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Sensitive liquid chromatography/tandem mass spectrometry method for the simultaneous determination of olanzapine, risperidone, 9-hydroxyrisperidone, clozapine, haloperidol and ziprasidone in rat brain tissue

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Abstract

One prerequisite for therapeutic effects of psychiatric drugs is the ability to pass the blood brain barrier. Hence, it is important to know the concentration of antipsychotic drugs in brain tissue. In general, determinations of lipophilic compounds from lipophilic matricies such as the brain are a challenge. Here we have adapted a plasma assay for antipsychotics for the target organ the brain. Using modified sample preparation and chromatographic strategies, the analytes were extracted from rat brain homogenate and analyzed by LC-MS/MS. The method used a Waters Atlantis™ dC-18 (30 mm × 2.1 mm i.d., 3 μm) column with a mobile phase of acetonitrile/5 mM ammonium formate (pH 6.1 adjusted with formic acid) and gradient elution. All analytes were detected in positive ion mode using multiple-reaction monitoring. The method was validated and the linearity, lower limit of quantitation, precision, accuracy, recoveries, specificity and stability were determined. This method was then successfully used to quantify the rat brain tissue concentration of the analytes after chronic treatment with these antipsychotic drugs.

Keywords

Brain; Risperidone; 9-Hydroxyrisperidone; Olanzapine; Clozapine; Ziprasidone; Haloperidol

1. Introduction

It has long been known that chronic exposure to certain antipsychotics, such as haloperidol, often results in cholinergic imbalances in the striatum and consequently abnormalities in motor function. More recently, cholinernergic imbalances leading to impairment in cognition have been shown from exposure to several antipsychotics [1,2]. Given that cognition is now recognized as a key factor that influences the long term functional outcome in schizophrenia, it is important to determine if there is a correlation between antipsychotic brain tissue levels, the target compartment for therapeutic action, and cognitive function. Accurate measurements

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of antipsychotic levels in brain tissue will also facilitate more precise estimates of dopamine D2 receptor occupancy, a factor that has been suggested to serve as a cross-species guideline for deriving clinically relevant antipsychotic doses for animal models [3].

Since the antipsychotic drugs are very active, they are usually administered at low daily dosages. Oral doses of antipsychotics for the treatment of chronic schizophrenia are on the order of a few milligrams per day. At steady state these doses result in plasma levels in the low ng/ml range [4]. However, the concentrations for many antipsychotics, such as risperidone and its active metabolite, 9-hydroxyrisperidone, are very low in brain tissue. The concentration ratios of brain/plasma have been reported as 0.22 for risperidone and 0.04 for 9-hydroxyrisperidone [5]. In order to quantify relevant brain tissue levels of these and other antipsychotics, an analytical method with high sensitivity is required. At present, the determination of some of these drugs in plasma has been established by high performance liquid chromatography (HPLC) with UV detection [6–8], coulometric detection [9], fluorescence detection [10] or electrochemical detection [11,12]. But, to date, very few papers have reported the determination of antipsychotic drugs in brain tissue [5,13–17]. Most of these methods were not validated and did not deal with more than one agent. The usefulness of liquid chromatography-electrospray-tandem mass spectrometry (LC-ESI-MS/MS) with improved sensitivity, selectivity and specificity has been demonstrated for a wide range of applications in the bioanalytical, environmental and pharmaceutical fields [18,19]. Several LC-MS/MS methods were reported for the quantification of the antipsychotic drugs in biological fluids [20–22]. However, no comprehensive method is available to study olanzapine, risperidone, 9-hydroxyrisperidone, clozapine, ziprasidone and haloperidol simultaneously in brain tissue by LC-MS/MS. To our knowledge, this paper is the first validated comprehensive method for determination of antipsychotics in brain tissue and the first method to determine ziprasidone in the brain.

