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ORIGINAL RESEARCH

Liquid chromatography tandem mass spectrometry method for the quantification of amisulpride with LLOQ of 100 pg/mL using 100 µL of plasma

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ABSTRACT: A sensitive and selective high-performance liquid chromatography–positive ion electrospray tandem mass spectrometry method was developed and validated for the quantification of amisulpride in 100 µL of 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* 370–242 for amisulpride and *m/z* 341–112 for the internal standard. The assay exhibited a linear dynamic range with a lower range of 0.1–100 ng/mL and a higher range of 1–500 ng/mL of amisulpride in human plasma. The lower limit of quantification was 0.1 ng/mL with a relative standard deviation of less than 10%. Acceptable precision and accuracy were obtained for both linearity ranges. A run time of 2.0 min for each sample made it possible to analyze more than 275 human plasma samples per day. The validated method has been successfully used to analyze plasma samples for application in pharmacokinetic studies. Copyright © 2008 John Wiley & Sons, Ltd.

KEYWORDS: amisulpride; liquid chromatography–tandem mass spectrometry; plasma

INTRODUCTION

Amisulpride (Fig. 1) is an atypical antipsychotic drug with benzamidic structure, that is active against both positive (hallucinations, delusions) and negative (anergia, flat affectivity) symptoms of schizophrenia (McKeage and Plosker, 2004; Peuskens and Moller, 2002; Racagni *et al.*, 2004). Its pharmacological activity is based on the selective binding to D_2 and D_3 dopaminergic receptors (Curran and Perry, 2001). It has a lower risk of extrapyramidal side effects and it is relatively better tolerated than conventional antipsychotic drugs (Mortiner, 2004).

At low doses, amisulpride binds preferentially to presynaptic receptors, increasing dopaminergic transmission, and at high doses the postsynaptic receptor blockade induces a decrease in dopaminergic transmission. In clinical studies, amisulpride, at high doses, was shown to be effective in treating positive symptoms of schizophrenia (Davis *et al.*, 2003; Lecrubier *et al.*, 2000; Peuskens *et al.*, 1999) and also at low doses, was shown to be effective in treating negative symptoms of schizophrenia (Danion *et al.*, 1999; Colonna *et al.*, 2000; Leucht *et al.*, 2002; Muller *et al.*, 2000). A positron emission tomography (PET) imaging study (Castelli *et al.*, 2001) reported a curvilinear relationship between doses and plasma concentrations of amisulpride and the dopamine receptor binding whereby plasma concentrations were related more accurately to receptor binding than doses. Similar to the two dose ranges, two plasma ranges were found. Plasma concentrations below 92 ng/mL were associated with low striatal binding but were sufficiently high to block extrastriatal dopamine receptors, whereas at plasma concentrations above 153 ng/mL marked binding to both extrastriatal and striatal regions was found.

Amisulpride is rapidly absorbed after oral administration with a bioavailability of about 48% because of its low first-pass metabolization. The drug shows a weak protein binding (11–17%) and a large volume of distribution (Noble and Benfield, 1999; Rosenzweig *et al.*, 2002). The elimination half-life is 7–8 h after the i.v. route and about 11–12 h after the oral route. A steady state is reached after 2–3 days. Amisulpride is metabolized in the liver to only a minor degree, yielding only two inactive metabolites (Noble and Benfield,

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Abbreviations used: PET, positron emission tomography.

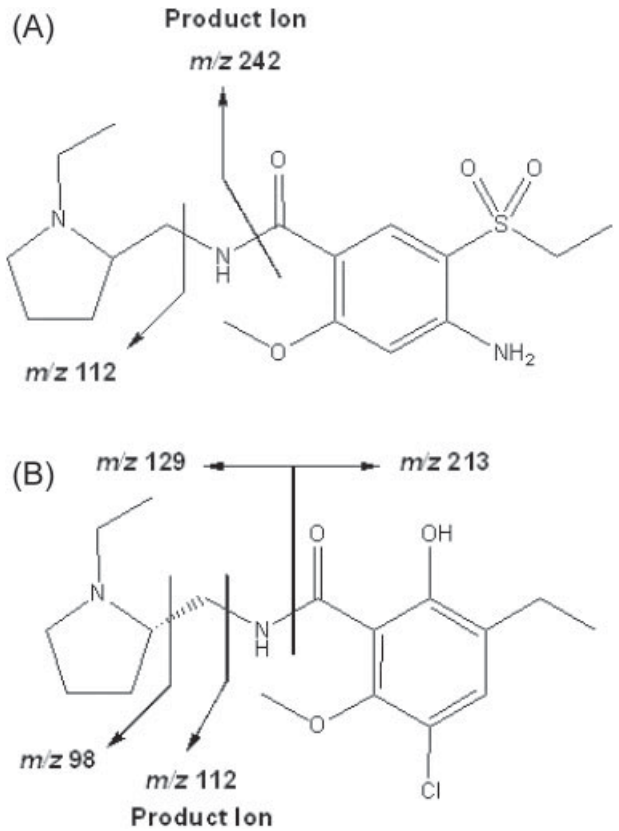

Figure 1. Chemical structures of (A) amisulpride and (B) eticlopride (IS).

