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Development and validation of rapid LC-MS with electrospray ionization for the quantification of pramipexole in human plasma

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ABSTRACT

A rapid, accurate and precise LC-MS method is described for the quantitative determination of pramipexole in human plasma matrix using ropinirole as internal standard. Pramipexole and ropinirole were extracted from plasma by liquid-liquid extraction technique. The method was validated over the concentration range of 100-2514 pg/mL. The method was found to have acceptable accuracy, precision, linearity and selectivity. The mean extraction recovery from spiked plasma samples was in the range of 79.415-87.00 %. The intra-day accuracy of the assay ranged from 98.924 to 112.236 % and intra-day precision ranged from 3.489 to 6.756 %. Inter-day accuracy and precision results for quality control samples ranged between 100.340 and 107.443% of nominal and precision is observed to be 3.970-5.714 %. The pramipexole was found to be stable after several stability studies. The proposed method yielded a quick, simple and reliable protocol for estimating pramipexole concentrations in human plasma.

***Keywords*:** Pramipexole; LC-MS; human plasma; bioanalytical method

1. INTRODUCTION

Pramipexole (PPE) is a aminothiazole dopamine agonist approved in the US (1997) for management of the symptoms of early and advance stages of Parkinson's disease [1-3] and restless legs syndrome [4,5]. Preferential affinity of PPE for the D_3 receptor could contribute to efficacy in the treatment of both the motor and psychiatric symptoms of Parkinson's

disease. The precise mechanism of action of PPE in the treatment of Parkinson's disease is unknown, although it is believed to be related to its ability to stimulate dopamine receptors in the striatum.

Generally PPE is administered three times daily (dosage: 0.375-1.5 mg/day) for Parkinson's disease. Single daily administration (dosage: 0.125-0.50 mg/day) is suggested for restless legs syndrome. Accumulation of the drug can occur in patients with renal dysfunction as PPE is eliminated primarily by renal clearance. Therefore dosage reduction must be envisaged. It is essential to develop sensitive and rapid bioanalytical method for the quantification of PPE in biological matrices like urine and human plasma to minimize the risk of PPE accumulation and to lessen the occurrence of adverse effects.

Lau *et al.,* [6] developed a HPLC method for the assay of PPE in human plasma and urine. Separation is achieved by ion-pair chromatography on a Zorbax Rx C_8 column with electrochemical detection at 0.6 V for plasma and ultraviolet detection at 286 nm for urine. The retention time of PPE is approximately 14.4 min. Lau *et al.,* [7] proposed a sensitive HPLC with atmospheric pressure chemical ionization tandem mass spectrometry for estimating PPE in human plasma. The chromatographic analysis time was < 5 min, but the method used a large volume (1 mL) of plasma for processing. Further more, the method was applied to study the pharmacokinetics of PPE administered to only one human volunteer (0.25 mg oral dose).

A high performance liquid chromatography/electrospray ionization tandem mass spectrometry method has been proposed by Nirogi *etal.,* [8] for the estimation of PPE in human plasma. This method has high throughput but is less sensitive. Bharathi *etal.,* [9] have developed a highly sensitive and rapid LC–MS/MS method for determination of PPE in 0.5 mL human plasma. Solid-phase extraction was applied to extract PPE from human plasma. The chromatographic run time was 3.0 min. Uma *etal.,* [10] developed a highly sensitive LC-MS method for the estimation of PPE in 0.5 mL human Plasma. PPE and internal standard were ionized by positive-ion electro spray ionization mode and solid phase extraction technique was used for extraction of PPE from plasma using Strata-X 33 μm cartridges. The method was not completely validated.

Due to the drawbacks or disadvantages in the earlier reported bioanalytical methods, there is a need to develop and validate a sensitive and rapid bioanalytical method for the assay of PPE. The present paper describes the development and validation of a sensitive and rapid LC-MS method for the bioanalysis of PPE in human plasma.

2. EXPERIMENTAL

2. 1. Instrumentation

1. The chromatographic experiments were performed on a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) consisting of a LC-10ADvp pump, a SCL-10Avp system controller (accompanied by an auto sampler), a CTO-10Avp column oven, an FCV-10ALvp low pressure gradient unit and DGU- 14A degasser.
2. Mass spectrometric detection was performed on an API 4000 triple quadrupole tandem mass spectrometer (Applied Biosystems MDS SCIEX, CA, USA) equipped with an electro spray ionization source in positive ionization mode.
3. Analyst software version 1.4.2 (Applied Biosystems MDS SCIEX, CA, USA) was used for data processing.

