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# UPLC-MS/MS Method for Simultaneous Quantification of Pramipexole, Ropinirole and Rasagiline In Human Plasma and Its Application to A Pharmacokinetic Study

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## ABSTRACT

A selective, sensitive and rapid UPLC-MS/MS method has been developed and validated for simultaneous quantification of pramipexole, ropinirole and rasagiline in human plasma using trimetazidine as internal standard (IS). The analytes and IS were extracted from 200 µL of plasma by solid phase extraction technique using strata cartridges which offers high sensitivity, wide linearity without interferences form endogenous matrix components. Chromatographic separation was achieved in 3.00 min run time on a Synergi Polar RP column using a 5 mM ammonium acetate/methanol mobile phase in gradient mode. The quantification of target compounds was performed in a positive electrospray ionization mode and multiple reaction monitoring (MRM). The proposed method was validated over the concentration ranges of 5-50000 pg/mL for each analyte. The intra- and inter-day precision and accuracy results were acceptable as per FDA guidelines. Stability of compounds were established in a battery of stability studies, i.e. bench top, auto sampler, dry extract and long term storage stability as well as freeze-thaw cycles. The validated method has been successfully used to analyze human plasma samples for application in pharmacokinetic studies.

**Keywords:** Pramipexole; Ropinirole; Rasagiline; UPLC-MS/MS.

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## INTRODUCTION

Parkinson's disease is the second most common neurodegenerative disease. Approximately 1 to 2% of people over 60 years old have parkinson's disease. The life time risk to develop parkinson's disease is estimated to be 2%, but a positive family history increases that risk to 4%[1]. In parkinson's disease, there is a loss of dopaminergic neurons in the substantianigra; decreased dopamine in the striatum; a loss of pigmentation in the neurons of the brain stem, but especially in substantianigra; and the development of gliosis and lewy bodies. A decrease in dopaminergic neurons leads to increased firing of neurons in the globuspallidus and subthalamic nuclei. As a result, the thalamus is inhibited and there is less activation of the motor cortex, which results in the clinical features of parkinson's disease[2]. The clinical symptoms of parkinson's disease include motor and nonmotor, with nonmotor symptoms starting 4 to 20 years before the motor symptoms are noticeable. The diagnosis however is based upon the classic motor symptoms of resting tremor, postural instability, badykinesia and rigidity. No treatment that can stop the progression of parkinson's disease is currently available; antiparkinson medication merely ameliorates the motor symptoms. Although initially effective, dopaminergic therapies are eventually complicated by motor fluctuations, including off time (periods of return of parkinson disease symptoms where medication effect wears off) and dyskinesia (drugs induced involuntary movements including chorea and dyskinesia) in most patients[3].

These problems are effectively reduced by adjunctive therapy with pramipexole, ropinirole and rasagiline. Pramipexole is a non ergot dopamine against which stimulates the D3 receptors. It is especially effective in treating patients who are experiencing the "on-off phenomenon". Ropinirole is a non-ergot dopamine agonist. It stimulates the D2 receptor and is effective in mild cases. It is also effective in those patients in an advanced stage who have "on- off phenomenon"[4]. Rasagiline is MAO-B inhibitor which by interfering with dopamine metabolism, increase the concentration of dopamine at the neuronal synapse. It was very useful in early or mild parkinson's disease[5].

Literature survey reveals that several methods have been reported for the quantitative determination of pramipexole[6-9], ropinirole[10] and rasagiline[11, 12] individually in biological fluids. However, till date no LC-MS/MS method has been reported in literature for the simultaneous determination of pramipexole, ropinirole and rasagiline in human plasma. Hence, authors attempted to develop a simple and reproducible LC-MS/MS method for simultaneous

quantification of these three analytes which helps the researchers for therapeutic drug monitoring and pharmacokinetics.

The aim of this work is, to develop a simple, selective and sensitive method, which employs solid-phase extraction technique for sample preparation and liquid chromatography with electrospray ionisation-tandem mass spectrometry for simultaneous quantitation of pramipexole, ropinirole and rasagiline in human plasma. The proposed method has significant advantages over earlier reported methods like 1) simultaneous quantification of three analytes, 2) Shorter run time, 3) Wider linearity range with a more sensitivity, 4) Simple reproducible extraction. The present method has been validated as per the current USFDA guidelines[13].The application of this assay in a clinical pharmacokinetic study following oral administration of pramipexole, ropinirole and rasagiline were described.

