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DETERMINATION OF ANTIPSYCHOTIC MEDICATIONS IN HUMAN PLASMA BY UPLC: PHARMACOKINETIC STUDY

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ABSTRACT

A simple and sensitive ultra-performance liquid chromatography (UPLC) method has been developed and validated for simultaneous estimation of olanzapine (OLZ), risperidone (RIS) and 9-hydroxyrisperidone (9-OHRIS) in human plasma in vitro. The sample preparation was performed by simple liquid–liquid extraction technique. The analytes were chromatographed on a Waters Acquity H class UPLC system using isocratic mobile phase conditions at a flow rate of 0.3 mL/min and Acquity UPLC BEH shield RP18 column maintained at 40°C. Quantification was performed on a photodiode array detector set at 277 nm and clozapine was used as internal standard (IS). OLZ, RIS, 9-OHRIS and IS retention times were found to be 0.9, 1.4, .1.8 and 3.1 min, respectively, and the total run time was 4 min. The method was validated for selectivity, specificity, recovery, linearity, accuracy, precision and sample stability. The calibration curve was linear over the concentration range 1–100 ng/mL for OLZ, RIS and 9-OHRIS. Intra- and inter-day precisions for OLZ, RIS and 9-OHRIS were found to be good with the coefficient of variation <6.96%, and the accuracy ranging from 97.55 to 105.41%, in human plasma. The validated UPLC method was successfully applied to the pharmacokinetic study of RIS and 9-OHRIS in human plasma.

Keywords: UPLC; risperidone; 9-hydroxyrisperidone; olanzapine; pharmacokinetics

INTRODUCTION

Schizophrenia is a common chronic neuropsychiatric illness affecting patients worldwide. This condition requires the initiation of treatment with antipsychotic medications, of which second- generation drugs [risperidone (RIS) and olanzapine (OLZ)] are the most commonly prescribed in patients. Second-generation antipsychotic drugs exhibit beneficial effects on anxiety, depression and mania, and exert reduced extra-pyramidal side effects (Leucht *et al.*, 2009; Ansari and Mulla, 2014; Lieberman *et al.*, 2005). Both risperidone and olanzapine are widely prescribed second generation antipsychotic drugs, and olanzapine is often co-administered with risperidone [1]. 9-Hydroxyrisperidone (9-OHRIS) is the major metabolite of RIS, formed in-vivo through hepatic oxidation, and itself is biologically active. Correlation of its plasma levels, parent drug levels and clinical effects is complicated (Sheehan *et al.*, 2010). Of the numerous metabolic enzymes, the cytochrome P450 family account for the metabolism of the majority of psychotropic medications. Cytochrome P450 has a variable expression pattern and these microsomal enzymes are subjected to genetic, pharmacogenetic and epigenetic

variations, which leads to pharmacokinetic variations and results in unexpected alterations and clinical response [2]. Quantification of antipsychotic medications in human biological matrices requires high precision and greater sensitivity since most of these compounds are present in low concentrations in plasma.

Moreover, as most of the antipsychotic medications are prescribed along with other drugs in schizophrenia patients, drug interactions can be expected.

In the past, various high-performance liquid chromatography (HPLC) methods with UV detection [3], coulometric [4] and fluorescence detection [4] have been reported to detect and quantify antipsychotic medications. Recently, several studies have utilized methods based on liquid chromatography coupled with mass spectrometry (LC-MS/MS) to increase sensitivity in detection with shorter run times [5]. Although LC-MS/MS methods are advantageous in many ways, the expensive instrumentation and de-creased accessibility to routine hospital practice and laboratories are their major limitations.

Considering these facts, an ultrahigh

performance liquid chromatography (UPLC) with diode array detection (DAD) for OLZ, RIS and 9-OHRIS was developed and validated. Further, the method was designed using a simple mobile phase in isocratic conditions, devoid of strong buffers and ion pairing agents to achieve better reproducibility with less interference. The method has a shorter run time, and can even be transferred to LC-MS/MS if needed as compatible buffer and chromatographic conditions were used. The proposed method was successfully applied to a pharmacokinetic study of risperidone in human volunteers. The developed method can also be utilized for therapeutic drug monitoring of OLZ, RIS and 9-OHRIS.

