Discussion

Method development

The present study was conducted using electrospray ionization (ESI) for MRM UHPLC–MS–MS analyses to attain high sensitivity and a good linearity in regression curves. As PPX and IS have primary, secondary and/or tertiary amine groups that can be protonated in solution under the experimental conditions, the intensity found was much higher in the positive mode compared to the negative mode. Q1 MS full scan spectra for PPX and IS predominantly contained protonated precursor $[M+H]^+$ ions at *m/z* 212.1 and 315.0, respectively. The most abundant product ions in Q3 MS spectra for PPX and IS were observed at *m/z* 153.0 (corresponding to 2-amino benzothiazole) and 176.1 respectively at 21 eV collision energy. The source-dependent and compound-dependent parameters were suitably optimized to obtain a consistent and sufficient response for the analyte.

Nirogi et al. (14) have presented a LLE procedure with tert-butylmethylether–dichloromethane (8:2, v/v) to extract PPX under alkaline conditions. Their extraction recovery for PPX and tamsulosin hydrochloride (IS) was 96.1% and 77.6%, respectively, from 0.5 mL human plasma. Solid-phase extraction (SPE) of PPX on Oasis HLB cartridges has also been reported by Bharathi et al. (15); however, their mean extraction recovery was only 59.2% for PPX. Thus, based on sensitivity, matrix effect, and reproducibility requirements, both these extraction techniques were tried during method development. Reproducibility and recovery data for both the drugs supported LLE to be used as the extraction technique. The SPE under alkaline condition gave reproducible results in terms of accuracy and precision (< 15%) but showed significant ion suppression (> 20%) at LLOQ and LLOQ QC levels. LLE was tested to isolate the drug from plasma under alkaline (0.1–1.0 N NaOH) conditions using diethyl ether (alone and in combination with dichloromethane), methyl *tert* butyl ether, *n*-hexane and ethyl acetate as extracting solvents. Quantitative and consistent recoveries were obtained at all QC levels for PPX and IS with ethyl acetate using 50 µL, 0.5 N NaOH. The recovery in other solvent systems was > 70% but was inconsistent with some ion suppression (greater than 15% CV).

Reported procedures have employed 5 µm particle size and 4.6 mm inner diameter columns with run times ≥ 3.0 min (14,15). Thus, in the present work, chromatographic separation of PPX and IS was initiated on Waters Acquity UPLC BEH C-18 (100 × 2.1 mm, 1.7 µm particle size) column to achieve a short run time, good peak shapes, minimum matrix interference, and solvent consumption. The column has a wide pH working range (1–12) with a surface area of 187 m^2/g and an average pore diameter of 147 Å. To find the best eluting solvent system, various combinations of methanol–acetonitrile with acidic and alkaline buffers (formic acid–acetic acid-ammonium formate–ammonium acetate, ammonia-ammonium formate) in different volume ratios were tested. Higher sensitivity, efficiency, and symmetric peak shapes were obtained with ammonium formate (10 mM, pH 7.50 adjusted with ammonia)–acetonitrile (15:85, v/v) as the mobile phase. The total run time of 1.5 min ensured elution of PPX and IS at 1.05 and 0.95 min, respectively. Representative chromatograms in Figure 2 of extracted blank human plasma (double blank), blank plasma fortified with IS (*m/z* 315.0 → 76.1), PPX at LLOQ (*m/z* 212.1 → 153.0), and an actual subject sample at 2.0 h demonstrates the selectivity of the method. The extraction procedure together with mass detection gave very good selectivity for the analysis of PPX and IS in the blank plasma. None of the commonly used medications by human volunteers showed interfering signals at the retention time of PPX or the IS. Results of post-column infusion experiment in Figure 3 indicate no ion suppression or enhancement at the retention time of PPX and IS. Significant ion suppression (> 15%) was observed around 1.35 min, however, it did not affect the quantitation of PPX in subsequent measurements. The average matrix

factor value calculated as the response of post spiked sample/response of neat solutions in mobile phase at the LLOQ levels was 0.97, which indicates a minor suppression of 3%.