2. Experimental

2.1 Chemicals and reagents

Haloperidol (HAL), risperidone (RISP), clozapine (CLOZ) 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). Midazolam (internal standard, I.S.) was from Sigma (St. Louis, MO, USA). Ethyl acetate, methyl tert-butyl 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 (St. Louis, MO, USA). 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 (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™ dC-18 (30 mm × 2.1 mm i.d.; 3 μm) with a 4.0 × 2.0 mm Phenomenex Security Guard C8 guard column. Mobile phase A consisted of

5 mM ammonium formate in water with pH adjusted to 6.1 using formic acid and mobile phase B was acetonitrile. The flow rate was set 0.3 ml/min. A 15 μl injection of each sample was loaded on to the column, separated and eluted using the following gradient (minutes, % mobile phase B) (0, 15) (1, 15) (5, 50) (10, 72) (10.5, 80) (13.5, 80) (13.6, 15) (18, 15). The column temperature was maintained at 25°C. The LC flow was introduced directly to the mass spectrometer from 5.0–12.0 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. The precursor to product ion transitions along with the cone voltage and collision energy for each analyte and the internal standard are as follows: Olanzapine *m/z* 313 → *m/z* 256, 32 V, 23 eV; Risperidone *m/z* 411 → *m/z* 191, 35 V, 24 eV; 9-Hydroxyrisperidone *m/z* 427 → *m/z* 207, 35 V, 27 eV; Clozapine *m/z* 327 → *m/z* 270, 35 V, 22 eV; Ziprasidone *m/z* 413 → *m/z* 194, 37 V, 26 eV; Haloperidol *m/z* 376 → *m/z* 165, 30 V, 21 eV; Midazolam *m/z* 326 → *m/z* 291, 32 V, 30 eV. The source temperature and capillary voltage were set at 130°C and 3.5 kV, respectively.

2.4 Sample collection

Antipsychotic doses were based on previous rodent studies in which time dependent behavioral and neurochemical effects were detected [1,2,23]. These doses produced plasma levels that approximated those often associated with antipsychotic effects in human [24]. 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/day), RISP (2.5 mg/kg/day), OLZ (10.0 mg/kg/day), ZIP (12 mg/kg/day) and CLOZ (20 mg/kg/day) orally in drinking water for periods above two weeks to reach steady-state concentration of the analytes. 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 OLZ, RISP, 9-OH RISP, CLOZ, HAL, ZIP 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. Combined standard solutions with concentrations of 0.5, 1.0, 2.0, 4.0, 10.0, 20.0, 40.0, 100.0, 200.0, 400.0 and 1000.0 ng/ml were prepared by serial dilution with 5 mM ammonium formate – acetonitrile solutions (30:70). Precision and accuracy standards with concentrations of 0.5, 3.0, 30.0, and 300.0 ng/ml for OLZ, RISP, 9-OH RISP and CLOZ and 1.0, 3.0, 30.0, and 300.0 ng/ml for HAL and ZIP were also prepared in the same manner. A 40.0 ng/ml I.S. standard solution was prepared with 5 mM ammonium formate – acetonitrile solutions (30:70) 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. Fresh standard solutions were 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 200 μl blank brain homogenate. This yielded calibration standard concentrations of 0.208, 0.416, 0.832, 1.664, 4.16, 8.32, 16.64, 41.6, 83.2, 166.4 and 416.0 ng/g. The final concentrations of QCs were 0.208, 1.25, 12.5, 125.0 ng/g for OLZ, RISP, 9-OH

RISP and CLOZ and 0.416, 1.25, 12.5, 125.0 ng/g for HAL and ZIP. The spiked brain homogenate samples (standards and quality controls) were extracted for each analytical batch along with the unknown samples.

2.7 Sample preparation

To 200 μl of rat brain homogenate, 25 μl of internal standard (40.0 ng/ml, midazolam) and 0.4 ml 0.5 M Na_2HPO_4 (pH 10.69) were added. The samples were briefly mixed and extracted with 3 ml isopropyl ether twice. 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 methanol: 20 mM ammonium formate (pH 3.86, adjusted by formic acid) (70:30) was added, ultrasonicated for 1 min, then vortexed and centrifuged at 16000×g prior to LC-MS/MS analysis.

3. Results and discussion

3.1 Method development

We have previously developed and validated a method for the determination of these antipsychotics from plasma [22]. However, we were unable to use this method for the determination of these analytes from brain samples. There were three major obstacles that had to be addressed to adapt this method to brain samples. First, the increased lipophilicity of this matrix greatly reduced the recovery of the analytes. Second, there was a greater level of ion suppression for the analytes during the electrospray process. Finally, tissue samples had a higher buffering capacity which made it more difficult to adjust the pH prior to the liquid-liquid extraction.

