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Simultaneous determination of five antipsychotic drugs in rat plasma by high performance liquid chromatography with ultraviolet detection

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Abstract

A significant percentage of psychiatric patients who are treated with antipsychotics are treated with more than one antipsychotic drug in the clinic. Thus, it is advantageous to use a rapid and reliable assay that is suitable for determination of multiple antipsychotic drugs in plasma in a single run. A simple and sensitive HPLC-UV method was developed and validated for simultaneous quantification of olanzapine, haloperidol, chlorpromazine, ziprasidone, risperidone and its active metabolite 9-hydroxyrisperidone in rat plasma using imipramine as an internal standard (I.S.). The analytes were extracted from rat plasma using a single step liquid–liquid acid solution back extraction technique with wash procedure, which provided the very clear baseline for blank plasma extraction. The compounds were separated on an Agilent Eclipse XDB C8 (150 mm × 4.6 mm i.d., 5 μm) column using a mobile phase of acetonitrile/30 mM ammonium acetate including 0.05% triethylamine (pH 5.86 adjusted with acetic acid) with gradient elution. All of the analytes were monitored using UV detection. The method was validated and the linearity, lower limit of quantitation (LLOQ), precision, accuracy, recoveries, selectivity and stability were determined. The LLOQ was 2.0 ng/ml and correlation coefficient (R^2) values for the linear range of 2.0–500.0 ng/ml were 0.998 or greater for all the analytes. The precision and accuracy for intra-day and inter-day were better than 7.44%. The recovery was above 74.8% for all of the analytes. This validated method has been successfully used to quantify the plasma concentration of the analytes for pharmacological and toxicological studies following chronic treatment with antipsychotic drugs in the rat.
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Keywords: Risperidone; 9-Hydroxyrisperidone; Olanzapine; Haloperidol; Chlorpromazine; Ziprasidone

1. Introduction

Antipsychotic agents are used in psychiatric patients for the management of psychotic episodes as well as for other behavioral symptoms such as agitation. Second generation antipsychotics (SGAs) (such as olanzapine, ziprasidone and risperidone) and first generation antipsychotics (FGAs) (such as haloperidol and chlorpromazine (CPZ)) (Fig. 1) are popular for the treatment of schizophrenia and other psychoses in the clinic [1]. It is reported that the SGAs olanzapine, risperidone, clozapine and ziprasidone are effective in the treatment of both positive and negative symptoms of schizophrenia and that they are less likely to produce extrapyramidal side effects when compared with FGAs such as haloperidol and chlorpromazine [2,3]. However, suicide and several intoxications of antipsychotics have been published [4–6]. It has long been known that chronic exposure to FGAs such as haloperidol often result in cholinergic imbalances in the striatum and consequently abnormalities in motor function. Furthermore, given that cognition is now recognized as a key factor that influences the long term functional outcome in schizophrenia [7–9], it is important to determine if there is a correlation between antipsychotic plasma levels (particularly in association with chronic drug exposure) and cognitive function in the rat model. Such a correlation would allow for improved clinical monitoring of these compounds. Since the antipsychotic drugs are very active, they are usually administered at low daily dosages. Therefore, the concentration of these drugs in plasma is very low. For example, the therapeutic plasma levels of olanzapine are in the range of 8–80 ng/ml [10–12]. In the case of haloperidol, levels from 5.0 to 15.0 ng/ml have

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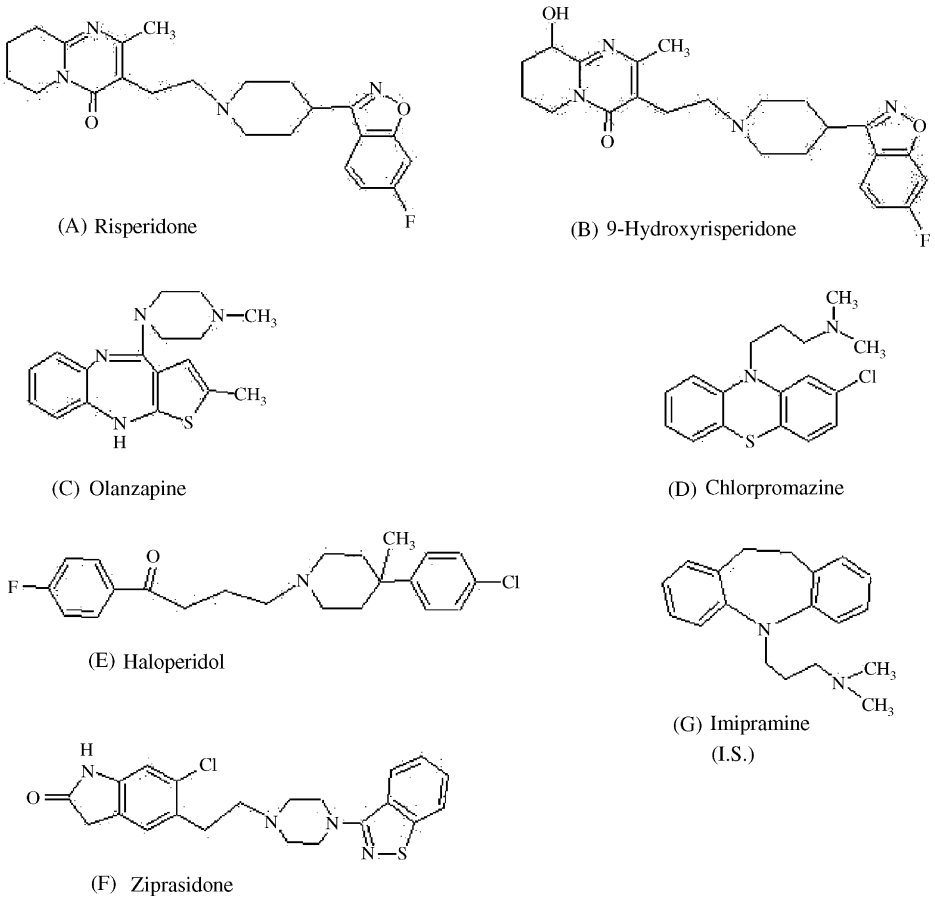

