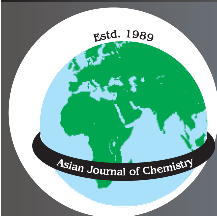
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A New Validated Stability Indicating Ion-Pair HPLC Method for Evaluation of Impurities of Pramipexole from Low Dose Extended Release Formulation

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A new validated stability indicating ion-pair HPLC method was developed for evaluation of impurities of pramipexole from low dose extended release tablet formulation. Challenges related to extraction and recovery of impurities along with active ingredient-pramipexole from inactive ingredient matrix which contain HPMC-polymer, high placebo to drug ratio (100:1), were fully addressed. Tablets were subjected for forced degradation studies and oxidative impurities (N- and S-oxides) were identified by LC-MS. All impuritiies as well as placebo peaks were well resolved from pramepexole peak. The method was developed by using Inertsil ODS-3V column with a pre-guard column. Sodium salt of octane sulphonic acid as ion pair was used along with phosphate buffer (pH 2.7) with flow rate of 1 mL/min, linear gradient using acetonitrile as organic modifier. Pramipexole and its related impurities were monitored at UV wavelength of at 264 nm. The finalized method consists was fully validated as per ICH Q2 (R1) guideline.

Keywords: Pramipexole, Ion pair HPLC, Extended release formulation, LC-MS, Stability, Validation.

INTRODUCTION

Pramipexole, a non-ergot dopamine agonist, is used to treat symptoms of Parkinson's disease such as stiffness, tremors, muscle spasms and poor muscle control and also used to treat restless legs syndrome (RLS). Pramipexole has some of the same effects as a chemical called dopamine, which occurs naturally in our body. Low levels of dopamine in the brain are associated with Parkinson's disease [1].

The chemical name of pramipexole dihydrochloride is (S)-2-amino-4,5,6,7-tetrahydro-6-(propylamino)benzothiazole dihydrochloride monohydrate. Its empirical formula is $C_{10}H_{17}N_3S{\cdot}2HCl{\cdot}H_2O$ and its molecular weight is 302.26. Tablets with extended release formulation are administered orally, which are available in dosage strengths of 0.375, 0.75, 1.5, 2.25, 3, 3.75 or 4.5 mg of pramipexole dihydrochloride monohydrate. Inactive ingredients include hypromellose, corn starch, carbomer homopolymer, colloidal silicon dioxide and magnesium stearate [2].

Despite of many advantages like sustained drug levels in blood, attenuation of adverse effects and improved patient compliance in formulating drugs as extended release dosage forms, analytical scientists face numerous challenges in developing analytical methods which were sensitive and should separate all the degradants and process related impurities from high polymer matrix, used as extended release agents. Even the situation becomes worse, when the placebo to drug ratio is high for low dose formulations and the developed methods should separate peaks due to placebo.

With current work, challenges related to extraction and recovery of impurities along with active ingredient-pramipexole from inactive ingredient matrix which contain HPMC-polymer, high placebo to drug ratio (100:1), were fully addressed and a stability indicating RP-HPLC method was developed. Desired and consistent separation was achieved between impurities; unknown impurities formed during stability studies and forced degradants from main peak along with negligible placebo interferences.

Few analytical methods were available for the determination of impurities in pramipexole. One study was reported about experimental design in chromatographic analysis of pramipexole and its impurities [3]. One more study discusses about development of a validated liquid chromatography method for separation of process-related impurities including the R-enantiomer of S-pramipexole on polysaccharide chiral stationary phases [4]. Another study was reported for the establishment of inherent stability of pramipexole and development of validated stability indicating LC-UV and LC-MS method [5]. Several other spectroscopic and LC-MS methods were published for determination of pramipexole in human plasma

and dosage forms [6-15]. All the above mentioned studies reveal the impurities related to drug substance and even official monographs of pramipexole like USP and Phr.EP contain estimation of impurities related to drug substance.