When analyzing plasma samples we had used 3 mL of isopropyl ether to extract 250 uL of plasma. This extraction method had provided an absolute recovery of 80–95% of the analytes from the samples. However, when we tried this method with 200 uL of brain homogenate our recoveries were only 30–50%. We found that extracting using a second 3 mL volume of isopropyl ether significantly increased the recovery of our analytes. The high concentration of co-extracted biological materials had an impact on the ion suppression of the system. We found that the sample signal decreased with each successive injection of extracted brain sample which indicated that there was a build-up of biological material, most likely lipids, in the system. Therefore, we altered the chromatographic conditions so that from 10.5 to 13.5 min, high acetonitrile concentration (80 % of mobile phase B) was used to wash the biological residue from the column following each run. This greatly method improved the reproducibility for the analytes from the brain matrix.

We had previously established that adding 0.5 M Na_2HPO_4 (pH 10.69) to samples prior to the liquid-liquid extraction would neutralize the antipsychiotics and improve their transfer to the organic layer. However, we found that brain samples required twice the amount of this basic phosphate solution to achieve comparable extraction efficiency to plasma. With these three changes, we next made sure that the method would provide results of a similar quality to the plasma method.

3.2 Linearity and sensitivity

The calibration curves showed good linear response ($R^2 > 0.997$) over the range from 0.208–416.0 ng/g for OLZ, RISP, 9-OH RISP and CLOZ and 0.416–416.0 ng/g for HAL and ZIP using a $1/x^2$-weighting scheme. The LLOQ, defined as the lowest concentration of analyte with an accuracy within 20% and a precision < 20%, was 0.208 ng/g for OLZ, RISP, 9-OH RISP and CLOZ and 0.416 ng/g for HAL and ZIP in rat brain homogenate. A signal-to-noise (S/N) > 10 at the LLOQ was observed for all analytes.

3.3 Precision and accuracy

Precision and accuracy measurements were acquired for the QC points for each compound. The values for the intra-day precision and accuracy were better than 4.43% and 7.67% for all the analytes. The inter-day precision and accuracy were determined by pooling all validation data (n=15) QC samples. The values for the inter-day precision and accuracy were better than 7.22% and 6.02%.

3.4 Recovery and matrix effect

Absolute recoveries range from 73.6% to 95.0% and relative recoveries from 83.7% to 94.2% for all of the analytes and I.S. The matrix effects of brain homogenate for all of the analytes in this work were less than 16.2% suppression or enhancement.

3.5 Specificity

Representative chromatograms obtained from blank brain homogenate and brain homogenate spiked with the LLOQ standard (0.208 ng/g for OLZ, RISP, 9-OH RISP and CLOZ and 0.416 ng/g for HAL and ZIP) are shown in Figure 1. No interfering peaks from endogenous compounds were observed at the retention times of the analytes or I.S. in blank brain homogenate. Utilization of the major precursor-to-product transition for each compound enhanced method selectivity.

3.6 Stability studies

Stability testing is important for validated methods in biological samples. The stock solutions were stable at the storage conditions (−20°C) for three months (data not shown). All the other stability studies were conducted at two concentration levels (1.25 and 12.5 ng/g) with five determinations for each. Brain homogenate extracts were stable in mobile phase in the HPLC autosampler for at least 24h, indicating that samples can be processed within this period of time. The freeze/thaw stability tests indicate the analytes were stable in rat brain for three freeze/thaw cycles. The results of bench-top stability indicate that spiked samples were stable for all of the analytes for 2h. The RE% was from 1.01% to 6.59% (< 15%) and RSD was from 0.75% to 5.64% for all the analytes. We found the storage of OLZ in rat brain homogenate at room temperature for 12h produced significant degradation (18%) of OLZ. Saracino et al. [16] reported that Vitamin C was added to the brain homogenate to prevent OLZ degradation. In this work, OLZ did not show significant degradation in rat brain homogenate kept at room temperature for up to 2h without Vitamin C addition. However, for longer storage of OLZ, freezing rat brain homogenate is recommended. In addition, the storage of brain homogenate at room temperature over 8h increased the viscosity of brain homogenate. The increased viscosity of brain homogenate resulted in the reduced recovery of HAL, ZIP and the I.S. So freshly prepared brain homogenate should be analyzed during 2h or kept frozen in plastic vials until analysis.