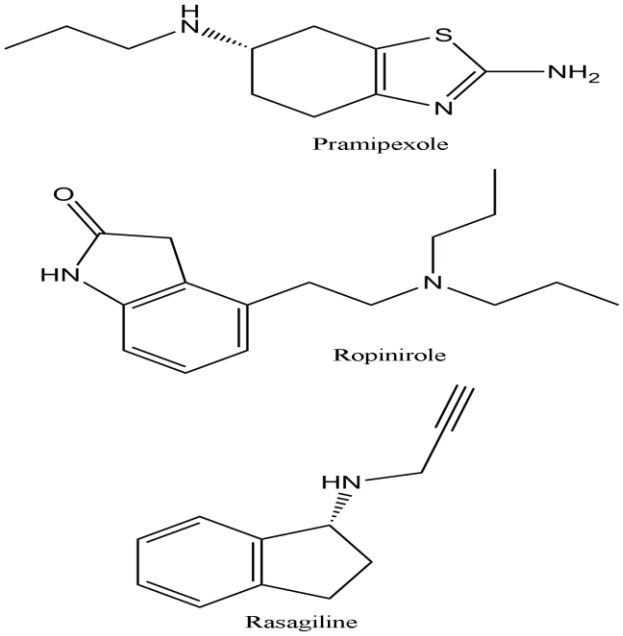


**Figure 1: Chemical structures of pramipexole, ropinirole and rasagiline**

## MATERIALS AND METHOD

### Materials and reagents

Reference standards of pramipexole dihydrochloride, ropinirole hydrochloride, rasagiline mesylate (Figure. 1), internal standard (trimetazidine hydrochloride) were purchased from Sigma-Aldrich (Hyderabad, India). HPLC grade ammonium acetate was procured from Thermo Fisher Scientific (Mumbai, India). HPLC grade methanol was procured from J.T Baker (Phillipsburg, USA). Milli-Q water (18.2 mΩ and TOC≤50 ppb) from Milli-Q purification system, Millipore (Bangalore, India) was used throughout the study. The solid phase extraction

cartridges (Strata, 1 $cm^3$ / 30 mg) were purchased from Phenomenex (Hyderabad, India). Drug free human plasma was procured from King George Hospital (Visakhapatnam, India).

**Chromatographic and mass spectrometric conditions**

Waters Acquity UPLC system (Waters corporation, Milford, USA) consisting of binary solvent manager, sample manager and column manger was used for solvent and sample delivery. Mobile phase A consisted of 5 mM ammonium acetate in Milli-Q water and mobile phase B was methanol. The analytes and IS were separated by using the following gradient (minutes, % mobile phase B) (0.01, 10) (0.50, 10) (1.00, 90) (1.80, 90) (2.10, 10) (3.00, 10) delivered with flow rate of 1 mL/min on to a Synergi Polar RP (30 mm×2.0 mm, 2.5 µm; Phenomenex, Hyderabad, India) column maintained at 40°C.The sample manager was maintained at 5°C and injection volume was 10 µL. The total chromatographic run time was 3.00 min. The analytes and IS were detected using a Waters XEVO TQ mass spectrometer (Waters corporation, Milford, USA) equipped with Z spray source. The quantification of analytes and IS were achieved by operating the mass spectrometer in positive ion ESI with multiple reaction monitoring (MRM) mode. Nitrogen gas was used as both cone gas and desolvation gas with a flow rate of 50 L/Hr and 800 L/Hr respectively. The source dependent parameters capillary voltage, extractor voltage, source temperature and desolvation temperature were set at 3.50 KV, 3 V, 150°C and 400°C respectively. The precursor to product ion transitions along with the cone voltage and collision energy for each analyte and IS were as follows: Pramipexole m/z 212.10→m/z 153.01, 24 V, 20 eV; Ropinirole m/z 261.22→m/z 114.08, 32 V, 18 eV; Rasagiline m/z 172.08→m/z 117.01, 10 V, 18 eV; Trimetazidine m/z 267.18→m/z 181.09, 20 V, 22 eV with dwell time 100 ms. Data acquisition and calculations were performed using Masslynx software, version 4.0

**Preparation of calibration standards and quality control samples**

Individual standard stock solutions of pramipexole, ropinirole and rasagiline were prepared by dissolving requisite amounts in methanol to obtain final drug concentration of 1mg/ml respectively. A series of combined working stock solutions with concentrations in the range of 0.25 to 2500 ng/mL were prepared by serial dilutions with methanol: water (50:50). Calibration standards were prepared by spiking (2% of total plasma volume) in blank human plasma with combined working stock solutions. A nine point calibration curve standards were made at 0.005, 0.01, 0.05, 0.10, 0.50, 1.00, 5.00, 25.00 and 50.00 ng/mL concentrations respectively. The quality control (QC) samples were similarly prepared at concentration of 40 ng/mL (high quality control, HQC), 2.5 ng/ml (middle quality control), 0.015 ng/mL (low quality control) and 0.005 ng/ml (lower limit of quantification quality control, LLOQ QC) with blank human plasma by a

separate weighing of standards. Stock solution (1 mg/mL) of the internal standard (Trimetazidine) was prepared by dissolving appropriate amount in methanol. Its working stock solution (25 ng/mL) was prepared by diluting the stock solution in methanol.

**Sample preparation**

A simple solid phase extraction method was developed for extraction of analytes and IS from human plasma. Prior to analysis all frozen subject samples, calibration standards and quality control samples were thawed at ambient temperature. In the following order 0.02 mL of IS working stock solution (25 ng/ml trimetazidine) was added into each 1.5 mL eppendorf tube except for blank plasma. 0.2 mL of standards, QCs, study samples and blank plasma were transferred into eppendorf tubes. After vortex for 30 s, 0.5 mL of Milli-Q water was added to each tube and vortexed to mix. The sample mixture was loaded into strata cartridge that was preconditioned with 1 mL of methanol followed by 2 mL of water. The cartridge was washed with 1 ml of water followed by 1 ml of 5% methanol in water after draining the sample from cartridge. The analytes and IS were eluted from cartridge with 1 ml of methanol. The eluent was evaporated to dryness at 40°C under a gentle stream of nitrogen in the Turbo vap evaporator (Caliper life sciences, USA).The dried extract was reconstituted with 0.2 ml of mobile phase and 10 µL of aliquot was injected onto the LC-MS/MS system for analysis.