A general IS was used to minimize any analytical variation due to solvent evaporation, extraction efficiency, integrity of the column, and ionization efficiency of PPX. Ranitidine used as an IS in the present study had similar chromatographic behavior, similar protein binding, and was easily extracted with ethyl acetate. There was no effect of IS on analyte recovery, sensitivity, or ion suppression.

Assay performance and validation

Throughout the method validation, the precision (%CV) of system suitability test was observed in the range of 0.07% to 0.14% for the retention time and 1.01% to 1.5% for the area response of both the drugs (PPX and IS), which is not more than the acceptance criteria of 4%.

Carry-over evaluation was performed in each analytical run so as to ensure that it does not affect the accuracy and the precision of the proposed method. There was no carry-over observed during autosampler carryover experiment. No enhancement in the response was observed in double blank after subsequent injection of highest calibration standard (aqueous and extracted) at the retention time of PPX and IS, respectively.

All five calibration curves were linear over the concentration range of 20.0–4020 pg/mL. A straight-line fit was made through the data points by least square regression analysis to give the mean linear equation $y = 0.00100x + 0.00243$, where y is the peak area ratio of the analyte/IS, and x the concentration of the analyte. The mean standard deviation value for slope, intercept and correlation coefficient (r^2) observed were 0.00039, 0.00167, and 0.0008, respectively. The accuracy and precision (%CV) observed for the calibration curve standards ranged from 91.2% to 106.2% and 3.1 to 7.1, respectively (Table I). The lowest concentration (LLOQ) in the standard curve that can be measured with acceptable accuracy and precision was found to be 20 pg/mL in plasma at a signal-to-noise ratio (S/N) of ≥ 100.

The intra- and inter-batch precision and accuracy were established from validation runs performed at HQC, MQC, LQC, and LLOQ QC levels (Table II). The intra-batch precision (%CV) ranged from 1.6% to 6.0%, and the accuracy was within 96.4% to 105.5%. For the inter-batch experiments, the precision varied from 3.1% to 8.6%, and the accuracy was within 100% to 103.6%.

The relative recovery, absolute matrix effect, and process efficiency data for PPX and IS at LQC, MQC, and HQC levels is pre-

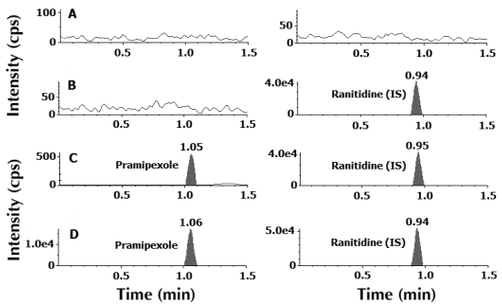

Figure 2. MRM ion-chromatograms of (A) double blank plasma (without IS) (B) blank plasma with ranitidine (IS), (C) pramipexole at LLOQ (*m/z* 212.1 → 153.0) and IS (D) real subject sample at 2.0 h after administration of 0.25 mg dose of pramipexole.

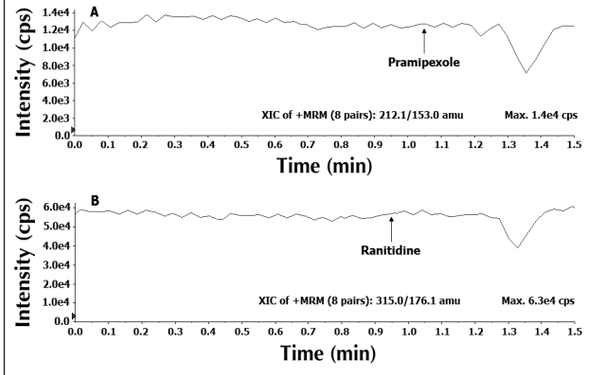

Figure 3. Post column analyte infusion experiment for (A) pramipexole and (B) ranitidine.