3.7 Application of the method

The validated method was next used to quantify antipsychotic drug concentrations in rat brain tissue following chronic treatment with antipsychotic drugs. The steady-state concentration data for antipsychotic drugs in rat brain tissue are reported in Table 1. The representative MRM chromatograms resulting from the analysis of brain tissue samples are shown in Figure 2. In general, the concentration of RISP and 9-OH RISP from rat brain homogenate were relatively low. However, all the analytes were observed above the LLOQ. Plasma and brain 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 for antipsychotics at a given dose. In order to get the more accurate values of brain to plasma ratio

of the concentration for all of the analytes, the corresponding plasma and brain concentration of the analytes were determined for the same batch of rats. The values of brain to plasma ratio were summarized in Table 1 and compared with published values. The values are similar to previously published values [5,25–27]. In general, the concentration of RISP and 9-OH RISP in brain tissue is much lower than the corresponding concentration in plasma. However, the concentration of CLOZ and HAL is much higher in brain tissue than in plasma. We are the first to report the value for the brain to plasma ratio for ZIP which we observe to be 2.38 (see Table 1). This value is similar to OLZ and intermediate among the various antipsychotics tested.

4. Conclusions

A simple, specific, reliable and sensitive LC-MS/MS analytical method for the simultaneous determination of OLZ, RISP, 9-OH RISP, HAL, CLOZ and ZIP in rat brain tissue has been developed and validated. This method provided a wide linear range and a LLOQ of 0.208 ng/g for OLZ, RISP, 9-OH RISP and CLOZ and 0.416 ng/g for HAL and ZIP. Liquid-liquid extraction sample preparation was used for 0.20 ml of rat brain homogenate with low matrix effects and high recovery. The method was successfully applied to study the effect of chronic treatment of antipsychotic drugs in rats. An advantage of this method was that only 0.2 ml brain homogenate was necessary for sample preparation. The persistence and distribution of antipsychotic drugs in the brain may better explain their long-term effects as well as their side effects [28]. This method appears to be sensitive enough to determine the concentration of these antipsychotic drugs in different regions of the brain.