Fig. 1. Chemical structures of RISP, 9-OH RISP, OLZ, CPZ, HAL, ZIP and I.S. (imipramine).

also been described as the therapeutic range [13]. Plasma levels of psychoactive drugs resulting under a given dose are highly variable between individual patients. This is primarily due to inter-individual variations in compliance and in the activities of drug metabolizing enzymes. This leads to poor predictability of drug concentrations at a given dose. Approximately 30 antipsychotic drugs are currently available in the clinic. A significant percentage of psychiatric patients who are treated with antipsychotics are treated with more than one antipsychotic drug in the clinic. Also, it is advantageous to have a method that enables determination of more than a single antipsychotic drug not only because of polypharmacy but also because of the use of so many different drugs in different patients. Laboratories therefore have to establish and validate many methods. So, for a broad and complete determination it is advantageous for the laboratory to have methods as described here that may be used for more than a single compound. At present, determination of some of these drugs has been established by high performance liquid chromatography (HPLC) with UV detection [2,14–21], HPLC with coulometric detection [22,23] or fluorescence detection [24,25]. Although electrochemical detection [26–30] offers enhanced sensitivity in the low ng/ml range, electrochemical detectors require optimal working conditions, sample preparation is critical and interference from co-medications is often unavoidable. Capillary electrophoresis (CE) methods [31,32] were reported to detect the antipsychotic drugs, but are not sensitive and robust for biological samples. Recently, several LC–MS/MS methods were reported for the quantification of the antipsychotic drugs in biological fluids [33–39]. In toxicity research and routine clinical monitoring, however, HPLC-UV may be advantageous because of lower cost and greater robustness. Few HPLC-UV methods [2,15,20,21] offer the ability to measure multiple antipsychotics (i.e. both FGAs and SGAs) simultaneously in biological samples in a single run. The lower limit of quantitation (LLOQ) for most of these methods are above 10 ng/ml. Here we describe the validation and application of a HPLC-UV method for simultaneous determination of risperidone, 9-hydroxyrisperidone, olanzapine, haloperidol, chlorpromazine and ziprasidone in rat plasma in a single run. The LLOQ of all the analytes was as low as 2 ng/ml.

2. Experimental

2.1. Chemicals and reagents

Haloperidol (HAL), risperidone (RISP) and olanzapine (OLZ) were kindly provided by Eli Lilly (Indianapolis, IN, USA). 9-Hydroxyrisperidone (9-OH RISP) was donated by the Janssen Research Foundation (Beers, Belgium). Ziprasidone was obtained from Pfizer Central Research (Groton, CT, USA).

Chlorpromazine and imipramine (internal standard, I.S.) were from Sigma (St. Louis, MO, USA). Ethyl acetate, methyl *tert*-butyl ether, chloroform, hexane, diethyl ether, isopropyl ether, isoamyl alcohol, HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Pittsburgh, PA, USA). Acetic acid used was reagent grade purchased from J.T. Baker (Phillipsburg, NJ, USA). Ammonium acetate and sodium phosphate dibasic were purchased from Sigma (St. Louis, MO, USA). The deionized water used was generated from a Continental deionized water system (Natick, MA, USA).

2.2. Instruments and chromatographic conditions

An Agilent 1100 series HPLC system, consisting of a degasser, quaternary pump, autosampler, and a variable wavelength UV detector with a thermostatted column compartment (TC-50 controller, Wisconsin, USA) was used in this study (Agilent, Palo Alto, CA, USA). The analytes were separated on an Agilent Eclipse XDB C-8 column (150 mm × 4.6 mm i.d., 5 μm) with a 4.0 mm × 2.0 mm Phenomenex Security Guard C8 guard column. Mobile phase A consisted of 30 mM ammonium acetate in water including 0.05% triethylamine (pH 5.86 adjusted with acetic acid) and mobile phase B was acetonitrile. The flow rate was set 1.0 ml/min. An 80 μl injection of each sample was loaded on to the column, separated and eluted using the following gradient (minutes, % mobile phase B) (0, 29) (18, 60) (20, 60) (20.5, 29) (27, 29). The column temperature was maintained at 35 °C. The UV-detector program consisted of a 0–5.30 min sequence set at 277 nm for RISP and 9-OH RISP acquisition, 5.31–7.90 min sequence set at 255 nm for OLZ acquisition, 7.91–20.0 min sequence set at 245 nm for HAL, CPZ and ZIP acquisition.

