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Short Communication

Quantitative analysis of olanzapine in rat brain microdialysates by HPLC–MS/MS coupled with column-switching technique

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ABSTRACT

A rapid and sensitive liquid chromatography–tandem mass spectrometric (LC–MS/MS) method coupled with column-switching technique was developed for the determination of olanzapine in rat brain microdialysates. A C8 guard column was used to desalt the samples before analytical separation on a C18 column and detection with tandem mass spectrometry. The mobile phase consisted of methanol/acetonitrile/water (v/v/v, 22.5/22.2/55) was used for desalting and the mobile phase consisted of methanol/acetonitrile/water (v/v/v, 43/43/14) was for analytical separation, water in both mobile phases contained 0.1% ammonium acetate. The lower limit of quantification (LLOQ) for olanzapine was 0.085 ng/ml. The method was linear from LLOQ to 34 ng/ml with a coefficient of determination >0.998. Intra- and inter-day accuracy and precision were determined with variability less than 13.24% (R.S.D). This sensitive method was successfully applied to quantify the concentration of olanzapine in rat brain microdialysates. With this study, the effect of the alcohol extract of *Schisandra sphenanthera* Rehd. et Wils on the concentration of olanzapine in brain was investigated.

1. Introduction

Olanzapine, a thienobenzodiazepine compound, is one kind of the newer antipsychotic drugs used in the treatment of schizophrenia and other psychotic disorders. It is effective in reducing positive and negative symptoms of schizophrenia with low incidence of extra pyramidal symptoms [1]. Many antipsychotic drugs are substrates of P-glycoprotein (P-gp) including olanzapine [2,3]. Researchers argue that the efficacy of antipsychotics is determined by the capability penetrating into blood brain barrier and the capability binding to the action targets. The efflux effect of P-gp in BBB is one of the most important factors which influence the antipsychotics' penetration into the brain [4–6].

Recently studies indicate that dibenzocyclooctadiene lignans, the major constituents of *Schisandra sphenanthera* Rehd. et Wils, are a novel class of P-gp inhibitors, which can fully restore the intracellular drug accumulation in multidrug resistance cell lines and overcome the P-gp mediated drug resistance [7–9]. Schisandrin A and schisandrin B have the strongest effect on inhibiting P-gp among all of the dibenzocyclooctadiene lignans. Such results suggest that *S. sphenanthera* Rehd. et Wils could potentially increase the drugs' capability penetrating into blood brain barrier by inhibiting the efflux effect of P-gp on its substrates. Five Esters Capsule, the pharmaceutical preparation of the alcohol extract of *S. sphenanthera* Rehd. et Wils, contains 11.25 mg schisandrin A in each capsule. As its strong hepatoprotective effect, Five Esters Capsule is widely used by schizophrenia patients in China by oral administration at a dosage of 22.5 mg once, three times a day. But there is no report about metabolism kinetics of olanzapine when co-administrated with *S. sphenanthera* Rehd. et Wils. This work is to study the effect of *S. sphenanthera* Rehd. et Wils on the concentration of olanzapine in the brain when co-administrating.

Verapamil, an antiarrhythmic agent to control supraventricular tachyarrhythmia, is also a short-term inhibitor of P-glycoprotein, which has been used to increase the therapeutic effectiveness of cytotoxic anticancer drugs in cancer chemotherapy [10]. More recently, P-glycoprotein reversal agents including verapamil have been demonstrated to alter the pharmacokinetic properties of coadministrated agents in therapeutic areas other than oncology [11]. Now verapamil has been widely used as an inhibitor of P-gp in both animal and *in vitro* studies.

Microdialysis is a powerful technique for collecting free drug from any tissues including the brain. Furthermore, the cleanup procedure of the sample often can be omitted [12]. However, since

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the samples' sizes of microdialysates are generally in the microliter range with low concentrations, a sensitive analytical method is required.

For the determination of olanzapine, high-performance liquid chromatography (HPLC) methods with UV [13,14] or photodiode array detection [15], electrochemical detection (ECD) [16,17], mass spectrometry (MS) detection [18,19] are widely used. The shortcoming of all these methods is their limited specificity. Quite recently, HPLC methods combined with MS/MS for very low-level quantification of olanzapine in biological samples have been described with subnanogram per milliliter quantitation [20–23]. In all these reported methods, complicated sample cleanup procedure is needed. And such methodology mentioned above needs large sample volume to get a satisfactory sensitivity, which is obviously not adapted for analysis in microdialysates with volumes smaller than 100 μl. As far as we known, there is no method reported about quantification of olanzapine in microdialysates.

