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Sensitive liquid chromatography/tandem mass spectrometry method for the determination of the lipophilic antipsychotic drug chlorpromazine in rat plasma and brain tissue

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Abstract

A simple, sensitive and robust liquid chromatography/electrospray ionization tandem mass spectrometry (LC–ESI-MS/MS) method was developed and validated for quantification of chlorpromazine in rat plasma and brain tissue. Chlorpromazine was extracted from rat plasma and brain homogenate using liquid–liquid extraction. The compounds were separated on a Waters Atlantis^{TM} dC-18 (30 mm × 2.1 mm i.d., 3 μm) column using a mobile phase of acetonitrile/20 mM ammonium formate (pH 4.25 adjusted with formic acid) with gradient elution. Chlorpromazine was detected in positive ion mode using multiple reaction monitoring (MRM). The method was validated and the specificity, linearity, lower limit of quantitation (LLOQ), precision, accuracy, recoveries and stability were determined. The LLOQ was 0.2 ng/ml for plasma and 0.833 ng/g for brain tissue. The method was linear over the concentration range from 0.2 to 200.0 ng/ml for plasma and from 0.833 to 833.3 ng/g for brain tissue. The correlation coefficient (R^2) values were more than 0.998 for both plasma and brain homogenate. The precision and accuracy for intra-day and inter-day were better than 7.54%. The relative and absolute recovery was above 84.9% and matrix effects were lower than 5.6%. This validated method has been successfully used to quantify the rat plasma and brain tissue concentration of chlorpromazine after chronic treatment.

Keywords: Chlorpromazine; Plasma; Brain tissue; LC–MS/MS

1. Introduction

Chlorpromazine (CPZ, Fig. 1), one of the First Generation Antipsychotics (FGAs), was discovered in the early 1950s. The advent of powerful phenothiazine psychopharmacological agents represents a landmark achievement in the history of medical and psychiatric sciences [1]. CPZ is the most important compound in the large group of phenothiazine derivatives. It is widely used as a therapeutic agent for controlling excitement, agitation and other psychomotor disturbances in schizophrenic patients and reduces the manic phase of manic-depressive conditions. It is used to treat hyperkinetic states and aggression and is sometimes given in other psychiatric conditions for the control of anxiety and tension. The antipsychotic effect of CPZ is believed to be closely related to its dopamine receptor blocking activity [2]. However, CPZ often produces extrapyramidal side effects. Recently, the chronic effects of FGAs and Second Generation Antipsychotics (SGAs) on cognitive function are attracting more attention [3–5]. One prerequisite for the therapeutic effects of CPZ is its ability to pass the blood brain barrier. Given that cognition is now recognized as a key factor that influences long-term functional outcome in schizophrenia, it is important to determine the concentration of CPZ in both plasma and brain (the target compartment for therapeutic action). Knowing these concentrations would allow the determination of extent to which there is a correlation between the concentration of CPZ in the plasma or brain tissue and alterations in cognitive function when CPZ is given chronically in rat model. Such a correlation would allow for improved clinical monitoring of CPZ. Since the antipsychotic drugs are very active, they are usually administered at low daily dosages. In addition, CPZ is widely metabolized in the body. Therefore, the concentration of

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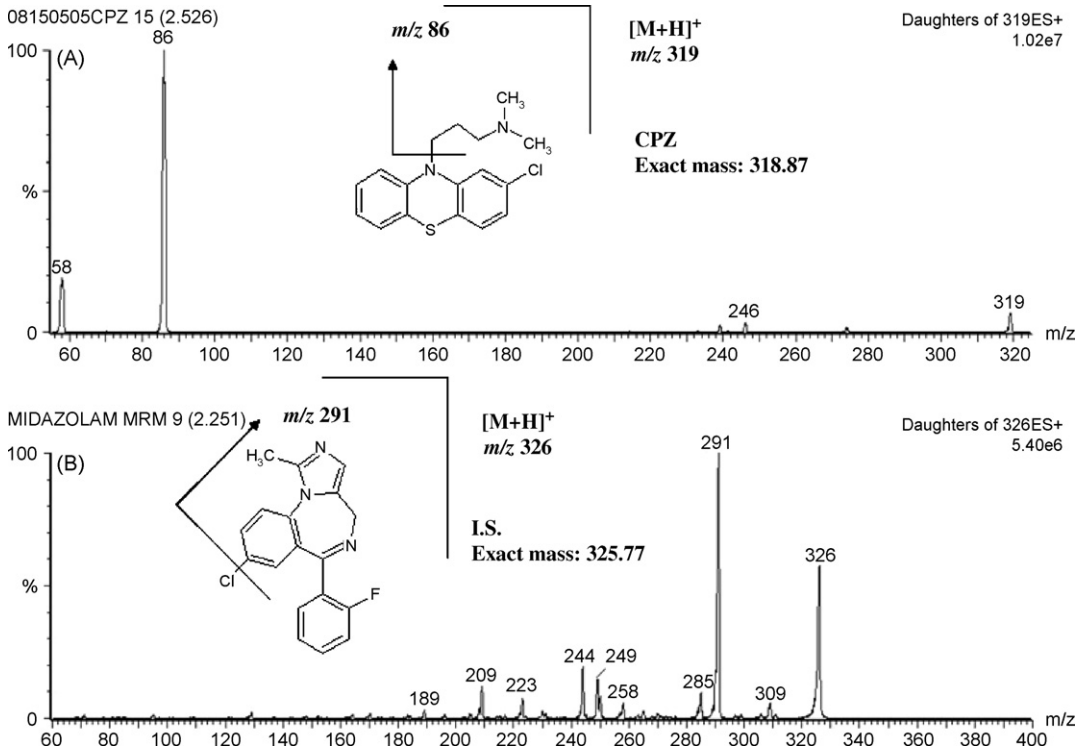

Fig. 1. Product ion mass spectra of: (A) CPZ and (B) I.S. and respective chemical structures.

