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Development and validation of GC/MS method for determination of pramipexole in rat plasma

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ABSTRACT: A simple, rapid and sensitive method has been developed and validated for the determination of pramipexole in rat plasma by using gas chromatography mass spectrometry. The lower limit of quantification (LLOQ) is superior to the other reported LC-MS/MS methods. After being made alkaline with NaOH, plasma samples (0.1 mL) were subjected to liquid–liquid exteraction using methyl-*t*-butyl ether. Analytes were determined using electron impact ionization in a single quadrupole mass spectrometer. GC/MS was performed in the selected ion monitoring mode using target ions at *m/z* 211, 212 and 152 for pramipexole and *m/z* 194 and 165 for caffeine as internal standard. A linear calibration curve was plotted over the range of 20–1000 pg/mL for pramipexole ($r^2 > 0.996$). The LLOQ was 20.0 pg/mL, respectively, which offered high sensitivity and selectivity enough for bioanalytical investigation. Inter- and intraday precisions ranged from 0.3 to 8.8% and from 0.9 to 11.33%, respectively. The recovery of pramipexole from plasma ranged from 82.4 ± 7.1 to 87.8 ± 5.7%. The method fulfills all standards required for bioanalytical methods and can be successfully applied to a pharmacokinetic study of pramipexole in rats.

Keywords: pramipexole; plasma; gas chromatography mass spectrometry; bio-analytical; pharmacokinetic

Introduction

Pramipexole ($C_{10}H_{17}N_3S$, Fig. 1a) is chemically (6*R*)-*N*′-propyl-4, 5, 6, 7-tetrahydro-1, 3-benzothiazole-2, 6-diamine, a non-ergot dopamine auto-receptor agonist. Pramipexole is a selective and specific non-ergot dopamine (DA) receptor agonist with high affinity and selectivity for the D2 receptor subfamily of dopamine receptors, particularly with highest affinity to dopamine D2 and D4 receptor subtype (Piercey *et al*., 1996; Partinen *et al*., 2006). Pramipexole can protect dopaminergic neurons via a receptor-dependent pathway at nanomolar concentrations (Ling *et al*., 1999; Ramirez *et al*., 2003; Pan *et al*., 2005), and at higher than 10 µmol concentrations it has shown to be neuroprotective *in vitro* independently of the dopaminergic agonism (Gu *et al*., 2004). The drug has proved to be an effective agent for patients with Parkinson's disease and drug-resistant tremor (Pogarell *et al*., 2002) and had a beneficial effect on mood and motivational symptoms in Parkinson's disease patients who did not have major depressive disorder. Pramipexole is also clinically valuable in the treatment of depressive and apathetic syndromes is (Leentjens *et al*., 2009) and has proven a suitable alternative in patients with moderate to severe restless legs syndrome (RLS), particularly when their therapy has to be switched to a dopamine agonist (Kolster and Oertel, 2004). A single dose of 0.125–0.75 mg pramipexole (mean 0.3 ± 0.2 mg) in the evening resulted in a significant improvement of subjective RLS symptoms as rated by the International RLS Study Group Severity Scale (Kolster and Oertel, 2004; Jama *et al*., 2009).

Literature survey reveals the lack of sensitive methods concerning analysis of pramipexole. Analysis of pramipexole in biological samples was performed using HPLC with atmospheric pressure chemical ionization tandem mass spectrometry (Lau *et al*., 1996a) and HPLC with electrochemical and UV detection (Lau *et al*., 1996b). Both methods used 1 mL of plasma samples, which is considered a large quantity of sample. An enantiomeric separation of pramipexole was reported by LC (Pathare *et al*., 2006). Analysis of pramipexole and its impurities in bulk substances and pharmaceuticals has also (Jancic *et al*., 2007a, b; Srinubabu *et al*., 2006) been reported. There are studies on analysis of pramipexole (Jancic *et al*., 2007a; Srinubabu *et al*., 2006), by experimental design and also to determine the dissociation constants (pK_a) of pramipexole and its impurities (Jancic *et al*., 2007b). Nirogi *et al*. (2007) developed an LC-MS-MS method for the determination of pramipexole in plasma with LOQ of 200 pg/mL. Capillary electrophoresis with laser-induced fluorescence detection was reported with limits of detection and quantitation of 10.0 and 25.0 ng/mL respectively (Musengaa *et al*., 2008). Recently Gurupadayya *et al*. (2009) developed a spectrophotometric method based on the diazotization of primary amine group of pramipexole.

