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Rapid and sensitive liquid chromatography–mass spectrometry method for determination of ropinirole in human plasma

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Abstract

A rapid and robust liquid chromatography–mass spectrometry (LC–MS/MS) method was developed for non-ergoline dopamine D_2-receptor agonist, ropinirole in human plasma using Es-citalopram oxalate as an internal standard. The method involves solid phase extraction from plasma, reversed-phase simple isocratic chromatographic conditions and mass spectrometric detection that enables a detection limit at picogram levels. The proposed method was validated with linear range of 20–1200 pg/ml. The extraction recoveries for ropinirole and internal standard were 90.45 and 65.42%, respectively. The R.S.D.% of intra-day and inter-day assay was lower than 15%. For its sensitivity and reliability, the proposed method is particularly suitable for pharmacokinetic studies.

Keywords: Ropinirole; LC–MS/MS

1. Introduction

As a non-ergoline dopamine D_2-receptor agonist, ropinirole known as 4-[2-(dipropylamino) ethyl]-l,3-dihydro-2H-indol-2-one, is indicated for the treatment of Parkinson's disease [1,2]. Ropinirole has been extensively studied in healthy volunteers and patients for the better understanding of individual variability. The systemic pharmacokinetics of ropinirole is highly variable. Maximal plasma concentration (C_{max}) is generally reached after about 1.5 h but time to reach maximal concentration (t_{max}), range is from 0.5 to 4 h [1,3]. Ropinirole is extensively metabolized to form *N*-despropyl metabolite in man. About 32–45% of ropinirole gets converted to *N*-despropyl metabolite, major metabolite of ropinirole [1,4,5]. The disposition and metabolic fate of ropinirole was studied in the animals and man [4]. Very few chromatographic methods have been reported for the determination of ropinirole in biological fluids. The reported HPLC method with UV detection has relatively high detection limit [4,6,7]. Thermo spray LC–MS/MS methods were developed for the structural analysis of drug metabolites [3,8,9] and pharmacokinetics evaluation. Due to relatively low dosing range of this ropinirole it was necessary to develop simple and sensitive analytical method for the quantification of ropinirole in human plasma at picogram level. This paper describes the development and validation of the specific and sensitive LC–MS/MS method for the determination of ropinirole in human plasma with a lower limit of quantification (LLOQ) 20 pg/ml. The structure of analytes is shown in Fig. 1.

2. Experimental

2.1. Materials and reagents

Ropinirole hydrochloride and Es-citalopram oxalate were procured from Torrent Pharmaceuticals Ltd. HPLC grade acetonitrile; methanol and water were purchased from Ranbaxy (India) Ltd. (Mumbai, Maharastra, India). Suprapure ammonium acetate and glacial acetic acid were purchased from Merck (Darmstadt, Germany). The solid phase extraction cartridges (Oasis HLB, 1 cm^3/30 mg) were purchased from Waters India Ltd. (Banglore, Karnataka, India). Drug free human plasma sam-

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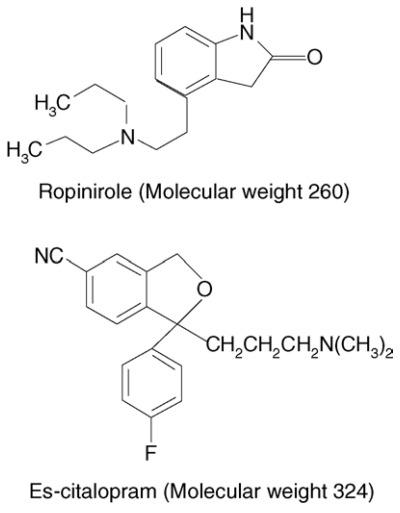

Fig. 1. Structure of ropinirole and Es-citalopram.

ples were purchased from Green cross hospital (Ahmedabad, Gujarat, India) and stored at −20 °C prior to use.

2.2. Equipments

LC–MS/MS analysis was performed using Surveyor HPLC system coupled with TSQ Quantum, a Thermofinnigan triple stage quadrupole LC–MS/MS instrument equipped with an electrospray ionization (Thermofinnigan Ltd., Herts, UK). The HPLC column used was Hypersil BDS C-18 (5 μm particle size, 4.6 mm × 50 mm) purchased from Thermo electron corporation, Herts, UK.

