

Quantification of pramipexole in human plasma by liquid chromatography tandem mass spectrometry using tamsulosin as internal standard

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ABSTRACT: A high-performance liquid chromatography/electrospray ionization tandem mass spectrometry method was developed and validated for the quantification of pramipexole in human plasma. Following liquid–liquid extraction, the analytes were separated using an isocratic mobile phase on a reverse-phase column and analyzed by MS/MS in the multiple reaction monitoring mode using the respective $[M + H]^+$ ions, m/z 212/152 for pramipexole and m/z 409/228 for the IS. The method exhibited a linear dynamic range of 200–8000 pg/mL for pramipexole in human plasma. The lower limit of quantification was 200 pg/mL with a relative standard deviation of less than 8%. Acceptable precision and accuracy were obtained for concentrations over the standard curve range. A run time of 3.5 min for each sample made it possible to analyze more than 200 human plasma samples per day. The validated method has been successfully used to analyze human plasma samples for application in pharmacokinetic, bioavailability or bioequivalence studies. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: pramipexole; liquid chromatography–tandem mass spectrometry; human plasma; pharmacokinetic study

INTRODUCTION

Pramipexole (Fig. 1) is an orally active, non-ergoline, dopamine agonist (Bennett and Piercey, 1999). It is a novel aminobenzothiazole compound that is potent agonist at the D_2 subfamily of dopamine receptors (Mierau and Schingnitz, 1992; Mierau *et al.*, 1995). It has little activity at other receptor families and, within the D_2 subfamily, it binds with highest affinity to D_3 receptors. Moreover, in contrast to the ergot dopamine agonists traditionally used to treat Parkinson's disease, pramipexole fully stimulates the dopamine receptors it binds to; the ergots only partially activate these receptors (Piercey *et al.*, 1996). Clinical trails with pramipexole as monotherapy and as an adjunct to levodopa have shown the compound to be safe, well tolerated, and efficacious (Relja and Klepac, 2006; Wright *et al.*, 1997).

The bioanalytical component of a pharmacokinetic study requires a drug assay with simplicity, selectivity, sensitivity, small sample size and rapid turnaround time. Very few methods for the quantification of

pramipexole in biological fluids have been reported. High-performance liquid chromatography (HPLC) methods for the determination of pramipexole in human plasma and urine with electrochemical detection and ultraviolet detection have been reported (Lau *et al.*, 1996a). However these HPLC methods need longer chromatographic run times. A liquid chromatography–tandem mass spectrometry (LC-MS/MS) method utilizing atmospheric pressure chemical ionization (APCI) has been described (Lau *et al.*, 1996b); this method was applied to determine pramipexole in human plasma after liquid–liquid extraction of 1 mL plasma samples using BHT-920 as an internal standard. The sensitivity (50 pg/mL) was obtained for 70 μ L injection volume corresponding to 35 fg on-column. The sample preparation was time-consuming and a strong polar interference was observed in the chromatograms at 1.4 min with ter-butylmethylether (TBME) as an extraction solvent. However, most laboratories are not equipped with APCI and the internal standard is not a commercially available compound.

The present paper describes the development and validation of an LC-electrospray ionization (ESI)-MS/MS method using a commercially available compound tamsulosin as an internal standard. The sample preparation was rapid using a 0.5 mL plasma sample, making it an attractive procedure for the bioanalysis of pramipexole.

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Abbreviations used: TBME, ter-butylmethylether.

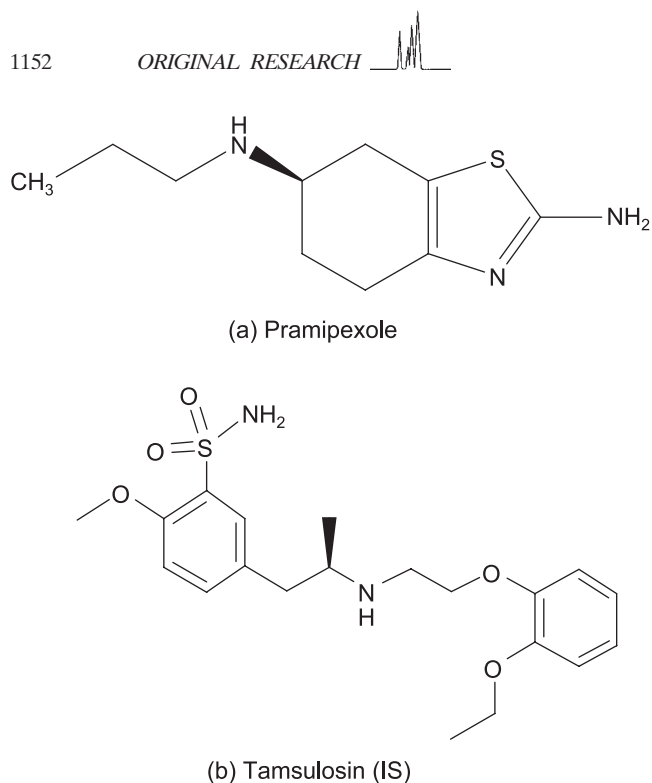


Figure 1. Chemical structures for pramipexole and IS (tamsulosin).