**Method validation**

The bioanalytical method was thoroughly validated to meet the acceptance criteria of industrial guidance for the bioanalytical method validation (US Food and Drug Administration, 2001).The method was validated for selectivity, linearity, precision and accuracy, recovery, matrix effect, dilution integrity and stability. Selectivity is the ability of analytical method to differentiate and quantify the analytes in the presence of other expected components in the sample. This was evaluated by comparing the chromatograms of six different blank human plasma with corresponding spiked plasma at LLOQ QC level. Peak areas of endogenous compounds co eluting with the analytes should be less than 20% of the peak area of LLOQ response. Peak areas of endogenous compounds co eluting with IS should be less than 5% of the mean response of internal standard in LLOQ samples. The matrix effect was evaluated by comparing the peak areas obtained from each analyte in post extraction blank plasma samples with those of the respective analyte dissolved at the same concentration in reconstitution solution (mobile phase). The matrix effect was determined at MQC level using six replicates at each level for each analyte, where IS was determined at a single concentration of 25 ng/mL. Linearity was tested for each analyte in the concentration range of 0.005 to 50 ng/mL. For the determination of linearity

five standard calibration curves containing at least nine non-zero standards were constructed by a weighed ($1/x^2$) least squares linear regression method through the measurement of the peak area ratio of analyte to IS. In addition blank and zero sample (only spiked with IS) were analyzed to conform the absence of direct interferences, these data were not included to construct calibration curves. The acceptance limit of accuracy for each of the back calculated concentrations were ±15% except for LLOQ, where it was ±20%. For a calibration run to be accepted at least 75% of the standards, including the LLOQ and ULOQ were required to meet the acceptance criterion, otherwise the calibration curve was rejected. Intra-day precision and accuracy were determined by analyzing six replicate analysis of each quality control (LLOQ, LQC, MQC and HQC) samples of two different batches on same day.Inter-day precision and accuracy were determined by analyzing six replicate analysis of each quality control (LLOQ, LQC, MQC and HQC) samples of five different batches.The acceptance criteria included accuracy with in ±15% deviation from the nominal values, except the LLOQ where it should be ±20% and a precision of ±15% coefficient of variance (%CV), except for LLOQ, where it should be ±20%. The extraction recovery of each analyte was estimated at three different QC levels (six replicates of each LQC, MQC and HQC) by comparing the peak area response of extracted analytes with unextracted analytes (extracted blank sample spiked with the analytes) that represents 100% recovery. Similarly recovery of IS was estimated by comparing the mean peak area of extracted QC samples (n=18) with mean peak area of unextracted QC samples. Recovery of the analytes and IS need not be 100% but it should be precise and reproducible at all QC levels. Dilution integrity was performed to extend the upper concentration limits with acceptable precision and accuracy. The dilution integrity experiment was carried out for each analyte by analysing six replicate samples at concentration of two times the ULOQ concentration was prepared and diluted to 2- and 4-fold with blank plasma.Stability tests were conducted to evaluate the analyte stability in stock solutions and in plasma samples under different conditions. The stock solution stability at room temperature and refrigerated conditions (2–8°C) was determined by comparing the area response of the analytes (stability samples) with the response of the sample prepared from fresh stock solution. Bench-top stability (8 h), processed sample stability (Autosampler stability for 24 h), dry extract stability (8 h), freeze–thaw stability (four cycles) and long-term stability (60 days) were tested at LQC and HQC levels using six replicates at each level. Samples were considered to be stable if assay values were within the acceptable limits of accuracy (±15%) and precision (±15% CV).

**Pharmacokinetic study**

The pharmacokinetic study was carried out in healthy male volunteers (n = 18). Eighteen volunteers were randomized into three groups and administereda single oral dose of pramipexole (0.25 mg), ropinirole (0.25 mg) and rasagiline (0.5 mg) respectively. Blood samples were collected at pre-dose and 0.18, 0.33, 0.50, 0.67, 0.83, 1, 1.25, 1.5, 2, 2.5, 3, 4, 5, 6, 8, 10 and 12 hours in K2-EDTA vacutainer collection tubes (BD, Franklin, NJ, USA).The tubes were centrifuged (Thermo Scientific, Germany) at 3200 rpm for 10 min and the plasma was collected. The collected plasma samples were stored at –80°C till their use. This study was carried out as per the approval and guidelines of the local ethical committee. Plasma samples were spiked with the IS and processed as per the extraction procedure described earlier. Along with the clinical samples, the QC samples at low, middle and high concentration levels were also assayed in triplicate. Plasma concentration-time profile of pramipexole, ropinirole and rasagiline was analyzed by non-compartmental method using WinNonlin Version 5.3 (Pharsight Corporation, CA, USA).