2.3. Standard and quality control (QC) preparation

The stock solutions of ropinirole and Es-citalopram were prepared in methanol at a concentration of 1.0 mg/ml each. Working solution of 100 and 40 ng/ml was prepared by appropriately diluting the stock solutions of ropinirole and Es-citalopram. Ropinirole working solution was used to prepare the spiking stock solutions for preparation of nine-point calibration curve (20–1200 pg/ml) and quality control samples at three concentration levels (50,360,840 pg/ml). All stock solutions were kept refrigerated (2–8 °C) when not in use. Bulk volume of calibration standards and quality control samples were prepared by spiking 500 μl of respective spiking stock solutions to 9.5 ml of control human plasma and aliquoted. Aliquots were stored at −70 °C until analyzed.

2.4. Sample preparation

A 1.0 ml aliquot of plasma containing ropinirole was pipette in micro tube, 50 μl of internal standard working solution (40 ng/ml Es-citalopram) was added and vortexed to mix. The sample mixture was loaded in to an Oasis HLB extraction cartridge that was preconditioned with 1 ml methanol followed by 2 ml water. The extraction cartridge was washed with 2 ml of water followed by 1 ml of 30% methanol in water. The analytes were eluted from the cartridge with 0.6 ml methanol and transferred to polypropylene auto sampler vials.

2.5. Chromatographic and mass spectrometric conditions

The analytes were chromatographically separated using reversed-phase high-performance liquid chromatography (HPLC) with isocratic elution. The mobile phase consisted of methanol–ammonium acetate (pH 4.5; 10 mM) (90:10, v/v) at a flow rate of 1 ml/min through the analytical column. For all analyses 20 μl of sample was injected. The total run time was 2.0 min.

The mass spectrometer was operated in the electro spray ionization mode with positive ion detection. The electro spray ion temperature was maintained at 350 °C and a voltage of 3200 V was applied to the sprayer needle. Nitrogen was used as the ion spray gas and the collision energy for the ropinirole and Es-citalopram was 24 and 28 eV, respectively. The analytes were monitored by multiple reaction monitoring (MRM) of the collision-induced dissociation (CID) of precursor ion to its corresponding product ion.

2.6. Data processing and regression

The MRM chromatographic peaks were integrated using Xcalibur Version 1.3 after which peak area ratios of ropinirole to Es-citalopram were plotted versus concentration and a linear curve fit, weighted by $1/x$ (where x = concentration) was used to produce the regression line.

2.7. Validation

The method has been validated for selectivity, sensitivity, recovery, linearity, precision and accuracy, stability, dilution integrity and matrix effect.

Selectivity is the ability of the analytical method to differentiate and quantify the analyte in the presence of other expected components in the sample. This test was performed by analyzing the blank plasma samples from different sources to test for the interference at the retention time of ropinirole and Es-citalopram.

Sensitivity was determined by analyzing control human plasma in replicates ($n = 5$) spiked with the analyte at the lowest level of the calibration curve, 20 pg/ml. Accuracy and precision of the quality control samples were calculated against the calibration curve. The recovery of ropinirole and Es-citalopram was calculated by comparing the peak area response of extracted analytes with unextracted standards (extracted blank sample spiked with the analyte and internal standard) that represent 100% recovery. Recovery was carried out at three concentrations (low, medium and high quality control sample).

As a part of method validation stability was evaluated. The stock solution stability was evaluated at room temperature and 2–8 °C by comparing with freshly prepared stock solution during analysis of the stability samples. The processed sample stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0), with samples that were

re-injected after sitting in auto sampler at 5 °C for 24 h. The stability of spiked human plasma stored at room temperature (bench-top stability) was evaluated for 6 h and compared with freshly prepared extracted samples. The freeze–thaw stability was conducted by comparing the stability samples that had been frozen and thawed three times with freshly prepared calibration standards and quality control samples. The long-term stability was conducted by analyzing low, medium and high quality control samples stored at −70 °C for 79 days with freshly prepared calibration standards and quality control samples. All stability evaluations were based on back-calculated concentrations.