EXPERIMENTAL

Chemicals. Pramipexole drug substances were obtained from Anazeal Analytical Laboratories (Mumbai, India) and tamsulosin hydrochloride (internal standard, IS) was obtained from this R&D Institute (Hyderabad, India). Chemical structures are presented in Fig. 1. HPLC-grade LiChrosolv methanol was purchased from Merck (Darmstadt, Germany). Ammonium acetate, sodium hydroxide pellets, tert-butylmethylether and dichloromethane were purchased from Merck (Worli, Mumbai, India). HPLC-grade water from Milli-Q system (Millipore, Bedford, MA, USA) was used. All other chemicals were of analytical grade.

LC-MS/MS instrument and conditions. The HPLC SIL HTC system (Shimadzu Corporation, Kyoto, Japan) was equipped with an LC-AD VP binary pump, a DGU20A5 degasser and a SIL-HTC auto sampler equipped with a CTO-10AS VP thermostated column oven. The chromatography was performed using LiChrospher[®] RP-select B column (5 μ m, 100 \times 4.0 mm i.d.) at 40°C temperature. The isocratic mobile phase composition was a mixture of 10 mM ammonium acetate–methanol (30:70, v/v), which was pumped at a flow-rate of 1.2 mL/min with a split ratio of load to waste 10:90.

Mass spectrometric detection was performed on an API 3000 triple quadrupole instrument (MDS-SCIEX, Concord, Ontario, Canada) using MRM. A turboIonSpray interface operating in positive ionization mode was used. Typically, setting source conditions were as follows: the turbo-gas temperature was set at 300°C and the ion spray needle voltage was adjusted to 5500 V. The common parameters, viz.

nebulizer gas, curtain gas and collision gas were set at 10, 15 and 6, respectively. The mass spectrometer was operated at unit resolution for both Q1 and Q3 in the MRM mode, with a dwell time of 200 ms per MRM channel. The precursor/product ion pairs monitored were m/z 212/152 for pramipexole and m/z 409/228 for IS. The collision energy was set at 20 and 30 V for pramipexole and IS, respectively. Data acquisition was performed with Analyst software (Version 1.4.1).

Sample preparation. Standard stock solutions of pramipexole (1 mg/mL) and the IS (1 mg/mL) were separately prepared in methanol. Working solutions for calibration and controls were prepared by appropriate dilution in water–methanol (50:50, v/v; diluent). The IS working solution (90 ng/mL) was prepared by diluting its stock solution with diluent. Working solutions (0.2 mL) were added to drug-free human plasma (9.8 mL) as a bulk, to obtain pramipexole concentration levels of 200, 500, 1000, 2000, 3000, 5000 and 8000 pg/mL as a single batch at each concentration. Quality control (QC) samples were also prepared as a bulk on an independent weighing of standard drug, at concentrations of 200 (LLOQ), 600 (low), 4000 (medium) and 6000 pg/mL (high) as a single batch at each concentration. The calibration and control bulk samples were divided into aliquots in microcentrifuge tubes (Tarson, 2 mL) and stored in the freezer at below –50°C until analysis.

A plasma sample (0.5 mL) was pipetted into a 15 mL glass tube and then 20 μ L of IS working solution (90 ng/mL) and 50 μ L of sodium hydroxide solution (1 M) were added. After vortex mixing for 10 s, 4 mL aliquot of the extraction mixture, tert-butylmethylether-dichloromethane (8:2), was added and the sample was vortex-mixed for 4 min. The organic layer (3 mL) was transferred to a glass tube and evaporated to dryness using an evaporator at 40°C under a stream of nitrogen. Then the dried extract was reconstituted in 150 μ L of reconstitution solvent (5% water and 95% methanol) and a 15 μ L aliquot was injected into the chromatographic system.

Bioanalytical method validation. A calibration curve was constructed from a blank sample (a plasma sample processed without the IS), a zero sample (a plasma processed with the IS) and seven non-zero samples covering the total range 200–8000 pg/mL for pramipexole including the LLOQ. The calibration curves were generated using the analyte to IS peak area ratios by weighted (1/x) least-squares linear regression on consecutive days. The acceptance criterion for a calibration curve was a correlation coefficient (r) of 0.99 or better, and that each back-calculated standard concentration must be within 15% deviation from the nominal value except at the LLOQ, for which the maximum acceptable deviation was set at 20%. At least 67% of non-zero standards were required to meet the above criteria, including acceptable LLOQ and upper limit of quantification.