1999; Rosenzweig *et al.*, 2002). The drug is predominantly eliminated via the kidneys (Lambert and Naber, 1999).

The high interindividual variability of amisulpride levels in blood at a given dose (Bergemann *et al.*, 2004; Moulin *et al.*, 1991; Xiberas *et al.*, 2001) and the good relation between drug levels in blood and dopamine receptor occupancy in the brain (Xiberas *et al.*, 2001) make it useful to monitor blood levels of patients treated with amisulpride. Several analytical methods dealing with the determination of amisulpride in biological fluids have been reported. Most of them have been applied for the simultaneous determination of several drugs for, e.g., screening, toxicological and forensic purposes (Frahnert *et al.*, 2003; Kirchherr and Kuehn-Velten, 2006; Kratzsch *et al.*, 2003). The analysis of amisulpride alone (or together with other benzamides) in biological fluids has been carried out using liquid chromatographic methods (Bohbot *et al.*, 1987; Gschwend *et al.*, 2006; Malavasi *et al.*, 1996; Pehourcq *et al.*, 2003; Sachse *et al.*, 2003). Recently Gschwend *et al.* (2006) reported a liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the determination of amisulpride in human plasma in the concentration range 0.5–500.52 ng/mL. The lower limit of quantification (LLOQ) of 0.5 ng/mL was obtained using a plasma volume of 250 μL.

The purpose of the present investigation was to explore the high selectivity and sensitivity of a triple-quadrupole MS system operated in MS/MS mode with an electrospray interface for the development and validation of a robust reversed-phase LC-MS/MS method for the quantification of amisulpride in 100 μL of plasma. It was essential to establish a method capable of quantifying amisulpride at concentrations down to 0.1 ng/mL. At the same time, it was expected that this method would be efficient in analyzing a large number of plasma samples obtained for pharmacokinetic studies after therapeutic doses of amisulpride. We believe that development of a method in human plasma would facilitate the ease of adaptability of amisulpride assay in rat plasma. The plasma volume required is only 100 μL; therefore, this method can also be employed in pre-clinical pharmacokinetic investigations of amisulpride.

EXPERIMENTAL

Chemicals and reagents. The standard substance amisulpride and eticlopride (internal standard) were obtained from the R&D department, Suven Life Sciences Ltd, Hyderabad. Molecular structures of the both compounds are shown in Fig. 1. HPLC-grade lichrosolv acetonitrile and HPLC-grade lichrosolv methanol were purchased from Merck (Darmstadt, Germany). Formic acid was purchased from Fluka (Sigma Aldrich, Steinheim, Germany). Diethyl ether, dichloromethane, ammonium formate and sodium hydroxide pellets were purchased from Merck (Worli, Mumbai, India). HPLC-grade water from a Milli-Q system (Millipore, Bedford, MA, USA) was used. All other chemicals were of analytical grade.

Liquid chromatography. The experiments were performed by using Perkin-Elmer HPLC 200 series (Shelton, CT, USA) consisting of a quaternary pump, an auto sampler and a column compartment. The column was a Waters® symmetry C_{18} (100 × 4.6, 3.5 μm particle size). The mobile phase consisted of 10 mM ammonium formate (pH was adjusted to 3.0 with formic acid) and acetonitrile, 35:65 (v/v), with a flow rate of 1.0 mL/min. The flow was split before the mass spectrometer in the ratio of 10:90 and the run time was 2.0 min. Water:methanol (1:1, v/v) was used as the injector rinsing solvent.

Mass spectrometry. A Sciex API-3000 triple quadrupole mass spectrometer (Applied Biosystems, Toronto, Canada) with a turboionspray source was used for detection. The ionspray, declustering potential, entrance potential and focusing potential were optimized to 4500, 55, 7 and 240 V respectively. Purified air was used as a nebulising agent and high-purity nitrogen generated with peak scientific NM20Z-A (Fountain Crescent, Scotland, UK) was used as the turbo, curtain and collision gas. The source temperature was kept at 250°C. Analysis was carried out in positive ion mode with multiple reaction monitoring (MRM) with the following reactions: m/z 370 → 242 for amisulpride and m/z 341 → 112 for the internal standard.

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The dwell time for each reaction was kept to 200 ms. Collision energy and collision cell exit potential were set at 35 (arbitrary units) and at 18 (arbitrary units) respectively. The data was acquired and processed in Analyst 1.4.1 software.

Sample preparation. Standard stock solutions of amisulpride (1 mg/mL) and internal standard (1 mg/mL) were prepared in methanol. Working solutions were prepared with water:methanol (50:50 v/v, diluent) with proper dilutions. Analytical standard samples were prepared by spiking known quantities of working solutions into blank human plasma. The concentration range for amisulpride in the spiked standard human plasma was 0.1, 0.2, 0.5, 1, 5, 10, 50 and 100 ng/mL for the lower calibration range and 1, 2, 5, 10, 20, 50, 100, 200 and 500 ng/mL for the higher calibration range. Quality control (QC) samples were also prepared on an independent weighing of standard drug, at concentrations of 0.1 and 1 (LLOQ), 0.3 and 3 (low), 50 and 250 (medium) and 80 and 400 ng/mL (high) as a single batch at each concentration for both calibrations.