Literature survey reveals that, no reverse phase liquid chromatography method available for the determination of the purity of pramipexole in pharmaceutical dosage forms, that too from extended release tablets. Hence, a stability indicating RP-HPLC Gradient method was developed and fully validated as per ICH guideline for the purity determination of pramipexole from extended release tablets in presence of impurities namely P1 and P2 along with forced degradation products. LC-MS studies were carried to identify oxidative degradants and evaluated their presence in drug product.

EXPERIMENTAL

Standards of pramipexole and its 2 impurities namely impurity P1 and P2 (Fig. 1) were supplied by Dr. Reddy's laboratories limited, Hyderabad, India. HPLC grade 1-octane sulfonic acid sodium monohydrate salt, potassium dihydrogen phosphate, ammonium acetate, orthophosphoric acid (85 %) and glacial acetic acid (99 %) were procured from Merck, India. HPLC gradient grade methanol and acetonitrile were procured from Merck, Germany. The purity of all chemicals was above 98 %. High purity, deionized water filtered through 0.22 µ membrane filter was obtained from Millipore Milli-Q Plus water purification system (Millipore, Milford, MA, USA).

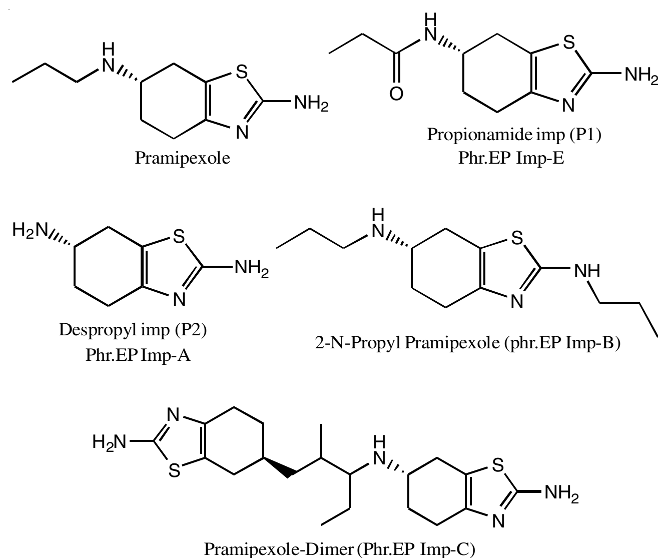

Fig. 1. Structure of pramipexole and its impurities

HPLC system (Model: Alliance 2695, Make: Waters, Milford, USA) was used, consists of a quaternary pump, auto sampler and a photo-diode array detector (PDA 2998). The output signal was monitored and processed using empower-2 software. Applied Biosystems 4000 Q Trap triple quadrupole mass spectrometer with Analyst 1.4 software (MDSSCIEX, USA) was used for LC-MS studies. Sonicator (Labtech, Korea), Centrifuge (Thermo electron GmbH, Germany) was used during sample preparation. Photo stability studies were carried out in a photo stability chamber (Sanyo, UK) and thermal stability studies were performed in a dry air oven (Thermolab, India).

Chromatographic conditions: The method was developed using Inertsil ODS-3 V, 250 mm × 4.6 mm, 5 µm column with a pre-guard column of Hypersil Gold 10 mm × 4.6 mm, 5 µm. Buffer was prepared by dissolving 1-octane sulfonic acid sodium monohydrate salt (0.5 % w/v) and potassium dihydrogen phosphate salt (9.1 g/L, 67 mM), pH adjusted to 2.7 with orthophosphoric acid and filtered through 0.45 µ membrane filter. Mobile phase-A was prepared by mixing buffer-acetonitrile (90:10, v/v) and mobile phase-B was prepared by mixing buffer-acetonitrile (50:50, v/v). The gradient program (Time in min/%B) was set as 0.0/10, 55.0/60, 70.0/85, 72.0/100, 76.0/100, 78.0/10, 95.0/10 with flow rate of 1.0 mL/min. The column oven temperature was maintained at 40 °C and sample cooler at 5 °C. HPLC was equipped with 1000 µL loop and the injection volume was 700 µL. Pramipexole and its related impurities were monitored at UV wavelength of at 264 nm.