Table I. Summary of Calibration Curve with Back-Calculated Concentration for Pramipexole*

	STD-1	STD-2	STD-3	STD-4	STD-5	STD-6	STD-7	STD-8			
	Nominal concentration (pg/mL)								Regression Parameters		
ID No.	20.0	40.0	125	251	502	1005	2010	4020	Slope	Intercept	r^2
1	19.2	43.8	126	245	551	1047	1876	3648	0.00074	0.00018	0.9968
2	20.5	39.2	116	262	553	1003	1934	3945	0.00081	0.00417	0.9981
3	20.3	39.0	125	268	508	1013	1721	4365	0.00089	0.00135	0.9969
4	20.3	38.7	127	275	519	1015	1734	4100	0.00088	0.00264	0.9972
5	20.8	36.4	138	233	536	1091	1903	3762	0.00168	0.00381	0.9959
Mean	20.2	39.4	126	257	533	1034	1834	3964	0.00100	0.00243	0.9970
S.D.	0.6	2.7	8.0	17.3	19.6	36.0	98.9	283.0	0.00039	0.00167	0.0008
% CV	3.1	6.9	6.3	6.7	3.7	3.5	5.4	7.1			
% Nominal	100.5	98.1	100.5	102.1	106.2	102.9	91.2	98.6			

* CV = coefficient of variance; S.D. = standard deviation; r^2 = correlation coefficient.

Table II. Intra-batch and Inter-batch Precision and Accuracy for Pramipexole*

		Intra-batch				Inter-batch			
QC ID	Nominal conc. (ng/mL)	*n*	Mean conc. observed (pg/mL)†	% CV	% Accuracy	*n*	Mean conc. observed (pg/mL)‡	% CV	% Accuracy
HQC	3663	5	3791	1.6	103.5	25	3795	3.1	103.6
MQC	308	5	325	2.8	105.5	25	315	6.8	102.3
LQC	55.0	5	53.1	2.6	96.4	25	55.0	8.6	100.0
LLOQ QC	20.5	5	21.2	6.0	103.4	25	20.7	8.2	101.0

* CV = coefficient of variance; n = total number of observations.
† Mean of 5 replicates at each concentration.
‡ Mean of 5 replicates for five precision and accuracy batches.

Table III. Absolute Matrix Effect, Relative Recovery, and Process Efficiency for Pramipexole

QC				(% ME)**		(% RE)††		(% PE)‡‡	
Level	A* (% CV)†	B‡ (% CV)†	C§ (% CV)†	PPX	IS	PPX	IS	PPX	IS
LQC	0.036 (1.5)	0.034 (3.7)	0.033 (4.0)	94.4	91.3	97.1	95.5	91.7	87.2
MQC	0.202 (2.5)	0.188 (2.4)	0.185 (5.0)	93.1	90.7	98.4	93.2	91.6	84.5
HQC	2.427 (1.30)	2.263 (2.9)	2.245 (3.4)	93.2	89.9	99.2	94.9	92.5	85.3

* Mean area ratio (analyte/IS) response of six replicate samples prepared in mobile phase (neat samples)
† Coefficient of variation
‡ Mean area ratio (analyte/IS) response of six replicate samples prepared by spiking in extracted blank plasma
§ Mean area ratio (analyte/IS) response of six replicate samples prepared by spiking before extraction
** B/A × 100 †† C/B × 100 ‡‡ C/A × 100 = (ME x RE)/100

Table IV. Relative Matrix Effect in Six Different Lots of Human Plasma (Spiked After Extraction) for Pramipexole at LLOQ level (*n* = 6)

Lot No.	Mean area ratio (analyte/IS) response	% CV	% Accuracy
1	0.0126	1.35	96.9
2	0.0128	1.82	98.5
3	0.0125	1.50	96.2
4	0.0127	2.10	97.7
5	0.0125	2.58	96.3
6	0.0126	1.95	96.8

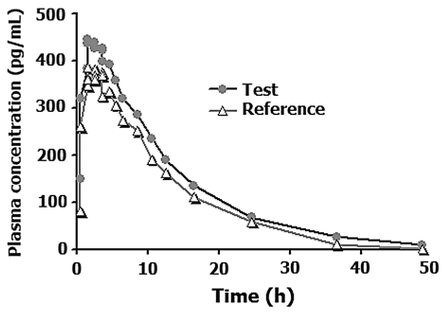

Figure 4. Mean plasma concentration-time profile of pramipexole after oral administration of test formulation of pramipexole hydrochloride (0.25 mg tablets of an Indian Company) and a reference formulation (Sifrol tablets containing 0.25 mg pramipexole hydrochloride) to 30 healthy volunteers under fasting condition.