The dosing limit of pramipexole is very low between 125 and 750 µg/day (Kolster and Oertel, 2004; Jama *et al*., 2009). Therefore, a highly sensitive and selective method for the

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Abbreviations used: DA, dopamine; DCM, dichloromethane; MTBE, methyl-*t*-butyl ether; RLS, restless legs syndrome.

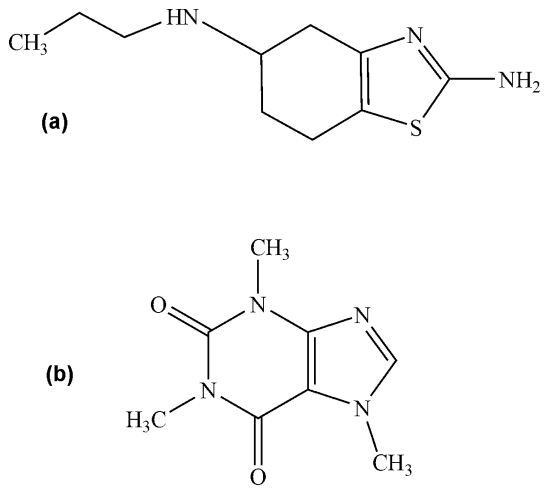

Figure 1. Chemical structure of (a) pramipexole and (b) caffeine (IS).

determination of pramipexole in plasma is necessary to support pharmacokinetic evaluation. The purpose of the present study is to develop a sensitive and selective method for the determination of pramipexole in rat plasma.

The combination of gas chromatography coupled with mass spectrometry using selected ion monitoring (GC/MS-SIM) provides many benefits, including analytical ruggedness as well as enhanced sensitivity and selectivity. The current method demonstrates a simple and rapid sample preparation method as well as significantly low volume of (0.1 mL) plasma samples required for the analysis.

Experimental

Reagents and Chemicals

Pramipexole (purity >98.6%) reference standard was a gift from Claris Lifesciences Limited (Ahmedabad, India). Caffeine (purity >98.0%) was purchased from Sigma–Aldrich (St Louis, USA). HPLC-grade methanol was purchased from Merck Fine Chemicals (Mumbai, India). HPLC-grade dichloromethane (DCM) and methyl-*t*-butyl ether (MTBE) was obtained from Thomas baker (India). Purified water was prepared using a Millipore (Synergy) system and was used throughout the study. All other chemicals and reagents used were of analytical grade and supplied by Merck (India).

GC-MS Instrument and Conditions

All analyses were performed using a Shimadzu GC-17A gas chromatograph interfaced with a Shimadzu QP-5050*A* quadrupole mass spectrometer (Shimadzu Corp., Kyoto, Japan). The GC/MS was operated with an interface temperature of 250°C, and an ionization source temperature of 300°C. The mass spectrometer was tuned every day using PFTBA (perfluorotributylamine). The solvent delay before the MS filament turned on was set to 4 min to protect the filament from oxidation. Chromatographic separation was achieved by using a Phenomenex Zebron ZB-5ms (5% phenyl–methylsilicone, 15 m × 0.25 mm i.d., 0.25 µm film thickness) capillary column. Helium with a minimum purity of 99.9% was used as carrier gas at a flow rate of 4 mL/min. The gas chromatograph was equipped with a split/splitless injection port operated at 200°C. Samples were injected in the splitless mode at a column temperature of 150°C, then the splitter was opened after 1 min sampling time. The gas chromatograph oven temperature was programmed as follows: initial temperature, 150°C for 1 min; from 150 to 300°C at a rate of 35°C/min and temperature was held for 1 min. The mass spectrometer was operated in the positive-ion electron impact (EI) mode. EI mass spectra were obtained at an ionizing energy of 70 eV, and at an emission current of 60 µA. Quantification was carried out by the SIM mode. In order to select the stable ion for monitoring, the mass spectra of pramipexole and IS were obtained by injecting 0.1 µL of the analyte standards into the GC/MS.