CPZ in plasma is low. From a bioanalytical and clinical point of view, sensitive and accurate methods are needed to determine CPZ in biological fluids for obtaining optimum therapeutic concentrations and controlling its side effects. At present, determination of CPZ in plasma has been accomplished by GC [6,7], high performance liquid chromatography (HPLC) with UV detection [8–12] and HPLC with fluorescence detection [13]. Radioimmunoassay [14,15] provides the sensitive assay, but little was known about the metabolites due to assay selectivity. Although electrochemical detection [16] offers enhanced sensitivity in the low ng/ml range, electrochemical detectors require optimal working conditions and sample preparation is critical. Interference from co-medications is often unavoidable. Another apparent limitation of electrochemical detection is the presence of wide solvent fronts, which may be due to the response of endogenous materials co-extracted from plasma. This limitation prevents operation of the detector at its lowest limits of detection [16]. An additional draw back of most of these methods was that at least 1 ml of plasma is needed to obtain the reported detection limit. So, most of these methods were not suitable for the study of the pharmacokinetics and metabolism of CPZ. The usefulness of liquid chromatography–electrospray tandem mass spectrometry (LC–ESI-MS/MS) has been demonstrated for a wide range of applications in the bioanalytical, environmental and pharmaceutical fields [17,18]. LC–MS methods offer several significant advantages when compared with the previous methods, such as the small sample volume required, minimization of mobile phase, rapid analytical run time and improved sensitivity, selectivity and specificity [19]. To date, very few LC–MS/MS methods have been reported for the quantitation of CPZ in plasma [20]. In addition, a few papers have reported the determination of CPZ in brain tissue using HPLC with electrochemical detection [21–23] or UV detection [24]. However, most of these methods were not validated. Also, due to the lipophilic nature of CPZ, it is readily absorbed by myelin and other lipid constituents of brain homogenates. Hence, the recovery of CPZ in brain tissue was very low for most methods. It was a challenge to improve the recovery of the hydrophobic CPZ from lipophilic brain tissue.

The purpose of this investigation was to develop and validate a highly selective, sensitive and robust LC–MS/MS method for the determination of the lipophilic drug CPZ in rat plasma and brain tissue. To our knowledge, our paper is the first to report a validated LC–MS/MS method for determination of CPZ in brain tissue.

2. Experimental

2.1. Chemicals and reagents

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

2.2. Instrumentation

An Agilent 1100 series HPLC system, consisting of a degasser, binary pump, autosampler and thermostatted column compartment, was used in this study (Agilent, Palo Alto, CA, USA). The mass spectrometer utilized for this work was a Quattro Micro triple-quadrupole mass spectrometer equipped with a Z-spray source and a built-in syringe pump (Waters, Manchester, UK). MS control and spectral processing were carried out using Masslynx software, version 4.0 (Waters, Beverly, MA, USA).

2.3. Liquid chromatographic and mass spectrometric conditions

The analytes were separated on a Waters Atlantis^{TM} dC-18 (30 mm × 2.1 mm i.d., 3 μm) with a 4.0 mm × 2.0 mm Phenomenex Security Guard C8 guard column. Mobile phase A consisted of 20 mM ammonium formate in water with pH adjusted to 4.25 using formic acid and mobile phase B was acetonitrile. The flow rate was set to 0.3 ml/min. A 10 μl injection of each sample was loaded on to the column, separated and eluted using the following gradient (minutes, %mobile phase B, flow rate (ml/min)) (0, 15, 0.3) (5, 70, 0.3) (7.5, 77, 0.3) (7.6, 90, 0.6) (8.8, 90, 0.6) (8.9, 15, 0.3) (12.5, 15, 0.3). From 7.6 to 8.8 min, it was very important to use a high concentration of acetonitrile (90%) in the mobile phase to wash the column at a flow rate of 0.6 ml/min. This procedure can remove the remaining brain extract residue from the column following each injection. Otherwise there would be decreased response of CPZ in next injection because of ion suppression coming from the brain extract residue of the previous injection. The column temperature was maintained at 25 °C. The LC flow was introduced directly to the mass spectrometer from 5.5–8.5 min and diverted to waste at other times using a six-port switching valve. The autosampler injection needle was washed with methanol to reduce carry-over after each injection. The mass spectrometer was run in positive ion ESI mode using multiple reaction monitoring (MRM) to monitor the mass transitions. Nitrogen gas was used as the desolvation gas and was set to a flow rate of 500 l/h with a temperature of 350 °C. The cone gas flow was set to 50 l/h. Argon was the collision gas and the collision cell pressure was 2.4×10^{-3} mbar. For quantitation, an MRM transition from *m/z* 319 → 86 was performed for CPZ with a cone voltage of 32 V and a collision energy of 20 eV. An MRM transition from *m/z* 326 → 291 was performed for the I.S. (midazolam) with a cone voltage of 32 V and collision energy of 30 eV. The source temperature and capillary voltage were set at 130 °C and 3.5 kV, respectively.