The plasma samples were prepared using liquid–liquid extraction technique. A 100 µL aliquot of the plasma sample was taken in a 15 mL glass test tube, and on that 10 µL of IS (2 µg/mL) was spiked and 50 µL of 0.1 M sodium hydroxide solution was added. To this 2.5 mL of extraction solvent (diethyl ether:dichloromethane 70:30; v/v) was added and vortex-mixed on a multiple vortexer for 3.0 min. The supernatant organic layer (2 mL) was transferred quantitatively into a 5.0 mL glass test tube and dried under nitrogen stream at 40°C. The dried residue was reconstituted in 250 µL of mobile phase and a 5 µL aliquot of this was injected into LC-MS/MS.

RESULTS AND DISCUSSION

Method development

HPLC coupled with a tandem mass spectrometer (MS/MS), which provides excellent sensitivity for monitoring new chemical entities, has become standard equipment in support of various *in vitro* and *in vivo* experiments (Hsieh and Korfmacher, 2006). The inherent selectivity of MS/MS detection was expected to be beneficial in developing a selective and sensitive method. $[M + H]^+$ was the predominant ion in the Q1 spectrum and was used as the precursor ion to obtain product ion spectra. The product ion mass spectra, and their proposed rationalizations in terms of fragmentation patterns of amisulpride and IS are illustrated in Fig. 2. By monitoring the most stable and sensitive transition *m/z* 370–242 for amisulpride, it as possible to detect LLOQ up to 0.1 ng/mL. For the MS analysis deuterated internal standard was preferred as it behaves exactly like analyte in recovery and ionization process. It was not available commercially; therefore, a structural analogue eticlopride was used as internal standard, which served the purpose. For the internal standard MRM transition *m/z* 341–112 was selected.

Liquid–liquid extraction (LLE) was used for sample preparation as it is economical and its preparation is easy. LLE is useful in giving spectroscopically clean samples, hindering the introduction of non-volatile material onto the column and MS system. Clean samples are required to avoid the matrix effects and ion suppression in MS analysis. The pK_a of the amisulpride was 9.37, hence plasma samples were alkalized to prevent ionization in the plasma itself and 10% higher recovery was obtained. The extraction solvent diethyl ether–dichloromethane (70:30 v/v) was useful in obtaining clean blank plasma samples. The average absolute recoveries of amisulpride and IS were about 78 and 82% respectively. LLE sample pretreatment enabled analyte preconcentration and was shown to have the advantages of simplicity and rapidity.

Validation of the amisulpride assay

Specificity and selectivity. Plasma samples from six different drug-free persons were tested for the presence of endogenous components, which might interfere with detection of amisulpride or IS. Chromatograms of the blank plasma [Fig. 3(A)] and plasma spiked with internal standard [2 µg/mL; Fig. 3(B)] were compared to show the specificity and selectivity of the proposed procedure.

Limit of detection and Limit of quantitation. The limit of detection (LOD) was estimated as the amount of amisulpride, which caused a signal (S) that was three times the noise (N) (S/N = 3:1). The value of LOD was calculated according to the equation LOD = (3N/S) × amount found. The data used for the calculation of the LOD of proposed method are summarized in Table 1. They were obtained by analyzing six different samples with the nominal concentration of 0.1 ng/mL, and the S/N chromatograms (IS not shown) are presented in Fig. 4. The obtained value of LOD was ~25 pg/mL.

The lower limit of quantitation (LLOQ) was defined as lowest concentration that could be analyzed with acceptable accuracy and precision (20%). LLOQ was 0.1 ng/mL, which was sufficient for the purpose of pharmacokinetic study (Tables 2 and 3). The validation data was not shown for the higher calibration range.

Linearity. The eight-point calibration curve obtained by weighted linear regression ($1/x^2$) showed good linearity over

Table 1. Signal-to-noise ratio used for calculation of LOD

Sample name	Signal/noise, S/N	Amount found (ng/mL)	LOD (ng/mL)
LLOQ-1	11.9	0.10	0.025
LLOQ-2	11.0	0.10	0.027
LLOQ-3	15.7	0.11	0.021
LLOQ-4	13.0	0.09	0.021
LLOQ-5	10.6	0.10	0.028
LLOQ-6	11.6	0.10	0.026