## RESULTS AND DISCUSSION

### Method development and optimization

### Optimization of the mass spectrometric conditions

Mass spectrometric conditions were optimized so as to achieve the maximum stable response of the precursor ions and the major product ions of the analytes. All the analytes in this study were basic in nature so mass spectrometer was operated in positive ion mode to get good response. The inherent selectivity of MRM mode for quantification of analytes was expected to be beneficial in developing a selective and sensitive method. All analytes showed the singly charged protonated ions $[M+H]^+$as the prominent ion in the full scan of Q1 spectrum and was used as the precursor ion to obtain Q3 product ion spectra. The cone voltage and collision energy was optimized to get highest intensity for precursor ion and product ion respectively. The mass transition ion pair was selected as m/z 212.10→m/z 153.01 for pramipexole, m/z 261.22→m/z 114.08 for ropinirole, m/z 172.08→m/z 117.01 for rasagiline. The product ion mass spectra for each analyte were presented in Figure. 2(A, B and C).

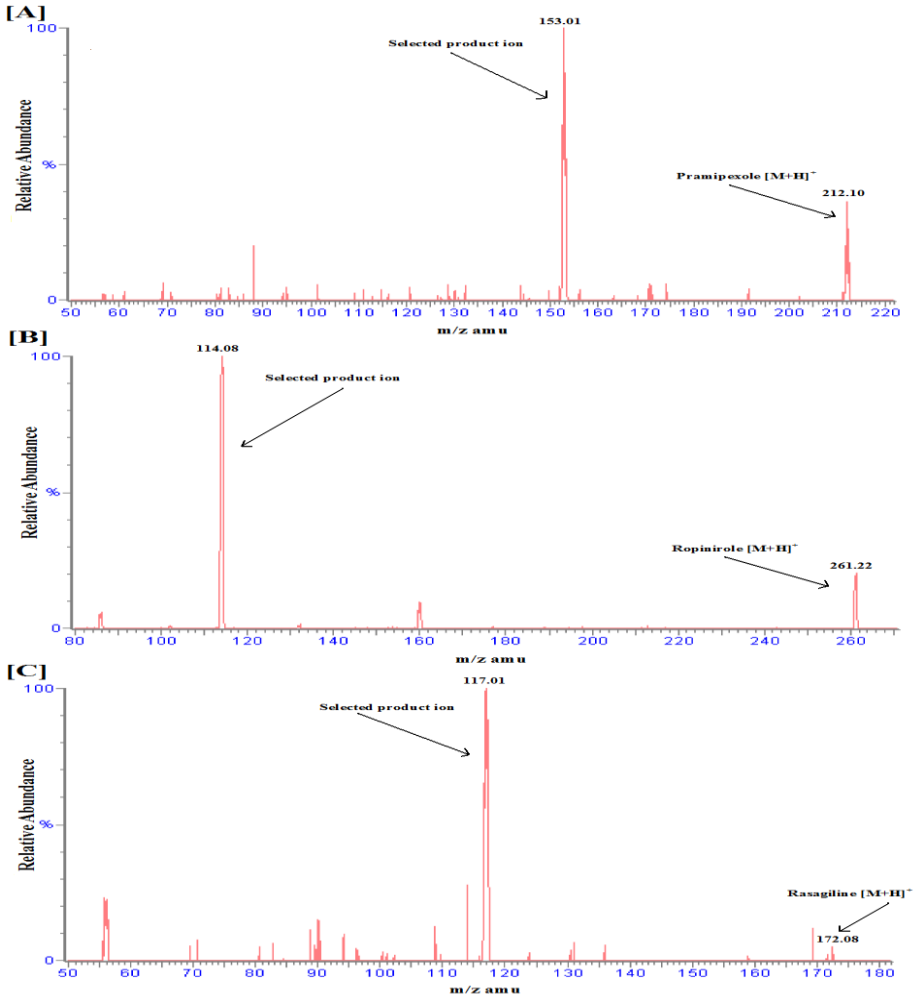


**Figure 2: [A] Product ion mass spectra of $[M+H]^+$ of pramipexole; [B] Product ion mass spectra of $[M+H]^+$ of ropinirole; [C] Product ion mass spectra of $[M+H]^+$ of rasagiline.**

**Optimization of the chromatographic conditions**

Chromatographic conditions, especially the composition of mobile phase and column were optimized inorder to achieve good chromatographic resolution; symmetric analyte peak shapes with in a shorter run time. The feasibility of various mixtures of solvents such as methanol and acetonitrile with different buffers such as ammonium acetate, ammonia solution with altered flow rates on different types of columns such as C18 and C8 were tested for complete chromatographic resolution of analytes and IS from interfering biological matrix. Finally the gradient mobile phase system consisting of 5 mM ammonium acetate- methanol mixture (minutes, % mobile phase B) (0.01, 10) (0.50, 10) (1.00, 90) (1.80, 90) (2.10, 10) (3.00, 10) delivered with 1 mL/min flow rate on to a Synergi Polar RP column achieved the good chromatographic separation of analytes and IS with desired response. The retention times of pramipexole, ropinirole, rasagiline and IS were 1.67, 1.57, 1.17 and 1.30 min respectively.

Several compounds were investigated to find a suitable IS and finally trimetazidine was found to be compatible with targeted analytes in terms of extraction efficiency, chromatographic behavior and ionization yield.