2.4. Sample collection

CPZ chronic dosing was derived from previously published data [4,5]. Furthermore, the selected dose produced plasma levels that approximated those often associated with antipsychotic effects in human [25]. 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/12-h dark cycle with free access to food. Rats were thus treated with CPZ (10.0 mg/kg/day) orally in drinking water for periods of up to 180 days. Plasma samples were collected at various days between 15 and 180 days of treatment in separate groups of rats ($N = 6$). (Steady-state concentrations of CPZ were obtained after 2 weeks of chronic dosing.) 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. The whole brains of sacrificed animals were removed and kept frozen at −70 °C until analysis.

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

Individual stock solutions of CPZ and I.S. (midazolam) 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. Standard solutions with concentrations of 2.0, 4.0, 10.0, 20.0, 40.0, 100.0, 200.0, 400.0, 1000.0 and 2000.0 ng/ml were prepared by serial dilution with acetonitrile–methanol solutions (60:40). Precision and accuracy standards with concentrations of 2.0, 6.0, 150.0 and 1500.0 ng/ml were also prepared in the same manner. An 80.0 ng/ml I.S. standard solution was prepared with acetonitrile–methanol solutions (60:40) from the 1.0 mg/ml I.S. stock solution. The 1.0 mg/ml stock solutions were kept at −20 °C when not in use and replaced every 3 months. A fresh standard solution was prepared for each day of analysis or validation.

2.6. Preparation of calibration and QC samples

The brains were minced and homogenized in a volume of deionized water (in ml) equal to twice the weight (in g) of the tissue using a Brinkmann (Westburg, NY, USA) Polytron TP 1020 homogenizer. Samples for the calibration curves and QCs were prepared by adding 25.0 μl of each standard into 250 μl blank rat plasma or 200 μl blank brain homogenate. This yields calibration standard concentrations of 0.2, 0.4, 1.0, 2.0, 4.0, 10.0, 20.0, 40.0, 100.0 and 200.0 ng/ml for plasma and 0.833, 1.666, 4.165, 8.33, 16.66, 41.65, 83.33, 166.6, 416.5 and 833.3 ng/g for brain tissue. The final concentrations of QCs were 0.2, 0.6, 15.0 and 150.0 ng/ml for plasma and 0.833, 2.5, 62.5 and 625.0 ng/g for brain tissue. The spiked plasma and brain homogenate samples (standards and quality controls) were extracted with each analytical batch along with the unknown samples.

2.7. Sample preparation

To a 250 μl rat plasma or 200 μl brain homogenate sample, 25 μl of internal standard (80.0 ng/ml, midazolam), 0.2 ml of 0.5 M Na_2HPO_4 (pH 10.69) for plasma and 0.4 ml of 0.5 M Na_2HPO_4 (pH 10.69) for brain homogenate were added. The samples were briefly mixed and extracted in 3 ml isopropyl ether once for plasma and twice for brain homogenate. 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, 200 μl of acetonitrile–methanol solutions (60:40) solution was added, ultrasonicated for 1 min, then vortexed and centrifuged at 16,000 × *g* prior to LC–MS/MS analysis.

2.8. Method validation

The method was validated for linearity, recovery, matrix effect, accuracy and precision. Brain homogenate calibration curves were constructed using the peak area ratios of CPZ 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. Five replicates of each QC points 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. Absolute and relative recoveries, and matrix effects were calculated for spiked samples at 0.2, 0.6, 15.0 and 150.0 ng/ml for plasma and 0.833, 2.5, 62.5 and 625.0 ng/g for brain tissue and 8.0 ng/ml I.S. in plasma samples and 16.64 ng/g I.S. in brain tissue ($n = 5$). Absolute recovery was calculated as the peak area for CPZ and I.S. in plasma or brain homogenate spiked before extraction divided by the peak area of the pure drugs in the acetonitrile–methanol solutions (60:40) solution at the same concentration. Relative recovery was calculated by dividing the peak area for CPZ and I.S. spiked before extraction by the peak area for an equal concentration of the sample in the same matrix spiked after extraction. The matrix effects were calculated by dividing the response for a CPZ sample in biological matrix spiked after extraction by an equal concentration of CPZ in an acetonitrile–methanol (60:40) solution [26]. The stability of the stock solutions was determined at its storage conditions of −20 °C for 3 months. CPZ was considered stable if the relative error (%RE) of the mean test responses were within 15% of appropriate controls [27]. 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 brain homogenate samples that were injected immediately (time 0), with the samples that were re-injected after storage in the autosampler for up to 24 h. The stability testing was performed at 0.6 and 150.0 ng/ml for plasma and 2.5 and 625.0 ng/g for brain tissue concentration levels.