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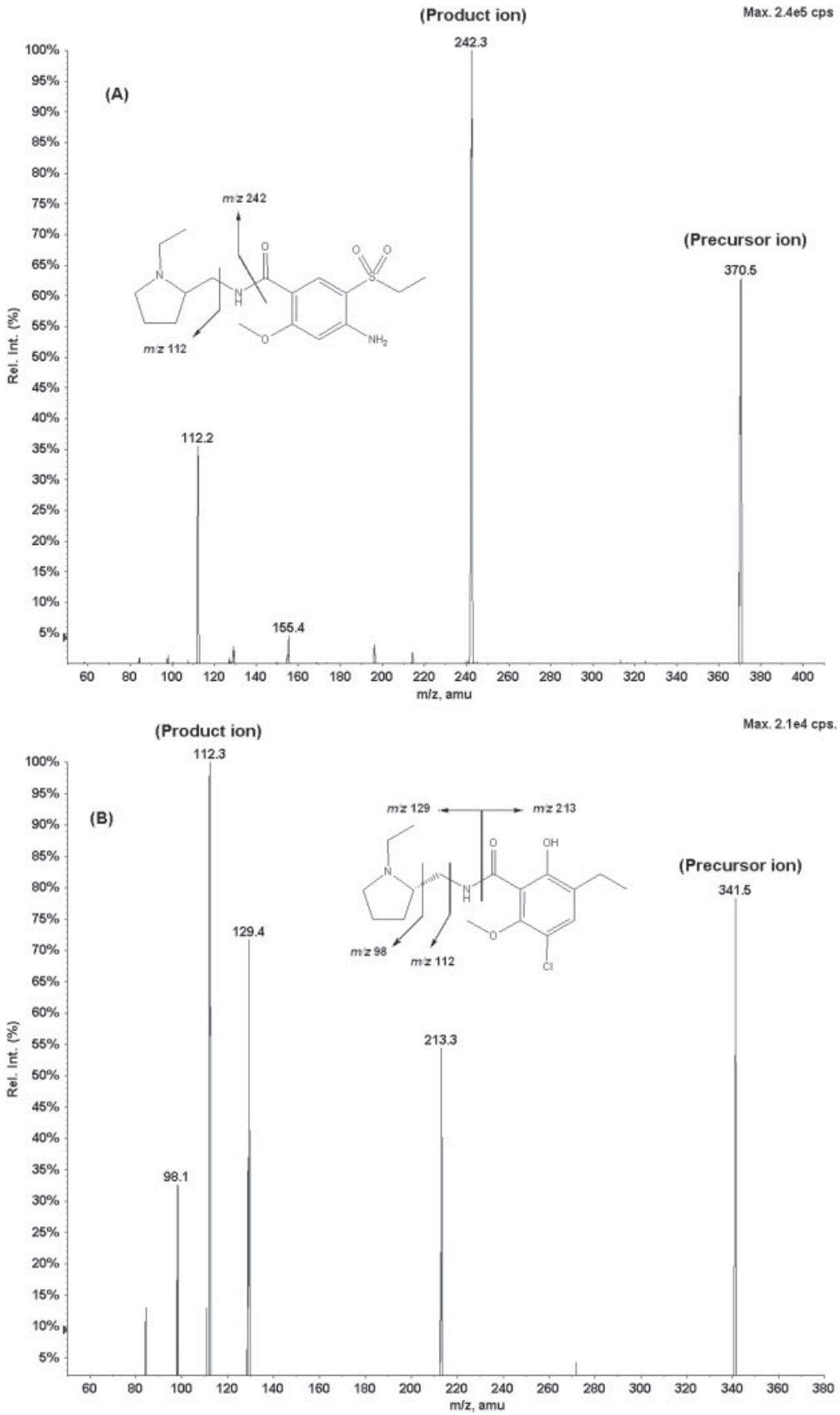

Figure 2. Full-scan positive turboionspray product ion mass spectra and the proposed patterns of fragmentation of (A) amisulpride and (B) eticlopride (internal standard).