Dilution integrity was performed to extend the upper concentration limits with acceptable precision and accuracy. Five samples each at concentration two times and four times the upper most concentration were prepared and diluted to 2- and 4-fold with blank plasma. These samples were processed and analyzed.

Matrix effect check was performed to demonstrate that no considerable endogenous contribution from human blank plasma affects the measurement of the analytes. Matrix effect check was conducted by analyzing three replicates of low and high quality control samples in five different lots of blank human plasma. No significant matrix effect was considered if the deviation of the mean test responses were within 15% of nominal concentration.

3. Results and discussion

3.1. Sample preparation and LC–MS/MS conditions

Since the ropinirole exists as a non-ionic form in the plasma. In this assay, the sample was applied directly on to the HLB SPE cartridge without buffering the plasma. In the state of non-ionic forms, the strong binding of analytes to the copolymer of SPE cartridge enables sufficient clean up. However, the analytes were easily eluted by 0.6 ml methanol and injected directly in to the system. Extraction procedure involved less technical difficulty by eliminating the evaporation and reconstitution steps. The analytes ionized by the ESI method were analyzed with positive ion detection because of efficiency of ionization of analytes. In general, positive ion detection is selective and highly sensitive to compounds, which readily accepts the proton. Ropinirole accepts the proton in an acidic mobile phase (pH 4.5) and produced a protonated precursor ion ($[M+H]^+$) at m/z 261.1. The strongest fragment of each compound, as shown in the Fig. 2 was selected and used as product ions to be monitored for better sensitivity and specificity. The mass transition ion pair was selected as 261.1 → 114.1 for ropinirole and 325.1 → 262.1 for Es-citalopram. Difference in response with changing the column was observed. The Hypersil BDS C-18 (5 μm particle size, 4.6 mm × 50 mm) column gave good peak symmetry with less column bleeding.

3.2. Selectivity

No interfering endogenous compound peak was observed at the retention time of analytes in the chromatograms obtained from control human plasma of six individuals, lipemic and heamolysed plasma. Therefore, it was expected that the assay for clinical samples would be not prevented by interference peak in this method. Under the chromatographic conditions described, the retention times of ropinirole and internal standard were 1.08 and 1.11 min, respectively. Representative chromatogram obtained from extracted control human plasma and ropinirole spiked plasma is shown in Fig. 3. The predominant product ion of m/z 114.1 and 262.1 was specific for ropinirole and escitalopram, respectively.

Table 1
Inter-run accuracy and precision of plasma calibration standard for ropinirole

Standard concentration (pg/ml)	Mean calculated concentration (pg/ml)	R.S.D. (%)	R.E. (%)
20.000	19.054	8.58	−4.73
40.000	39.193	6.93	−2.02
60.000	61.190	2.43	1.98
120.000	123.873	2.73	3.23
240.000	243.317	5.02	1.38
480.000	489.400	3.66	1.96
720.000	713.055	2.81	−0.96
960.000	955.819	0.77	−0.44
1200.000	1195.096	1.34	−0.41

3.3. Linearity

The calibration curves were linear over the range of 20–1200 pg/ml. The correlation coefficient was > 0.9992. The mean (±S.D.) slope of calibration curves for ropinirole was 0.003648 (±0.000249). The mean intercept of calibration curves for ropinirole was 0.026964 (±0.012195). Calibration curve data of ropinirole is listed in Table 1.

3.4. Sensitivity (lower limit of quantification)

The sensitivity experiment was carried out at LLOQ level. The mean percentage deviation from the nominal concentration was ≤7.93% and precision was within 11.0% relative standard deviation (R.S.D.) and interference of the plasma endogenous constituents were found to be negligible.

3.5. Precision and accuracy

Both the intra-day and inter-day accuracy and precision of the method were determined by analysis of the control human plasma spiked with ropinirole at lowest limit of quantification, low quality control, medium quality control and high quality control (LLOQ, LQC, MQC and HQC) levels and calculated against the calibration curve. The accuracy and precision of the method were described as a relative error % (R.E.) of theoretical versus measured concentrations and the percentage of the relative standard deviation, respectively.