**Optimization of the sample extraction procedure**

Due to the complex nature of plasma, a sample pre treatment was often needed to remove protein and potential interferences prior to LC-MS/MS analysis. Currently, the most widely employed biological sample preparation methodologies were protein precipitation (PPT), solid phase extraction (SPE) and liquid-liquid extraction (LLE). SPE procedure has the advantage of minimizing ion suppression and matrix effect compared to other techniques. Hence solid phase extraction was used for sample preparation in this study. It produces a clean chromatogram of a blank sample and yield good, reproducible recovery for analytes from the plasma.

**METHOD VALIDATION**

**Selectivity**

Representative chromatograms obtained from blank plasma sample and blank plasma spiked with LLOQ standard of each analyte and IS were presented in Figure. 3(A, B and C) and Figure. 4(A, B and C) significant endogenous interferences observed in the respective MRM channel at the retention time of each analyte and IS in blank plasma sample.

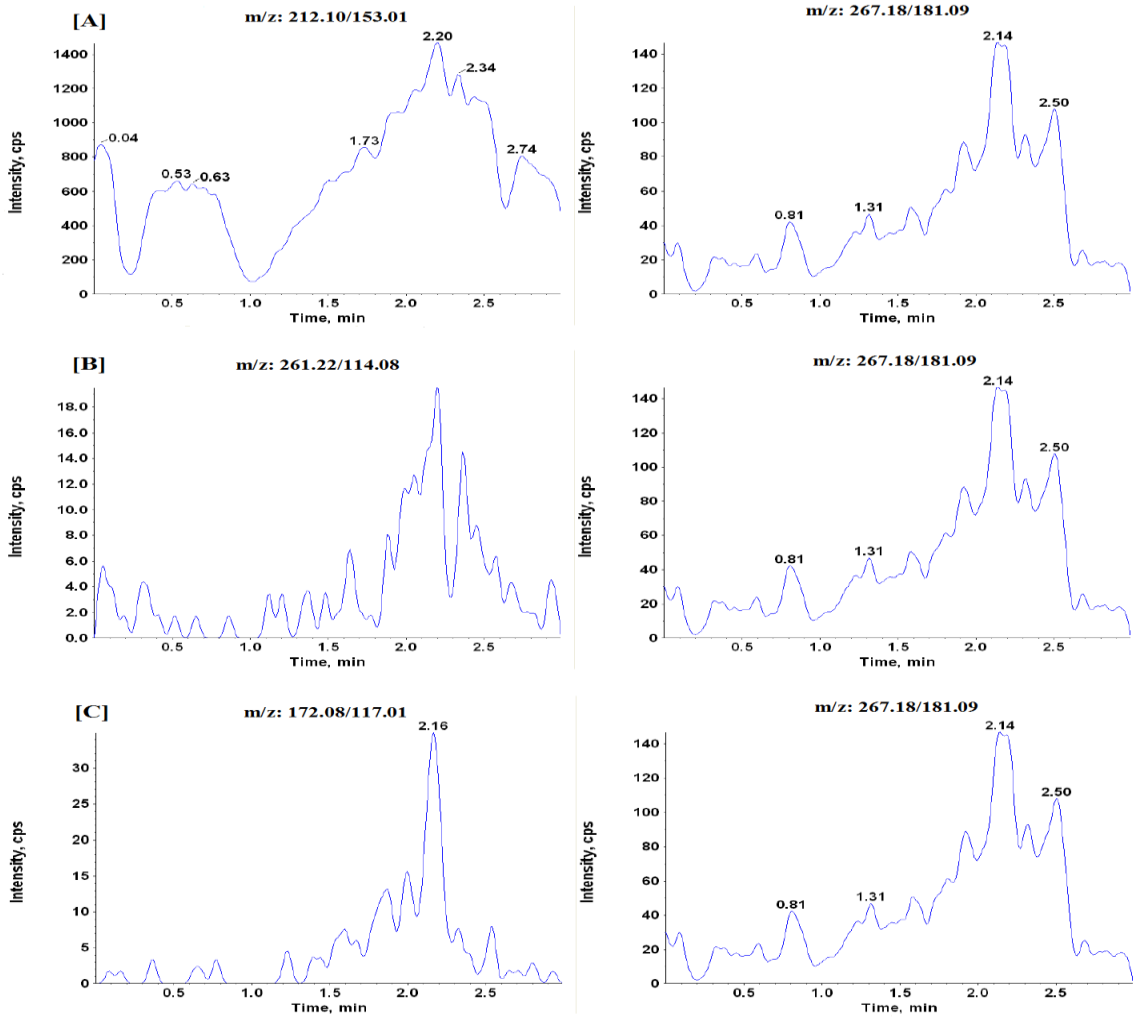


**Figure 3: Typical multiple reaction monitoring mode chromatograms of analytes (left panel) and IS (right panel) [A] human blank plasma of pramipexole; [B] human blank plasma of ropinirole; [C] human blank plasma of rasagiline.**

**Linearity**

Linearity of each calibration curve was determined by plotting the peak area ratio of analyte to IS (y) versus the nominal concentration(x) of the calibration points, and fitted to the y = mx+c using a regression factor ($1/x^2$). The nine point calibration curve was found to be linear over the concentration range of 0.005 to 50 ng/mL for all the analytes. Correlation coefficients were in the range $0.980<r^2<0.995$ for all the analytes. The percentage accuracy values ranged from 99.22-105.03%, while the precision (%CV) values ranged from 1.47-9.82 for all the analytes. Table 1 summarizes the calibration curve results for all the analytes.