3. Results and discussion

3.1. Method development

In order to develop and validate a highly sensitive and selective method with the designed LLOQ (0.2 ng/ml for plasma and 0.833 ng/g for brain tissue), during method development different options were evaluated to optimize the detection (MRM) parameters, chromatography and sample preparation methodology. MS/MS detection provided improved sensitivity for trace mixture analysis. ESI(+) MS/MS product ion spectra were produced by CAD of the protonated molecular ion $(M+H)^+$. The most favorable transition was selected and the instrument parameter settings were optimized individually for CPZ and I.S. by constant infusion at 10 μl/min of a 2 μg/ml solution. The major MS/MS transitions utilized for LC–MS/MS quantitate analysis were m/z 319 → 86 for CPZ and m/z 326 → 291 for I.S. (midazolam).

The chromatographic conditions, especially the analytical column and the composition of mobile phase, were optimized through several trials to achieve the desired sensitivity, separation, run time and symmetric peak shapes for CPZ and I.S. Agilent XDB C8 (150 mm × 2.1 mm i.d., 5 μm), Waters XTerra C18 (50 mm × 2.1 mm i.d., 5 μm) and Waters Atlantis™ dC-18 (30 mm × 2.1 mm i.d., 3 μm) columns were evaluated. The peak shape of CPZ on the XTerra C18 (50 mm × 2.1 mm i.d., 5 μm) column showed tailing. As a result, Waters Atlantis™ dC-18 (30 mm × 2.1 mm i.d., 3 μm) was selected as it produced the satisfactory separation, peak shape and a shorter analytical run time than the other columns. Twenty millimolars ammonium formate (pH 4.25 using formic acid) buffer combination with acetonitrile resulted in the sensitive signals with optimized gradient elution. CPZ is a lipophilic drug and produces low concentrations in biological samples. We initially pursued isocratic elution to obtain shorter run times but this was not suitable for brain tissue samples. Brain samples contained substantial amount of lipid residue after evaporation of the extraction solvent to dryness. Next, we pursued gradient elution with a low concentration of acetonitrile in the mobile phase at the start of the run to improve the peak shape and thus the assay sensitivity. Another reason we used gradient elution was that the separation capacity between CPZ and lipid residue could be improved which reduced ion suppression from brain tissue samples. When using an isocratic method, the peak shape of CPZ was broad which decreased sensitivity. We have optimized the total method run time to 12.5 min. From 7.6 to 8.8 min, it was very important to use a high concentration of acetonitrile (90%) in the mobile phase to wash the column with a flow rate of 0.6 ml/min. This procedure can remove the brain extract residue from the column following each run. Failure to remove the residue matrix would decrease the response of CPZ for subsequent injections because of ion suppression coming from the brain extract residue from the previous injection on the column. It is necessary to wash out the brain extract residue from the column during each running. Initially, we evaluated a one-step protein precipitation method using methanol or acetonitrile for sample preparation. However, the LLOQ was poorer because of dilution of the sample. Other authors [20] found recoveries of 96% for CPZ using protein precipitation for plasma. However, we found the recovery using protein precipitation was very low for CPZ and the I.S. in brain tissue. Next, we evaluated solid phase extraction (SPE) methods using different cartridges such as the Waters Oasis HLB and Varian C18. Although Ohkubo et al. [28] reported about 80–88% recovery of CPZ was obtained in serum and human breast milk, we found that the recovery of CPZ and I.S. was still very low using SPE from brain tissue. Finally, we evaluated liquid–liquid

Table 1
Statistical data for linearity including standard deviation (S.D.) (linear range 0.2–200.0 ng/ml for plasma and 0.833–833.3 ng/g for brain)

Biological matrix	R^2	Slope	Intercept
Plasma	0.9984 ± 0.00035	0.22176 ± 0.00748	0.003593 ± 0.002256
Brain tissue	0.9985 ± 0.00101	0.04916 ± 0.00273	0.00264 ± 0.001146

extraction (LLE) for the sample preparation. LLE can be helpful in producing a clean sample and avoiding the introduction of non-volatile materials on the column and MS system. Clean samples are essential for minimizing ion suppression and matrix effects in LC–MS/MS analysis [20]. Different organic solvents, ethyl acetate, *tert*-butyl methyl ether, chloroform, hexane, diethyl ether and isopropyl ether, and their mixtures in different combinations and ratios were evaluated. Finally, isopropyl ether was found to optimal, because it was able to produce a clean chromatogram for a blank brain homogenate sample and yielded the highest recovery for the analytes by liquid–liquid extraction.

3.2. Linearity and sensitivity

Table 1 shows the calibration curves for each day of validation. The curves showed good linear response ($R^2 > 0.9984$) over the range of 0.2–200.0 ng/g for plasma and 0.833–833.3 ng/g for brain tissue. Microsoft Excel or SAS JMPIN statistical software was used to generate linear regression equations for the calibration curves. A $1/x^2$-weighting scheme was used for each day of the validation and analysis for CPZ. Table 1 showed the slope, intercept 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 0.2 ng/g for plasma and 0.833 ng/g for brain tissue as shown in Table 2. A signal-to-noise (S/N) > 10 at LLOQ was observed for CPZ in biological matrix.

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 4.58 and 7.54%. 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 4.78 and 5.95% (Table 2).