The intra-day deviation from the nominal concentration was ≤7.93% and the intra-day precision was ≤14.11% at all QC level. The inter-day deviation from the nominal concentration ≤5.0% and the inter-day precision was ≤10.27% at all QC level. The results of accuracy and precision are enumerated in Table 2.

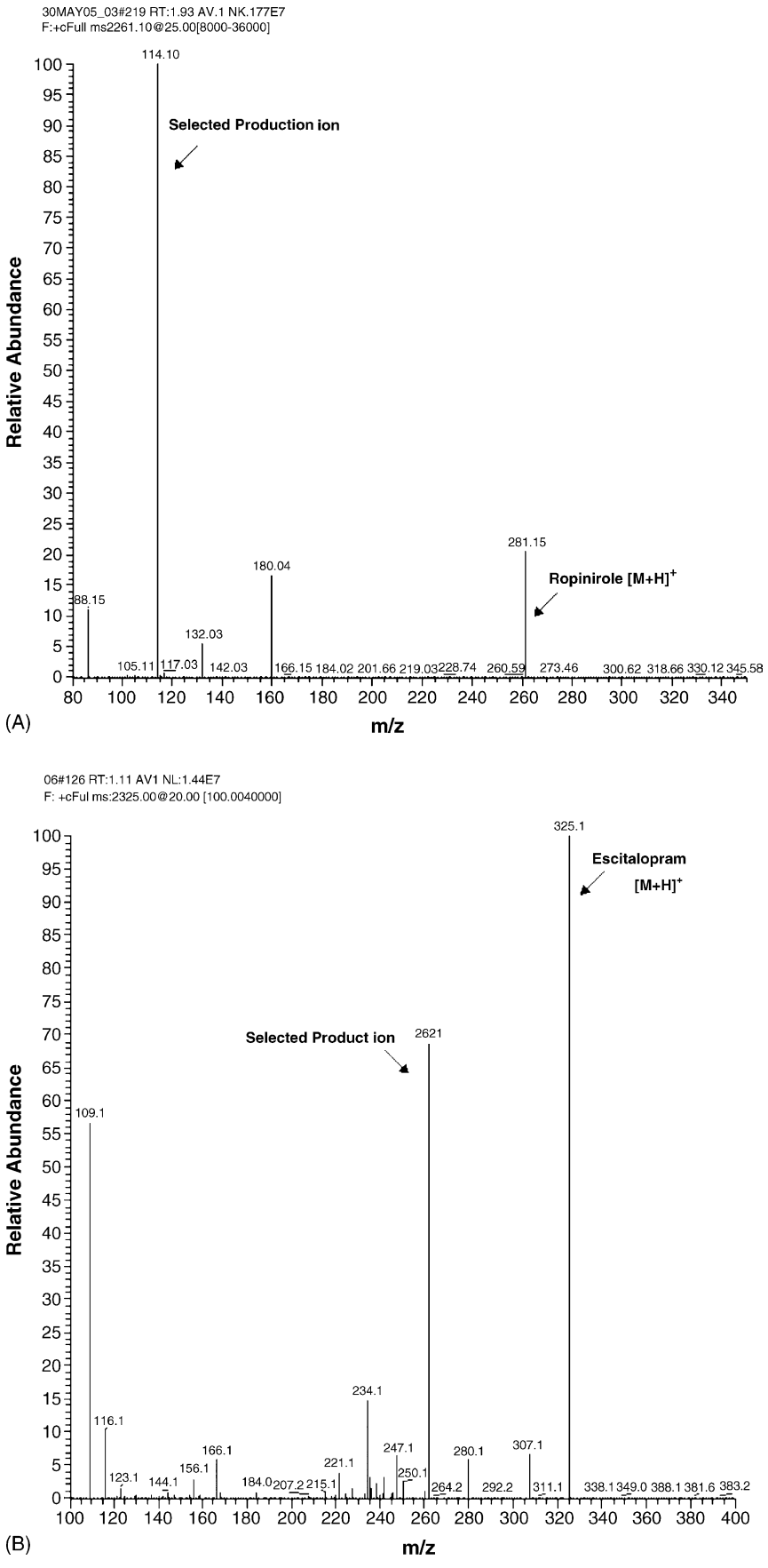

Fig. 2. Electrospray product ion mass spectra of the precursor ion of ropinirole (A) and Es-citalopram (B).