The within-batch precision and accuracy were determined by analyzing four sets of QC samples (LLOQ, low, medium and high concentrations) each comprised five replicates in a batch. The between-batch precision and accuracy were determined by analyzing such five different batches. The acceptance criteria for within- and between-batch precision were 20% or better for LLOQ and 15% or better for the other concentrations, and the accuracy was 100 \pm 20% or better for

LLOQ and $100 \pm 15\%$ or better for the other concentrations (Shah *et al.*, 1991).

Recovery of pramipexole from the extraction procedure was determined by a comparison of the peak area of pramipexole in spiked plasma samples (five each of low, medium and high QCs) with the peak area of pramipexole in samples prepared by spiking extracted drug-free plasma samples with the same amounts of pramipexole at the step immediately prior to chromatography. Similarly, recovery of IS was determined by comparing the mean peak areas of extracted QC samples ($n = 6$) to mean peak areas of IS in samples prepared by spiking extracted drug-free plasma samples with the same amounts of IS at the step immediately prior to chromatography.

The stability of the analyte and IS in human plasma under different temperature and timing conditions, as well as their stability in the stock solutions, was evaluated. QC samples were subjected to short-term room temperature conditions, to long-term storage conditions (-50°C), and to freeze–thaw stability studies. All the stability studies were conducted at two concentration levels (600 and 6000 pg/mL as low and high QC values) with five replicates for each.

RESULTS AND DISCUSSION

Mass spectrometry

Pharmacokinetic applications require highly selective assays with high sample throughput capacity. Quantification of drugs in biological matrices by LC-MS/MS is becoming more common due to the improved sensitivity and selectivity of this technique. The product ion mass spectra, and their proposed rationalizations in terms of fragmentation patterns, of pramipexole and IS are illustrated in Fig. 2. $[\text{M} + \text{H}]^+$ was the predominant ion in the Q1 spectrum and was used as the precursor ion to obtain product ion spectra. The most sensitive mass transition was from m/z 212/152 for pramipexole and m/z 409/228 for the IS.

Method development

Choosing the appropriate internal standard is an important aspect of achieving acceptable method performance, especially with LC-MS/MS, where matrix effects can lead to poor analytical results. In the initial stages of this work, several compounds were investigated to find a suitable IS, and finally tamsulosin was found to be suitable for the present purpose and commercially available. Clean chromatograms were obtained and no significant direct interferences in the MRM channels at the relevant retention times were observed. However, in ESI, signal suppression or enhancement may occur due to co-eluting endogenous components of the sample matrix. The importance of including the evaluation of the matrix effect in any LC-MS/MS method is outlined in an excellent paper by Matuszewski *et al.* (2003). Their data strongly emphasize the need to use a

blank matrix from (at least five) different sources/individuals instead of using one blank matrix pool to determine method precision and accuracy. Therefore, all validation experiments in this method were performed with matrices obtained from different individuals. In addition, validation experiments were performed using hemolytic and strongly lipemic matrices. As all data falls within the guidelines, we conclude that the degree of matrix effect was sufficiently low to produce acceptable data and the method can be considered as valid.

Liquid–liquid extraction (LLE) was used for the sample preparation in this work. LLE can be helpful in producing a spectroscopically clean sample and avoiding the introduction of non-volatile materials onto the column and MS system. Clean samples are essential for minimizing ion suppression and matrix effect in LC-MS/MS analyses. Five organic solvents, diethyl ether, hexane, ethyl acetate, dichloromethane and tert-butylmethylether, and their mixtures in different combinations and ratios were evaluated. Finally, a mixture of tert-butylmethylether and dichloromethane (8:2, v/v) was found to be optimal, which can produce a clean chromatogram for a blank plasma sample and yield the highest recovery for the analytes from the plasma.

The extraction recovery of pramipexole was $96.1 \pm 1.4\%$ and the recovery of the IS was $77.6 \pm 2.9\%$ at the concentration used in the assay (90 ng/mL). Recoveries of the analyte and IS were high, and were consistent, precise and reproducible. Therefore, the assay has proved to be robust in high-throughput bioanalysis.

The chromatographic conditions, especially the composition of the mobile phase, were optimized through several trials to achieve better sensitivity good resolution and symmetric peak shapes for the analytes and IS, as well as a short run time. It was found that a mixture of 10 mM ammonium acetate–methanol (30:70, v/v) could achieve this and was adopted as the mobile phase. The high proportion of organic solvent eluted the analyte and the IS at retention times of 2.2 and 2.4 min, respectively. A flow rate of 1.2 mL/min produced good peak shapes and permitted a run time of 3.5 min.