2.3. Sample collection

Antipsychotic doses were based on previous rodent studies in which time dependent behavioral and neurochemical effects were detected [7,8]. Furthermore, the selected dose produced plasma levels that approximated those often associated with antipsychotic effects in human [40]. Male albino Wistar rats (Harlan Inc.) 2–3 months old were housed individually in a temperature-controlled room (25 °C), maintained on a 12-h light/dark cycle with free access to food. Rats were thus treated with HAL (2.0 mg/kg per day), RISP (2.5 mg/kg per day), OLZ (10.0 mg/kg per day), CPZ (10.0 mg/kg per day) and ZIP (12 mg/kg per day) orally in drinking water for periods of at least 14 days to achieve a steady-state concentration of the antipsychotic drugs. Dosing antipsychotics by drinking water avoids stress of forced drug application. However, there is reduced water consumption associated with some of the antipsychotics (particularly olanzapine). In these cases (and in the study in question) we added saccharin 0.1% (w/v) to increase water consumption to normal levels. Plasma samples were collected over the course of treatment in separate groups of rats for measurement of the antipsychotic concentrations. Rats were anesthetized with isofluorane and 3.0 ml of blood was collected via cardiac puncture to heparined tubes. The blood was centrifuged for 15 min at 2500 × *g* at 8 °C and the resulting plasma was frozen until analysis.

2.4. Preparation of stock, working standard and quality control solutions

Individual stock solutions of OLZ, RISP, 9-OH RISP, HAL, CPZ and ZIP and I.S. (imipramine) were prepared by dissolving approximate amounts of drugs in absolute methanol to obtain final drug concentrations of 1.0 mg/ml, respectively, and were stored at −20 °C. Combined standard solutions with concentrations of 40.0, 100.0, 200.0, 400.0, 1000.0, 1500.0, 2000.0, 4000.0 and 10000.0 ng/ml were prepared by serial dilution with 0.02 M HCl water solutions. Precision and accuracy standards with concentrations of 40.0, 300.0, 3000.0 and 6000.0 ng/ml were also prepared in the same manner. A 2000.0 ng/ml of I.S. standard solution was prepared with 0.02 M HCl water solution from the 1.0 mg/ml I.S. stock solution. The 1.0 mg/ml stock solutions were kept at −20 °C when not use and replaced every 3 months. Fresh standard solution was prepared for each day of analysis or validation.

2.5. Preparation of calibration and QC samples

Sample for the calibration curves and QCs were prepared by adding 50.0 μl of each standard solution into 1.0 ml of blank plasma. This yields calibration standard concentrations of 2.0, 5.0, 10.0, 20.0, 50.0, 75.0, 100.0, 200.0 and 500.0 ng/ml. The final concentrations of QCs were 2.0, 15.0, 150.0 and 300.0 ng/ml. The spiked plasma samples (standards and quality controls) were extracted with each analytical batch along with the unknown samples.

2.6. Sample preparation

To a 1.0 ml of rat plasma sample, 50 μl of internal standard (2000.0 ng/ml, imipramine) and 0.4 ml 0.5 M Na_2HPO_4 (pH 10.69) were added. The samples were briefly mixed and extracted in 8 ml of isopropyl ether:pentane (70:30) solvent for 5 min. After centrifugation at 2000 × *g* for 10 min, the upper organic layer was removed and 100 μl of 0.05N HCl was added to the organic layer. The mixture was shaken for 3 min and centrifuged at 2000 × *g* for 10 min. The upper organic layer was discarded and the aqueous phase was washed by 0.3 ml of isopropyl ether and 0.1 ml of pentane, respectively. Then, the upper organic layer was aspirated to waste and the aqueous phase was placed in a vacuum centrifuge under reduced pressure for 15 s to evaporate traces of the organic solvent. Eighty microliters of the final aqueous phase was injected into the HPLC unit for analysis.

2.7. Method validation

The method was validated for linearity, recovery, accuracy, precision and selectivity. Plasma calibration curves were constructed using the peak area ratios of OLZ, RISP, 9-OH RISP, HAL, CPZ or ZIP to that of I.S., and applying a weighted ($1/x^2$)

least squares linear regression analysis, precision (expressed as % relative standard deviation, R.S.D.) and accuracy (expressed as % error) were calculated for four quality control (QC) samples (2.0, 15.0, 150.0 and 300.0 ng/ml). Five replicates of each QC point were analyzed to determine the intra-day accuracy and precision. This process was repeated three times over 3 days in order to determine the inter-day accuracy and precision. Recovery was calculated as the peak area for the analytes at 2.0, 15.0, 150.0, 300.0 and 100.0 ng/ml for the I.S. in plasma spiked before extraction divided by the peak area of the pure drugs in the 0.05 M HCl water solution at the same concentration. The stability of the stock solutions was determined at their storage conditions of −20 °C for 3 months. Analytes were considered stable if the relative error (% RE) of the mean test responses were within 15% of appropriate controls [35]. The bench-top stability of spiked plasma samples stored at room temperature was evaluated for 2 h. The freeze/thaw stability was investigated by comparing the stability samples following three freeze/thaw cycles against freshly spiked samples. The autosampler stability was evaluated by comparing the extracted plasma samples that were injected immediately (time 0), with the samples that were re-injected after storage in the autosampler for up to 12 h. The stability testing was performed at 15.0 and 300.0 ng/ml concentration levels for all of the antipsychotic drugs.