Preparation of standard solutions: A mixture of methanol-glacial acetic acid (90:10, v/v) was used as diluent-1 and buffer pH 2.7 was used as diluent-2. A primary stock solution of 200 µg/mL was prepared by dissolving appropriate amount of pramipexole standard in methanol. Intermediate stock of 40 µg/mL was prepared by diluting primary stock with diluent-1, followed by preparing final working standard solution of 0.08 µg/mL in diluent-2.

Preparation of sample solution: Not less than 30 tablets were finely crushed using mortar and pestle, weighed content equivalent to 7.5 mg of drug and transferred to a 200 mL volumetric flask. Added 140 mL diluent-1 and sonicated for 30 min with intermediate shaking to disperse the content and diluted to volume with diluent-1 to give a solution containing 37.5 µg/mL. This solution was centrifuged at 4000 rpm for 10 min and clear supernatant was diluted to meet the concentration of 7.5 µg/mL and filtered through 0.45 µm pore size PVDF syringe filter.

Impurity blend stocks of P1 and P2 were prepared in diluent-1 and spiked on test solution to meet 0.4 % of target concentration and were used for method validation.

Forced degradation and LC-MS studies for identification of degradants: From structure and literature, it is evident that pramipexole is prone to oxidation at N and S positions of benzothiazole ring, to form respective N-oxide and S-oxide degradants [5]. Hence, LC-MS studies were carried to confirm these degradants mass numbers (*m/z*) and their presence in drug product. Inertsil ODS 3V 250 × 4.6 mm, 5 µ column, MS grade 0.02 M ammonium acetate solution was used as mobile phase-A and water-methanol (50:50, v/v) was used as mobile phase-B. Flow rate was set at 1.2 mL/min with gradient program of time (min)/%B as 0.0/0, 10.0/5, 15.0/10, 20.0/10, 22.0/0, 30.0/0 and column temperature was maintained at 45 °C. Detection of degradants was monitored by photodiode array detector (PDA) at 264 nm.

Drug substance (1 mg/mL) was stressed in 3 % H_2O_2 solution for 4 h at 50 °C and diluted a portion of sample with equal portion of mobile phase-A. The analysis was performed on liquid chromatography coupled to Applied Biosystems 4000 Q Trap triple quadrupole mass spectrometer, ESI at positive mode with Analyst 1.4 software, MDSSCIEX, USA. The ion source voltage, temperature were maintained at 5000 V, 450 °C and curtain gas flow at 20 psi.

Method validation: The proposed method was validated as per ICH guidelines [16].

Specificity: Specificity is the ability of the method to measure the analyte response in the presence of its potential impurities. Forced degradation studies were performed for pramipexole extended release tablets to evaluate stability-indicating capability and specificity of the proposed method and specificity in presence of its impurities namely P1 and P2. Stress conditions were optimized which include exposure of drug product to UV light (1.2 Million Lux hours), heat (105 °C, 24 h) and humidity (25 °C/90 % RH, 2 days). To stock solution of test sample acid stress was carried by adding 5 mL of 5 N methanolic HCl and kept at 55 °C for 20 h and alkali stress by adding 5 mL of 0.5 N methanolic NaOH and kept at 55 °C for 20 h. Hydrolysis was carried by adding 5 mL water and refluxed at 55 °C for 20 h and oxidative stress was carried by adding 5 mL of 1 % H_2O_2 and kept at room temperature in dark for 24 h. After stress time, sample solutions were prepared from stressed stocks to evaluate the ability of the proposed method to separate pramipexole from its degradation products. Spectral purity of pramipexole peak was evaluated by using PDA detector in all stressed samples. Same stress conditions were also applied for Placebo to assess the impact of interferences with degradants and main peak.