sented in Table III. The process efficiency/absolute recovery obtained for PPX and IS was greater than 91% and 84%, respectively, at all QC levels. Further, the more important parameter in the evaluation and validation of a bioanalytical method using biofluids is the demonstration of absence of "relative" matrix effect, which compares the precision (%CV) values between different lots (sources) of plasma (spiked after extraction) samples. The precision results varied from 1.35–2.58% for different plasma lots with accuracy between 96.2–98.5% at the LLOQ level (Table IV).

The stability of the PPX and IS in human plasma and stock solutions was examined under different storage conditions. Samples for short-term stability remained unchanged up to 6 h, while the stock solutions for long term stability of PPX and the IS were stable for a minimum of 6 days at refrigerated temperature below 8°C. PPX in control human plasma (bench top) at room temperature was stable at least for 6 h at 25°C and for minimum of three freeze and thaw cycles at –20°C and –70°C. Spiked plasma samples stored at –20°C and –70°C for long term stability experiment were found stable for a minimum period of 139 days. Dry extract stability of the spiked quality control samples stored at –20 °C was determined up to 27 h. Autosampler stability (wet extract) of the spiked quality control samples maintained at 5°C was determined up to 16 h without significant drug loss. The percentage change for different stability experiments in plasma at two QC levels varied from –9.9% to 1.7% as shown in Table V.

The dilution integrity experiment was performed with an aim to validate the dilution test to be carried out on higher analyte concentration above the upper limit of quantification (ULOQ), which may be encountered during real subject sample analysis. The precision for dilution integrity of 1/5 and 1/10th dilution were 1.5% and 3.5%, while the accuracy results were 100.9 and 101.6%, respectively, which is within the acceptance limit of 15% for precision (%CV) and 85% to 115% for accuracy.

Method ruggedness was evaluated using re-injection of analyzed samples on two different columns of the same make and also with different analysts. The precision (%CV) and accuracy values for two different columns ranged from 1.6% to 7.0% and 96.2% to 105.5%, respectively, at all four quality control levels. For the experiment with different analysts, the results for precision and accuracy were within 5.4% to 6.2% and 90.0% to 101.4%, respectively, at these levels.

Application to a pharmacokinetic–bioequivalence study

The validated method has been successfully used to quantify PPX concentration in human plasma samples after the administration of a single 0.25 mg oral dose of PPX. Figure 4 shows the plasma concentration vs. time profile of PPX in human subjects under fasting condition. The method was sensitive enough to

monitor the PPX plasma concentration up to 48 h. In all ~ 2000 samples, including the calibration, QC, and volunteer samples, were run and analyzed during a period of 5 days, and the precision and accuracy were well within the acceptable limits. The mean pharmacokinetic parameters obtained for the test and reference formulation are presented in Table VI. The values for C_{max}, T_{max}, $t_{1/2}$, AUC_{0-t} and AUC_{0-inf} are comparable with a pharmacokinetic study in 14 Indian subjects reported by Bharathi et al. (15) with 0.25 mg dose of PPX. The 90% confidence interval of individual ratio geometric mean for test/reference was within 80–125% for AUC_{0-t}, AUC_{0-inf}, and C_{max}. The % change in the randomly selected subject samples for incurred samples (assay reproducibility) analysis was within ± 11% (Table VII). This authenticates the reproducibility and ruggedness of the proposed method. Further, there was no adverse event during the course of the study.

Comparison with reported methods

The method presented has the highest sensitivity and employs minimum plasma volume (100 µL) for processing compared to other procedures (11,13–15). Moreover, the total analysis time (extraction and chromatography) is the shortest compared to all other methods reported for PPX (11–15). Also, the on-column loading of PPX at LLOQ was only 0.04 pg per sample injection volume, which is 10 times lower for an equally sensitive method reported by Bharathi et al. (15). A detailed comparison of reported procedures with the present method for PPX determination in human plasma is given in Table VIII.