4. Chromatographic separation of PPE and internal standard, ropinirole, was carried out using a Zorbax SB-C18, 3.5 µm particle size, 4.6 x 150 mm analytical column.
5. The samples were weighed by Sartorius analytical balance (Goettingen, Germany).

2. 2. Chemicals

Pramipexole and ropinirole were obtained from Matrix laboratories, Hyderabad, India. Ammonium formate (reagent grade) was obtained from sd-fine chemicals (Mumbai, India). HPLC grade acetonitrile, methanol, ethyl acetate, n-hexane, formic acid and ammonia were obtained from Merck Specialities Pvt Ltd (Mumbai, India).

2. 3. Preparation of solutions

2. 3. 1. Diluent solution

Prepared by mixing water and methanol in the ratio of 50:50 *v/v*.

2. 3. 2. Extraction solvent

Prepared by mixing ethyl acetate and *n*-hexane in the ratio of 90:10 *v/v*.

2. 3. 3. Rinsing solution

Prepared by mixing water and acetonitrile in the ratio of 50:50 *v/v*.

2. 3. 4. Mobile phase

The mobile phase used for analysis was prepared by mixing 10 mM ammonium formate and acetonitrile in the ratio, 40:60 *v/v*. Ten mM ammonium formate was prepared by mixing 0.63 mg of ammonium formate and 2 mL of formic acid in a total volume of 1000 mL.

Diluent solution, extraction solvent, rinsing solution and mobile phase are used within 4 days of preparation.

2. 3. 5. Stock and working standard solutions

The stock standard solution of PPE (1 mg/mL) was prepared by dissolving 10 mg of PPE in methanol in a 10 mL volumetric flask. Working standard solution of PPE (10 ng/mL) was prepared by suitably diluting the PPE stock standard solution with diluent. The stock standard solution (1 mg/mL) of internal standard (ISTD), ropinirole was prepared by dissolving 10 mg of ISTD in methanol in a 10 mL volumetric flask. Working standard solution of ISTD (1 ng/mL) was prepared by suitably diluting the ropinirole stock standard solution with diluent.

These standard solutions were stored at 2-8 °C in a refrigerator (LG Electronics India Pvt. Ltd, New Delhi, India) until analysis. Before use these solutions are brought to room temperature.

2. 3. 6. Spiked plasma calibration curve standards

Pramipexole calibration curve standards with concentrations 100, 222, 317, 635, 1155, 1674, 2263 and 2514 pg/mL in drug free human plasma were prepared by spiking with appropriate volume of PPE standard working solution.

2. 3. 7. Spiked plasma quality control samples

Four quality control samples with concentrations 100, 264, 825 and 2230 pg/mL PPE were prepared by spiking drug free human plasma with appropriate volume of PPE standard working solution and they are treated as LLOQ, LQC, MQC and HQC, respectively.

Calibration curve standards and quality control samples were stored at -20 °C in a deep freezer (Haier, Delhi, India) until analysis. Before use these samples are brought to room temperature.

2. 4. Chromatographic conditions

The Zorbax SB-C18 (4.6 x 150 mm, 3.5 µm particle size) was selected as the analytical column. The mobile phase was composed of 10 mM ammonium formate and acetonitrile in the ratio of 40:60 (*v/v*). The flow rate of the mobile phase was set at 0.5 mL/min (split ratio 40:60). The injection volume was 20 µL. The column oven and auto sampler temperature was set at 40 ±1 °C and 10 ±1 °C, respectively. Ropinirole was found to be apt internal standard interms of chromatography and extractability. The run time was 3 min and 500 µL of rinsing solution was used during rinsing cycle or wash cycle.

2. 5. Mass spectrometer conditions

Electrospray ionization source in positive ionization mode with multiple reaction monitoring (MRM) was used for the detection. For PPE and ISTD the $[M+H]^+$ ions were monitored at *m/z* 212.10 and *m/z* 261.20 as the precursor ion, and a fragment at *m/z* 153.10 and *m/z* 114.20 as the product ion, respectively. Mass parameters were optimized as ion source temperature 500 °C, nebulizer gas 45 (nitrogen) psi, heater gas 50 (nitrogen) psi, curtain gas 20 (nitrogen) psi, collision gas 20 (nitrogen) psi, ionization voltage 5500 V. Entrance potential, collision cell exit potential and dwell time per transition were 10 V, 10 V and 400 milliseconds, respectively for PPE and ISTD. The declustering potential is 40 V for PPE and 60 V for ISTD. The collision energy is 19 eV and 26 eV for PPE and ISTD, respectively.