Precision: The precision of the method was verified by injecting six individual preparations of pramipexole from extended release tablets 7.5 µg/mL, spiked with 0.40 % of P1 and P2 impurities. Evaluated system suitability and % RSD of area for each impurity was calculated. Ruggedness of the method was determined using different instrument, column and analyst and performing the analysis on different day.

Limit of detection (LOD) and limit of quantification (LOQ): Limit of detection and limit of quantification for impurities P1 and P2, along with pramipexole were determined at a signal-to-noise ratio of 3:1 and 10:1, respectively, by injecting a series of dilute solutions with known concentrations. Precision at LOQ study was carried out by injecting six individual preparations of impurities and % RSD was calculated.

Linearity: Linearity test solutions for the method were prepared by diluting stock solution to the required concentrations. The solutions were prepared at six concentration levels from LOQ to 400 % of the specification level.

Accuracy: Accuracy of the related substance method was evaluated in triplicates using concentration levels ranging from LOQ to 400 % (specification 0.4 % of target concentration, *i.e.*, 7.5 µg/mL) by spiking impurities on test sample and % recovery was calculated. The same was performed with pramipexole also, to elucidate the recovery of unknown with respect to main peak.

Robustness: Robustness of the method was determined by deliberately altering the chromatographic conditions and to evaluate the impact on system suitability parameters like tailing and USP resolution between pramipexole and its impurities. The pH of the mobile phase was varied from 2.5 and 2.9 (original pH: 2.7), flow rate of the method was varied from 0.8 to 1.2 mL/min (original flow: 1.0 mL/min) and column oven temperature was varied from 35 °C to 45 °C (original temperature: 40 °C). Variation in mobile phase composition was performed by varying ± 10 % of organic solvent, from original.

Solution stability and mobile phase stability: Solution stability of pramipexole and its impurities in the related substance method was carried out by storing spiked sample solutions in tightly capped volumetric flasks in refrigerator (2-8 °C) and percent assay for impurities was determined at initial to till study period, along with monitoring formation of any unknown impurities. Bench top stability of mobile phase was conducted for a period of 1, 2 and 5 days by analyzing freshly prepared spiked sample and evaluated system suitability and % impurity results.

Filter validation: The study was performed to establish the suitability of filter by preparing spiked sample and filtered through 0.45 µ PVDF and 0.45 µ nylon membrane syringe filters. Percent impurities and total impurities were calculated and results were compared with results of clear centrifuged sample.

RESULTS AND DISCUSSION

The key objective of method development lies with separation of degradants, process related impurities and peaks due to placebo. Initial trials were performed using impurities mentioned in EPCRS, prepared impurity blend and spiked on drug product. Peaks due to placebo and their merging or co-elution with known impurities, unsatisfactory resolution between unknown impurities formed above reporting threshold stood as major challenges in selecting and screening of suitable chromatographic conditions. Study on variability of mobile phase pH and column temperatures was performed, where blunt, asymmetric peak shapes were observed. With Alltima C18 250 × 4.6 mm, 5 µ column, consistent placebo peaks and symmetric peak shapes for pramipexole and impurities were achieved with addition of ion-pairing agent (1-octane sulfonic acid sodium salt) to the mobile phase containing phosphate salt at lower pH and acetonitrile as organic modifier.

Extraction studies were performed using methanolic HCl to achieve dispersion of sample content and recovery of impurities from placebo, where unknown peaks were generated due to placebo and inconsistent trend was observed from sample to sample. This was rectified by using methanolic acetic acid with sonication, where extraction of drug and its impurities from finely crushed tablet powder found satisfactory. Due to high placebo content in sample and ion pair in mobile phase, column's life got ruined and resulted in poor separation. This was rectified by centrifuging the sample followed by dilution and further filtration, resulted in clear sample with low drug concentration due to which higher injection volume of 700 µL was selected to attain satisfactory response.