Table V. Stability of Pramipexole Under Different Conditions (*n* = 6)

Storage condition	Nominal Conc. (pg/mL)	Calculated conc. (pg/mL) Mean, stability samples + SD	% Change*
Bench Top Stability; 6 hours			
HQC	3663	3725 ± 195	1.7
LQC	55	53 ± 1.3	–3.6
Wet Extract Stability; 16 h			
HQC	3663	3301 ± 40	–9.9
LQC	55	53 ± 2.1	–3.6
Dry Extract Stability; 27 h			
HQC	3663	3916 ± 398	6.9
LQC	55	52 ± 0.9	–5.5
Freeze & Thaw Stability; 3 Cycles, –20°C			
HQC	3663	3552 ± 108	–3.0
LQC	55	55 ± 2.7	–0.1
Freeze & Thaw Stability; 3 Cycles, –70°C			
HQC	3663	3616 ± 120	–1.3
LQC	55	53 ± 1.7	–3.6
Long Term Matrix Stability; 139 days, –20°C			
HQC	3663	3750 ± 149	2.4
LQC	55	57 ± 3.4	3.6
Long Term Matrix Stability; 139 days, –70°C			
HQC	3663	3461 ± 136	–5.5
LQC	55	55 ± 3.3	–0.1

$$* \% \text{ Change} = \frac{\text{Mean stability samples} - \text{Mean comparison samples/Nominal conc.}}{\text{Mean comparison samples}} \times 100$$

Conclusions

To summarize, the UHPLC–MS–MS method for the quantitation of pramipexole in human plasma was developed and fully validated as per USFDA guidelines. The method offers significant advantages over those previously reported, in terms of lower sample requirements, simplicity of extraction procedure, and overall analysis time. The efficiency of LLE and a chromatographic run time of 1.5 min per sample make it an attractive procedure in high-throughput bioanalysis of pramipexole. With dilution integrity up to 10-folds, it is possible to extend the upper limit of quantification to 8040 pg/mL. In addition, the carry-over test, post column infusion study and the effect of commonly used medications by subjects is also studied in the present work. The current method has shown adequate sensitivity and selectivity for the quantification of pramipexole in human plasma in a clinical study.

Table VI. Mean Pharmacokinetic Parameters Following Oral Administration of 0.25 mg Tablet Formulation (Test and Reference) of Pramipexole in 30 Healthy Human Subjects

Parameter	Mean ± SD Test	Reference
C_{max} (pg/mL)	463 ± 144	456 ± 136
T_{max} (h)	2.1 ± 0.8	2.1 ± 1.1
$t_{1/2}$ (h)	9.1 ± 3.5	9.0 ± 2.8
$AUC_{0-48\ h}$ (h.pg/mL)	5319 ± 1620	5117 ± 1526
AUC_{0-inf} (h.pg/mL)	5825 ± 1715	5637 ± 1571

C_{max} = maximum plasma concentration.
T_{max} = time point of maximum plasma concentration.
$t_{1/2}$ = half life of drug elimination during the terminal phase.
AUC_{0-t} = area under the plasma concentration-time curve from zero hour to 48 h.
AUC_{0-inf} = area under the plasma concentration-time curve from zero hour to infinity.

Table VII. Sample Reproducibility Data for Pramipexole

Sr. No.	Initial Value (ng/mL)	Repeat Value (ng/mL)	% Change*
01	413	389	–5.9
02	52.9	54.0	2.1
03	495	515	3.9
04	418	378	–10.2
05	47.9	47.5	–1.0
06	365	360	–1.3
07	54.9	50.1	–9.2
08	53.7	50.4	–6.4
09	356	370	4.0
10	50.2	48.0	–4.4
11	419	447	6.4
12	351	321	–8.8
13	82.5	89.1	7.8
14	410	372	–9.6
15	79.9	88.2	9.9
16	52.0	52.9	1.7
17	69.7	66.6	–4.5
18	49.3	52.2	5.7
19	551	494	–11.0
20	86.2	82.4	–4.5

$$*\% \text{ Change} = \frac{(\text{Repeat value} - \text{Initial value})}{\text{Mean of initial and repeat values}} \times 100$$