Preparation of Calibration Standards and Quality Control Samples

The standard stock solution of pramipexole was prepared by dissolving the accurately weighed compound in methanol to give a final concentration of 4000 ng/mL. This solution was sequentially diluted with methanol to obtain working solutions at concentrations over 1–40 ng/mL. A standard stock solution of caffeine (IS) was prepared by dissolving the drug in methanol to a final concentration of 100 ng/mL. A 0.2 mL aliquot of this stock solution was diluted to 10 mL with methanol to a final concentration of 2 ng/mL. All the solutions were stored at 4°C until used.

The calibration standards were prepared by taking aliquots of working standard solutions and placed in an Eppendorff tube, and the solvent was evaporated under a compressed nitrogen stream. The dried analyte was reconstituted using blank plasma to final desired concentrations of 20, 30, 40, 50, 100, 200, 300, 400, 500 and 1000 pg/mL for pramipexole, and the solution was then vortex mixed for 1 min. Quality control (QC) samples were prepared by spiking working standard in to drug free plasma at concentrations of 100, 250 and 500.0 pg/mL for the analytes. For the determination of LLOQ of this method, QC standard was made at concentrations of 20 pg/mL for analyte and tested for accuracy and precision. Aliquots of calibration standards, internal standard and quality control plasma samples were dispensed into labeled Eppendorf tubes and stored at −25°C until required for assay. In all determinations, calibration standards, QC samples were prepared together.

Sample Preparation

Plasma samples were extracted employing a liquid–liquid extraction (LLE) technique. All frozen plasma samples (i.e. calibration standard and QC sample) were allowed to thaw at room temperature. A 100 µL plasma sample was dispensed to the 1.5 mL polypropylene micro-centrifuge tube, the 10 µL of internal standard (IS) (2 ng/mL caffeine) solution and 10 µL of sodium hydroxide (0.1 m) were added and vortex mixed for 30 s. A 2 mL aliquot of MTBE was then added and vortexed for 3 min. A 100 µL aliquot of MTBE was then added to the tubes, and extracted by vortex-mixing for 1 min. The mixture was centrifuged at 13,000 rpm for 5 min at 4°C. The separated organic phase was transferred to another clean glass tube and the solvent was evaporated at 45°C to dryness under nitrogen stream. The residue was reconstituted in 100 µL of methanol, followed by centrifugation at 13,000 rpm for 5 min. A 50 µL aliquot of the supernatant was transferred to a vial and 0.1 µL was injected into the GC/MS system.

Bioanalytical Method Validation

The method validation was performed in accordance with FDA (2001) guidelines for bioanalytical method validation.

Selectivity

The selectivity of the method was tested by analyzing blank plasma samples from six different individuals. All blank samples were tested for interferences by following the proposed extraction procedure and analyzing with the proposed GC/MS conditions, and the results were compared with those obtained for an aqueous solution of the analyte at a concentration near the LLOQ. The area response of analytes in blank extract should not be greater than 20% compared with the area response of LLOQ concentration.

Linearity

The linearity was tested by analyzing calibration standards at 10 concentration levels over the range 20–1000 pg/mL of the analyte. The samples were run in the order from low to high concentrations. A blank plasma

sample (without IS) and a zero sample (with IS) were also analyzed to confirm the absence of any interference; these data were not included to construct calibration plots. Standard curves based on peak area ratio of analytes to IS were prepared. Standard deviations of the slope and intercept were calculated to ensure the reliability of the calibration curve over a period of 1 week.