3. Results and discussion

3.1. Method development

The chromatographic conditions, especially the analytical column, the composition of mobile phase and gradient elution condition, were optimized through several trials to achieve the desired sensitivity, separation, run time, and symmetric peak shapes for the analytes and I.S. Agilent XDB C8 (150 mm × 4.6 mm i.d., 5 μm) and Waters XTerra C18 (150 mm × 4.6 mm i.d., 5 μm) were evaluated. As a result, Agilent XDB C8 (150 mm × 4.6 mm i.d., 5 μm) was selected as it produced a satisfactory separation, peak shape and shorter analytical run time. The peaks of HAL, CPZ and ZIP showed some tailing and resulted in a longer retention time on the Waters XTerra C18 column (150 mm × 4.6 mm i.d., 5 μm). Different mobile phase A (buffer), such as 30 mM ammonium acetate (pH 5.80 adjusted using acetic acid), 50 mM phosphate buffer (pH 5.80), 10 mM acetic acid, or 30 mM ammonium acetate in water including 0.05% triethylamine (pH 5.86 adjusted using acetic acid) were attempted to improve the method for these compounds. 30 mM ammonium acetate in water including 0.05% triethylamine (pH 5.86 adjusted using acetic acid) resulted in the best peak shape and separation for all of the analytes. The addition of 0.05% of triethylamine in the buffer played a key role in

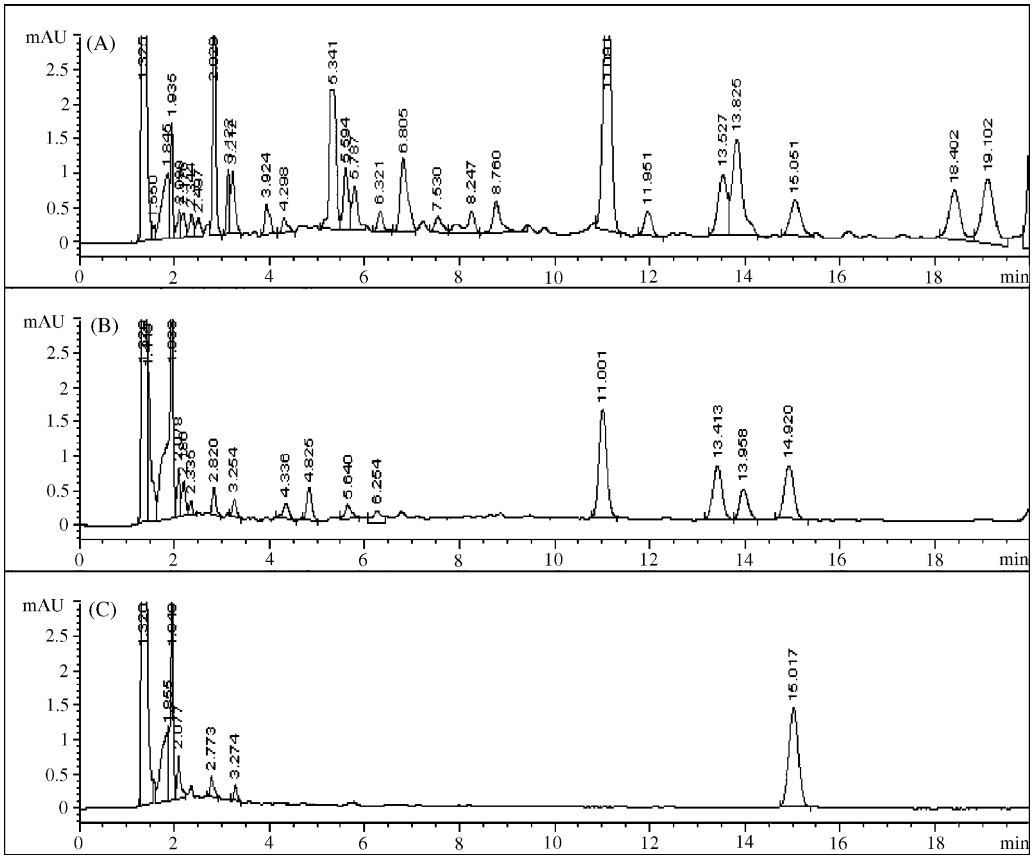

Fig. 2. Representative baseline chromatograms of blank plasma extraction obtained from (A) direct liquid–liquid extraction method; (B) normal liquid–liquid acid solution back extraction method (no wash procedure); (C) liquid–liquid acid solution back extraction with wash procedure using 0.3 ml of isopropyl ether and 0.1 ml of pentane, respectively.

Table 1
Statistical data for linearity including standard deviation (S.D.) (linear range 2.0–500.0 ng/ml) for all of the analytes

	RISP	9-OH RISP	OLZ	HAL	CPZ	ZIP
R^2	0.9984 ± 0.0005	0.9987 ± 0.0009	0.9993 ± 0.0005	0.9995 ± 0.0002	0.9986 ± 0.0004	0.9997 ± 0.0000
Slope	0.0093 ± 0.0002	0.0089 ± 0.0003	0.02397 ± 0.0012	0.0124 ± 0.0144	0.0196 ± 0.0006	0.0125 ± 0.0002

Table 2
The intra-day ($n = 5$) and inter-day ($n = 15$) precision (%R.S.D.) and accuracy (% error) of the HPLC-UV method used to quantitate antipsychotic drugs in rat plasma