Table VIII. Comparison of Analytical Methods Developed for Pramipexole in Human Plasma*

Sr. No.	Extraction procedure (plasma volume); internal standard; mean recovery (%)	Column; elution process; mobile phase; flow rate; injection volume; maximum on-column loading (LLOQ)	Analytical run time; detection technique	Linear dynamic range (pg/mL)	Ref. No.
1	LLE with EE, back extraction with HSA (1.0 mL plasma); BHT-920; (97.7%)	Zorbax Rx C18 (250 mm × 4.6 mm, 5 µm); Isocratic; KH_2PO_4+NaA+HSA+HA, pH 3.5–ACN (85:15, v/v);1.2 mL/min; 50 µL; 25 pg	16 min HPLC–EC	50–15000 pg/mL	11
2	LLE with MTBE (1.0mL); BHT-920; (81.6%)	Zorbax SB-CN (150 mm × 4.6 mm, 5 µm); Isocratic; H_2O-0.1M AA–MeOH (15:5:80, v/v/v); 1.2 mL/min; 70 µL; 35 pg	5.0 min LC–APCI-MS–MS	50–5000 pg/mL	13
3	LLE with MTBE–DCM (0.5mL); tamsulosin hydrochloride; (96.1%)	LiChrospher RP-select B (100 mm × 4 mm, 5 µm); Isocratic; 10mM AA-MeOH (30:70, v/v); 1.2 mL/min;15 µL; 10 pg	3.5 min LC–MS–MS	200–8000 pg/mL	14
4	SPE on Waters Oasis HLB Cartridge (0.5mL); memantine hydrochloride; (59.2%)	Discovery CN (50mm × 4.6mm, 5µm); Isocratic; 0.01M AA, pH 4.4–ACN (30:70, v/v); 0.5 mL/min; 20 µL; 0.4 pg	3.0 min LC–ESI-MS–MS	20–3540 pg/mL	15
5	LLE with EA (0.1mL); ranitidine hydrochloride; (98.2%)	Waters Acquity UPLC BEH C18 (100mm × 2.1mm, 1.7 µm); Isocratic; 10mM AF, pH 7.5-ACN (10:90, v/v); 0.3 mL/min; 2 µL; 0.04 pg	1.5 min UHPLC–ESI-MS–MS	20–4020 pg/mL	Present Method

* Abbreviations are as follows: LLE = liquid–liquid extraction; EE = ethyl ether; HSA = heptanesulphonic acid; NaA = sodium acetate; HA= acetic acid; ACN = acetonitrile; MTBE = methyl tert-butyl ether; AA = ammonium acetate; meOH = methanol; DCM = dichloromethane; SPE = solid phase extraction; EA = ethyl acetate; AF = ammonium formate.

Acknowledgements

The authors are thankful to scientists, Mr. Chandan Makwana, Mr. Sanket Mistri, and the management of Veeda Clinical Research, India for providing infrastructure facility to carry out this work.