The LLOQ is defined as the lowest concentration on the calibration curve at which an acceptable accuracy (RE) within ±20% and a precision (RSD) below 20% can be obtained. The lower limit of quantification (LLOQ) response should be 10 times the average noise level in the chromatogram.

Precision and Accuracy

Intra- and interday precisions were calculated as coefficient of variation (CV) (or %RSD) and accuracy as RE on the basis of five replicate sample analyses of each QC level. The intra-day precision and accuracy of the assay were measured by analyzing replicate analysis of each QC (LLOQ, low, medium and high concentration quality control samples) of analytes on the same day. Inter-day precision and accuracy were determined on three different days by analysis of three batches of QC samples at each QC levels (LLOQ, low, medium and high concentration quality control samples). The precision determined at each concentration level should not exceed 15% of the coefficient of variation (CV) except for the LLOQ, where it should not exceed 20% of the CV. The accuracy was required to be within ±15% relative error of the actual values.

Recovery and Effect of the Matrix

The recovery (extraction yield) of each analyte was determined by comparing the analytes and IS peak-area ratios obtained for each QC level subjected to the extraction procedure with those obtained from post-extraction blank plasma samples spiked (spiked after extraction) with equivalent amounts of each analyte at the same nominal concentrations (equivalent QC levels). The recovery of the IS was determined in the same way. Recovery of the analyte need not be 100%, but the extent of recovery of an analyte and that of the internal standard should be consistent, precise and reproducible.

The matrix effect was evaluated by comparing the peak areas obtained from analytes spiked in post-extraction blank plasma sample, with those for the respected compound dissolved at the same concentrations in methanol (Matuszewski *et al*., 2003; Matuszewski, 2006).

The blank plasma used in this study was obtained as three different batches. Three different concentrations (each QC levels) of each analyte with an appropriate concentration (200 pg/mL) of internal standard were evaluated by analysis of three different batches at each concentration. If peak-area ratios from the plasma extracts were ±15% those from methanolic solutions, a matrix effect was implied.

Stability

Stability of the analytes and IS in human plasma was tested at three concentration levels (low, medium and high QC levels) with three determinations for each. The samples were analyzed using freshly prepared calibration samples. Plasma samples of each QC levels were analyzed for short-term temperature stability. Long-term temperature stability of analytes was studied for 30 days by analyzing QC samples at the three different levels. Freeze–thaw stability QC samples containing analytes were tested after three freeze cycles (–20°C) and thaw (room temperature). Post-preparative stability was determined by re-analysis of extracted QC samples kept under in the autosampler at 4°C for 24 h. The stability of the standard and internal standard working solutions was tested for 6 h at room temperature.

Pharmacokinetic Application

Application to pharmacokinetic study in rats. After an overnight fasting period (10 h), 18 Wistar rats (weighing from 200 to 250 g) were given single oral dose of 10, 20 and 30 µg/kg pramipexole, respectively. No food was allowed until 4 h after dose administration, while water intake was free. About 200 µL of blood samples were collected into heparinized tubes from the tail vein of the rat prior to dosage and at 1 min–48 h thereafter. Plasma was separated by centrifugation at 13,000 rpm for 5 min and kept frozen at –20°C and analyzed within 1 week. This study was approved by the local animal ethics committee.

Results and Discussion

Sample Preparation

The mean extraction recovery of pramipexole was found to be below 70% when the other organic solvents (e.g. ether, ethyl acetate) were used as extracting solvents in our pilot studies, which did not enhance the sensitivity of the analytical method. However better results were obtained using MTBE as extracting solvent.