**Table 1. Precision and accuracy data for back - calculated concentrations of calibration standards.**

| Concentration added (pg/ml) | Pramipexole | | | Ropinirole | | | Rasagiline | | |
|---|---|---|---|---|---|---|---|---|---|
| | Mean (n=5) | CV (%) | Accuracy (%) | Mean (n=5) | CV (%) | Accuracy (%) | Mean (n=5) | CV (%) | Accuracy (%) |
| 5.000 | 5.123 | 4.10 | 102.46 | 5.137 | 9.82 | 102.73 | 5.223 | 7.72 | 104.46 |
| 10.000 | 10.422 | 5.78 | 104.22 | 10.164 | 6.22 | 101.64 | 10.159 | 8.37 | 101.59 |
| 50.000 | 51.167 | 3.88 | 102.33 | 51.487 | 4.11 | 102.97 | 50.785 | 6.42 | 101.57 |
| 500.000 | 506.085 | 3.32 | 101.22 | 517.732 | 5.17 | 103.55 | 510.048 | 3.92 | 102.01 |
| 5000.000 | 5081.250 | 2.49 | 101.62 | 5103.843 | 5.25 | 102.08 | 5125.254 | 3.17 | 102.51 |
| 10000.000 | 10502.568 | 5.15 | 105.03 | 10034.567 | 9.51 | 100.35 | 10288.500 | 4.82 | 102.89 |
| 20000.000 | 20471.086 | 5.53 | 102.36 | 20221.939 | 4.33 | 101.11 | 19844.735 | 1.47 | 99.22 |
| 40000.000 | 40724.708 | 3.32 | 101.81 | 40402.361 | 3.83 | 101.01 | 41181.170 | 2.95 | 102.95 |
| 50000.000 | 50971.165 | 3.98 | 101.94 | 50560.473 | 2.59 | 101.12 | 49705.842 | 2.81 | 99.41 |

**Table 2. Intra -day and inter -day accuracy and precision of pramipexole, ropinirole and rasagiline.**

| Concentration added (pg/mL) | Pramipexole | | | | Ropinirole | | | | Rasagiline | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | LLOQ 5.000 | LQC 15.000 | MQC 15000.000 | HQC 35000.000 | LLOQ 5.000 | LQC 15.000 | MQC 15000.000 | HQC 35000.000 | LLOQ 5.000 | LQC 15.000 | MQC 15000.000 | HQC 35000.000 |
| **Intra-day (n=6)** | | | | | | | | | | | | |
| Mean | 5.206 | 15.185 | 15623.005 | 35653.026 | 5.175 | 15.853 | 15308.250 | 34468.854 | 5.440 | 14.650 | 15413.012 | 36313.097 |
| CV (%) | 9.53 | 6.52 | 6.44 | 5.12 | 7.84 | 6.97 | 4.79 | 1.71 | 2.43 | 7.25 | 5.27 | 3.77 |
| Accuracy (%) | 104.13 | 101.23 | 104.15 | 101.87 | 103.49 | 105.68 | 102.06 | 98.48 | 104.64 | 104.88 | 99.40 | 103.38 |
| **Inter-day (n=30)** | | | | | | | | | | | | |
| Mean | 4.951 | 15.311 | 15122.654 | 35650.025 | 5.021 | 15.483 | 15520.274 | 33924.139 | 5.214 | 14.736 | 15196.286 | 35037.236 |
| CV (%) | 10.55 | 6.55 | 5.85 | 4.93 | 7.90 | 6.27 | 4.06 | 2.64 | 7.17 | 6.29 | 5.88 | 5.84 |
| Accuracy (%) | 99.03 | 102.07 | 100.82 | 101.86 | 100.42 | 103.22 | 103.47 | 96.93 | 104.29 | 98.24 | 101.31 | 100.11 |

**Precision and Accuracy**

The intra- and inter-day precision and accuracy values were within the acceptance limit for all the analytes and summarized in Table 2. The intra-day accuracy ranged between 98.48 and 105.68% with a precision of 1.71-9.53%, the inter-day accuracy between 96.93 and 104.29% with a precision of 2.64-10.55%.

**Recovery**

The extraction recoveries of all the analytes and IS were good and reproducible. The mean overall recoveries (with the precision) of all the analytes were summarized in Table 3.