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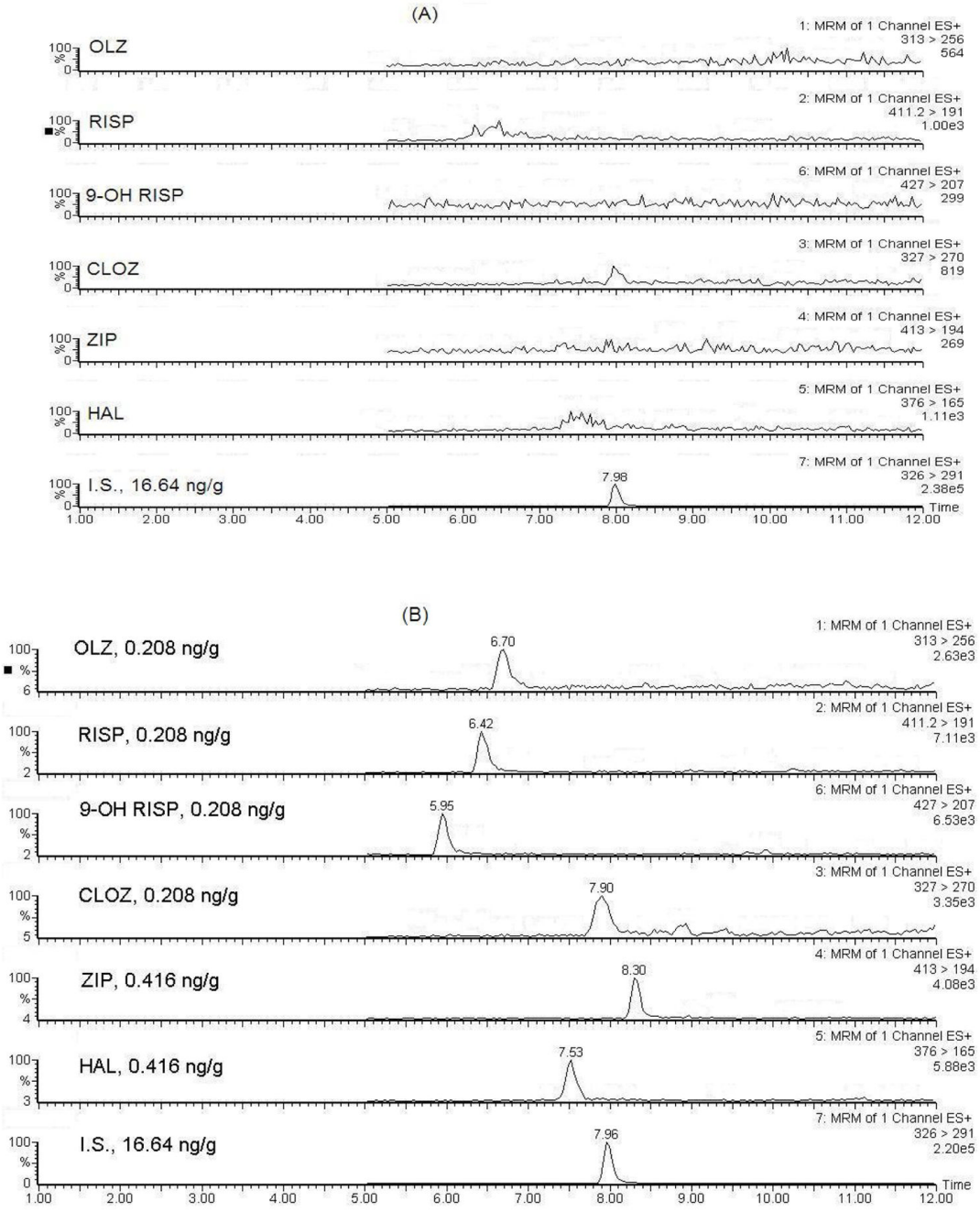

Figure 1.

Representative chromatograms obtained from (A) blank rat brain homogenate; (B) plasma spiked with LLOQ (0.208 ng/g for OLZ, RISP, 9-OH RISP and CLOZ and 0.416 ng/g for HAL and ZIP) concentration for all of the analytes and I.S. (16.64 ng/g).