MATERIALS AND METHODS

Chemicals and reagents

Acetonitrile, methanol and ammonium acetate of HPLC grade were purchased from Sigma-Aldrich, India. The reference standards of RIS, 9-OHRIS, OLZ and clozapine (IS) were purchased from Sigma-Aldrich, India. Methyl tert-butyl ether and acetic acid of HPLC grade were obtained from HI media, India. Ammonia solution (25% pure) was purchased from Merck, India. Blank human plasma was obtained from blood bank services XYZ

Hospitals, Hyderabad. Ultrapure water (18.2 MΩ cm) was obtained from a MilliQ water purification system from Millipore (Milford, USA).

Instrumentation and chromatography conditions

The UPLC instrument consisted of a Waters Acquity H class UPLC system equipped with a quaternary pump and 96-vial autosampler coupled with a diode array UV detector (Waters, Milford, MA, USA). The chromatographic separation was performed on an Acquity UPLC BEH C18 column from Waters (2.1 × 100 mm; 1.7 μm). The column temperature was set at 40°C and the autosampler was kept at 10°C. The mobile phase composed of a mixture of 10 mM ammonium acetate buffer at a pH of 3.5, which was adjusted with acetic acid (70%, v/v) and acetonitrile (30%, v/v) at a flow rate of 0.3 mL/min. Before analysis, the mobile phase was filtered through a 0.22 μm membrane filter and degassed by ultra-sonication. A 5 μL injection of each sample was loaded on to the system and total analysis time was 4 min. DAD was set at 277 nm. The sampling needle was washed with 400 μL of strong wash (ACN–water, 65:35) to reduce carry-over and 400 μL of weak wash (ACN–water, 30/70). Data

acquisition was done using Empower 3 software version 1.0 (Waters).

Preparation of stock and working standard solution

OLZ, RIS and 9-OHRIS stock solutions of 1 mg/mL were prepared by dissolving suitable amounts of the single drugs in methanol. Mixtures of stock solutions OLZ, RIS and 9-OHRIS (100 μg/mL) were prepared in methanol. Clozapine was selected as IS based on a previous study (Idris and Elgorashe, 2013). Further, clozapine has been used widely only in treatment-resistant schizophrenia (Meyer, 2011). Therefore we selected clozapine as IS. The IS stock solution of 200 μg/mL was prepared in methanol. The stock solution of OLZ, RIS, 9-OHRIS and IS were stable at 4°C for 1 month. Further dilution was made in methanol–water (50:50, v/v) to produce working stock solutions for the calibration standards and quality control (QC) standards. The IS working solution (5 μg/mL) was prepared in methanol–water (50:50 v/v). Calibration curve samples were prepared by spiking 240 μL of human blank plasma with the appropriate mixture of working solutions of OLZ, RIS and 9-OHRIS (10 μL) on the day of analysis. All the samples were stored together at 80 ± 10°C until analysis.

Sample preparation

Sample preparation was carried out by the liquid–liquid extraction procedure. To a 250 μL of aliquot of plasma, 25 μL of IS working solution and 250 μL of 10mM ammonium acetate (pH 9.0) were added and mixed for 20 s on a spinix vortex shaker (Tarsons, India). The mixture was vortexed for 30 s. A 3.0 mL aliquot of tert-butylmethylether was added and vortex mixed for 5 min, and then centrifuged at 10,000 rpm for 5 min at 4°C on an Eppendorf 5810R centrifuge (Eppendorf AG, Hamburg, Germany). The clear supernatant organic layer (2.5 mL) was transferred into 5 mL polypropylene tubes and evaporated to dryness at 40°C using a nitrogen evaporator (Turebovap®, Biotage, USA). The residue was reconstituted in 200 μL of the mobile phase, vortex mixed for 1.0 min and centrifuged at 10,000rpm for 5 min. Finally, 190 μL of the clear supernatant was transferred into a glass micro-vial and 5 μL was injected onto the UPLC system for analysis.