Drug	Concentration added (ng/ml)	Intra-day			Inter-day		
		Observed concentration ±S.D. (ng/ml)	R.S.D. (%)	Error (%)	Observed concentration (ng/ml)	R.S.D. (%)	Error (%)
RISP	2.0	2.05 ± 0.030	1.48	2.48	2.05 ± 0.075	3.66	3.71
	15.0	15.64 ± 0.558	3.56	4.67	15.02 ± 0.743	4.95	4.31
	150.0	154.83 ± 2.584	1.67	3.22	151.79 ± 3.997	2.63	2.23
	300.0	310.64 ± 2.381	0.77	3.55	303.16 ± 9.836	3.24	2.94
9-OH RISP	2.0	2.01 ± 0.024	1.18	0.95	1.99 ± 0.119	5.98	4.00
	15.0	16.05 ± 0.178	1.11	7.03	16.12 ± 0.178	1.11	7.44
	150.0	150.86 ± 1.678	1.11	1.07	150.10 ± 2.147	1.43	1.20
	300.0	293.24 ± 5.111	1.74	2.25	288.21 ± 6.016	2.09	3.93
OLZ	2.0	1.99 ± 0.0514	2.58	2.02	2.03 ± 0.0688	3.39	2.63
	15.0	15.25 ± 0.759	4.98	4.69	15.00 ± 0.659	4.39	4.02
	150.0	149.93 ± 2.574	1.72	1.11	150.72 ± 4.008	2.66	2.05
	300.0	303.75 ± 5.13	1.69	1.49	299.79 ± 9.338	3.12	2.51
HAL	2.0	1.97 ± 0.094	4.76	3.99	2.02 ± 0.0831	4.10	3.64
	15.0	16.00 ± 0.653	4.08	6.68	15.22 ± 0.814	5.35	4.13
	150.0	152.69 ± 2.434	1.59	1.91	151.52 ± 2.625	1.73	1.63
	300.0	304.36 ± 2.436	0.80	1.45	300.55 ± 7.998	2.66	2.11
CPZ	2.0	2.05 ± 0.0791	3.85	4.35	2.03 ± 0.0829	4.08	3.92
	15.0	15.81 ± 0.490	3.10	5.43	14.95 ± 0.824	5.51	4.35
	150.0	154.16 ± 3.972	4.26	4.35	153.10 ± 4.762	3.11	3.03
	300.0	307.56 ± 9.264	3.01	3.62	306.19 ± 13.165	4.30	3.98
ZIP	2.0	1.99 ± 0.0593	2.99	2.19	1.99 ± 0.0579	2.91	2.26
	15.0	15.51 ± 0.428	2.76	3.75	15.09 ± 0.525	3.48	2.88
	150.0	148.58 ± 2.864	1.93	1.41	149.35 ± 2.984	2.00	1.47
	300.0	298.01 ± 3.434	1.15	0.92	296.24 ± 5.861	1.98	1.70

enhancing the peak symmetry and separation capacity. In addition, the column temperature should be kept at 35 °C to obtain a baseline separation between CPZ and ZIP. Initially, we evaluated solid phase extraction (SPE) methods using different cartridges such as the Waters Oasis HLB and Varian C18 for sample preparation. However, the recovery of ZIP was very low and there were a lot of interferences from the matrix at low concentration. Finally, we evaluated a liquid–liquid acid solution back extraction method for sample preparation. We found that liquid–liquid acid solution back extraction [2,14] produced a cleaner baseline for blank plasma when compared with direct liquid–liquid extraction when injecting the reconstitution solution after evaporating the liquid–liquid extraction organic solvent to dryness. We modified the standard liquid–liquid acid back extraction method and washed the acid back extraction solution using 0.3 ml of isopropyl ether and 0.1 ml of pentane, respectively. As a result, the baseline of blank plasma was very clean as seen in Fig. 2(C) and there was no interference for any of the analytes compared with a 2.0 ng/ml spiked sample (Fig. 3(B)). Also, the wash procedure did not significantly reduce the recovery for the analytes (data not shown). First we evaluated the direct liquid–liquid extraction method. The procedure was as follows: to a 1.0 ml blank rat plasma sample, 100 μl of 0.02 M HCl solution and 0.4 ml 0.5 M Na_2HPO_4 (pH 10.69) were added. The samples were

Table 3
Recovery (%, mean ± S.D.) of analytes in rat plasma ($n = 5$); recovery of I.S. was 96.3 ± 3.9 ($n = 5$) at 100.0 ng/ml in rat plasma

Concentration (ng/ml)	RISP	9-OH RISP	OLZ	HAL	CPZ	ZIP
2.0	87.1 ± 2.0	84.0 ± 1.6	93.8 ± 3.3	102.3 ± 3.5	95.9 ± 3.5	100.3 ± 2.1
15.0	88.4 ± 1.2	82.5 ± 1.0	90.4 ± 3.3	97.4 ± 2.3	92.4 ± 3.0	95.3 ± 1.0
150.0	89.6 ± 1.5	74.8 ± 0.5	94.9 ± 1.9	97.4 ± 1.9	89.5 ± 2.3	94.2 ± 2.0
300.0	88.7 ± 0.7	75.0 ± 1.8	94.7 ± 1.5	95.1 ± 1.3	87.5 ± 2.6	91.6 ± 1.4