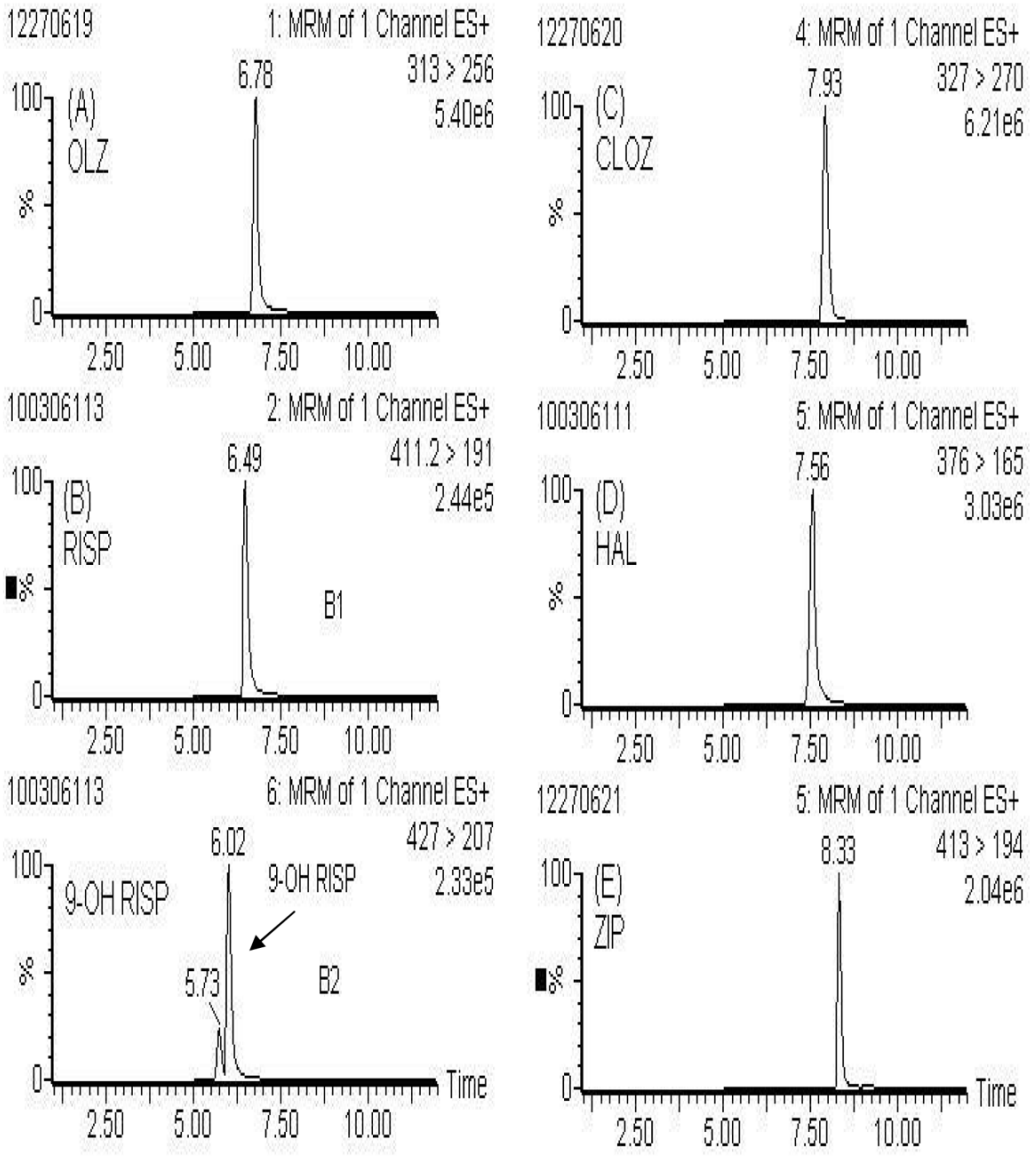

Figure 2.

Representative chromatograms of brain tissue samples from chronic treatment of antipsychotic drugs: (A) a rat treated with OLZ (10.0 mg/kg/day) the concentration of OLZ in brain tissue was 392.93 ng/g; (B) a rat treated with RISP (2.5 mg/kg/day) the concentration of RISP and its active metabolite (9-OH RISP) in brain tissue was 6.5 ng/g (B1) and 7.0 ng/g (B2), respectively; (C) a rat treated with CLOZ (20.0 mg/kg/day) the concentration of CLOZ in brain tissue was 341.56 ng/g; (D) a rat treated with HAL (2.0 mg/kg/day) the concentration of HAL in brain tissue was 300.0 ng/g; (E) a rat treated with ZIP (12.0 mg/kg/day) the concentration of ZIP in brain tissue was 206.25 ng/g.

Table 1

Steady-state brain tissue concentrations of the analytes after the chronic treatment of antipsychotic drugs for rats and the comparison of brain to plasma ratio (N=6).

Drugs administered	Concentrations in plasma (ng/ml ± S.D.)[a]		Concentrations in brain (ng/g ± S.D.)	Brain/plasma ratio	Published brain/plasma ratio[b]
RISP	RISP:	10.88 ± 13.77	5.17 ± 4.28	0.48	0.22
	9-OH RISP:	30.96 ± 24.87	4.85 ± 2.29	0.16	0.04
HAL		18.43 ± 4.88	334.66 ± 63.01	18.16	22
OLZ		175.74 ± 21.91	348.11 ± 44.06[c]	1.98	5.4
CLOZ		28.05 ± 7.60	286.57 ± 64.33[c]	10.22	24
ZIP		103.61 ± 70.79	246.22 ± 73.02[c]	2.38	--

[a] The concentration of all of the analytes in rat plasma were determined using the reported LC-MS/MS method developed by Zhang et al.22

[b] See references: Aravagiri et al.5 for RISP and 9-OH RISP; Sunderland and Cohen25 for HAL; Aravagiri et al.26 for OLZ; Weigmann et al.27 for CLOZ.

[c] n=4.