Chromatography and Mass Spectrometry

The retention times of pramipexole and IS were approximately 4.51 and 4.93 min, respectively. Caffeine was selected as the internal standard for its similarity in retention and extraction recovery. In order to enhance the sensitivity of method, SIM mode was selected at the dominant and characteristic ions for pramipexole (Fig. 2a) which were at *m/z* 211, 212 and 152 and, for IS (Fig. 2b) at *m/z* 194 and 165. The total run time was only 6 min, which was much shorter than those reported in the literature (Table 1). The method developed by Nirogi *et al*. (2007) and Lau *et al*. (1996a) provides a shorter run time; however the LLOQ is very high and also the sample quantity (1 mL) required is higher compared with the present method. After careful comparison of many temperatures, we finally adopted the temperatures of 150°C at the injection port and 200°C at the oven for optimal monitoring of the analyte, and the solvent cutoff time of 4.0 min to minimize any early eluting plasma interferences; in addition, it yielded suitable retention time and peak shape for pramipexole and IS, which offered relatively short analytical runtimes.

Bioanalytical Method Validation

Specificity. Specificity with respect to plasma components was determined by analyzing hyperlipemic and hemolyzed blank samples from six different bathes of plasma samples collected under controlled conditions. No interference was observed at the retention times of pramipexole and IS. The samples were analyzed using the proposed extraction procedure and chromatographic conditions in order to compare them with an aqueous solution of the analyte at a concentration near to the limit of quantification. Representative chromatograms of blank rat plasma, blank rat plasma spiked with known concentrations of pramipexole (250 pg/mL), and the IS (200 pg/mL) are shown in Fig. 3(a, b). No interference was observed at the retention time of the analyte at 4.51 min and IS at 4.93 min due to endogenous substances in blank plasma.

Linearity. The calibration curves showed good linearity within the range 20–1000 pg/mL. The representative linear equation of calibration curve for the analyte was $y = (0.01121 \pm 0.71)x + (0.05583 \pm 2.18)$ with a correlation coefficient of 0.9988, where y is the peak area ratio of the analyte to the IS and the x is the concentration of the analyte.

Precision and accuracy. The precision and accuracy of the method were evaluated by analyses of each QC level sample. The

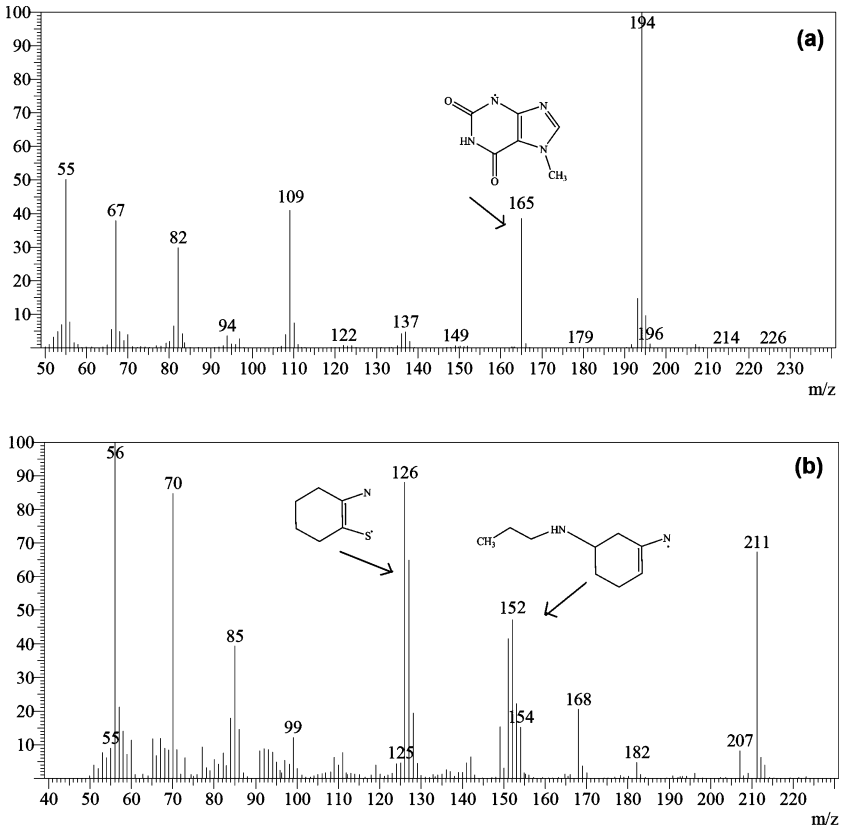

Figure 2. Full-scan mass spectra of (a) caffeine (IS) and (b) pramipexole.