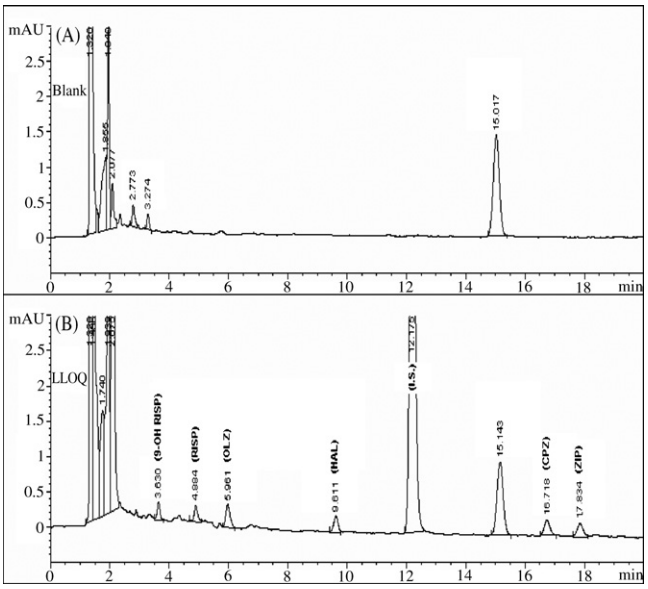

Fig. 3. Representative chromatograms obtained from (A) blank rat plasma; (B) plasma spiked with LLOQ (2.0 ng/ml) concentration for all of the analytes and I.S. (100.0 ng/ml).

briefly mixed and extracted in 8 ml of isopropyl ether:pentane (70:30) solvent for 5 min. After centrifugation at 2000 × *g* for 10 min, the upper organic layer was removed and evaporated to dryness under reduced pressure in a vacuum centrifuge. To the residue, 100 μl of 0.05 M HCl was added, ultrasonicated for 1 min, then vortexed and centrifuged at 16,000 × *g* for 10 min. Eighty microliters of the reconstitution solution (blank plasma) was injected into HPLC-UV system. As a result, the direct liquid–liquid extraction method produced a lot of interference for the analytes as Fig. 1(A). The liquid–liquid acid solution back extraction method (no wash procedure) produced an interference for RISP as seen in Fig. 1(B). Different organic solvents, ethyl acetate, methyl *tert*-butyl ether, chloroform, hexane, pentane, diethyl ether, isoamyl alcohol and isopropyl ether, and their mixtures in different combinations and ratios were evaluated for extraction solvents. Finally, isopropyl ether:pentane (70:30) was found to be optimal, because it is able to produce a clean chromatogram for a blank plasma sample and yielded the highest recovery for the analytes by a liquid–liquid acid solution back extraction method. Another advantage of isopropyl ether and pentane is lower solubility in water when compared to other solvents. Low solubility and high extraction strength of the organic solvents are key factors for liquid–liquid acid back extraction methods.

3.2. Linearity and sensitivity

The calibration curves in Table 1 showed good linear response ($R^2 > 0.998$) over the range from 2.0 to 500.0 ng/ml for all of the analytes. Microsoft Excel or SAS JMPIN statistical software was used to generate linear regression equations for all calibration curves. A $1/x^2$-weighting scheme was used for each day of the validation and analysis for the analytes. Table 1 showed the slope and R^2 values generated from the calibration curves used in the validation study. The LLOQ, defined as the lowest concentration of analyte with an accuracy within 20% and a precision <20%, was 2.0 ng/ml for determination of all of the analytes in rat plasma as shown in Table 3. Representative chromatograms obtained from blank plasma and plasma spiked with the LLOQ standard (2.0 ng/ml) are shown in Fig. 3. No interfering peaks from endogenous compounds were observed at the retention times of the analytes or I.S. in blank rat plasma from six different lots. A signal-to-noise (S/N) > 10 at LLOQ (2.0 ng/ml) was observed for all of the analytes.

3.3. Precision and accuracy

Precision and accuracy measurements were acquired for the QC points for each compound. The accuracy and precision data can be seen in Table 2. The values for the intra-day precision and accuracy were better than 4.98% and 7.03% for all the analytes. The inter-day precision and accuracy were determined by pooling all of the validation assay ($n = 15$) QC samples. The values for the inter-day precision and accuracy were better than 5.98% and 7.44% (Table 3).

3.4. Recovery and selectivity

The analytes and I.S. are basic compounds. Therefore, extraction recovery was, to a great extent, influenced by the pH of the plasma sample. Hence, alkalytic modifiers were used to adjust the pH of plasma samples. Several alkalytic modifiers, 0.5 M Na_2CO_3 (pH 10), 1 M NaOH and 0.5 M phosphate buffer (pH

Table 4
Retention times of some commonly used drugs and their metabolites

Drugs	Retention times (min)
Caffeine	1.84
9-Hydroxyrisperidone	**3.62**
Risperidone	**4.83**
Propranolol	5.24
Olanzapine	**5.90**
Lidocaine	6.33
Normethylclozapine	6.55
Oxazepam	9.09
Haloperidol	**9.52**
Desipramine	9.94
Clozapine-*N*-oxide	10.42
Promazine	10.65
Clonazepam	10.96
Imipramine (I.S.)	**12.05**
Clozapine	13.37
Verapermil	13.49
Amitriptyline	13.88
Trimipramine	15.16
Chlorpromazine	**16.55**
Midazolam	16.88
Ziprasidone	**17.71**
Diazepam	18.50
Morphine	N.D.
Codeine	N.D.
Loxapine	N.D.