Method validation

The method was validated for selectivity, specificity, recovery, linearity, accuracy, precision and stability using US Food and Drug Ad-ministration guidelines (US DHHS *et al.*, 2001) for assay in human

plasma.

Carry-over and limit of quantification

Carry-over effect was assessed by injecting the highest calibration standard just before the blank plasma. The lower limit of quantitation (LLOQ) was defined as the first point of the calibration standard with lowest concentration (signal-to-noise ratio $\geq 10:1$) that can be calculated with an acceptable precision and accuracy.

Selectivity and specificity

Selectivity of the method was assessed by analysing six different donor's human blank plasma injected at the beginning of the validation and investigating the potential interferences at the LC peak region for analytes and IS. At least five out of six lots should have responses less than five times the LLOQ level response in the same matrix.

Recovery

The recoveries of OLZ, RIS and 9-OHRIS were assessed by the analyses of the three concentrations (5, 45 and 80 ng/mL) whereas the recovery of IS was determined at a single concentration of 5 μg/mL. Recovery was calculated as the extracted spiked plasma peak area response compared with the response obtained for neat standard solution at equivalent concentrations by the liquid–liquid extraction process. The analyte responses from the extracted samples at known concentrations were compared with responses of un-extracted standards.

Linearity

Linearity was evaluated by linear regression analysis with the use of working standard solutions and spiked plasma samples containing the drugs of interest at different concentrations within the range 1–100 ng/mL for all analytes. The calibration curves were constructed by plotting the ratio of the peak area of each analyte to the peak area of internal standard vs the nominal drug concentration. The slopes and intercept were calculated with least square linear regression analysis of the data with the use of a $1/x^2$ (where x is the concentration) weighting factor. The acceptance criterion for each back-calculated standard concentration was ±15% deviation from the nominal value except at LLOQ, which was set as ±20% (US DHHS *et al.*, 2001).

Accuracy and precision

The validation method evaluation of the accuracy and precision in the experiments were performed for four independent series (including ruggedness) in plasma. The intra- and inter-day assay accuracy and precision were estimated by analyzing six

replicates containing analytes at three different QC levels, that is, 1, 5, 45 and 80 ng/mL. Accuracy represents the closeness of agreement between the mean values obtained from the series of measurements by the method and the actual value. The accuracy should be within 85–115% of the nominal value, except at LLOQ, where it should not deviate by no more than 20% (US DHHS *et al*., 2001). Precision, which represents the closeness of agreement among a series of measurements obtained from multiple sampling, was estimated with variances of repeatability (intra-day variances) and intermediate precision (sum of intra-day and inter-day variances). The precision should not exceed ±15% relative standard deviation (RSD), except for the LLOQ, where it should be within ±20% of RSD (US DHHS *et al*., 2001).

Stability

Stability tests were performed to evaluate the stability of analytes in plasma samples under different conditions. The bench-top stability study assessed the stability of analytes in plasma at room temperature for 8 h, whereas a post-preparative stability study assessed the stability of analytes in the treated samples (autosampler stability for 26 h at 10°C). These were determined at low (5 ng/mL) and high (80 ng/mL) QC concentrations. The freeze–thaw stability was determined after three freeze and thaw cycles and long-term stability was determined by accessing QC samples stored at 80°C for 30 days.

Pharmacokinetic study

Twenty healthy male volunteers aged between 24 and 31 years (26.8 ± 2.4 years) and weighing from 62 to 82 kg (70.7 ± 6.8 kg) were enrolled in the study and informed consent was obtained. The study was approved by the Institutional Human Ethics Committee prior to commencing and was performed in accordance with the principles of the World Medical Association's Declaration of Helsinki. The volunteers underwent screening examinations that included a medical history and physical examination. Volunteers received a single 2 mg risperidone tablet (Respidon-2, Torrent Pharmaceuticals Ltd, India) along with 200 mL water. Serial venous blood samples (2 mL) for the determination of plasma RIS and 9-OHRIS concentrations were obtained from forearm vein before dosing and at 0.5, 1.0, 2.0, 4.0, 8.0, 12.0 and 24 h after dosing. Samples were collected in K2.EDTA tube and plasma was separated by centrifugation at 4000 rpm and stored at 80°C until analysis. WinNonlin software