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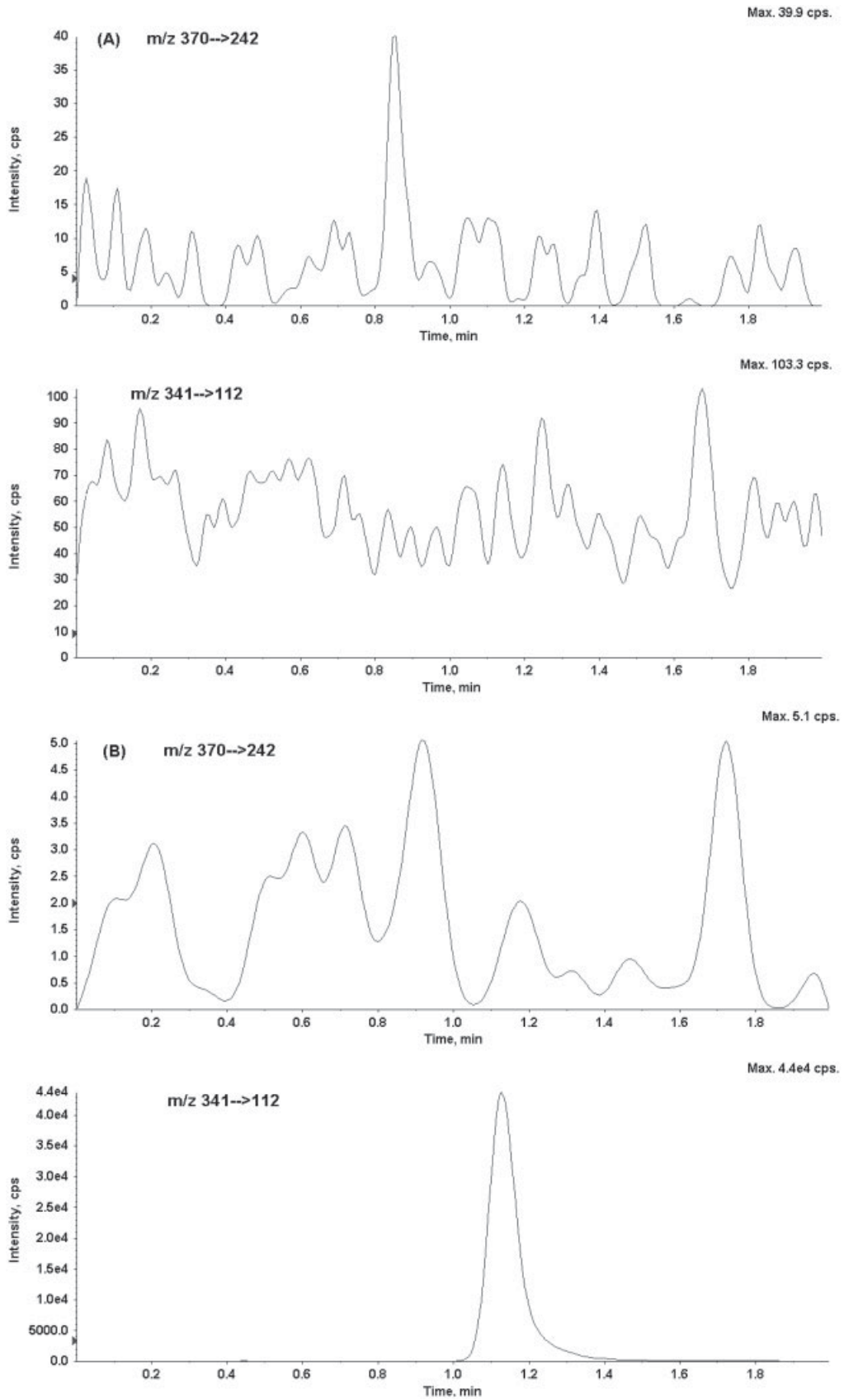

Figure 3. MRM chromatograms for amisulpride and IS resulting from analysis of (A) blank (drug- and IS-free) human plasma; (B) zero sample (drug-free spiked with IS) human plasma.

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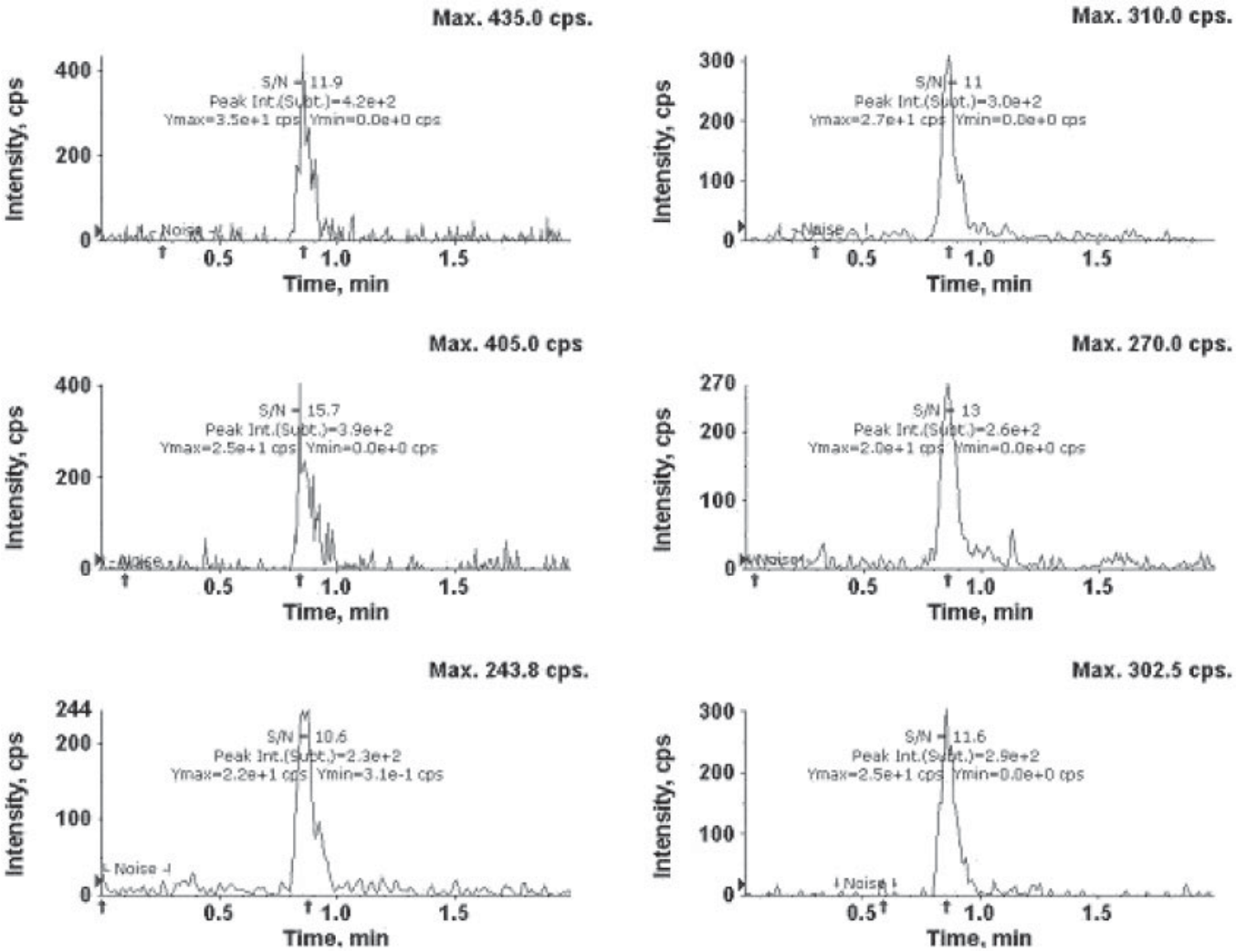