The analytes from the current assay are included in bold. N.D.: not detected within a 20.0-min run.

10.69), were evaluated. Finally, 0.5 M phosphate buffer (pH 10.69) was selected because it produced the highest recovery for all of the analytes. In addition, 1 M NaOH was not suitable because of the possible reduction of chlorpromazine-*N*-oxide to chlorpromazine from this solution [41]. The recoveries ranged from 74.8% to 102.3% for all of the analytes and I.S. (Table 3).

Several drugs commonly used in psychiatric practice were tested for interference comparing their retention times with those of the analytes and the IS. Some of the main metabolites of the analytes were also checked. The results of these assays are reported in Table 4. The results demonstrated that there is little interfere with the determination of the analytes, granting good method selectivity.

3.5. Stability studies

Stability testing is very important for validated methods in biological samples. The stock solutions were stable at the storage conditions (−20 °C) for 3 months (data not shown). All the other stability studies were conducted at two concentration levels (15.0 and 300.0 ng/ml) with five determinations for each. Plasma extracts were stable in mobile phase in the HPLC autosampler

Table 5
Stability testing of antipsychotic drugs used in this study ($n = 5$)

Drugs	Stability	Spiked concentration (ng/ml)	Observed concentration ± S.D. (ng/ml)	R.S.D. (%)	Relative error (%)
RISP	Three freeze-thaw cycles	15.0	15.31 ± 0.562	3.67	2.05
		300.0	306.79 ± 8.012	2.62	2.26
	Bench top (2 h)	15.0	15.47 ± 0.406	2.62	3.15
		300.0	313.77 ± 13.421	4.28	4.59
	Autosampler stability (12 h)	15.0	14.26 ± 0.500	3.50	−4.91
		300.0	300.32 ± 7.126	2.37	0.11
9-OH RISP	Three freeze-thaw cycles	15.0	15.97 ± 0.139	0.87	6.47
		300.0	297.88 ± 1.372	0.46	−0.71
	Bench top (2 h)	15.0	16.41 ± 0.169	1.03	9.37
		300.0	297.81 ± 6.588	2.21	−0.73
	Autosampler stability (12 h)	15.0	15.98 ± 0.218	1.37	6.54
		300.0	292.24 ± 2.635	0.90	−2.59
OLZ	Three freeze-thaw cycles	15.0	15.56 ± 0.505	3.25	3.71
		300.0	298.68 ± 11.505	3.85	−0.44
	Bench top (2 h)	15.0	15.53 ± 0.246	1.59	3.54
		300.0	305.37 ± 10.811	3.54	1.79
	Autosampler stability (12 h)	15.0	15.53 ± 0.525	3.38	3.54
		300.0	302.19 ± 3.502	1.16	0.73
HAL	Three freeze-thaw cycles	15.0	15.23 ± 0.261	1.71	1.53
		300.0	298.29 ± 2.906	0.97	−0.57
	Bench top (2 h)	15.0	14.97 ± 0.2494	1.67	−0.24
		300.0	304.93 ± 3.779	1.24	1.64
	Autosampler stability (12 h)	15.0	15.18 ± 0.702	4.62	1.19
		300.0	300.86 ± 0.564	0.19	0.29
CPZ	Three freeze-thaw cycles	15.0	15.37 ± 1.325	8.62	2.47
		300.0	303.49 ± 4.587	1.51	1.16
	Bench top (2 h)	15.0	15.96 ± 0.968	6.07	6.42
		300.0	301.52 ± 4.107	1.36	0.51
	Autosampler stability (12 h)	15.0	14.85 ± 0.714	4.81	−1.01
		300.0	295.74 ± 5.948	2.01	−1.42
ZIP	Three freeze-thaw cycles	15.0	15.58 ± 0.231	1.48	3.89
		300.0	311.02 ± 3.484	1.12	3.67
	Bench top (2 h)	15.0	14.69 ± 0.591	4.02	−2.06
		300.0	308.31 ± 9.837	3.19	2.77
	Autosampler stability (12 h)	15.0	15.22 ± 0.411	2.70	1.44

Table 6
Steady-state plasma concentrations of the analytes after the chronic treatment with antipsychotic drugs for rats ($n = 3$)

Drugs administered	Dose (mg/kg per day)	Concentrations (ng/ml ± S.D.)
RISP	2.5	RISP: 12.91 ± 4.608 9-OH RISP: 23.05 ± 2.456
HAL	2.0	11.15 ± 2.200
OLZ	10.0	22.24 ± 11.749
CPZ	10.0	11.85 ± 2.421
ZIP	12.0	111.09 ± 86.563

for at least 12 h, indicating that samples should be processed within this period of time (Table 5). The freeze/thaw stability tests indicate the analytes were stable in rat plasma for three freeze/thaw cycles. The results of bench-top stability indicate that spiked samples were stable for all of the analytes for at least 2 h. The RE% was from 0.11% to 9.37% (<15%) and R.S.D. was from 0.19% to 8.62% for all the analytes (see Table 5).

Zhou et al. [42] reported that storage of OLZ in human plasma at room temperature for 24 h produced significant degradation of OLZ. If Vitamin C was added to plasma, then OLZ was stable at room temperature for 24 h. In this work, OLZ did not show significant degradation in rat plasma kept at room temperature for up to 2 h without Vitamin C addition. However, for longer storage of OLZ, freezing rat plasma is recommended [38].