Assay performance and validation

The seven-point calibration curve was linear over the concentration range 200–8000 pg/mL for pramipexole. The calibration model was selected based on the analysis of the data by linear regression with/without intercepts and weighting factors ($1/x$, $1/x^2$ and none). The best linear fit and least-squares residuals for the calibration curve were achieved with a $1/x$ weighting factor, giving a mean linear regression equation for the calibration curve of:

$$y = 0.0002(\pm 0.0000)x + 0.0035(\pm 0.0017)$$

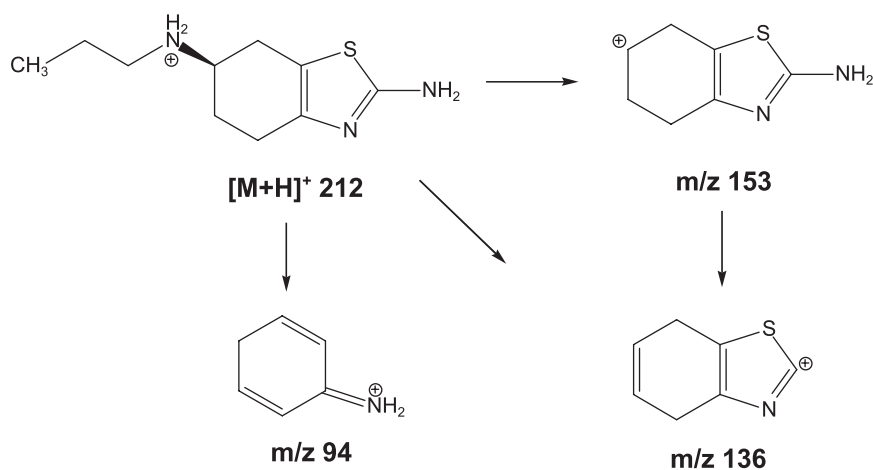
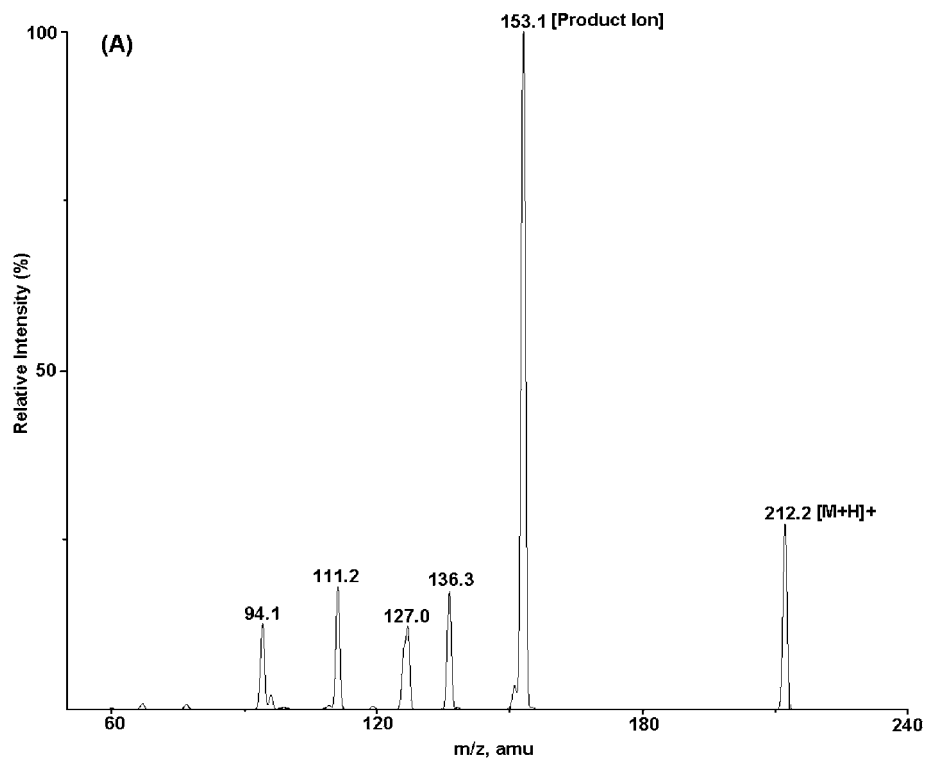


Figure 2. Full-scan positive ion turboionspray product ion mass spectra and proposed pattern of fragmentation of (a) pramipexole and (b) tamsulosin (internal standard).

where y was the peak area ratio of the analyte to the IS and x was the concentration of the analyte. The mean correlation coefficient of the weighted calibration curve generated during the validation was 0.9989 ± 0.0007 .

The selectivity of the method was examined by analyzing ($n = 6$) blank human plasma extract [Fig. 3(A)] and an extract spiked only with the IS [Fig. 3(B)]. As shown in Fig. 3(A), no significant direct interference in the blank plasma traces was observed from endogenous substances in drug-free human plasma at the retention time of the analyte. Similarly, Fig. 3(B) shows the absence of direct interference from the IS to the

MRM channel of the analyte. Figure 3(C) depicts a representative ion-chromatogram for the LLOQ (200 pg/mL).