Figure 4. Six chromatograms showing the LLOQ (IS not shown) for the determination of LOD and LOQ.

Table 2. Intra-day precision and accuracy of the method for determining amisulpride concentrations in plasma samples

Amount added (ng/mL)	0.1	0.3	50	80
Amount found (ng/mL)	0.09	0.30	48.1	76.0
	0.10	0.30	50.2	76.1
	0.09	0.31	49.4	78.5
Mean	0.10	0.30	49.3	76.9
SD	0.00	0.01	1.1	1.4
%CV	4.6	3.5	2.2	1.8

Table 3. Inter-day precision and accuracy of the method for determining amisulpride concentrations in plasma samples

Amount added (ng/mL)	0.1	0.3	50	80
Amount found (ng/mL)	0.10	0.30	51.4	76.3
	0.11	0.31	51.6	76.6
	0.09	0.28	50.0	74.0
	0.09	0.28	50.2	74.3
	0.09	0.28	49.7	73.6
Mean	0.10	0.29	50.6	75.0
SD	0.01	0.02	0.9	1.4
%CV	9.2	5.3	1.7	1.8

the lower concentration range 0.1–100 ng/mL [Fig. 5(A)] and over the higher concentration range 1–500 ng/mL [Fig. 5(B)], which covered the concentration typically found in human plasma after administration of amisulpride in the pharmacokinetic study. The best linear fit and least-squares residuals for the calibration curve were achieved with a $1/x^2$ weighing factor, giving a mean linear regression equation for the calibration curve of: $y = 0.0536x - 0.0003$ for lower calibration range and $y = 0.0209x - 0.0018$ for higher calibration range, where y is the peak area ratio of the analyte to the IS and x is the concentration of the analyte. The correlation coefficient was better than 0.997 ($n = 5$) for both calibration ranges. Table 4 gives a summary of linearity responses for lower calibration range.