5.1 was used to calculate the pharmacokinetics parameters using a noncompartmental model (Pharsight Corporation, Mountain View, CA, USA). Pharmacokinetic parameters including Cmax (maximum plasma concentration), Tmax (the time to reach Cmax), the area under the plasma concentration–time curve from time zero to last measurable time point (AUC0–t), the area under the plasma concentration–time curve from zero to infinity (AUC0–∞), Kel (first-order rate constant associated with the terminal (log–linear) portion of the curve) and T1/2 (the terminal half-life) were calculated.

RESULTS AND DISCUSSION

Optimization of the chromatographic conditions

A reverse-phase liquid chromatographic method was developed for the determination of OLZ, RIS and 9-OHRIS in human plasma samples using diode array detection. LC method development was carried out focusing on achieving sufficient resolution of target drugs and endogenous interferences in matrix within a short run time, with simple buffers. For this purpose, different mobile phases comprising several combinations of buffers (e.g. phosphate buffer and ammonium acetate buffer) and organic solvents

(acetonitrile and methanol) along with altered flow rates (range of 0.200–0.500 mL/min) were tested to optimize for an effective chromatographic resolution of OLZ, RIS, 9-OHRIS and IS. Using methanol in mobile phase produced broad peaks without symmetry and a long analysis time. When acetonitrile used as organic solvent in the mobile phase, the analysis time was reduced and peaks were more symmetrical and sharp. The best resolution of peaks achieved with isocratic mobile phase consisted of a mixture of acetonitrile–10 mM ammonium acetate (30:70; v/v) containing acetic acid with the pH adjusted to 3.5 at a flow rate of 0.3 mL/min on an Acquity UPLC BEH C18 column. The utilization of a volatile buffer like ammonium acetate in mobile phase gives better column life and can be compatible with mass spectrometry, unlike phosphate buffers.

Carry-over and limit of quantification

No carry-over was observed when the highest calibration standard was analyzed just before the blank plasma. This indicated that a flow through needle configuration of the injector and a washing step of the column at the end of the chromatography separation were adequate to remove potential residues of the

analytes. The LLOQ on the calibration curve was 1.0 ng/mL for all of the compounds and the signal-to-noise ratio in plasma was ≥10.

Selectivity and specificity

Selectivity was evaluated by comparing the chromatograms of blank plasma, blank plasma spiked with IS and with analytes (1 ng/mL) and IS, and a kinetic study sample obtained 2 h after oral administration (2 mg risperidone tablet). As shown in **Figure 1**, no interfering peaks from endogenous compounds were observed at the retention times of analytes and IS. The total chromato-graphic run time was 4 min. **Figure 1**. Representative UPLC chromatograms of olanzapine (OLZ), risperidone (RIS), 9- hydroxyrisperidone (9-OHRIS) and IS in human plasma.(a) Blank plasma sample; (b) blank plasma sample spiked with IS; (c) blank plasma spiked with analytes (1ng/mL) and IS; and (d) pharmacokinetic plasma samples collected from healthy volunteers at 2.0 h time point following oral administration of RIS along with IS.

Recovery

Liquid–liquid extraction technique gave adequate recovery and cleaner samples. The results of the evaluation of neat standards vs plasma extracted standards were estimated for OLZ, RIS and 9-OHRIS (5, 45 and 80 ng/mL) and the mean recoveries were 89.42 ± 2.89, 90.16 ± 2.06 and 90.13 ± 1.82%, respectively. The recovery of IS at 5 μg/mL was 84.25 ± 3.23%.