Table 1. Comparison of present method with other reported method

References	Techniques	Run time	Limit of quantification
Lau *et al.* (1996b)	HPLC	16 min	50 pg/mL
Lau *et al.* (1996a)	LCMS/MS	5.5 min	50 pg/mL
Srinubabu *et al.* (2006)	HPLC	7 min	4.5 µg/mL
Medenica *et al.* (2007a)	HPLC	16 min	—
Gurupadayya *et al.* (2009)	Spectrophotometric	—	0.2963 g/mL
Raggia *et al.* (2008)	Capillary electrophoresis	12 min	25.0 ng/mL
Nirogi *et al.* (2007)	LCMS/MS	3.5 min	200 pg/mL
Newly developed method	GC/MS	6.0 min	20 pg/mL

data for intra- and interday precision and accuracy from QC samples are summarized in Table 2. The intra- and interday precisions (%CV RSD, $n = 5$) at LLOQ-QC were 2.61 and 9.50%, respectively, with accuracies (RE) of ± 2.36 and ± 7.80. The precision and accuracy of the present method conform to the criteria for the analysis of biological samples according to the guidance of FDA, where the RSD determined at each concentration level is required to not exceed 15% (20% for LLOQ) and the RE must be within $\pm 15\%$ ($\pm 20\%$ for LLOQ) of the actual value (FDA, 2001). The results were within acceptable limits, showing satisfactory accuracy and precision.

Recoveries and matrix effect. The recovery in terms of extraction efficiency was determined by analyzing the QC samples. The recoveries were determined at four concentrations (LOQ, low, medium and high QC) by comparing peak areas obtained from plasma samples with those obtained by unextracted (spiked in post extracted blank plasma) sample at the same concentration and conditions. The recovery of IS was also tested using the same condition, and the mean recovery of IS was found to be 83.31 $\pm$ 4.07%. At the concentrations of 20, 100, 250 and 500 pg/mL the recoveries of pramipexole were 86.6 $\pm$ 4.2, 87.8 $\pm$ 5.7, 82.4 $\pm$ 7.1 and 85.4 $\pm$ 6.6% respectively.

Matrix effects were absent, as shown by the fact that concentrations of analytes as a percentage of nominal concentrations for low, medium and high QC samples were, respectively, 90.59 $\pm$ 1.54, 93.92 $\pm$ 2.21 and 92.99 $\pm$ 2.13 for pramipexole, and the percentage nominal concentration of IS was 92.10 $\pm$ 1.20%.

Stability. No significant decrease of the analyte concentration was observed when kept at room temperature for 48 h during short-term temperature stability, which indicated reliable