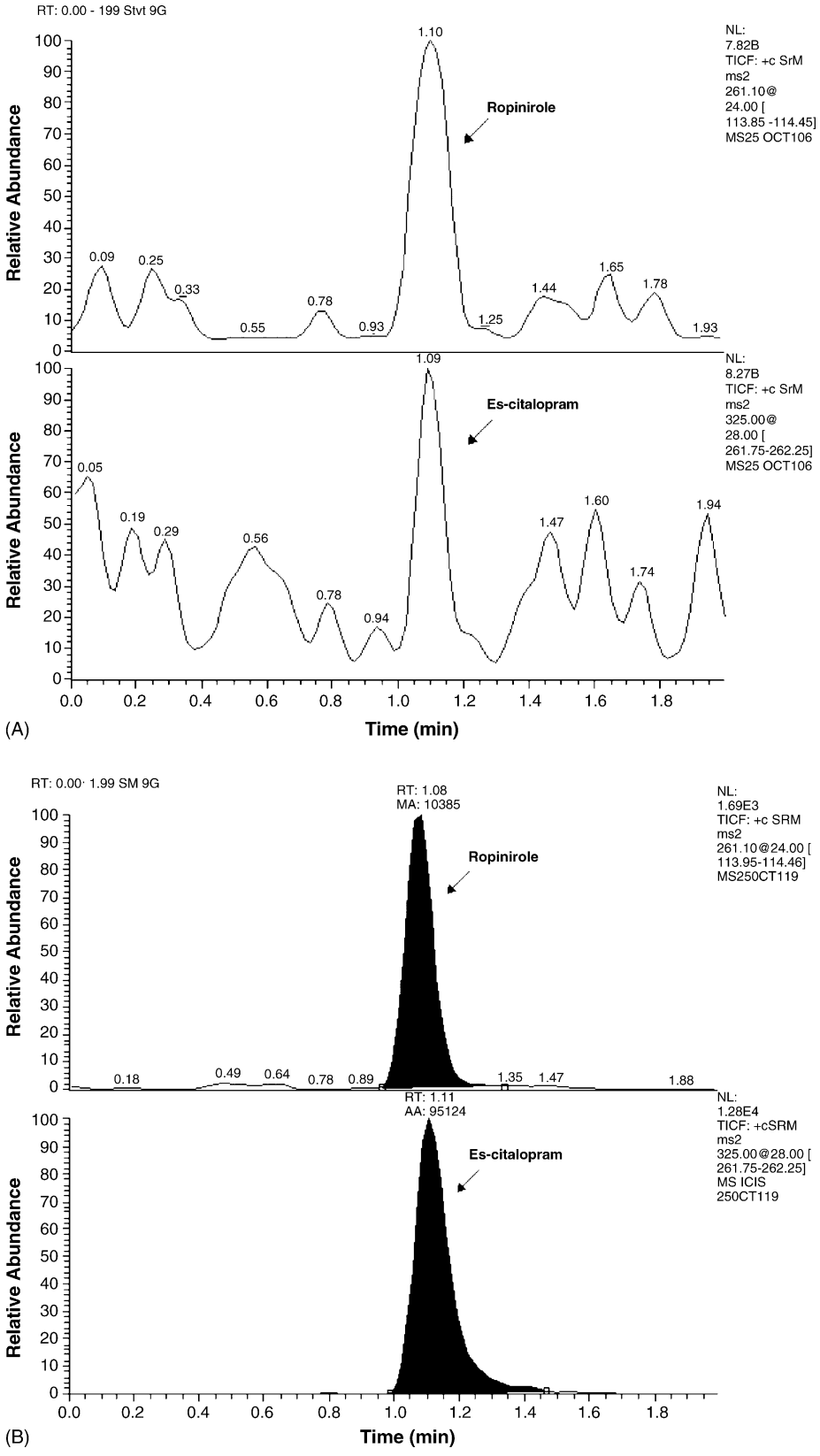

Fig. 3. Representative chromatograms of extracted blank plasma (A) and extracted plasma containing 50 pg/ml of ropinirole (B).