Further few more columns were screened, where Inertsil ODS-3 V, 250 mm × 4.6 mm, 5 µm column connected with a pre-guard column of Hypersil Gold 10 mm × 4.6 mm, 5 µm gave superior separation and enhanced column life. The final optimized gradient program (time in min/%B) was set 0.0/10, 55/60, 70/85, 72/100, 76/100, 78/10, 95/10 with flow rate of 1.0 mL/min. Using the finalized method, chromatograms of spiked test sample and EPCRS impurity mix standard were depicted in Figs. 2 and 3. Stress testing was performed and degradants peaks were well separated and found no interference of placebo peaks (Figs. 4-6). Peak purity assessed by PDA and passed for pramipexole in all the degradation conditions.

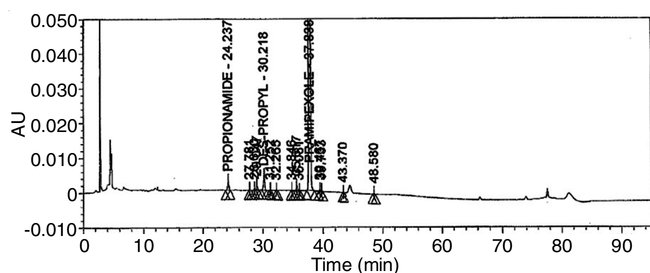

Fig. 2. Spiked test chromatogram

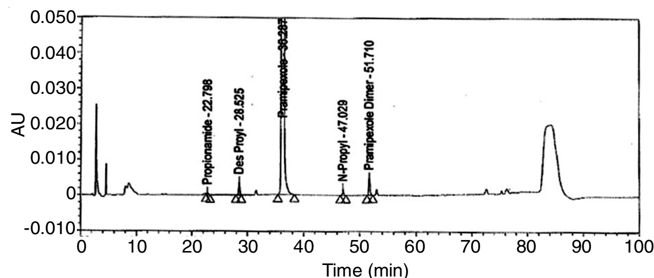

Fig. 3. EP CRS impurity mix chromatogram

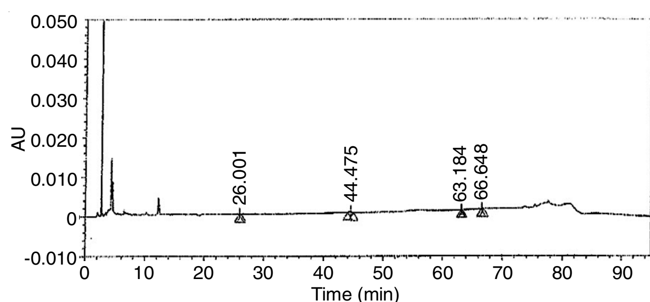

Fig. 4. Placebo chromatogram

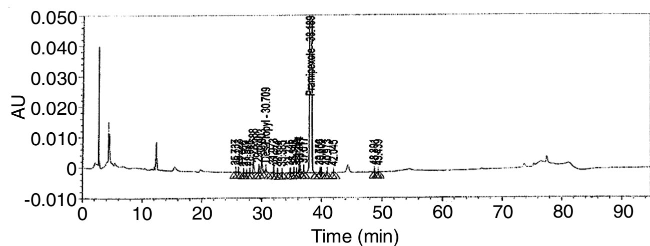

Fig. 5. Thermal stressed test chromatogram

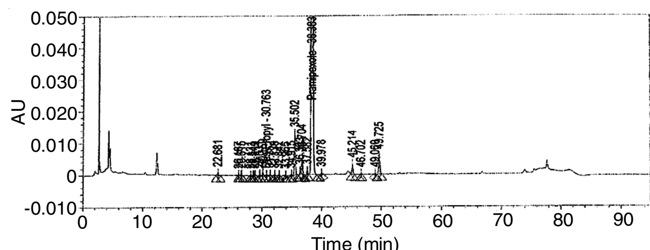

Fig. 6. Humidity stressed test chromatogram

Outcome of forced degradation and LC-MS studies: Unknown impurities due to oxidative stress were identified as N-oxide pramipexole with *m/z* 228.20 and S-oxide pramipexole with *m/z* 228.40 which eluted at RRT 0.65 and 0.80, in mentioned LCMS conditions (Figs. 7 and 8).