3.4. Recovery and matrix effect

CPZ and the I.S. are basic compounds. Therefore, extraction recovery was, to a great extent, influenced by the pH of brain homogenate samples. Hence, alkalytic modifiers were used to adjust the pH of brain homogenate. Several alkalytic modifiers, 0.5 M Na_2CO_3 (pH 10), 1 M NaOH and 0.5 M phosphate buffer (pH 10.69), were evaluated. 1.0 M NaOH is not suitable as a pH modifier because of the possible reduction from chlorpromazine-N-oxide (one metabolite of CPZ) to CPZ from this solution [29]. Finally, 0.5 M phosphate buffer (pH 10.69) was selected because it produced the highest recovery for CPZ and the I.S.

Due to non-specific binding of lipophilic CPZ to brain constituents [30], it is a challenge to improve the recovery of CPZ in brain homogenate. We found that reconstitution solution plays a key role for improved recovery and reduced matrix effects in brain homogenate residue after liquid–liquid extraction. Methanol:20 mM ammonium formate (pH 4.25) (80:20), pure methanol, acetonitrile:20 mM ammonium formate (pH 4.25) (80:20) and acetonitrile:methanol (60:40) were evaluated as reconstitution solutions, respectively. Table 3 showed the effect of different reconstitution solutions on absolute recovery, relative recovery (%) and matrix effects (mean ± S.D.) of CPZ in rat plasma (15.0 ng/ml) and brain tissue (62.5 ng/g) ($n = 3$). The peak shape of CPZ in different reconstitution solution

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

Matrix	Concentration added (ng/ml or ng/g)	Intra-day			Inter-day		
		Observed concentration (ng/ml or ng/g)	R.S.D (%)	Error (%)	Observed concentration (ng/ml or ng/g)	R.S.D. (%)	Error (%)
Plasma	0.2	0.196 ± 0.0071	3.63	3.41	0.197 ± 0.0057	2.88	2.57
	0.6	0.645 ± 0.0087	1.35	7.54	0.626 ± 0.02154	3.44	4.67
	15.0	15.88 ± 0.1534	0.97	5.87	14.963 ± 0.7026	4.70	4.16
	150.0	156.06 ± 0.991	0.64	4.04	153.75 ± 7.3508	4.78	4.68
Brain	0.833	0.800 ± 0.0367	4.58	4.96	0.824 ± 0.0331	4.01	3.29
	2.5	2.636 ± 0.0521	1.98	5.42	2.649 ± 0.0482	1.82	5.95
	62.5	61.935 ± 1.5940	2.57	2.08	62.933 ± 2.0714	3.29	2.71
	625.0	599.98 ± 9.1217	1.52	4.00	609.54 ± 18.384	3.02	3.45

Table 3
Effect of different reconstitution solutions on absolute recovery, relative recovery (%) and matrix effects (mean ± S.D.) of CPZ in rat plasma (15.0 ng/ml) and brain tissue (62.5 ng/g) ($n = 3$)

Matrix	Reconstitution solution[a] (v:v)	Absolute recovery (%)	Relative recovery (%)	Matrix effect (%)	Type of effect
Plasma	ACN:MeOH = 60:40	93.3 ± 1.47	90.8 ± 1.75	102.7 ± 0.37	2.7% enhancement
	ACN:buffer = 80:20	92.4 ± 1.03	91.2 ± 0.95	101.2 ± 0.37	1.2% enhancement
	MeOH:buffer = 80:20	88.9 ± 0.56	90.1 ± 0.83	98.7 ± 0.95	1.3% suppression
	MeOH	95.4 ± 1.48	97.7 ± 2.15	97.1 ± 0.94	2.9% suppression
Brain	ACN:MeOH = 60:40	90.8 ± 0.84	92.3 ± 0.86	97.9 ± 0.22	2.1% suppression
	ACN:buffer = 80:20	70.5 ± 1.55	81.4 ± 0.71	86.7 ± 1.16	13.3% suppression
	MeOH:buffer = 80:20	66.7 ± 2.69	82.8 ± 3.98	80.4 ± 0.64	19.6% suppression
	MeOH	76.1 ± 1.17	87.4 ± 2.31	87.1 ± 1.55	12.9% suppression

[a] ACN: acetonitrile; MeOH: methanol; buffer: 20 mM ammonium formate (pH 4.25 adjusted using formic acid).

was acceptable in plasma or brain tissue extracts. For plasma, the effect of different reconstitution solution on recovery and matrix effects was not significantly different. However, for brain tissue, the effect of different reconstitution solution on recovery and matrix effects was highly variable. Methanol:20 mM ammonium formate (pH 4.25) (80:20) as reconstitution solution produced the lowest absolute recovery (66.7%) and the highest ion suppression (19.6%). Finally, acetonitrile:methanol (60:40) solution was chosen because it produced the highest absolute recovery (90.8%) and the lowest matrix effects (2.1% ion suppression) for brain tissue. In addition, absolute recovery, relative recovery and matrix effect data of CPZ in different concentration in plasma or brain tissue using acetonitrile:methanol (60:40) solution as reconstitution solution are summarized in Table 4. Absolute recoveries range from 89.6 to 93.2% and relative recoveries from 84.9 to 98.2% for CPZ and the I.S. in plasma or brain homogenate. Matrix effects are an important issue in ESI. Matrix effects are the results of co-eluting components, generally from the matrix, that cause variable suppression or enhancement of analyte response [31]. In the positive ion mode, if the ionized analyte is transferred to the gas phase, gas phase proton transfer reactions may cause neutralization if another neutral species is present with a higher proton affinity. In addition, other ionic species, such as salts, in biological samples with high ionization efficiency or surface activity may compete with the analytes during ion evaporation. LLE used in this study can be helpful in producing clean samples, which are essential to reduce matrix effect. The matrix effect of plasma for all of the analytes in this work was lower than 5.6% suppression or enhancement (Table 4).