**Table 3. Mean overall recoveries of analytes and IS.**

| Analyte name | Sample conc. (pg/mL) | Response unextracted (Mean ± CV (%)) | Response extracted (Mean ± CV (%)) | Recovery | Overall recovery (Mean ± CV (%)) |
|---|---|---|---|---|---|
| Pramipexole | 15 | 3738 ± 2.28 | 3289 ± 1.56 | 87.98 | 89.44 ± 1.52 |
| | 15000 | 3178708 ± 1.76 | 2882399 ± 0.87 | 90.68 | |
| | 35000 | 6782538 ± 0.72 | 6081879 ± 0.64 | 89.67 | |
| Ropinirole | 15 | 5859 ± 1.12 | 5366 ± 1.47 | 91.59 | 89.36 ± 2.42 |
| | 15000 | 5834719 ± 1.46 | 5092190 ± 1.75 | 87.27 | |
| | 35000 | 11915894 ± 4.49 | 10628731 ± 2.74 | 89.20 | |
| Rasagiline | 15 | 4298 ± 0.79 | 3886 ± 1.75 | 90.42 | 90.49 ± 2.26 |
| | 15000 | 3774797 ± 1.26 | 3494474 ± 1.16 | 92.57 | |
| | 35000 | 9360897 ± 0.45 | 8283478 ± 3.75 | 88.49 | |
| IS | 25000 | 1630104 ± 0.24 | 1306719 ± 1.67 | 80.16 | |

**Matrix effect**

There was no effect of endogenous contribution from blank plasma in the measurement of all the analytes and IS. The average matrix factor values (matrix factor = peak area of post-spiked concentrations/peak area of neat concentrations) ranged from 0.952-0.996, while the precision (%CV) values ranged from 1.06-3.19 for all the analytes.

**Dilution integrity**

The upper concentration limit was extended to 100 ng/mL for all the analytes by a half and quarter dilution with screened human blank plasma. The mean back calculated concentrations for half and quarter dilution samples within 85-115% of nominal value, while precision values ranged from 1.74-2.74% for all the analytes.

**Stability studies**

Analysis of stock solution stability was performed at 50 ng/mL. After storage for 24 days at 2-8°C and at room temperature for 6 h, more than 97% for pramipexole and ropinirole, 98% for rasagiline remained unchanged. The results of bench-top stability (8 h), processed sample

stability (Auto sampler stability for 24 h), dry extract stability (8 h), freeze–thaw stability (four cycles) and long-term stability (60 days) were summarized in Table 4 and found to be within the acceptance limit.

**Table 4. Summary of stability data of pramipexole, ropinirole and rasagiline in human plasma**

| Stability | Pramipexole | | Ropinirole | | Rasagiline | |
|---|---|---|---|---|---|---|
| | LQC | HQC | LQC | HQC | LQC | HQC |
| **Bench top (27°C, 8 h)** | | | | | | |
| Mean (n=6) | 15.766 | 36211.531 | 15.383 | 35135.538 | 16.031 | 36409.292 |
| CV (%) | 4.63 | 1.55 | 4.27 | 3.44 | 2.30 | 4.35 |
| Change (%) | 1.38 | 5.02 | -2.52 | 3.43 | 1.89 | 2.39 |
| **Auto sampler (4°C, 24 h)** | | | | | | |
| Mean (n=6) | 15.622 | 35465.814 | 15.486 | 33715.550 | 15.799 | 36895.859 |
| CV (%) | 5.45 | 2.62 | 4.14 | 2.15 | 2.98 | 5.35 |
| Change (%) | 0.45 | 2.86 | -1.87 | -0.75 | 0.42 | 3.76 |
| **Dry extract (4°C, 24 h)** | | | | | | |
| Mean (n=6) | 15.589 | 36477.553 | 14.906 | 34354.213 | 15.882 | 36720.950 |
| CV (%) | 4.82 | 5.35 | 3.27 | 3.31 | 3.11 | 3.18 |
| Change (%) | 0.24 | 5.79 | -5.55 | 1.13 | 0.95 | 3.27 |
| **Freeze-thaw (-80°C, After 4$^{th}$ cycle)** | | | | | | |
| Mean (n=6) | 15.001 | 33976.456 | 15.638 | 35858.848 | 15.122 | 36347.597 |
| CV (%) | 4.27 | 3.19 | 2.83 | 1.64 | 6.14 | 3.55 |
| Change (%) | -3.54 | -1.46 | -0.91 | 5.56 | -3.88 | 2.22 |
| **Long term (-80°C, 60 days)** | | | | | | |
| Mean (n=6) | 15.030 | 35341.762 | 15.181 | 33701.188 | 15.552 | 35155.052 |
| CV (%) | 3.00 | 2.75 | 3.70 | 3.96 | 7.01 | 2.26 |
| Change (%) | -0.51 | -1.59 | 1.77 | -5.89 | -0.73 | -4.28 |

**APPLICATION OF THE METHOD IN PHARMACOKINETIC STUDY**

The established UPLC-MS/MS method was successfully applied to the determination of pramipexole, ropinirole and rasagiline concentrations in human plasma samples collected from healthy volunteers (n=6). The mean plasma concentrations vs time profiles of pramipexole, ropinirole and rasagiline were shown in Figure. 5. In addition, the pharmacokinetic parameters were presented in Table 5.