2. 6. Processing of plasma sample

Pramipexole and ropinirole were extracted from plasma samples by using liquid-liquid extraction technique. All frozen, calibration curve standards and quality control samples were thawed at room temperature and homogenized with a vortex shaker (Spinix, Mumbai, India). Five hundred µL plasma samples in polypropylene tubes were spiked with 50 µL of ISTD standard solution (1 ng/mL).

The samples were vortexed for 30 seconds. To all the tubes 100 µL of 50 % ammonia in water was added and again vortexed for few seconds. Then 2.5 mL of extraction solvent [Ethyl acetate: *n*-Hexane (90:10 *v/v*)] was added, closed with tight caps and vortexed for 10 min followed by centrifugation at 4500 rpm at 5 °C for 10 min.

The upper organic layer was transferred into another polypropylene tubes and evaporated, in a nitrogen evaporator (Caliper Lifesciences, Hopkinton, Massachusetts), to dryness under nitrogen atmosphere at 40 ºC temperature.

After evaporation was completed, samples were reconstituted with 300 µL of mobile phase and vortexed for 30 seconds. The samples were transferred into auto sampler vials for injection into LC-MS/MS system.

2. 7. Method validation

The method was validated as per the guidelines of United States Food and Drug Administration for validation of bioanalytical methods [11].

2. 7. 1. Method selectivity

The method selectivity was assessed by the comparison of chromatograms of six different blank human plasma with the corresponding chromatograms of plasma samples spiked at LLOQ (100 pg/mL) level of the PPE and 1 ng/mL of the ISTD using the proposed extraction procedure, chromatography and mass spectrometry conditions. The acceptance criteria for area of interference compounds in blank human plasma are ≥20 % in comparison to the spiked LLOQ and ≤5 % in comparison to internal standard.

2. 7. 2. Linearity

The performance of the method was assessed by determining the linearity. The linearity of the method was determined by preparing three calibration curves with eight different concentrations 100, 222, 317, 635, 1155, 1674, 2263 and 2514 pg/mL PPE. The peak area ratios of the PPE to ISTD were calculated. The calibration curves were obtained by fitting these ratios as a function of corresponding concentrations using weighted ($1/x^2$) least square linear regression. The lowest concentration of the PPE (100 pg/mL) on the calibration curve was considered as LLOQ with acceptable accuracy and precision. The acceptance criteria of back calculated standard concentration was lower than 15 % deviation from nominal concentration and lower than 20 % deviation from nominal concentration at the LLOQ level. The acceptance criterion for correlation coefficient (r^2) was 0.99 or more.

2. 7. 3. Accuracy and precision

The precision and accuracy of the method were assessed under repeatable (intra-day) and reproducible (inter-day) circumstances. The intra- and inter-day precision and accuracy were evaluated at four different concentration levels [100 pg/mL (LLOQ), 264 pg/mL (LQC), 825 pg/mL (MQC) and 2230 pg/mL (HQC)]. For intra-day precision and accuracy, each quality control sample was analyzed six times on the same day whereas for inter-day precision and accuracy same samples were analyzed on three different days. The precision and accuracy of the method was expressed as percentage of coefficient of variation (CV) and percentage recovery, respectively. The intra- & inter-day accuracy determined at each concentration level should not exceed 15 % of nominal concentration for all the quality control samples except for LLOQ, where it should not exceed 20 %. The intra- & inter-day precision for all the quality control samples should be <15 % of CV.

2. 7. 4. Extraction recovery

The extraction recovery of PPE and ISTD were evaluated by comparing peak area of plasma spiked with PPE and ISTD prior to extraction with the peak area of plasma spiked with PPE and ISTD after the extraction. The extraction recovery of PPE was evaluated at three different concentration levels [264 pg/mL (LQC), 825 pg/mL (MQC) and 2230 pg/mL (HQC)] and extraction recovery of ISTD was evaluated at a concentration of 1 ng/mL. The recovery of the analyte and ISTD need not be 100 %, but the extent of recovery of an analyte and ISTD should be consistent, precise and reproducible.