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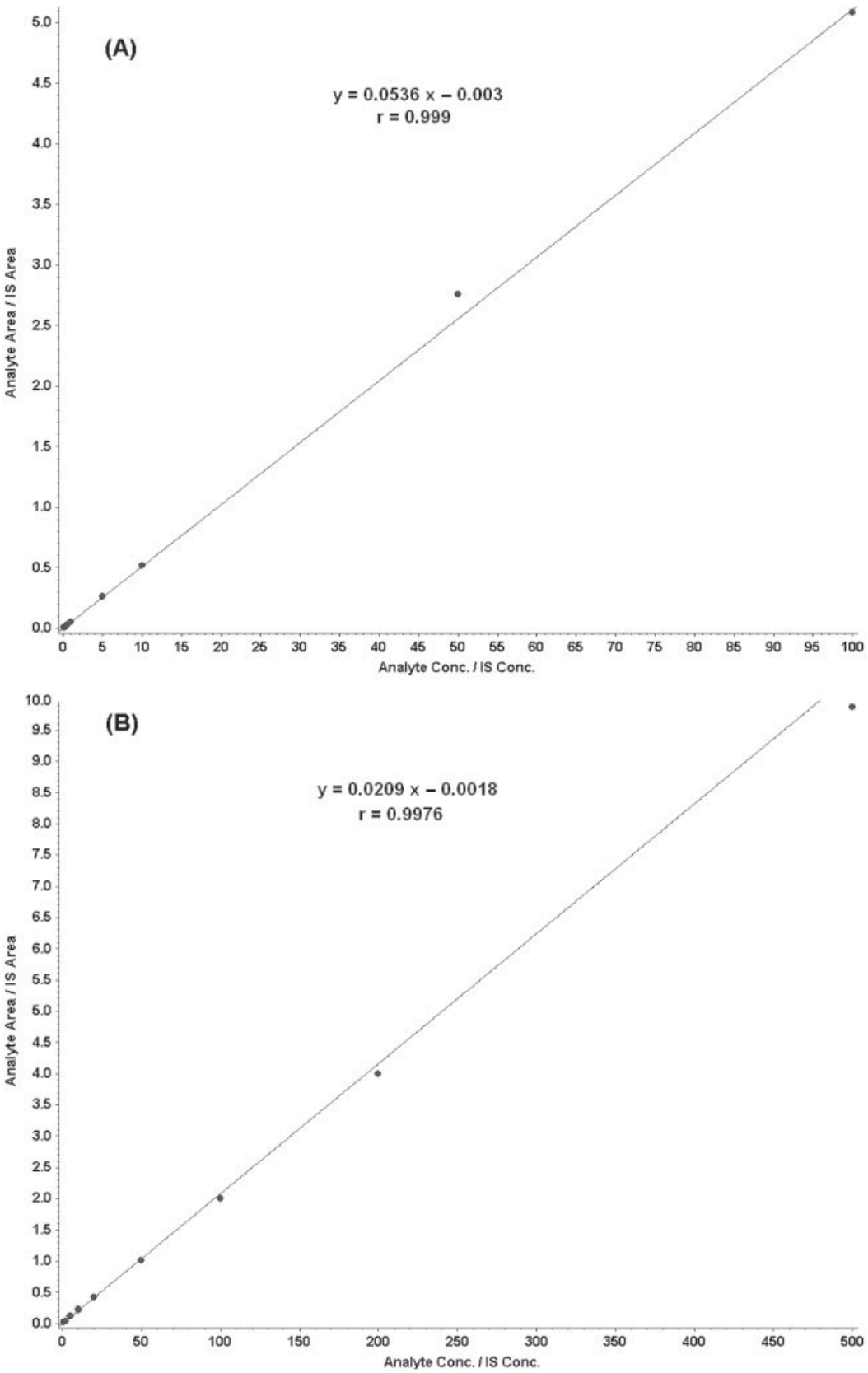

Figure 5. Representative calibration curves ranging from (A) 0.1 to 100 ng/mL and (B) 1 to 500 ng/mL in human plasma.

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Table 4. Linearity summary for lower calibration range from 0.1 to 100 ng/mL

	Equation form: $y = mx + c$		
Batch number	m	c	Correlation coefficient (r)
MV-01	0.0511	−0.0005	0.9972
MV-02	0.0509	−0.0012	0.9978
MV-03	0.0532	−0.0007	0.9993
MV-04	0.0531	0.0001	0.9989
MV-05	0.0536	−0.0003	0.9990
Mean	0.0524	−0.0005	0.9984
SD	0.0013	0.0005	0.0009

Accuracy and precision. Inter-day and intra-day assays were performed to evaluate precision and accuracy. Inter-day precision and accuracy were analyzed using four QC samples (LLOQ, low QC, nid QC and high QC) in five batches for four days consecutively. Intra-day and inter-day assay results for the lower calibration range are summarized in Tables 3 and 4 respectively, and precision and accuracy of the proposed method were acceptable (≤15%). The validation data is not shown for the higher calibration range.

Dilution integrity

Dilution integrity was performed to quantify the amount of amisulpride in human plasma samples if the concentration of amisulpride is above the upper limit of quantification. Six aliquots of 10-fold dilution were processed, analyzed and calculated for percentage nominal concentrations (data was not shown). Percent nominal concentrations and %CV were well within the limits for dilution integrity of 10-folds dilution, extending the range from 500 to 5000 ng/mL.

Stability

Freeze–thaw stability (–50°C) was determined as percentage recovery compared with the nominal value of low QC and high QC in six aliquots. The samples were thawed for analysis and frozen again. The results showed all samples of freeze–thaw cycles were well within the acceptance limits (data not shown) up to three freeze–thaw cycles.

Long term stability at –50°C was performed in three batches over 3 months at 15, 30 and 90 days. The percentage accuracy of low QC and high QC in six aliquots of each was determined and compared with the nominal value. The data showed no loss of amisulpride for the entire 3 months at below –50°C.

Room temperature stability was assessed by analyte determination in six sets of low QC and high QC. Each set was left at room temperature for various time periods (4, 8 and 24 h). The data showed that there was no significant loss of amisulpride at room temperature for 24 h.

In-process stability of amisulpride was evaluated by determination of six sets of low and high QC. After alkalization by sodium hydroxide samples were left for various intervals of time (0, 15, 30, 60 and 120 min). All the samples were within the limits. Hence it was concluded that amisulpride in plasma was stable up to 2 h after alkalization.