Table 2
Intra-day and inter-day accuracy and precision of ropinirole in human plasma

Added concentration (pg/ml)	Mean calculated concentration (pg/ml)	R.S.D. (%)	R.E. (%)
Intra-day ($n = 5$)			
20.000	18.415	11.00	−7.93
50.000	47.933	14.11	−4.13
360.000	367.083	4.26	1.97
840.000	866.269	3.41	3.13
Inter-day ($n = 3$)			
20.000	19.000	10.27	−5.00
50.000	47.681	9.19	−4.64
360.000	354.481	4.24	−1.53
840.000	846.403	3.79	0.76

3.6. Recovery

Peak areas from unextracted analyte with those of extracted analyte determined recovery. The mean absolute recovery of ropinirole at LQC, MQC and HQC was 90.45%. The recovery of Es-citalopram was found to be 65.42%.

3.7. Stability

Analysis of stock solution stability was performed at 1200 pg/ml. After storage for 12 days at 2–8 °C and at room temperature for 6 h, more than 97.0% of ropinirole remained unchanged, based on their peak areas in comparison with freshly prepared solution of ropinirole (1200 pg/ml). This suggests that the ropinirole in standard solution was stable for at least 12 days when stored at 2–8 °C and for 6 h at room temperature.

Bench top stability of ropinirole in plasma and auto sampler (5 °C) stability after processing of ropinirole was investigated at LQC and HQC levels. This revealed that the ropinirole in plasma was stable for at least 6 h at room temperature and 24 h at auto sampler with mean percentage change of ≤8.90 and ≤13.21%, respectively. It was confirmed that repeated freeze and thawing (three cycles) of plasma samples spiked with ropinirole at LQC and HQC level did not effect the stability of ropinirole with mean percentage change of ≤13.75%. Long-term stability of the ropinirole in plasma at −70 °C was also performed for 79 days at LQC, MQC and HQC level with mean percentage change of ≤2.73%. The results of the stability studies are enumerated in Table 3. The above results indicate that ropinirole is stable enough to be analyzed using this assay method.

3.8. Dilution integrity

The upper concentration limit of ropinirole can be extended to 2400 pg/ml with acceptable precision and accuracy of 15% by a 2- or 4-fold dilution with blank human plasma. The results demonstrate a precision of ≤3.87% and the mean percentage deviation from the nominal concentration was ≤3.31%.

3.9. Matrix effect

To evaluate the effect of endogenous contribution of different source of blank plasma in the measurement of the analytes the matrix effect was checked. Three replicate of low quality control and high quality control samples of five different lots were processed and analyzed along with a set of calibration standard. Matrix effect results summarized in Table 4 show that at each level of quality control the mean difference in the back-calculated concentration across the sample was ≤6.42%, which demonstrates that no considerable endogenous contribution from blank plasma for the measurement of analytes.

4. Application of method

The proposed method was applied to the determination of ropinirole in plasma samples from an on going abbreviated new drug application (ANDA) project for the development of conventional formulation. Plasma samples were periodically collected upto 24 h after oral administration of 0.25 mg tablet to 24 healthy male volunteers. The mean maximum plasma concentration obtained for ropinirole in test and reference formulation was 403.55 and 426.17 pg/ml, respectively. Representative chromatogram obtained from extracted study sample plasma is shown in Fig. 4. The AUC measured from 0 h to the last sampling point was higher than 90% of the value of AUC extrapolated from zero time to infinity, which indicates a suitability of the analytical method for pharmacokinetic studies. The application of method to 0.25 mg dose tablet concluded bioequivalency of

Table 3
Stability sample results for ropinirole ($n = 5$)