Thus, the goal of present work is to develop a sensitive detection method for determination of olanzapine in rat brain microdialysates, and use microdialysis technology to monitor extracellular levels of olanzapine in the rat brain following coadministration of *S. sphenanthera* Rehd. et Wils.

2. Experimental

2.1. Chemicals and reagents

Olanzapine and quetiapine (the internal standard, IS) were purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Verapmil was obtained from Sigma–Aldrich Company (St. Louis, MO, USA). The alcohol extract of *S. sphenanthera* Rehd. et Wils was donated by Sichuan HYGIEN Pharmaceutical Company (Sichuan, China) which contained 20.9% schisandrin A. Acetonitrile and menthol (HPLC grade) were purchased from Merck (Darmstadt, Germany). Ammonium acetate (HPLC grade) was obtained from Tedia (Ohio, American). Water used in the experiment was deionized and purified by a Milli-Q system (Millipore, Bedford, MA, USA). Chemical structures of olanzapine and quetiapine are shown in Fig. 1.

2.2. Instrument

The HPLC system was Waters Acquity ultraperformance liquid chromatography system (Waters, Milford, MA, USA) coupled with an auxiliary Waters 515 pump (Waters, Milford, MA, USA). The mass spectrometer was a Micromass Quattro Premier XE tandem quadrupole mass spectrometer (Waters, Manchester, UK) equipped with an electrospray ionization (ESI) interface. Data acquisition was carried out by MassLynx 4.1 software.

2.3. Liquid chromatographic and mass spectrometric conditions

An Ultimate XB-C8 column, 2.1 mm × 30 mm, 5 μm particle size (Welch, Shanghai, China) was used as the purification column and an Ultimate AQ-C18 column, 2.1 mm × 150 mm, 3.0 μm particle size (Welch, Shanghai, China) was used for analytical separation. The outlet of the analytical column was directly connected to the mass spectrometer.

Both of the two pumps were operating at a flow rate of 0.3 ml/min. One pump (pump A) was used for the sample purification with the mobile phase consisted of methol/acetonitrile/water (v/v/v, 22.5/22.2/55), the auxiliary Waters 515 pump (pump B) was used for analytical separation with the mobile phase consisted of methol/acetonitrile/water (v/v/v, 43/43/14), water in both mobile phases contained 0.1% ammonium acetate.

A 20 μl sample was loaded on the Ultimate XB-C8 column using pump A with the switching valve in position 1 and desalted for 1.55 min. Concurrently isocratic flow at 0.3 ml/min *via* the analytical column, which was eluted with methol/acetonitrile/water (0.1% ammonium acetate) (v/v/v, 43/43/14), was maintained to the mass spectrometer by means of pump B. Thereafter, the switching valve was switched to position 2 allowing the analyte being eluted from the purification column onto the analytical column by pump B. The flow from pump A was diverted directly to waste. At 2.65 min, the switching valve was switched back to position 1 and the configuration of the online column switching system reverted back to initial conditions as described for the sample loading above. Total run time was 4.5 min.

The detector was used in the positive ion mode with ESI using multiple-reaction monitoring (MRM) to monitor the mass transitions. The MRM transitions were *m*/*z* 313 to *m*/*z* 256 for olanzapine and *m*/*z* 385 to *m*/*z* 253 for quetiapine (Fig. 2). The source temperature was set to 120 °C and the desolvation temperature to 350 °C. The cone gas (N_2) flow and the desolvation gas (N_2) flow were 70 and 900 L/h, respectively. The collision gas was argon, operating at 0.12 ml/min. The capillary voltage was set to 0.8 kV. The cone voltage was set to 28 V for olanzapine and 42 V for IS. The collision energy was 21 eV for olanzapine and 22 eV for IS. The dwell time established for each transition was 0.5 s.

2.4. Stock solutions and spiked samples

Ringer's solution, which was used as perfusate for the microdialysis probes, consisted of 154 mM sodium chloride, 1.6 mM potassium chloride and 2.2 mM calcium chloride. Individual stock solutions of olanzapine and IS (quetiapine) were prepared by dissolving approximate amounts of drugs in absolute methanol to obtain final concentrations of 1.70 μg/ml and 1.55 μg/ml respectively, and they were stored at −20 °C. The internal standard (IS) working solution, quetiapine, was prepared by diluting 0.2 ml IS