The LLOQ was defined as the lowest concentration in the standard curve that can be measured with acceptable accuracy and precision, and was found to be 200 pg/mL in human plasma. Lau *et al.* (1996b) reported a sensitivity of 50 pg/mL for a 70 μ L injection volume corresponding to 35 pg on-column. In the present method the sensitivity was observed for a 15 μ L injection volume corresponding to 10 pg on-column. By increasing the injection volume higher sensitivity can be

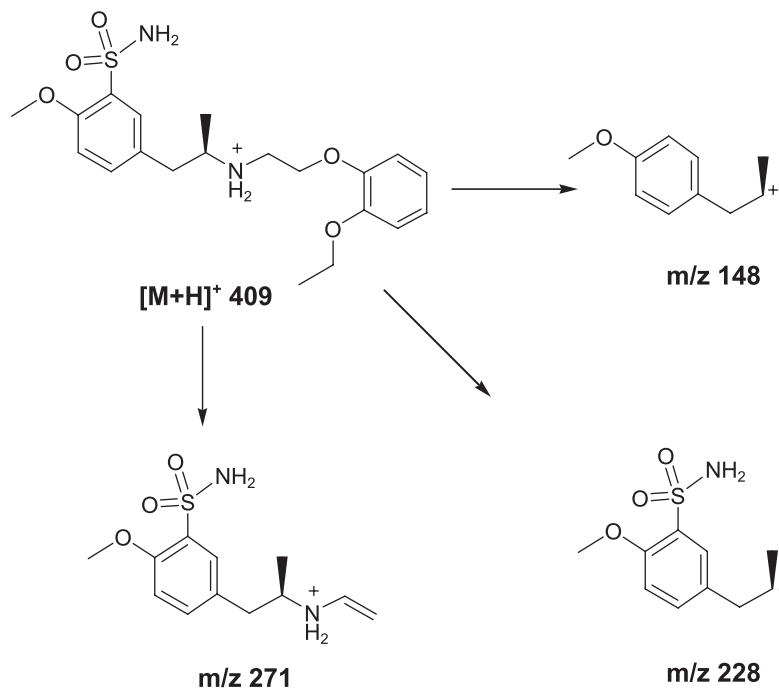
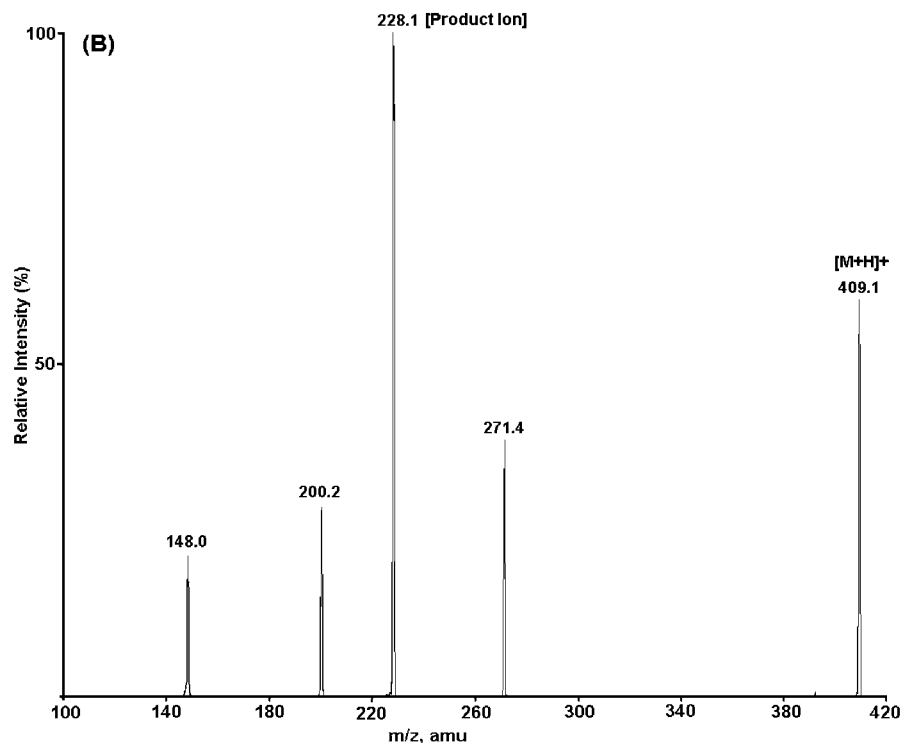


Figure 2. (Continued)

obtained. The between-batch precision at the LLOQ was 5.7% and the accuracy was 105.1% (Table 1). The within-batch precision was 8.0% and the accuracy was 98.9%.

The middle and upper quantification levels of pramipexole ranged from 600 to 6000 pg/mL in human plasma. For the between-batch experiments the precision ranged from 3.4 to 7.2% and the accuracy from

99.7 to 101.3% (Table 1). For the within-batch experiments the precision and accuracy for the analyte met the acceptance criteria ($\leq \pm 15\%$).