Stability	Spiked concentration (ng/ml)	Mean calculated comparison sample concentration (ng/ml)	Mean calculated stability sample concentration (pg/ml)	Mean % change
Process[a]	50.000	46.159	52.25	13.21
	840.000	753.277	838.52	11.32
Bench top[b]	50.000	46.159	49.46	7.15
	840.000	753.277	820.35	8.90
Freeze and thaw[c]	50.000	46.159	52.51	13.75
	840.000	753.277	849.52	12.78
Long-term[d]	50.000	48.187	49.153	2.00
	360.000	350.666	341.109	−2.73
	840.000	860.934	875.258	1.66

Where a, after 24 h in autosampler at 5 °C; b, after 6 h at room temperature; c, after 3 freeze and thaw cycles at −70 °C; d, at −70 °C for 79 days.

Table 4
Results for effect of matrix on ropinirole in human plasma

Analyte	Lot	Lower quality control ($n = 3$)				High quality control ($n = 3$)			
		Concentration (pg/ml)	Mean calculated concentration (pg/ml)	R.S.D. (%)	R.E. (%)	Concentration (pg/ml)	Mean calculated concentration (pg/ml)	R.S.D. (%)	R.E. (%)
Ropinirole	1	50.000	53.089	2.24	6.18	840.000	893.939	2.04	6.42
	2		50.586	6.90	1.17		857.123	1.77	2.04
	3		48.935	2.07	−2.13		833.352	4.00	−0.79
	4		52.491	8.79	4.98		865.041	1.77	2.98
	5		45.431	4.12	−9.14		834.785	3.57	−0.62

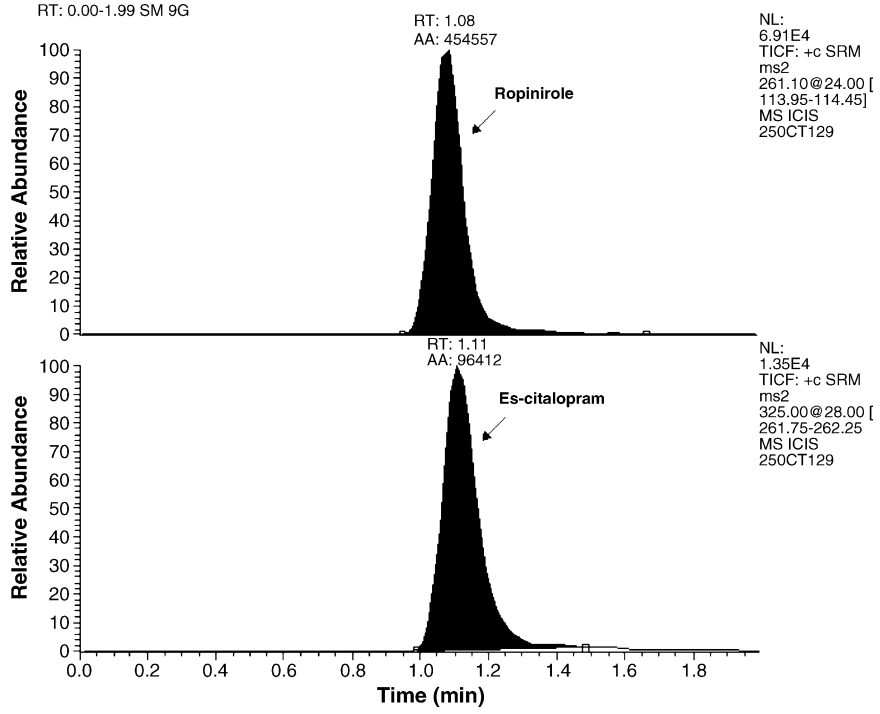

Fig. 4. Representative chromatogram obtained from extracted study sample plasma.

test formulation to reference formulation. These results enabled to file an ANDA application for ropinirole in regulatory market.

5. Conclusion

A simple, rapid and sensitive analytical method for the determination of ropinirole was developed. Significantly lower limit of quantification achieved in the plasma compared to previously published method. The run time of only 2.0 min was adequate to achieve the required chromatographic separation of ropinirole. The sample preparation involved very simple and results are reproducible. The good validation criteria results of the method and sensitivity allowed its use in pharmacokinetic studies.

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