3.5. Specificity

Representative chromatograms obtained from blank biological matrix and spiked with LLOQ standard (0.2 ng/ml for plasma and 0.833 ng/g for brain homogenate) are shown in Figs. 2 and 3. No interfering peaks from endogenous compounds were observed at the retention times of CPZ or I.S. in blank plasma or brain homogenate. Three MRM transitions have been used: one transition (319 → 86) for quantification and two transitions (319 → 58 and 319 → 246) for identification. Table 5 contains the data for the first identification MRM transition (319 → 58), the peak area ratio (319 → 86/319 → 58) in plasma or brain tissue is from 4.60 to 4.79 over the calibration curve range. The peak area ratio (319 → 86/319 → 58) from real samples was 4.71 for plasma and 4.78 for brain tissue, respectively. Also, for the second identification MRM transition (319 → 246), the peak area ratio (319 → 86/319 → 246) in plasma or brain tissue is from 20.89 to 21.77 over the calibration curve range. The peak area ratio (319 → 86/319 → 246) from real samples was 21.12 for plasma and 21.08 for brain tissue, respectively. These results showed that no significant interference existed for CPZ in plasma or brain tissue. The addition of the two MRM transitions (319 → 58 and 319 → 246) from CPZ for identification improved the specificity of the validated method.

Table 4
Absolute recovery, relative recovery (%) and matrix effects (mean ± S.D.) of analytes and I.S. in rat plasma and brain tissue at different concentrations ($n = 5$) using acetonitrile:methanol (60:40) as the reconstitution solution

Matrix	Concentration (ng/ml or ng/g)	Absolute recovery (%)	Relative recovery (%)	Matrix effect (%)	Type of effect
Plasma	0.2	93.2 ± 1.37	84.9 ± 1.25	104.5 ± 4.57	4.5% enhancement
	0.6	91.0 ± 1.59	89.3 ± 1.56	101.7 ± 0.39	1.7% enhancement
	15.0	91.5 ± 0.85	89.1 ± 0.83	102.0 ± 0.84	2.0% enhancement
	150.0	90.2 ± 1.18	89.1 ± 1.16	101.8 ± 1.33	1.8% enhancement
I.S. (plasma)	8.0	89.8 ± 1.08	91.1 ± 1.10	97.8 ± 0.67	2.2% suppression
Brain	0.833	90.0 ± 2.02	91.9 ± 2.06	96.8 ± 4.18	3.2% suppression
	2.5	90.6 ± 1.33	98.2 ± 1.44	94.4 ± 2.80	5.6% suppression
	62.5	91.2 ± 3.40	94.8 ± 3.54	95.6 ± 0.56	4.4% suppression
	625.0	89.6 ± 1.51	93.1 ± 1.57	96.5 ± 1.82	3.5% suppression
I.S. (brain)	33.33	95.1 ± 1.23	97.3 ± 1.26	97.7 ± 0.58	2.3% suppression

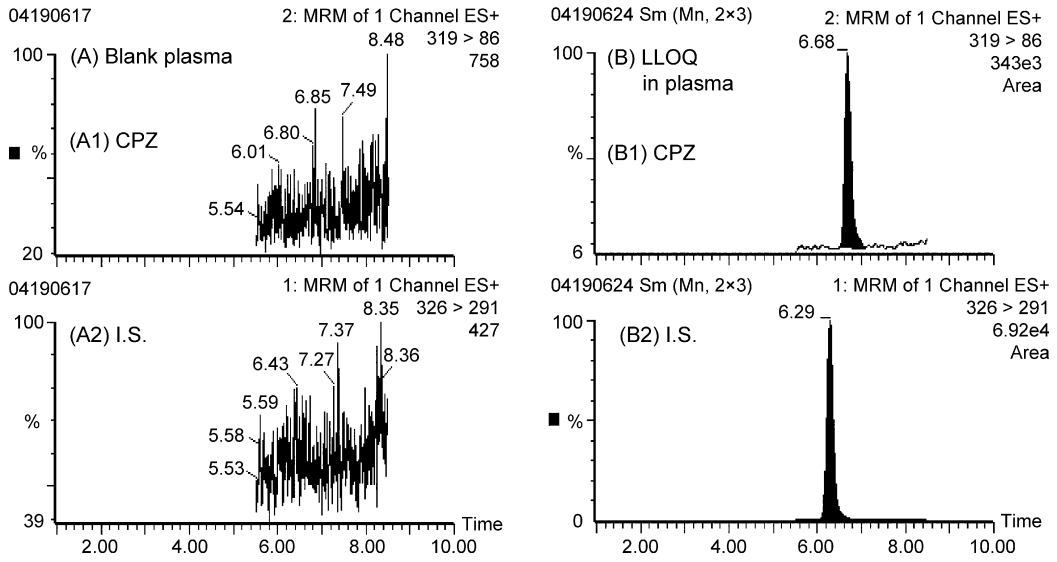

Fig. 2. Representative chromatograms obtained from rat plasma: (A) blank rat plasma; (B) plasma spiked with LLOQ for CPZ at 0.2 ng/ml (B1) and I.S. at 8.0 ng/ml (B2).