Stability studies

For short-term stability determination, stored plasma aliquots were thawed and kept at room temperature for

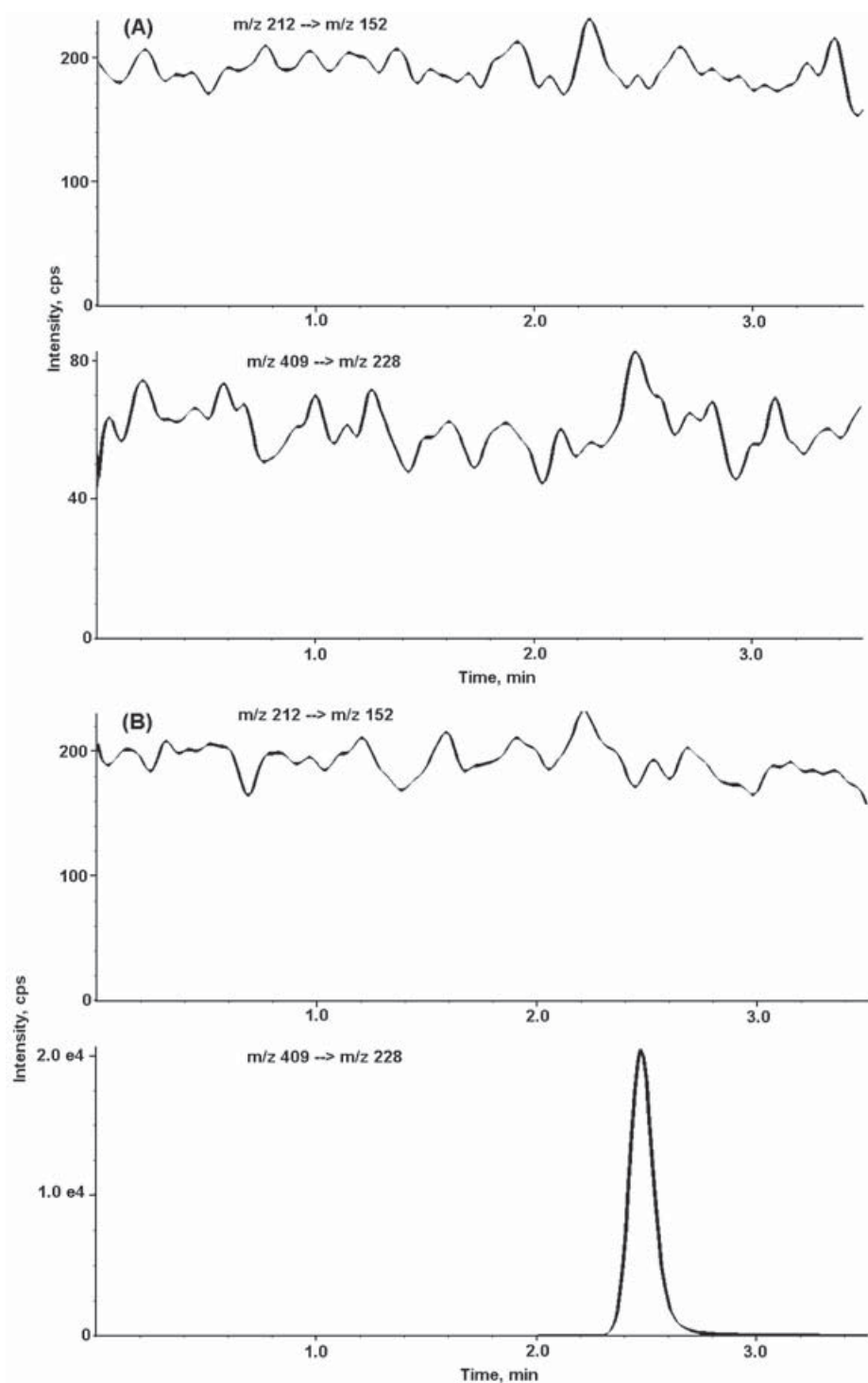


Figure 3. MRM chromatograms for pramipexole and IS resulting from analysis of: (a) blank (drug and IS free) human plasma; (b) zero sample (drug-free spiked with IS) human plasma; (c) 200 pg/mL (LLOQ) of pramipexole spiked with the IS.

a period of time exceeding that expected to be encountered during routine sample preparation (around 24 h). Samples were extracted and analyzed as described above and the results indicate reliable stability behavior under the experimental conditions of the regular analytical procedure. The stability of QC samples kept in

the autosampler for 25 h was also assessed. The results indicate that solutions of the analyte and the IS can remain in the autosampler for at least 25 h without showing significant loss in the quantified values, indicating that samples should be processed within this period of time.