2. 7. 5. Stability of drug in stock solution

The stability of the PPE in solution was investigated in two ways: short term stability and long term stability. For short term stability, stock solutions of PPE and ISTD were prepared and left at room temperature (stability samples) for 10 hrs. The freshly prepared stock solution was used as comparison sample. For long term stability, stock solutions of PPE and ISTD were prepared and stored in refrigerator below 10 °C (stability samples) for 8 days. On the day of analysis, freshly prepared stock solution was used as comparison sample. The short-term and long term stock solution stability of PPE and ISTD in diluent solution was successfully assessed by comparing mean responses of six replicates of stability samples versus six replicates of comparison samples. For both the stability studies, the samples were considered stable in stock solution if the deviation from the mean peak area is within ±15 %.

2. 7. 6. Stability of drug in human plasma

The stability of the PPE in human plasma was investigated in five ways: Freeze thaw stability, Bench top stability, Long term stability, In-injector stability and Effect of anticoagulant. All the stability studies were assessed by analyzing six replicates of LQC (264 pg/mL) and HQC (2230 pg/mL).

Freeze thaw stability was determined after six cycles of freeze-thaw, bench top stability was assessed after keeping the plasma samples at room temperature for 7 hrs, long term stability was assessed after storage of the test samples at -70 °C for 30 days, in-injector stability was determined by storing the reconstituted quality control samples for 48 hrs at 10 °C in auto sampler before being analyzed and effect of anticoagulant was assessed by comparing the percentage stability of PPE in human plasma containing ethylenediaminetetraacetic acid with human plasma containing citrate phosphate dextrose adenine.

The stability samples were run along with the freshly spiked calibration curve. The values were calculated against calibration curve of the day. The freshly prepared quality control samples (LQC and HQC) were used as comparison sample. The stability of the PPE in human plasma was successfully assessed by comparing mean value of six replicates of stability samples with six replicates of comparison samples. The samples were considered stable in human plasma at each concentration level if the deviation from the mean calculated concentration of quality control samples were within ±15 %.

3. RESULTS AND DISCUSSION

3. 1. Method development

3. 1. 1. Optimization of mass spectroscopy conditions

The LC-MS/MS operating parameters were carefully optimized for the sensitive and selective determination of PPE. A standard solution (1 ng/mL) of PPE and the ISTD were directly infused along with the mobile phase into the mass spectrometer with ESI as the ionization source and tuned initially in both positive and negative ESI modes. The outcomes indicated that positive ESI mode was found to be more sensitive than negative ESI mode. The mass spectra for PPE and ISTD revealed peaks at *m/z* 212.10 and 261.20, respectively, as protonated molecular ions $[M+H]^+$. The product ion mass spectrum for PPE shows the formation of major fragment ion at m/z 153.10. For ISTD, the fragment at m/z 114.20 was dominant and was consequently used for quantification.

The mass spectrometric parameters, such as ion source temperature, ion source gas 1 (nebulizer gas), ion source gas 2 (heater gas), curtain gas, collision gas, ionization voltage and collision energy were optimized to obtain the optimum intensity of deprotonated molecules of PPE and ISTD. The results are summarized in Tables 1 and 2.

Table 1. Optimized source gas parameters of the proposed LC-MS method.

Parameter	Value
Curtain gas (nitrogen) (psi)	20
Collision (nitrogen) (psi)	6
Gas1 (nebulizer gas) (psi)	45
Gas2 (heater gas) (psi)	50
Ion source temperature (^{0}C)	500
Ionization voltage (V)	5500

Table 2. Optimized compound parameters of the proposed LC-MS method.

Parameters	PPE	ISTD
Declustering potential (V)	40	60
Entrance potential (V)	10	10
Collision energy (eV)	19	26
Collision cell exit potential (V)	10	10
Dwell time (millisecond)	400	400

3. 1. 2. Optimization of chromatography conditions

To achieve the effective chromatographic resolution of PPE and ISTD, various mixture(s) of organic solvents such as acetonitrile and methanol along with 10 mM ammonium formate with altered flow rates (in the range of 0.20-1.0 mL/min) were tested.

The best resolution of peaks was achieved with an isocratic elution by the mobile phase consisting of acetonitrile and 10 mM ammonium formate in the ratio of 60:40 (*v/v*) at a flow-rate of 0.5 mL/min, on Zorbax SB-C18 analytical column (3.5 µm particle size, 4.6 x 150 mm).

The column oven temperature was maintained at 40 °C. Under the optimized chromatographic conditions, the retention times of PPE and the ISTD were around 1.344 and 1.534 min, respectively.