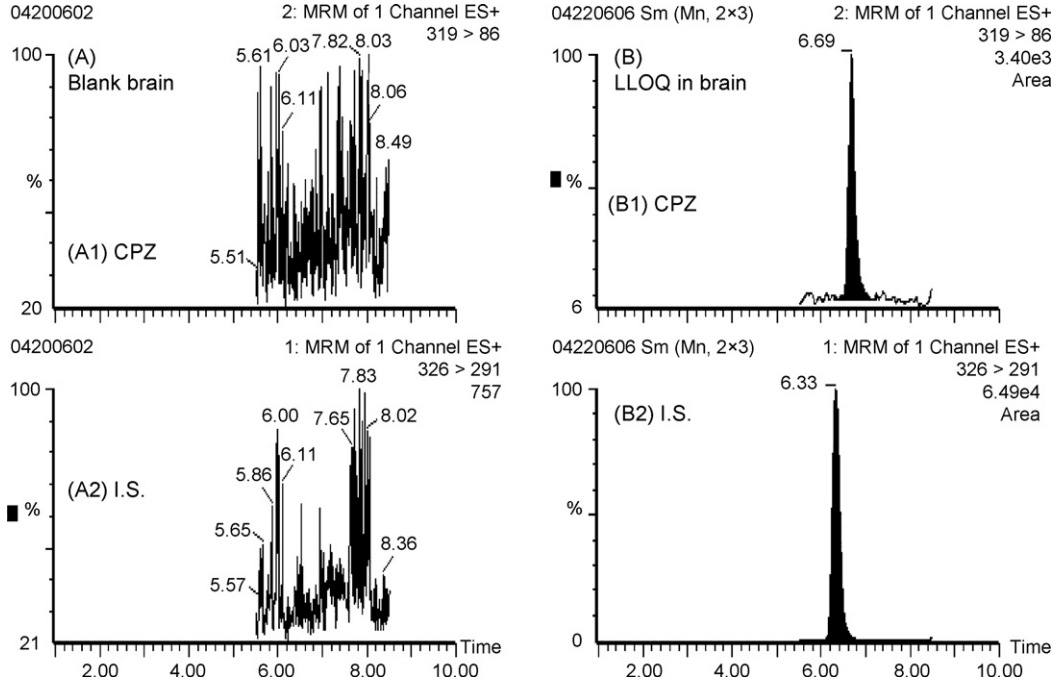

Fig. 3. Representative chromatograms obtained from rat brain tissue: (A) blank rat tissue; (B) brain tissue homogenate spiked with LLOQ for CPZ at 0.833 ng/g (B1) and I.S. at 33.33 ng/g (B2).

Table 5
Enhanced specificity of CPZ by multiple MRM transitions (319 → 86 for quantification; 319 → 58 and 319 → 246 for identification for CPZ in plasma and brain tissue)

	Concentration (ng/ml in plasma/ng/g in brain tissue)						Real samples plasma/brain
	0.2/0.833	0.4/1.666	1.0/4.165	4.0/16.66	40.0/166.6	200.0/833.3	
Peak area ratio in plasma (319 → 86/319 → 58)	n.d.	4.68	4.73	4.60	4.71	4.71	4.71
Peak area ratio in brain (319 → 86/319 → 58)	n.d.	4.70	4.71	4.79	4.74	4.72	4.78
Peak area ratio in plasma (319 → 86/319 → 246)	n.d.	n.d.	n.d.	21.72	21.05	20.89	21.12
Peak area ratio in brain (319 → 86/319 → 246)	n.d.	n.d.	n.d.	21.77	21.11	21.53	21.08

n.d.: not detected with 319 → 58 or 319 → 246 MRM transition.

Table 6
Stability testing of CPZ in rat plasma and brain homogenate ($n = 5$)

Matrix	Stability	Spiked concentration (ng/ml or ng/g)	Observed concentration ± S.D. (ng/g)	R.S.D. (%)	Relative error (%)
Plasma	Three freeze–thaw cycle	0.6	0.584 ± 0.0115	1.97	−2.59
		150.0	150.58 ± 1.8110	1.20	0.39
	Bench top (2 h)	0.6	0.589 ± 0.0117	1.98	−1.76
		150.0	152.099 ± 2.596	1.71	1.40
	Autosampler stability (24 h)	0.6	0.594 ± 0.0090	1.51	−0.95
		150.0	151.70 ± 0.3392	0.22	1.14
Brain	Three freeze–thaw cycle	2.5	2.427 ± 0.0567	2.34	−2.93
		625.0	613.08 ± 9.788	1.60	−1.91
	Bench top (2 h)	2.5	2.45 ± 0.0550	2.25	−1.99
		625.0	610.11 ± 1.727	0.28	−2.38
	Autosampler stability (24 h)	2.5	2.54 ± 0.0964	3.79	1.80
		625.0	605.09 ± 0.8125	0.13	−3.19

Table 7
Steady-state plasma and brain tissue concentrations of CPZ after the chronic treatment of CPZ in rats