Linearity

The calibration curves exhibited excellent linearity with regression correlation coefficient ($r2 > 0.998$) over the concentration range of 1.0–100ng/mL for all of the drugs in human plasma. The standard calibration curve had a consistent reproducibility over the standard concentrations across the calibration range. A typical regression equation was prepared by determining the best fit of peakarea ratio (peak area analyte/peak area IS) vs concentration, and fitted to $y = mx + c$ using a weighting factor ($1/x2$). The lowest concentration with RSD < 20% was taken as the LLOQ and was found to be 1.0 ng/mL for all the drugs. The percentage accuracies observed for the mean of back-calculated concentration for four calibration curves for the entire drugs were within 96.10–102.50.

Table 1. Intra- and inter-day precision and accuracy of olanzapine (OLZ), risperidone (RIS) and 9-hydroxyrisperidone (9-OHRIS) in human plasma *in vitro*

| Nominal concentration (ng/mL)	Intra-day ($n = 6$)			Inter-day ($n = 18$)		
	Measured concentration (mean ± SD, ng/mL)	CV (%)	Accuracy (%)	Measured concentration (mean ± SD, ng/mL)	CV (%)	Accuracy (%)
RIS						
1.12	1.13 ± 0.06	5.31	100.89	1.15 ± 0.08	6.96	102.68
5.12	5.18 ± 0.13	2.51	101.17	5.28 ± 0.36	6.82	103.13
45.82	46.88 ± 0.72	1.54	102.31	48.30 ± 2.20	4.55	105.41
80.78	80.61 ± 1.62	2.01	99.79	80.12 ± 3.29	4.11	99.18
9-OHRIS						
1.09	1.07 ± 0.04	3.74	98.17	1.14 ± 0.07	6.14	104.59
5.10	5.06 ± 0.13	2.57	99.22	5.18 ± 0.31	5.98	101.57
45.21	46.72 ± 0.80	1.71	103.34	45.68 ± 2.34	5.12	101.04
81.48	80.49 ± 0.74	0.92	98.78	84.15 ± 3.44	4.09	103.28
OLZ						
1.21	1.20 ± 0.02	1.67	99.17	1.26 ± 0.07	5.56	104.13
4.98	5.15 ± 0.16	3.11	103.41	4.94 ± 0.22	4.45	99.20
44.74	46.02 ± 1.04	2.26	102.86	45.35 ± 1.94	4.28	101.36
81.32	79.33 ± 1.65	2.08	97.55	81.27 ± 2.92	3.59	99.94
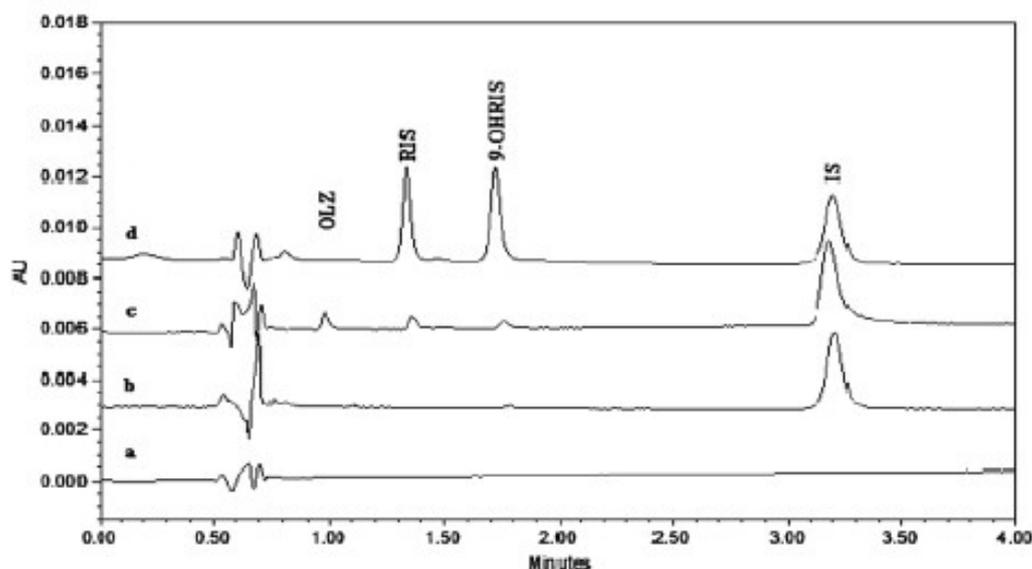
Figure 1. Representative UPLC chromatograms of olanzapine (OLZ), risperidone (RIS), 9-hydroxyrisperidone (9-OHRIS) and IS in human plasma. (a) Blank plasma sample; (b) blank plasma sample spiked with IS; (c) blank plasma spiked with analytes (1ng/mL) and IS; and (d) pharmacokinetic plasma samples collected from healthy volunteers at 2.0 h time point following oral administration of RIS along with IS.