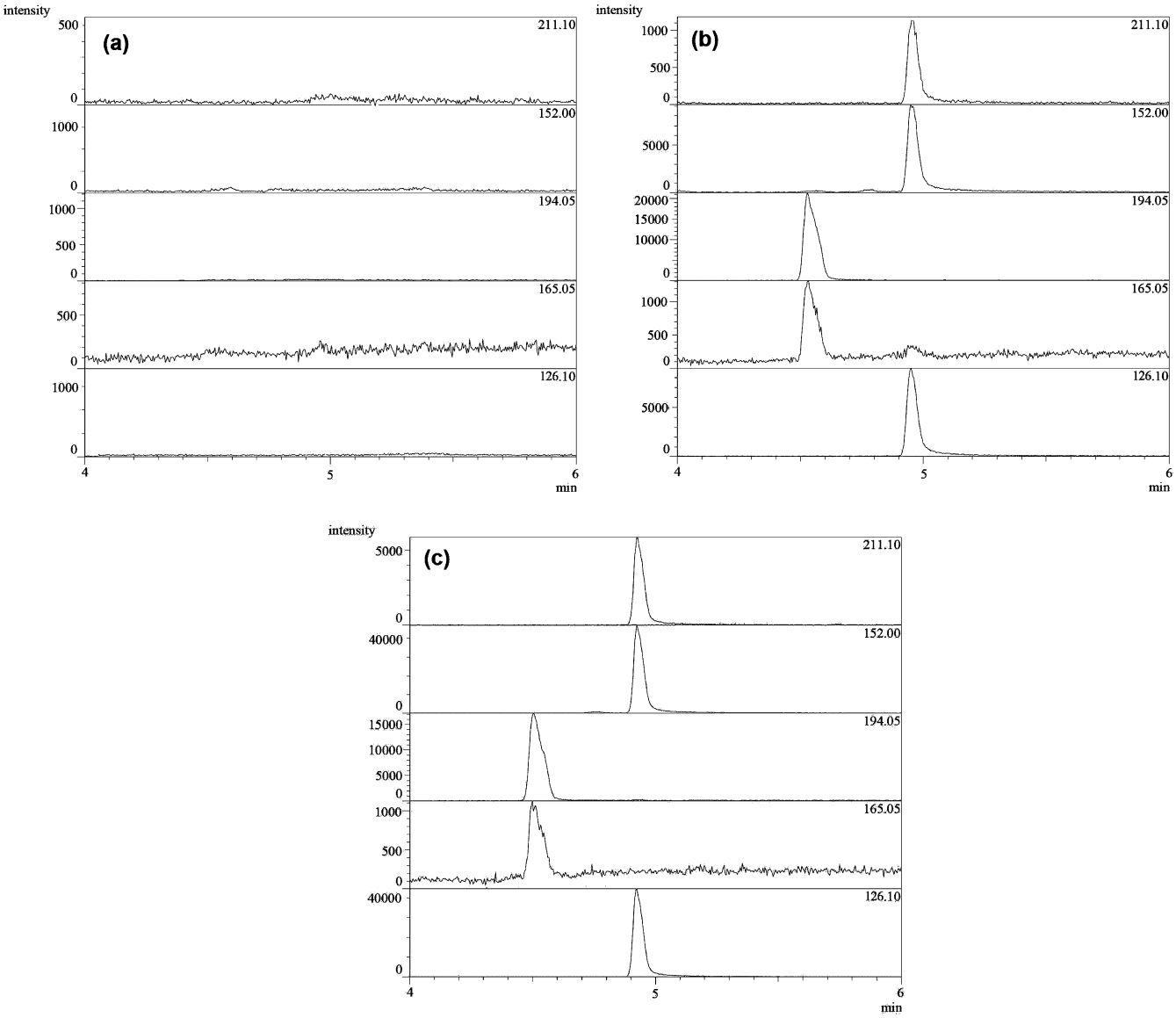

Figure 3. Representative SIM chromatograms for pramipexole (SIM *m/z* 211, 212 and 152; R_t 4.51 min) and IS (SIM *m/z* 194 and 165; R_t 4.93 min) obtained after extraction and GCMS analysis: (a) blank rat plasma; (b) blank rat plasma spiked with known concentrations of pramipexole (250 pg/mL), and the IS (200 pg/mL); (c) plasma sample obtained from a rat after 4 h of oral dose of pramipexole (20 µg/kg).