Matrix	Dose (mg/kg/day)	Steady-state concentrations (ng/ml or ng/g ± S.D.)	Concentration range (ng/ml or ng/g)
Plasma ($n = 18$)	10.0	11.78 ± 7.06	2.98–26.58
Brain tissue ($n = 6$)	10.0	251.02 ± 66.14	125.46–312.60

3.6. Stability studies

Stability testing is very important for validated methods in biological samples. The stock solution was stable at the storage conditions (−20 °C) for 3 months (data not shown). All the other stability studies were conducted at two concentration levels (0.6 and 150.0 ng/ml for plasma or 2.5 and 625.0 ng/g for brain tissue) with five determinations for each. Plasma and brain homogenate extracts were stable in the mobile phase in the HPLC autosampler for at least 24 h, indicating that samples could be processed within this period of time (Table 6). The freeze/thaw stability tests indicate CPZ was stable in rat plasma and brain homogenate for three freeze/thaw cycles. The results of bench-top stability indicate that spiked samples were stable for 2 h. The %RE was from 0.39 to 2.59% for plasma and from 1.80 to 3.19% for brain homogenate, and R.S.D. was from 1.20 to 1.98% for plasma and from 0.13 to 2.34% for brain homogenate (see Table 6). Finally, the storage of brain homogenate at room

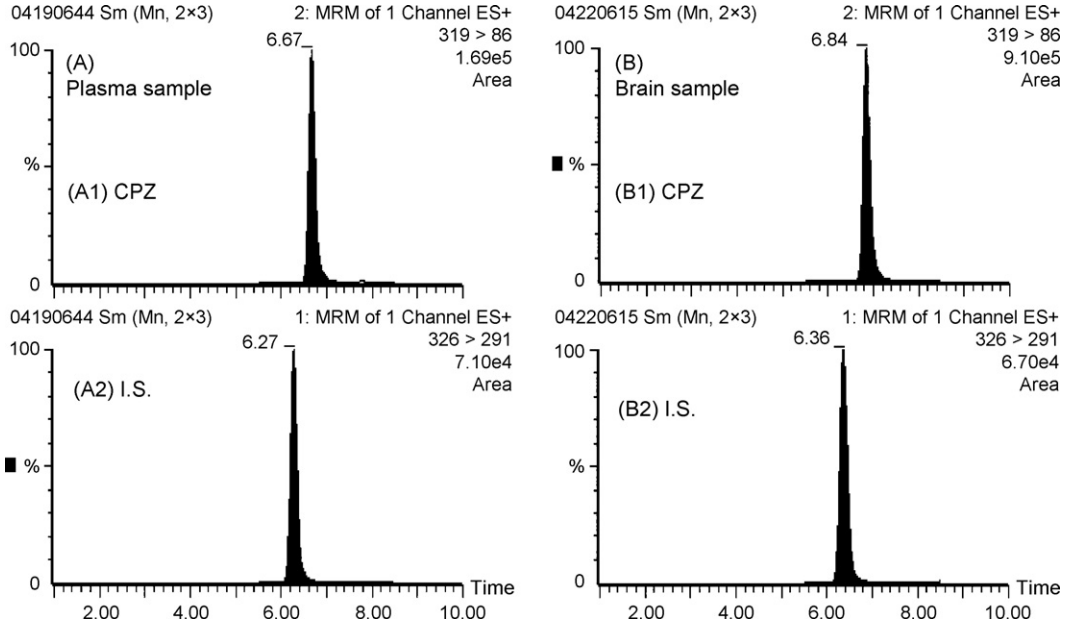

Fig. 4. Representative chromatograms of plasma and brain tissue samples from rats following chronic treatment with CPZ for 90 days at 10.0 mg/kg/day: (A) the concentration of CPZ in the plasma sample was 9.98 ng/ml (A1); (B) the concentration of CPZ in brain tissue sample was 254.73 ng/g (B1).

temperature over 8 h caused an increase in the viscosity of the brain homogenate samples. This increased viscosity of the brain homogenate resulted in reduced recovery of CPZ and the I.S. Therefore, fresh brain homogenate should be analyzed within 2 h or frozen immediately for later analysis.

3.7. Application of the method

The validated method has been successfully used to quantify CPZ concentrations in rat plasma and brain tissue after the chronic treatment of rats with CPZ in their drinking water. The concentration data for CPZ in rat plasma and brain tissue are reported in Table 7. The representative of MRM chromatograms resulting from the analysis of real plasma and brain tissue samples after the chronic treatment of antipsychotic drugs is shown in Fig. 4. The concentration of CPZ in brain tissue is much higher than in plasma. Titier et al. reported the same result [24].

4. Conclusions

A simple, specific and sensitive LC–MS/MS analytical method for the determination of CPZ in rat plasma and brain tissue has been developed and validated. This method provided excellent specificity, wide linear range and a LLOQ of 0.2 ng/ml for plasma and 0.833 ng/g for brain tissue. Liquid–liquid extraction sample preparation was used for 0.25 ml plasma and 0.20 ml rat brain homogenate that provided low matrix effects and high recovery for CPZ. The method is currently being used in a study in which the effects of chronic CPZ treatment on cognitive function in rats are being assessed. Another advantage of this method was that only 0.25 ml for plasma and 0.2 ml for brain homogenate (small sample volume) are necessary for sample preparation. This method will also be used to determine the concentration of CPZ in different brain regions in the future.

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