Table 2. Stability of RIS, 9-OHRIS and OLZ in human plasma *in vitro* under different conditions ($n = 6$)

Nominal concentration (ng/mL)	Bench-top (room temperature for 8 h)		Autosampler (8°C for 26 h)		Freeze–thaw cycles		Long-term stability (−80°C for 30 days)	
	Measured concentration (mean ± SD, ng/mL)	Bias (%)	Measured concentration (mean ± SD, ng/mL)	Bias (%)	Measured concentration (mean ± SD, ng/mL)	Bias (%)	Measured concentration (mean ± SD, ng/mL)	Bias (%)
RIS								
5.12	4.97 ± 0.10	−2.93	5.19 ± 0.05	1.37	4.91 ± 0.08	−4.10	5.25 ± 0.08	2.54
80.78	79.83 ± 1.03	−1.18	81.97 ± 1.32	1.47	79.91 ± 0.95	−1.08	82.57 ± 1.07	2.22
9-OHRIS								
5.10	5.25 ± 0.14	2.94	5.04 ± 0.21	−1.18	5.23 ± 0.30	2.55	4.99 ± 0.23	−2.16
81.48	82.12 ± 2.51	0.79	79.73 ± 2.15	−2.15	80.04 ± 0.72	−1.77	82.41 ± 2.18	1.14
OLZ								
4.98	4.93 ± 0.17	−1.00	5.07 ± 0.19	1.81	5.03 ± 0.16	1.00	5.02 ± 0.26	0.80
81.32	79.87 ± 1.95	−1.78	79.04 ± 2.25	−2.80	80.04 ± 0.72	−1.57	78.91 ± 1.95	−2.96

Accuracy and precision

The accuracy and precision of the method were evaluated as intra-day (repeatability) and inter-day (intermediate) human plasma for entire drug samples and are summarized in **Table 1**. The intra- and inter-day precisions were <6.96% and the accuracy ranged from 97.55 to 105.41 at all levels. All the values were within the accepted range and the method was accurate and precise.

Stability

The stability of OLZ, RIS, and 9-OHRIS in human plasma were investigated under a variety of storage and process conditions: in plasma at room temperature for 8 h; in the autosampler for 26 h at 10°C; after three freeze–thaw cycles; and after long-term storage at 80°C for 30 days. Results are summarized in **Table 2** and indicated that the samples of OLZ, RIS and 9-OHRIS were stable under the conditions described.

Applicability of the validated method

We successfully validated the novel method of quantification of analytes, viz. RIS and 9-OHRIS, after a single oral dosing of 2 mg RIS (Respidon-2, Torrent Pharmaceuticals Ltd, India)

tablets in healthy volunteers. The maximum concentration of RIS in our study was almost twice that of its major metabolite measured in our assay method, viz. 9-OHRIS, both compounds being quantified up to 24 h after a single oral dosing of RIS (16.48 ± 5.07 vs 10.33 ± 1.90 ng/mL). On similar lines of reference, the time taken to attain this maximum concentration in plasma for RIS was nearly one-quarter that for 9-OHRIS (0.80 ± 0.25 vs 4.0 ± 1.12 h). The AUCs of RIS from 0 to 12 h and 0 to ∞ h were 92.64 ± 27.79 and103.93 ± 32.38 (ng h/mL), which were less than the corresponding values for its metabolite (141. 80 ± 32.41 and 191.80 ± 49.81 ng h/mL, respectively). Moreover, the corresponding plasma half-lives for the parent and its major metabolite were 6.17 ± 1.76 and 11.54 ± 3.11 h, respectively. Finally, the first-order elimination rate constants associated with the terminal part of plasma concentration–time curves were 0.12 ± 0.04 and 0.06 ± 0.02/h, respectively. Additionally, we calculated the apparent volumes of distribution and clearance of the parent to be 2.49 ± 0.53 L/kg and 4.95 ± 1.36 mL/min kg, respectively. The corresponding values