Table 2. Intra- and interday precision data of QC samples for pramipexole

Precision type	Nominal concentration (pg/mL)	Precision (mean ± SD)	RSD (%)	Accuracy Mean relative (%)
Intra-day				
	20.00	20.459 ± 0.53	2.61	2.36
	100.00	98.635 ± 3.86	3.91	3.63
	250.00	247.325 ± 12.98	5.25	4.16
	500.00	494.683 ± 24.55	4.96	4.48
Inter-day				
	20.00	19.568 ± 1.86	9.50	7.80
	100.00	102.882 ± 3.22	3.13	3.10
	250.00	246.269 ± 11.48	4.66	3.40
	500.00	500.717 ± 28.34	5.66	4.57

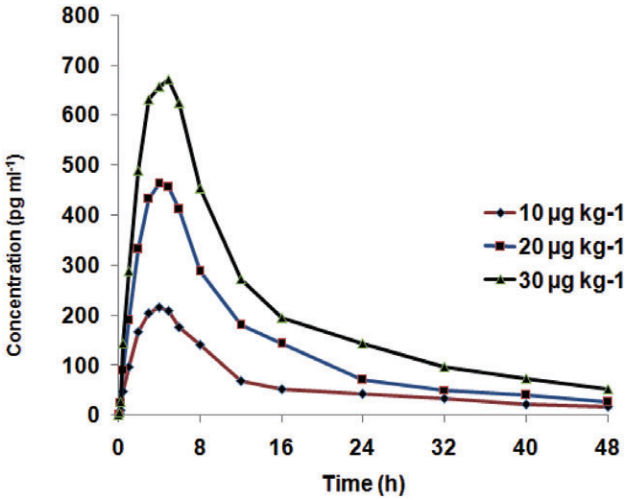

Figure 4. Mean plasma concentration–time profile of pramipexole in Wistar rats after a single oral dose of 10, 20 and 30 µg/kg.

Table 3. Mean pharmacokinetic parameters for pramipexole in rat plasma after oral administration

	Dose (µg/kg)		
Parameters	10	20	30
AUC_{0-t} (pg/mL/h)	2614.55	5691.45	8725.6
$AUC_{0-\infty}$ (pg/mL/h)	2998.55	6291.45	9949.60
C_{max} (pg/mL)	216	462	671
T_{max} (h)	4	4	4

stability behavior under the experimental conditions of the analytical runs. The stability data of the analytes in plasma over three freeze–thaw cycles indicated that the analytes were stable in rat plasma for three freeze–thaw cycles, when stored at −20°C and thawed to room temperature. The samples were stable for a period of 30 days at −20°C. The samples were stable in autosampler at 4°C for 24 h. The stability of the working solutions was tested at room temperature. Based on the results obtained, these working solutions were stable over 6 h. The results from all stability tests demonstrate good stability of each analyte over all steps of the determination and no stability-related problems are expected during the routine analyses. The method is therefore proved to be applicable for routine analysis for the pharmacokinetic, bioavailability or bioequivalence studies samples.

Pharmacokinetic Study

The developed GC/MS method yielded satisfactory results for the determination of pramipexole in rat plasma samples and was used successfully in a pilot bioequivalence study of pramipexole in rat following oral administration. The mean plasma concentration–time profiles for pramipexole are shown in Fig. 4. The plasma concentration data were used to assess key pharmacokinetic parameters (Table 3) such as the mean peak concentration C_{max}, area under the curve AUC_{0-t} and $AUC_{0-\infty}$ values. The time to maximum plasma concentration (T_{max}) was linearly related to dose ($r > 0.997$, $p < 0.05$), while no significant differences in other pharmacokinetic parameters among the various dose groups were found.

Conclusion

A fast, sensitive, specific analytical method has been developed and validated for the determination of pramipexole in rat plasma using GC/MS-SIM, which is superior to the other reported methods. The LLOQ of the present method was 20 pg/mL. The assay involved relatively simple sample preparation. Acceptable precision and accuracy were obtained within the standard curve range of 20–1000 pg/mL. The method fulfills all requirements for a bioanalytical method and can be successfully applied to the pharmacokinetic study of pramipexole.

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