**Table 5. Pharmacokinetic data of pramipexole, ropinirole & rasagiline (n-6, Mean ± SD).**

| Parameter | Estimated value | | |
|---|---|---|---|
| | Pramipexole | Ropinirole | Rasagiline |
| $C_{max}$ (ng/ml) | 0.486±0.07 | 3.937±0.20 | 2.400±0.22 |
| $T_{max}$ (h) | 0.70±0.12 | 1.75±0.27 | 1.63±0.31 |
| $T_{1/2}$ (h) | 3.64±3.26 | 2.92±1.64 | 3.64±1.66 |
| $AUC_{0-t}$ (ng.h/mL) | 0.708±0.05 | 9.300±1.56 | 12.579±2.50 |
| $AUC_{0-\alpha}$ (ng.h/mL) | 0.791±0.12 | 9.494±1.68 | 13.997±4.24 |

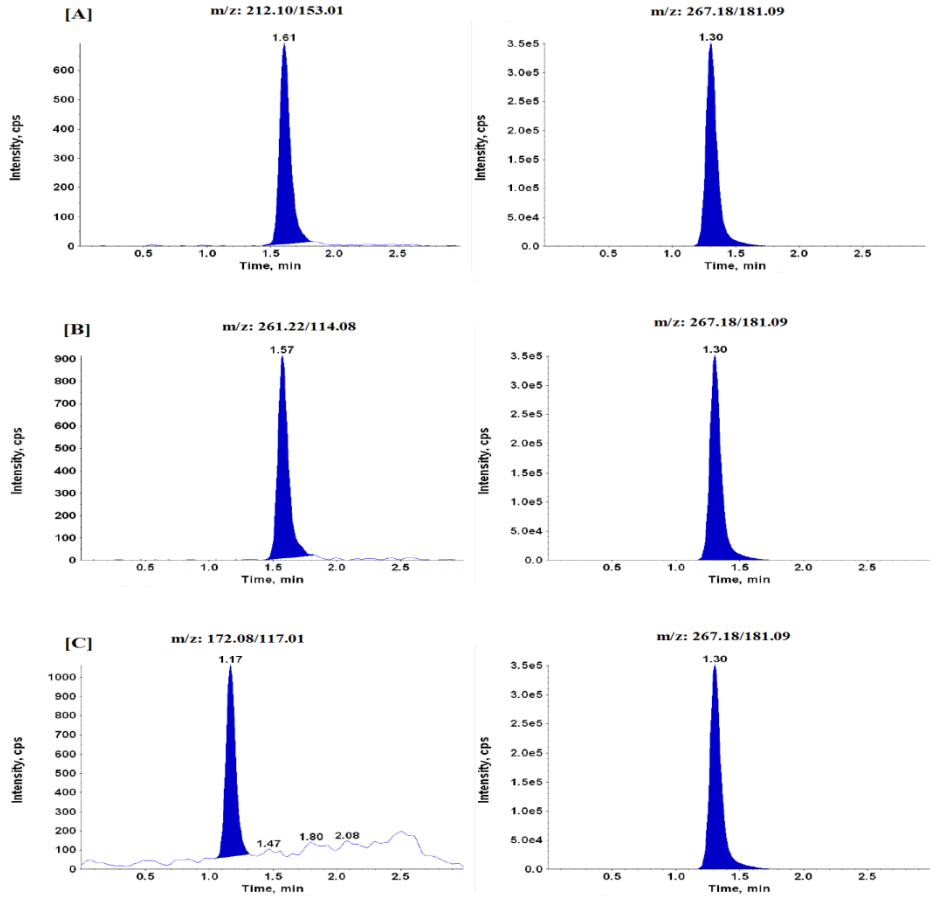


**Figure 4: Typical multiple reaction monitoring mode chromatograms of analytes (left panel) and IS (right panel) [A] human plasma spiked with pramipexole (at LLOQ level) along with internal standard; [B] human plasma spiked with ropinirole (at LLOQ level) along with internal standard; [A] human plasma spiked with rasagiline (at LLOQ level) along with internal standard.**

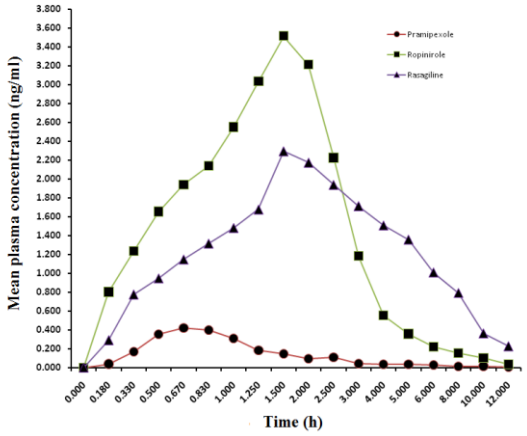


**Figure 5: Mean plasma concentration-time profile of pramipexole, ropinirole and rasagiline.**

## CONCLUSION

In summary, a selective, sensitive and rapid UPLC-MS/MS method for simultaneous quantification of pramipexole, ropinirole and rasagiline in human plasma was developed and fully validated as per FDA guidelines. Till date there was no reported LC-MS/MS method for simultaneous quantification of pramipexole, ropinirole and rasagiline in any biological matrix. This method offers significant advantages, in terms of wide range of linearity, recovery, rapid extraction and shorter run time. Moreover, this method provides superior sensitivity with the lower limit of quantification as low as 5 pg/mL for each analyte. Finally the simplicity of sample preparation and the shorter chromatographic runtime gives the method capability for high sample throughput. From the results of all the validation parameters we can conclude that the present method can be useful for pharmacokinetic/bioequivalence studies with desired precision and accuracy.

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