for the active metabolite of RIS in the participants were 2.56 ± 0.60 L/kg and 2.67 ± 0.79 mL/min kg. The values reported for these pharmacokinetic parameters were comparable to previously reported ones (Liu *et al.*, 2013). The profile of mean plasma concentration of RIS and 9-OHRIS vs time is shown in **Figure 2**.
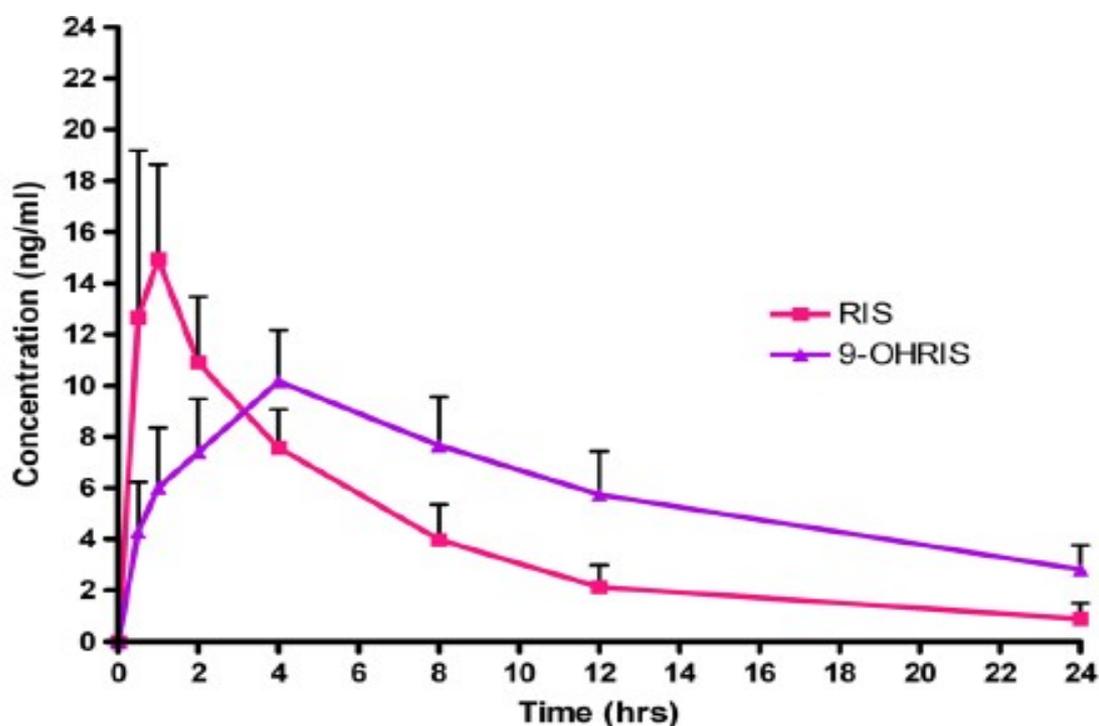

Figure 2. Mean plasma concentration–time profile of RIS and 9-OHRIS in human plasma after oral administration of 2 mg of risperidone tablet.

CONCLUSION

A simple, sensitive and reliable UPLC method has been developed and validated for simultaneous determination of OLZ, RIS and 9-OHRIS in human plasma in vitro. This UPLC method has significant advantages including good resolution between peaks and adequate extraction recovery with shorter chromatographic run time. The developed method was successfully adopted to evaluate the pharmacokinetic parameters of a second-generation antipsychotic drug, RIS, and its major metabolite, 9-OHRIS, in human plasma. It can be employed in therapeutic drug monitoring practices and clinical toxicological assays. This method has similar sensitivity to other methods developed using LC-MS/MS.

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