3. 1. 3. Selection of extraction solvent

Liquid-liquid extraction can be helpful in creating a clean sample and avoiding the insertion of non volatile materials onto the HPLC column and mass spectrophotometer system. Clean samples are essential for minimizing ion suppress ion and matrix effect in bioanalytical methods. Acetonitrile : dichloromethane, *n*-hexane : dichloromethane, *n*-hexane : dichloromethane : ethyl acetate and ethyl acetate : *n*-hexane mixtures were tried with different percentages as a solvent for the extraction of PPE and ISTD from the plasma. A mix of ethyl acetate : *n*-hexane in the proportion of 90:10 (*v/v*) was found to be optimal, which can produce a clean chromatogram for blank plasma blank and maximum recovery. As a result, this solvent mixture was preferred as a solvent for extraction.

3. 1. 4. Selection of internal standard

Selecting the appropriate ISTD is an important aspect to achieving acceptable method performance, especially with bioanalytical methods, where matrix effects can contribute to poor analytical results. Therefore, ropinirole was selected as apt internal standard, which is relevant to PPE. In addition, ropinirole retention behavior is similar to that of the PPE. Clean chromatograms were obtained and no significant direct interferences in multiple reaction monitoring channels at the relevant retention times were observed.

3. 2. Method validation

3. 2. 1. Method selectivity

No peaks were observed in blank plasma at the corresponding retention time of PPE (1.344 min) and internal standard (1.534 min). This suggests that there was no interference from endogenous substances present in the plasma and the method was found to be selective enough for the determination of PPE. The chromatograms of PPE and internal standard for blank plasma, showing no interference at the retention time of the PPE and the internal standard, plasma spiked at the LLOQ level of PPE are shown in Fig. 1 and 2.

3. 2. 2. Linearity

Table 3. Back calculated concentrations for calibration curve standards of PPE.

Calibration Standard	Concentration of PPE (pg/mL)		± SD	% CV
	Nominal	Mean calculated*		
S1	100	98.736	1.93	1.95
S2	222	233.633	15.83	6.78
S3	317	313.637	5.85	1.86
S4	635	614.021	30.44	4.96
S5	1155	1172.747	24.34	2.08
S6	1674	1623.062	73.01	4.50
S7	2263	2215.351	67.52	3.05
S8	2514	2633.206	167.81	6.37