In-injector stability was estimated by analysis of six sets low QC and high QC samples. Samples were analyzed at the beginning of the test and after 24 h by storing at 5°C. The difference in results was well within the limits, proving that analyte was stable for 24 h at 5°C.

All the samples were prepared as described in the Sample preparation section. All the results of stability tests showed good stability of amisulpride over all steps of determination; therefore the method proved to be applicable for routine analysis.

Application

The method was applied successfully to determine the plasma concentration of amisulpride following a single 50 mg oral dose administration to healthy subjects. The MRM chromatograms obtained for an extracted plasma sample of a healthy subject who participated in a pharmacokinetic study are depicted in Fig. 6.

CONCLUSIONS

The sensitive LC-MS/MS method was described for the quantification of amisulpride in human plasma covering the concentration range of 0.1–100 and 1–500 ng/mL using 100 µL of plasma. No interferences from endogenous plasma components or other sources were found in plasma samples. A simple extraction procedure and short retention time could allow determination of more than 275 samples per day. The analytical method presented here has proved useful for the clinical pharmacokinetic investigation of amisulpride.

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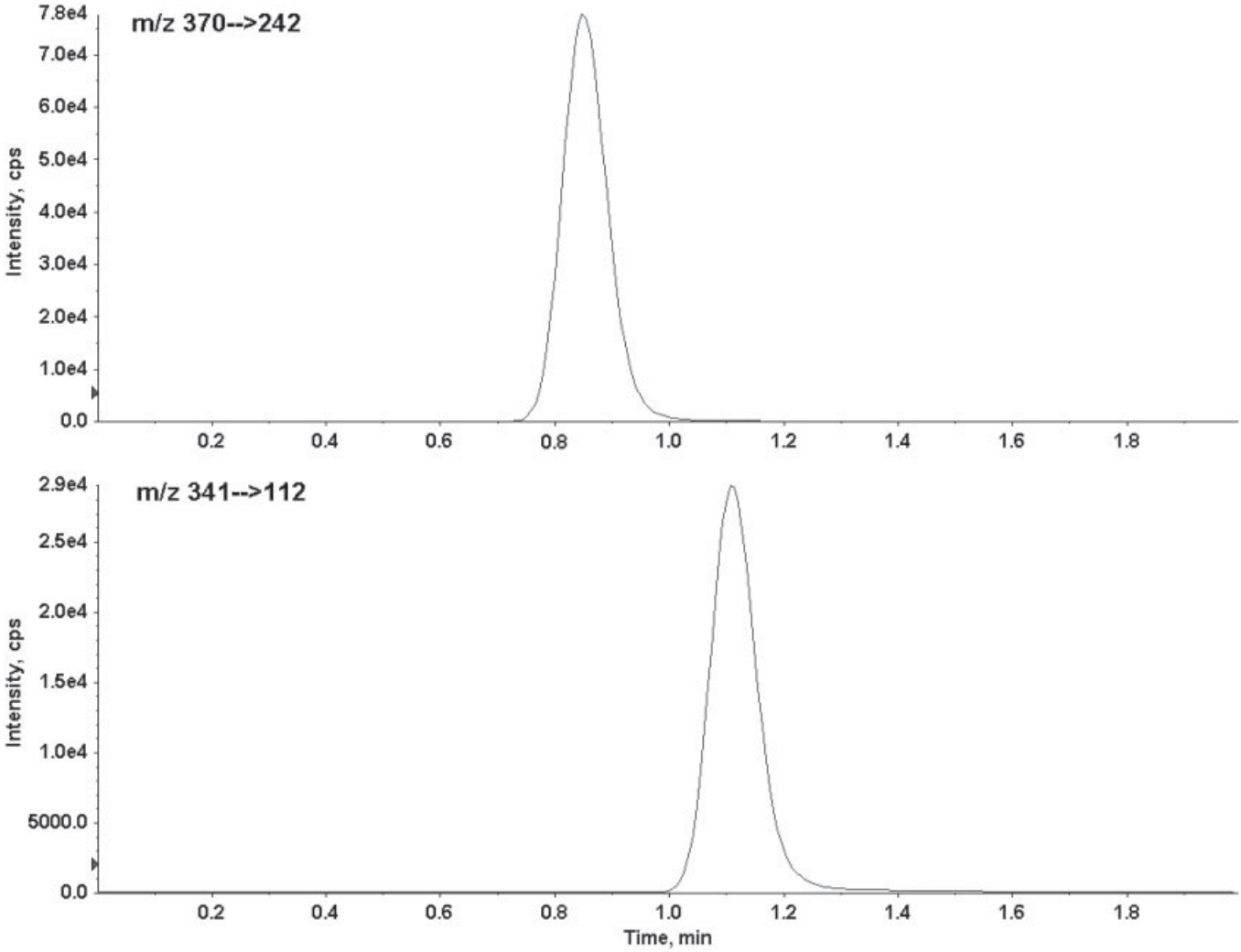

Figure 6. MRM chromatograms resulting from analysis of a subject plasma sample after the administration of a 50 mg oral single dose of amisulpride.

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