In newly developed method, confirmed the retention times of N-oxide and S-oxide impurities at RRT's 0.88 and 0.90. Pramipexole found to be degraded to despropyl impurity (P2) with *m/z* 169.25 acid, alkali and thermal stressed conditions.

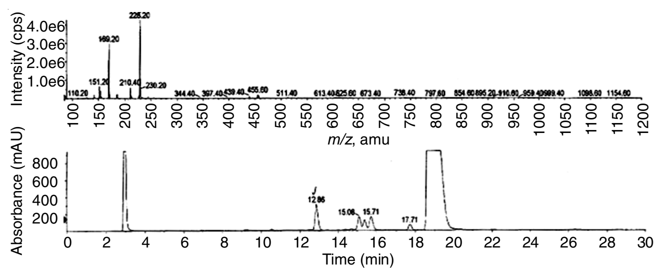

Fig. 7. UV and extracted mass spectrum of N-oxide impurity of pramipexole

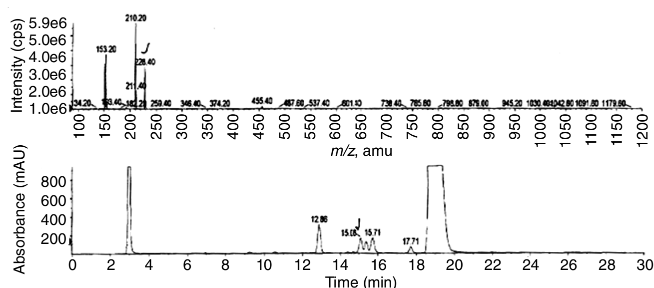

Fig. 8. UV and extracted mass spectrum of S-oxide impurity of pramipexole

Mild degradation was observed in water hydrolysis and photolytic degradation. At humidity stress, few unknown peaks were formed, but are below identification threshold. Photodiode array detector (PDA) was employed to check and ensure the homogeneity and purity of main peak in all the stressed sample solutions. Assay studies were carried out for stress samples against pramipexole qualified working standard. The mass balance (% assay + % impurities + % sum of all degradants) results are presented in Table-1. The purity and assay of pramipexole was unaffected by the presence of its impurities and degradation products and thus confirms the stability-indicating power of the method.

Outcome of stability studies: Stability studies for finished product, starting from initial to 6 months accelerated condition 40 °C/75 % RH were analyzed and evaluated impurity trending. No potential degradants were observed greater than or equal to identification threshold and rest of known impurities (dimer imp and 2-N-propyl imp from EPCRS mix) and unknown impurities formed in humidity stress were monitored. Hence, validation was performed including P1 and P2 impurities only.

Precision: System suitability found passed and % impurity for the peak areas of P1 and P2 in method precision and interprecision study was within acceptance criteria and results demonstrated that the method is precise (Table-2).

Limit of detection (LOD) and limit of quantification (LOQ): Signal to noise ratio for pramipexole, P1 and P2 impurities were determined at LOD and LOQ levels and precision at LOQ values were reported in Table-3.The % mean and % RSD for pramipexole, P1 and P2 impurities at LOQ level are within 6.7 %.The recoveries at LOQ level are in the range of 95.2-105.6 %

Linearity: Linear calibration plot for the related substance method was obtained over the calibration ranges tested, *i.e.*, LOQ to 400 % of the specification level (0.4 %). The correlation coefficient obtained was greater than 0.997 for pramipexole, P1 and P2 impurities. The slope and y-intercept values are also provided in Table-3, which confirmed good linearity between peak areas and concentration.