***** mean of three determinations

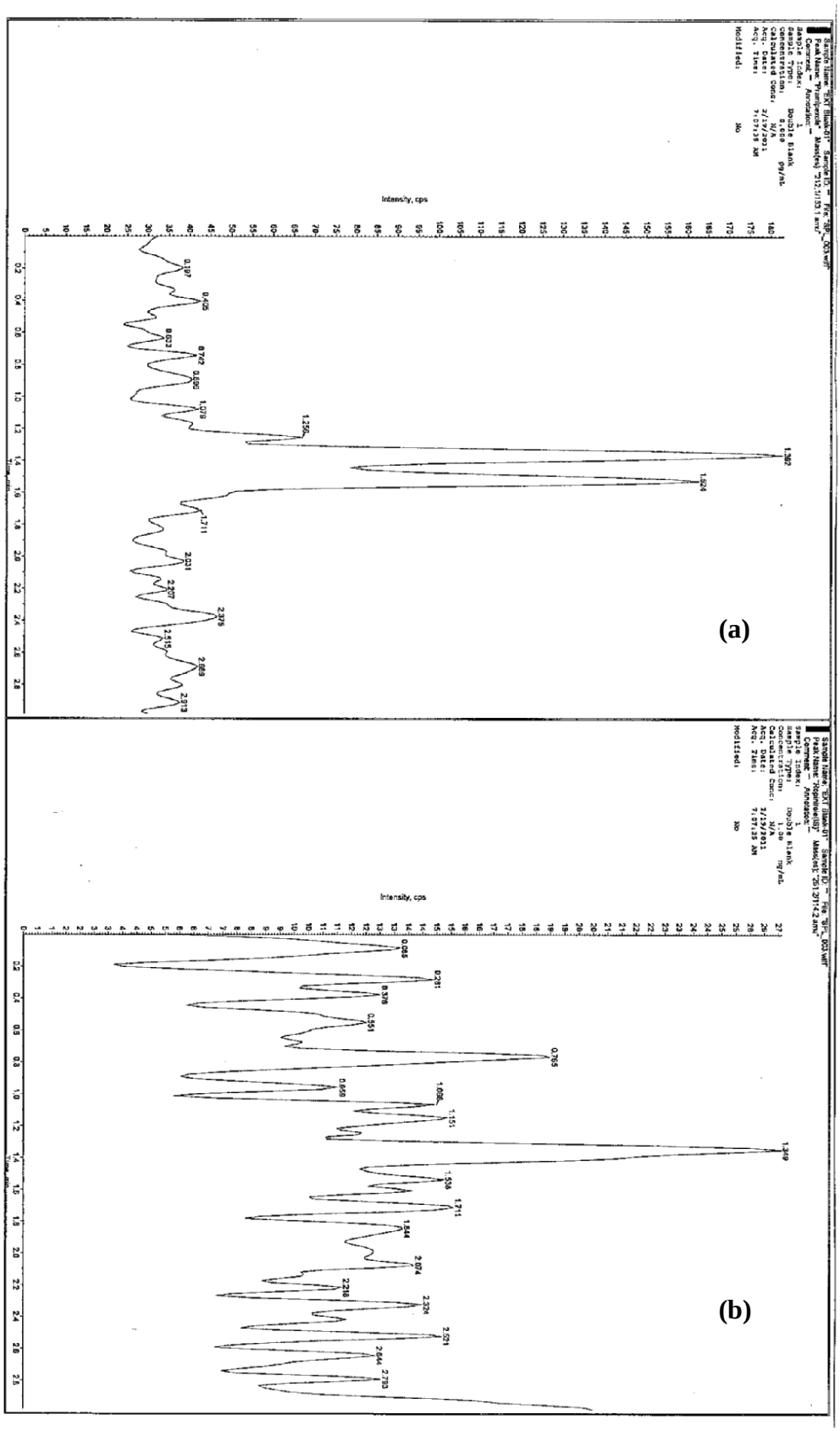

Fig. 1. Chromatogram of (a) blank plasma for PPE (b) blank plasma for ISTD.