3.6. Application of the method

The validated method has been successfully used to quantify antipsychotic drug concentrations in rat plasma after the chronic treatment of rats with the antipsychotic drugs in their drinking water. The steady-state concentration data for antipsychotic drugs in rat plasma are reported in Table 6. The representative

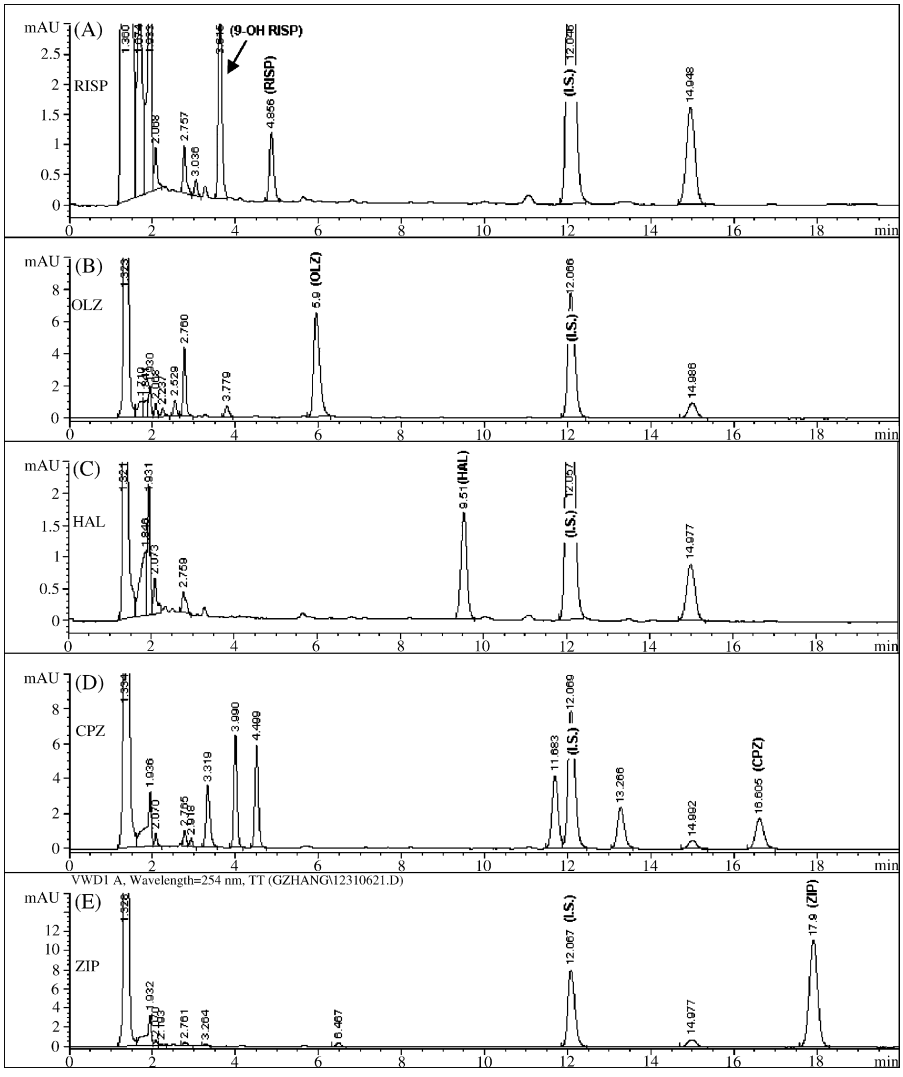

Fig. 4. Representative chromatograms of plasma samples from chronic treatment with antipsychotic drugs: (A) a rat treated with RISP (2.5 mg/kg per day) and the concentration in plasma was 8.37 ng/ml for RISP and 20.25 ng/ml for 9-OH RISP; (B) a rat treated with OLZ (10.0 mg/kg per day) and the concentration of OLZ in plasma was 34.52 ng/ml; (C) a rat treated with HAL (2.0 mg/kg per day) and the concentration of HAL in plasma was 13.26 ng/ml; (D) a rat treated with CPZ (10.0 mg/kg per day) and the concentration of CPZ in plasma was 13.89 ng/ml; (E) a rat treated with ZIP (12.0 mg/kg per day) and the concentration of ZIP in plasma was 140.58 ng/ml.

chromatograms resulting from the analysis of real samples after chronic treatment with antipsychotic drugs is shown in Fig. 4. There was no significant interference for any of the analytes from any other of the five analytes in the real plasma samples. In general, the concentration of HAL, RISP, OLZ and ZIP in rat plasma was relatively low, but clearly within the range that is generally considered therapeutic in humans. In addition, all the analytes were observed above the method LLOQ.

4. Conclusions

A simple, selective and sensitive HPLC-UV analytical method for the simultaneous determination of RISP, 9-OH RISP, OLZ, HAL, CPZ and ZIP in rat plasma has been developed and validated. A liquid–liquid acid solution back extraction method with wash procedure was evaluated and provided a clean baseline for blank plasma. This method provided good selectivity and a LLOQ of 2.0 ng/ml for all of the analytes. Liquid–liquid extraction sample preparation was used for 1.0 ml of rat plasma that provided high recovery for all of the analytes. The method was successfully applied to study the effect of chronic treatment of FGAs (HAL and CPZ) and SGAs (OLZ, RISP and ZIP) antipsychotic drugs on the cognitive function in rats.

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