TABLE-1
SUMMARY OF STRESS TESTING RESULTS

Stress condition	Duration (time)	Net % degradation	Mass balance* (% assay)	Purity angle**	Purity threshold**	Purity flag**
Thermal stress 105 °C	24 h	2.46	95.8	0.042	0.294	No
Humidity stress 25 °C/90 % RH	2 days	5.33	96.3	0.051	0.270	No
Acid Stress 5 N methanolic HCl at 55 °C	20 h	0.32	101.1	0.044	0.274	No
Alkali stress 0.5 N methanolic NaOH at 55 °C	20 h	0.66	98.2	0.035	0.279	No
Water and refluxed at 55 °C	20 h	0.00	100.8	0.075	0.275	No
Peroxide stress 1 % H_2O_2 at room temperature	24 h in dark	0.64	96.6	0.043	0.268	No
Photo stress-UV light	1.2 M Lux hours	0.94	97.1	0.041	0.290	No

*Mass Balance = % assay + % impurities + % sum of all degradants
**As per Empower software: Purity angle should be less than purity threshold with no flag

TABLE-2
SUMMARY OF SYSTEM SUITABILITY AND PRECISION

Compound name	RRT	USP tailing	Precision	RSD (%)	Inter-precision	RSD (%)
Imp-P1	0.64	1.1	0.433	1.4	0.440	1.7
Imp-P2	0.80	1.1	0.449	3.6	0.472	1.8
Pramipexole	1.00	1.1	0.457	3.3	–	–

RRTs must be comparable, USP tailing: NMT 2.0 %, %RSD for precision and inter-precision NMT 10.0 %

TABLE-3
SUMMARY OF LINEARITY, LOD, LOQ, PRECISION AND RECOVERY AT LOQ

Compound	LOD (%)	Slope (m)	Intercept (y)	Correlation coefficient (R)	% Bias	LOQ Precision (n = 6)		% LOQ recovery (n = 3)
						Mean (%)	RSD (%)	
Imp-P1	0.023	131400.59	-658.79	0.999	-1.6	0.073	6.7	95.2
Imp-P2	0.019	1666802.51	-528.07	0.999	-1.0	0.077	2.1	105.6
Pramipexole	0.028	1050076.72	907.87	0.998	-2.8	0.079	3.3	98.9

Obtained USP s/n = 3 for LOD and 10 for LOQ. % RSD for LOQ Precision is NMT 15 %, Recovery at LOQ should be within 85-115 %.
For Linearity: Correlation coefficient >0.997 and bias should be ± 5.0 %

Accuracy: Recovery of impurities from drug product in spiked studies ranged from 97.5 to 109.5 % and spiked studies of pramipexole on placebo was ranged from 98.4 to 105.6 % at five different levels (Table-4).

TABLE-4
SUMMARY OF ACCURACY

Compound	50 %	100 %	200 %	300 %	400 %
Imp-P1	109.7	105.6	101.0	98.4	107.5
Imp-P2	110.7	97.5	100.9	101.6	107.8
Pramipexole	105.6	105.5	103.3	99.2	98.4

% Recovery should be within 85 to 115 % for each level

Solution stability, mobile phase stability and filter validation: Refrigerated solution stability (at 2-8 °C) for spiked test sample was established from initial till 8 h and results found satisfactory and standard solution found stable till 5 days at bench top and refrigerated condition. No significant changes were observed in system suitability and estimation of content of P1 and P2 impurities during mobile phase stability from initial and up to 5 days. No extra peaks and compatibility issue found with samples filtered through PVDF and nylon membrane filters and results were satisfactory.

Robustness: In all the deliberate varied chromatographic conditions (pH, flow rate, column temperature and composition of organic solvent), RRT's and resolution between all pairs of compounds was greater than 2.0 and tailing factor for pramipexole and its impurities was less than 1.2.

Conclusion

The newly developed ion-pair gradient reversed phase liquid chromatography method for quantitative analysis of pramipexole and its impurities in extended release tablet formulation was found to be specific, precise, accurate, linear, rugged and robust. Satisfactory results were obtained from validation of the method. The method is stability-indicating and can be used for routine analysis of developmental, stability and production samples of pramipexole extended release tablets.

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