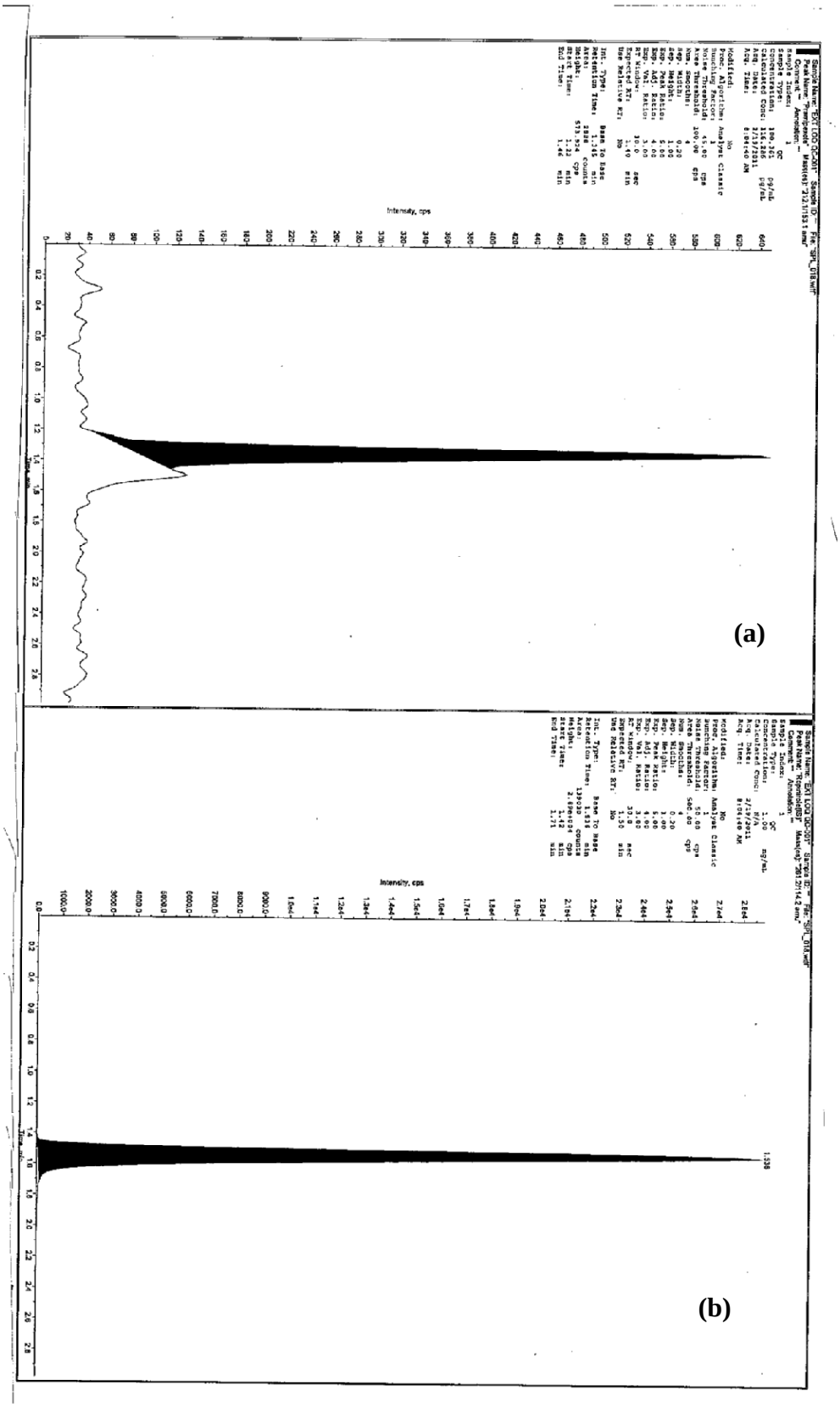

Fig. 2. Chromatogram of (a) LLOQ level of PPE (b) ISTD.

The linearity was calculated by using weighted least squares regression analysis ($1/X^2$) of PPE/ ISTD peak area ratios *vs* PPE concentration. The method was linear over the PPE concentration range from 100-2514 pg/mL. The calibration curve showed good linearity with correlation coefficient value of 0.9966. The calibration curve had a regression equation of y = 0.000196x + -0.00198, where y is the peak area ratio of PPE to ISTD and x is the plasma concentration of PPE. The back calculated concentration of calibration samples showed good precision & accuracy and they are within the acceptance limit (Table 3). The lowest point (100 pg/mL) on the standard curve was accepted as LLOQ.

3. 2. 3. Accuracy and precision

The percentage recovery and percentage coefficient of variation values for the assays in accuracy and precision study, respectively were calculated. The results are summarized in Table 4. The intra-day precision and accuracy were in the range of 3.489-6.756 % and 98.924-112.236 %, respectively. Like wise the inter-day precision and accuracy ranged from 3.970-5.714 % and 100.340-107.443 %, respectively. The precision and accuracy values are within the acceptable limit and indicating that the developed method was sufficiently precise and accurate.

Table 4. Intra- and Inter-day precision and accuracy of the proposed LC-MS method.

Level	Concentration of PPE (pg/mL)		Recovery (%)	CV (%)
	Nominal	Found* ± SD		
Intra-day				
LLOQ	100	112.236 ± 5.532	112.236	4.928
LQC	264	281.561 ± 19.024	106.651	6.756
MQC	825	836.785 ± 47.285	101.428	5.650
HQC	2230	2206.021 ± 76.976	98.924	3.489
Inter-day				
LLOQ	100	107.443 ± 5.765	107.443	5.365
LQC	264	280.707 ± 16.042	106.328	5.714
MQC	825	835.477 ± 41.474	101.269	4.964
HQC	2230	2237.603 ± 88.836	100.340	3.970

***** Average of six determinations

3. 2. 4. Extraction recovery

The extraction yield was calculated by dividing the mean peak area of the plasma sample spiked with PPE and ISTD prior to extraction by the corresponding mean peak area of the plasma sample spiked with PPE and ISTD after the extraction. The extraction recovery of the PPE at LQC, MQC and HQC levels was 87.0 %, 84.499 % and 82.08 %, respectively (Table 5).

The recovery of ISTD was 79.415 % (Table 5). These results indicate that the recovery of PPE and ISTD using liquid-liquid extraction technique by extraction solvent**,** ethyl acetate and *n*-hexane (90:10 *v/v*)*,* was satisfactory, consistent, precise and reproducible.

Table 5. Extraction recovery of PPE and ISTD.

Level	Concentration (pg/mL)	Drug spiked	Peak area* ± SD	CV (%)	Recovery (%)
LQC	264	Before extraction	7403.6 ± 488.33	6.6	87.00
		After extraction	8509.7 ± 748.01	8.8	
MQC	825	Before extraction	20177.0 ± 1512.35	7.5	84.499
		After extraction	23878.2 ± 571.72	2.4	
HQC	2230	Before extraction	60435.7 ± 3186.02	5.3	82.080
		After extraction	97351.2 ± 2426.13	2.5	
ISTD	1	Before extraction	135125.6 ± 8260.24	6.1	79.415
		After extraction	212746.7 ± 13969.10	6.6	

***** Average of six determinations

3. 2. 5. Stability of drug in stock solution

The results are summarized in Table 6. The percentage deviation for the mean peak area of PPE and ISTD left at room temperature for 10 hrs was found to be -7.07 % and -0.719 %, respectively. The percentage deviation for the mean peak area of PPE and ISTD stored in refrigerator below 10 °C for 8 days was found to be -2.013 % and -8.864 %, respectively. This is within the acceptance range of ±15 %. Results indicate that PPE and ISTD were stable for least 10 hrs at room temperature and for 8 days when stored in refrigerator below 10 °C.