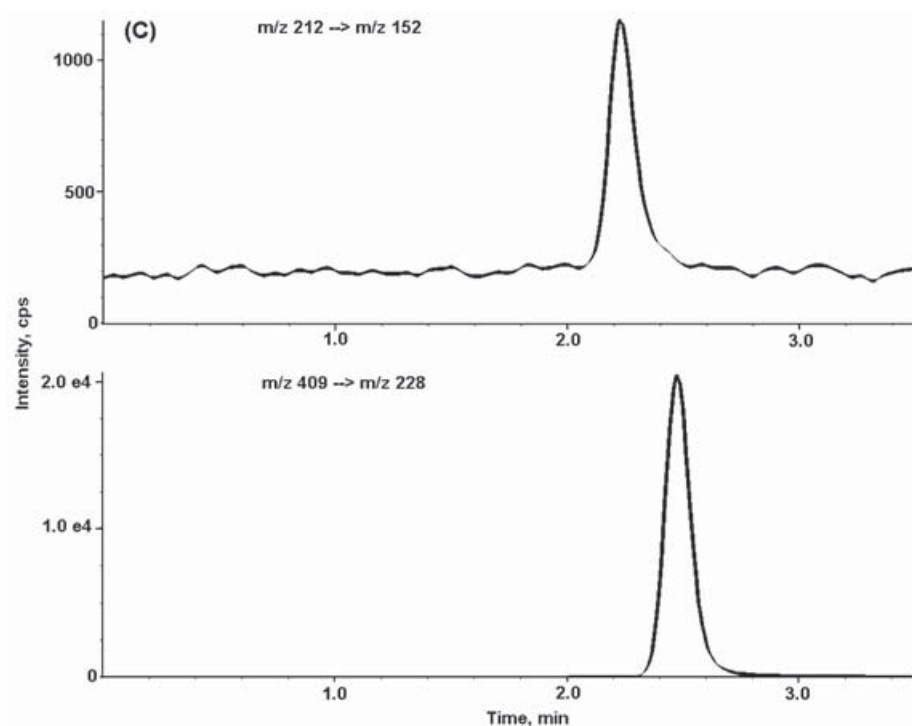


Figure 3. (Continued)

Table 1. Precision and accuracy of the method for determining pramipexole concentrations in plasma samples

Concentration added (pg/mL)	Within-batch ($n = 5$)			Between-batch ($n = 5$)		
	Concentration found (mean \pm SD; pg/mL)	Precision (%)	Accuracy (%)	Concentration found (mean \pm SD; pg/mL)	Precision (%)	Accuracy (%)
200	197.8 \pm 15.9	8.0	98.9	210.4 \pm 11.9	5.7	105.1
600	565.0 \pm 21.0	3.7	94.1	598.6 \pm 42.9	7.2	99.7
4000	3914.5 \pm 147.7	3.8	97.8	4056.4 \pm 138.0	3.4	101.3
6000	5647.1 \pm 236.1	4.2	94.1	5996.9 \pm 241.9	4.0	99.9

The stability data of the analyte in plasma over three freeze–thaw cycles indicate that the analyte is stable in human plasma for three freeze–thaw cycles, when stored at below -50°C and thawed to room temperature.

The long-term stability data of the analyte in human plasma stored for a period of 45 days at below -50°C showed reliable stability behavior, as the means of the results of the tested samples were within the acceptance criteria of $\pm 15\%$ of the initial values of the controls. These findings indicate that storage of the analyte in plasma samples at below -50°C is adequate, and no stability-related problems would be expected during routine analyses for pharmacokinetic, bioavailability or bioequivalence studies.

The stability of the stock solutions was tested and established at room temperature for 4 and 21 h, and under refrigeration (4°C) for 45 days (data not shown). The results revealed optimum stability for the prepared

stock solutions throughout the period intended for their daily use.

Application

The method was successfully applied to determine the plasma concentration of pramipexole following a single 1.5 mg oral administration to 12 healthy subjects. The MRM chromatograms obtained for an extracted plasma sample of a healthy subject who participated in a bioequivalence study are depicted in Fig. 4. Pramipexole was quantified as 3955 pg/mL.