References

1. R. Constantinescu. Update on the use of pramipexole in the treatment of Parkinson's disease. *Neuropsychiatr. Dis. Treat.* **4:** 337–352 (2008).
2. P.L. McCormack, and M.A. Siddiqui. Pramipexole: in restless legs syndrome. *CNS Drugs.* **21:** 429-437 (2007).
3. J. Mierau, F. Schneider, H. Ensinger, C. Chio, M. Lajiness, and R. Huff. Pramipexole binding and activation of cloned and expressed dopamine D2, D3 and D4 receptors. *Eur. J. Pharmacol.* **290:** 29–36 (1995).
4. M. Martínez-Corral, and J. Kulisevsky. Pramipexole and Parkinson's disease, an update. *Rev. Neurol.* **46:** 49–52 (2008).
5. R. Danzeisen, B. Schwalenstoecker, F. Gillardon, E. Buerger, V. Krzykalla, K. Klinder, L. Schild, B. Hengerer, A.C. Ludolph, C. Dorner-Ciossek, and L. Kussmaul. Targeted antioxidative and neuroprotective properties of the dopamine agonist pramipexole and its nondopaminergic enantiomer SND919CL2x [(+)2-amino-4,5,6,7-tetrahydro-6-L-propyl amino-benzathiazole dihydrochloride. *J. Pharmacol. Exp. Ther.* **316:** 189-199 (2006).
6. K.M. Biglan, and R.G. Holloway. A review of pramipexole and its clinical utility in Parkinson's disease. *Expert Opin. Pharmacother.* **3:** 197–210 (2002).
7. D. Deleu, M.G. Northway, and Y. Hanssens. Clinical pharmacokinetic and pharmacodynamic properties of drugs used in the treatment of Parkinson's disease. *Clin Pharmacokinet.* **41:** 261–309 (2002).
8. G. Srinubabu, K. Jaganbabu, B. Sudharani, K. Venugopal, G. Girizasankar, and J.V.L.N.S. Rao. Development and validation of a LC method for the determination of pramipexole using an experimental design. *Chromatographia* **64:** 95–100 (2006).
9. B. Jancic, M. Medenica, D. Ivanovic, and A. Malenovic. Experimental design in chromatographic analysis of pramipexole and its impurities. *Acta Chim. Slov.* **54:** 49–54 (2007).
10. D.B. Pathare, A.S. Jadhav, and M.S. Shingare. Validated chiral liquid chromatographic method for the enantiomeric separation of pramipexole dihydrochloride monohydrate. *J. Pharm. Biomed. Anal.* **41:** 1152–1156 (2006).
11. Y.Y. Lau, G.D. Hanson and N. Ichhpurani. Determination of pramipexole (U-98,528) in human plasma and urine by high-performance liquid chromatography with electrochemical and ultraviolet detection. *J. Chromatogr. B* **683:** 217–223 (1996).
12. A. Musenga, E. Kenndler, E. Morganti, F. Rasi, and M.A. Raggi. Analysis of the anti-Parkinson drug pramipexole in human urine by capillary electrophoresis with laser-induced fluorescence detection. *Anal. Chim. Acta* **626:** 89–96 (2008).
13. Y.Y. Lau, J.M. Selenka, G.D. Hanson, R. Talaat, and N.E. Ichhpurani. Determination of pramipexole (U-98,528) in human plasma by high-performance liquid chromatography with atmospheric pressure chemical ionization tandem mass spectrometry. *J. Chromatogr. B* **683:** 209–216 (1996).
14. R.V. Nirogi, V. Kandikere, W. Shrivastava, K. Mudigonda, S. Maurya, and D. Ajjala. Quantification of pramipexole in human plasma by liquid chromatography tandem mass spectrometry using tamsulosin as internal standard. *Biomed. Chromatogr.* **21:** 1151–1158 (2007).
15. D.V. Bharathi, K.K. Hotha, P.V. Sagar, S.S. Kumar, A. Naidu, and R. Mullangi. 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. *Biomed. Chromatogr.* **23:** 212–218 (2009).
16. S.M. Chesnut and J.J. Salisbury. The role of UHPLC in pharmaceutical development. *J. Sep. Sci.* **30:** 1183–1190 (2007).
17. N. Wu and A.M. Clausen. Fundamental and practical aspects of ultrahigh pressure liquid chromatography for fast separations. *J. Sep. Sci.* **30:** 1167–1182 (2007).
18. Guidance for Industry, Bionanlytical Method Validation, US Department of Health and Human Services, Food and Drug Administration Centre for Drug Evaluation and Research (CDER), Centre for Veterinary Medicine (CVM), May 2001.
19. B.K. Matuszewski, M.L. Constanzer, and C.M. Chavez-Eng. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Anal. Chem.* **75:** 3019–3030 (2003).
20. Guidance for Industry: ICH E6 Good Clinical Practice, U.S. Department of Health and Human Services, Food and Drug Administration, Centre for Drug Evaluation and Research (CDER), Centre for Biologics Evaluation and Research (CBER), April 1996.
21. M.L. Rocci, Jr., V. Devanarayan, D.B. Haughey, and P. Jardieu. Confirmatory Reanalysis of Incurred Bioanalytical Samples. *AAPS Journal* **9:** E336–E343 (2007).

Manuscript received May 5, 2009;
revision received July 13, 2009.