Table 6. Short term and long term stability of PPE and ISTD in stock solution.

Drug	Type of sample	Peak area*	Stability (%)	Deviation (%)
PPE	Stability	22060.5	92.930	-7.07
	Comparison	23738.7		
ISTD	Stability	217096.7	99.821	-0.719
	Comparison	217485.7		
PPE	Stability	23156.5	97.984	-2.016
	Comparison	23632.8		
ISTD	Stability	234919.2	91.136	-8.864
	Comparison	257765.5		

***** Average of six determinations

3. 2. 6. Stability of drug in human plasma

The stability results of freeze thaw, bench top, long term, in-injector and effect of anticoagulant are summarized in Table 7. When PPE stability at the LQC and HQC concentrations was evaluated after six freeze thaw cycles, the differences from freshly prepared quality control samples was about 1.774 % and 4.593 %, respectively. When bench top stability at the LQC and HQC concentrations at room temperature for 7 hrs was measured the differences from freshly prepared quality control samples were 1.609 % and -3.681 %, respectively.

Table 7. Stability of PPE in human plasma.

Stability	Type of sample	LQC (264 pg/mL)			HQC (2230 pg/mL)		
		Found* ± SD	Stability (%)	Deviation (%)	Found* ± SD	Stability (%)	Deviation (%)
Freeze thaw	Stability	261.590 ± 18.091	101.774	1.774	2270.612 ± 124.023	104.593	4.593
	Comparison	257.030 ± 23.917			2170.883 ± 216.776		
Bench top	Stability	272.185 ± 14.998	101.609	1.609	2153.347 ± 131.973	96.319	-3.681
	Comparison	267.874 ± 16.050			2235.624 ± 159.583		
Long term at -70^{0}C for 30 days	Stability	244.033 ± 14.939	97.573	-2.427	2012.031± 67.396	95.830	-4.170
	Comparison	250.102 ± 11.367			2099.576 ± 99.270		
In-injector	Stability	248.659 ± 16.003	98.727	-1.273	2169.922 ±35.178	99.720	-0.280
	Comparison	251.863 ± 7.333			2176.010 ± 38.406		
Effect of anticoagulant	With EDTA	271.026 ± 27.657	102.661	2.661	2260.878 ± 233.694	101.384	1.384
	With CDPA	276.559 ± 25.419	104.757	4.757	2336.776 ± 212.845	104.788	4.788

***** Average of six determinations; **EDTA** - Ethylenediaminetetraacetic acid; **CDPA** - citrate phosphate dextrose adenine

When PPE stability in the human plasma at -70 °C for 30 days was measured at the LQC and HQC concentrations, difference from freshly prepared samples were -2.427 % and -4.170 %, respectively. When in-injector stability at the LQC and HQC concentrations for 48 hrs at 10 °C was measured, the differences from freshly prepared samples were low with values -1.273 % and -0.280 % respectively. The values are within the acceptance range of ±15 %. The results indicate that PPE spiked into human plasma was stable during six freeze thaw cycles, at least 7 hrs at room temperature, 48 hrs at 10 °C in auto sampler and up to 30 days at around -70 °C.

The percentage deviation of the mean calculated for PPE in plasma with EDTA was 2.661 % and 1.384 % at LQC and HQC levels, respectively. Whereas, for PPE in plasma with CDPA at LQC and HQC levels are 4.757 % and 4.788 %, respectively. The results indicate that the PPE have greater stability in plasma with anticoagulant, EDTA.

4. CONCLUSION

A LC-MS method in positive electrospray ionization mode using multiple reaction monitoring is described for the quantification of PPE in human plasma. The developed method was fully validated according to guidelines of United States Food and Drug Administration for validation of bioanalytical methods. The method has shown satisfactory precision and accuracy. The reproducibility, specificity and sufficient sensitivity of this method allows for the selective and reliable determination of PPE in human plasma. Further more this method offers shorter run time over earlier methods.

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