CONCLUSIONS

In summary, a method is described for the quantification of pramipexole from human plasma by LC-MS/MS in positive electrospray ionization mode using multiple

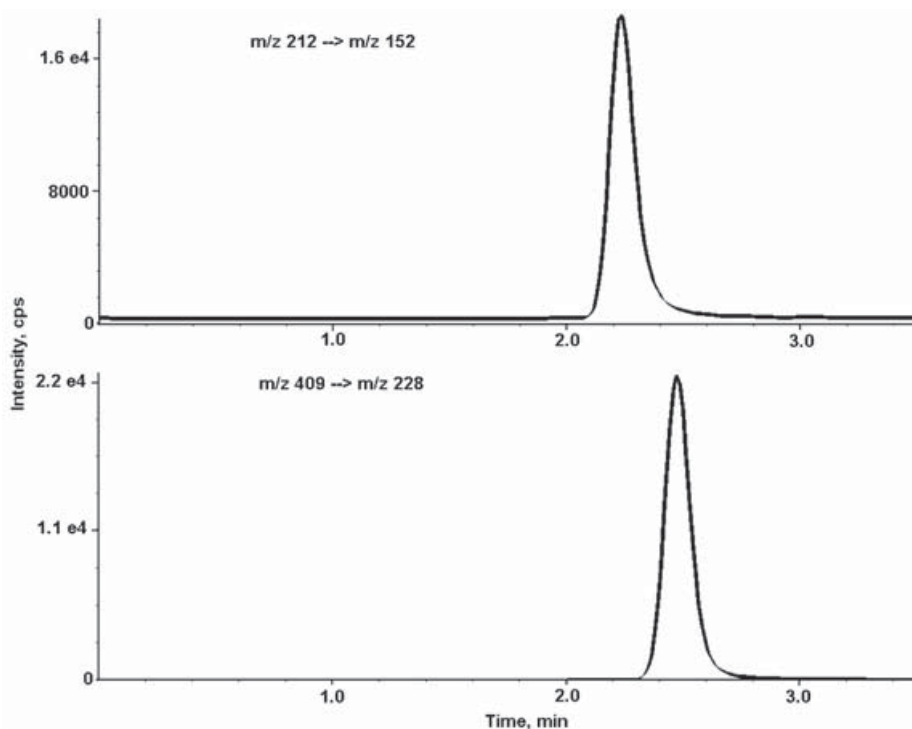


Figure 4. MRM chromatograms resulting from the analysis of a subject plasma sample after the administration of a 1.5 mg oral single dose of pramipexole. The sample concentration was determined to be 3955 pg/mL.

reaction monitoring and fully validated according to commonly accepted criteria (Shah *et al.*, 1991). The current method has shown acceptable precision and adequate sensitivity for the quantification of pramipexole in human plasma samples obtained for pharmacokinetic, bioavailability or bioequivalence studies. Many variables related to the electrospray reproducibility were optimized for both precision and sensitivity to obtain these results. The rapid extraction method makes it an attractive procedure in high-throughput bioanalysis of pramipexole. The method was successfully applied to quantify the concentrations of pramipexole in a clinical pharmacokinetic study.

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REFERENCES

- Bennett JP and Piercey MF. Pramipexole—a new dopamine agonist for the treatment of Parkinson's disease. *Journal of the Neurological Sciences* 1999; **163**: 25.
- Lau YY, Hanson GD and Ichhpurani N. Determination of pramipexole (U-98,528) in human plasma and urine by high-performance liquid chromatography with electrochemical and ultraviolet detection. *Journal of Chromatography B* 1996a; **683**: 217.
- Lau YY, Selenka JM, Hanson GD, Talaat R and Ichhpurani N. Determination of pramipexole (U-98,528) in human plasma by high-performance liquid chromatography with atmospheric pressure chemical ionization tandem mass spectrometry. *Journal of Chromatography B* 1996b; **683**: 209.
- Matuszewski BK, Constanzer ML and Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC-MS/MS. *Analytical Chemistry* 2003; **75**: 3019.
- Mierau J and Schingnitz G. Biochemical and pharmacological studies on pramipexole, a potent and selective dopamine D₂-receptor agonist. *European Journal of Pharmacology* 1992; **215**: 161.
- Mierau J, Schneider FJ, Ensinger HA, Chio CL, Lajiness ME and Huff RM. Pramipexole binding and activation of cloned and expressed dopamine D₂, D₃ and D₄ receptors. *European Journal of Pharmacology* 1995; **290**: 29.
- Piercey MF, Hoffmann WE, Smith MW and Hyslop DK. Inhibition of dopamine neuron firing by pramipexole, a D₂-preferring dopamine agonist: comparison to other dopamine agonists. *European Journal of Pharmacology* 1996; **312**: 35.
- Relja M and Klepac N. A dopamine agonist, pramipexole, and cognitive functions in Parkinson's disease. *Journal of Neurological Sciences* 2006; **248**: 251.
- Shah VP, Midha KK, Dighe S, McGilveray IJ, Skelly JP, Yacobi A, Layloff T, Vishwanathan CT, Cook CE and McDowall RD. Analytical methods validation: bioavailability, bioequivalence and pharmacokinetic studies. Conference report. *European Journal of Drug Metabolism and Pharmacokinetics* 1991; **16**: 249.
- Wright CE, Sisson TL, Ichhpurani AK and Peters GR. Steady-state pharmacokinetic properties of pramipexole in healthy volunteers. *Journal of Clinical Pharmacology* 1997; **37**: 520.