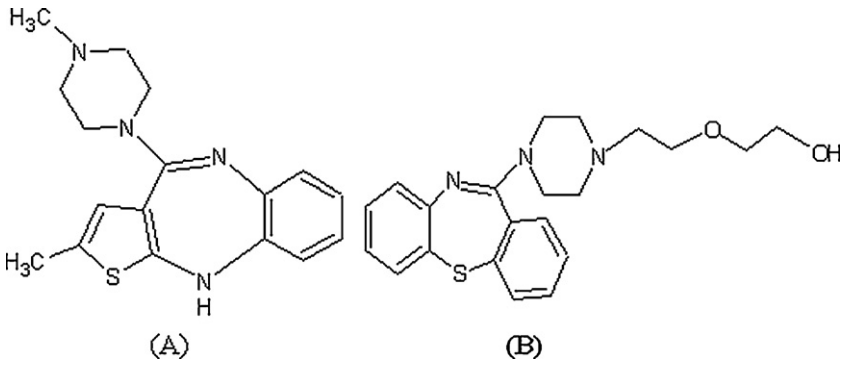

Fig. 1. Chemical structures of olanzapine (A) and quetiapine (B).

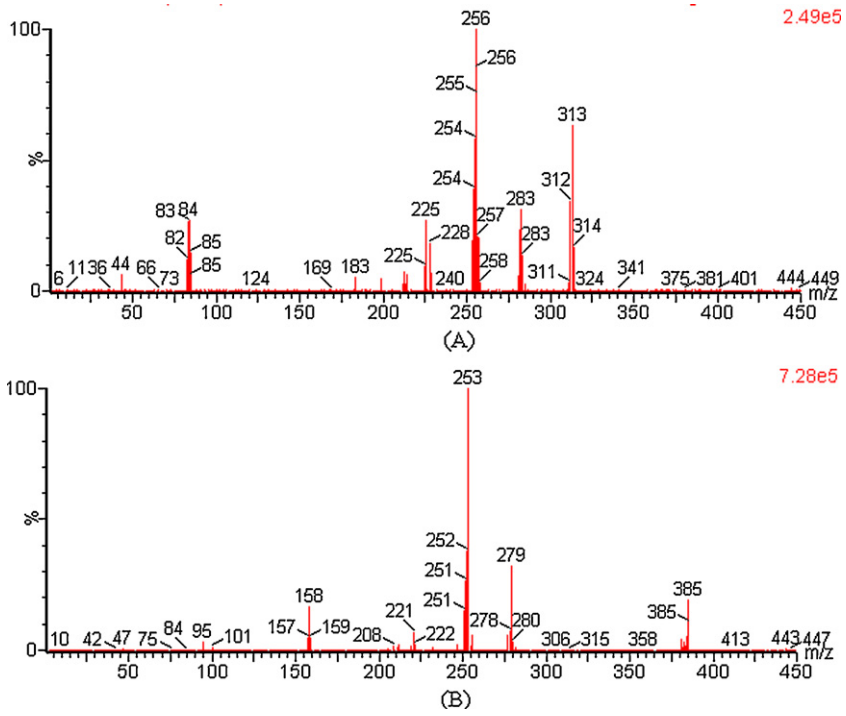

Fig. 2. Daughter ion scan of olanzapine (A) and quetiapine (B).

stock solution with 63.8 ml menthol, 80 ml water and 16 ml 25% Vit C. IS working solution was stored at −20 °C when not being used and replaced every 5 days. Standard curves for olanzapine at concentrations of 0.085, 0.17, 0.34, 0.85, 1.7, 3.4, 8.5, 17, 34 ng/ml were prepared by serial dilutions with drug free dialysates (Ringer's solution). Ninety microliters of IS (1.94 ng/ml) were added to 90 μl of the calibration standard solution. After vortex mixing for 3 min, the mixture was centrifuged at 4 °C for 10 min at 12,000 × *g*. 150 μl supernate was aspirated into vials, 20 μl was injected into instument for analysis. The samples were prepared as the same, when the volume of microdialysate was only 45 μl, 45 μl blank dialysate was added and vortexed, making the total volume to 90 μl, then its prepare procedure was just like above-mentioned. The samples prepared were kept at 15 °C in the autosampler.

2.5. Method validation

2.5.1. Accuracy and precision

The intra-day accuracy and precision were calculated by analyzing five replicates of Ringer's solution containing olanzapine at three concentrations: 0.17, 1.7 and 17 ng/ml. The inter-day accuracy and precision were determined by analyzing the three concentrations on five replicates on three separate days.

2.5.2. Stability

The stability of olanzapine in Ringer's solution was evaluated under different temperatures and storage conditions. Samples of olanzapine were subjected to room temperature, −70 °C, and three freeze-thaw cycles. All stability studies were conducted at three concentrations of olanzapine (0.17, 1.7 and 17 ng/ml) with three determinations each.

2.6. Microdialysis experiments and sample collection

2.6.1. Surgical techniques

Male Sparague-Dawley rats weighing 250–300 g were used for all experiments (Slac Laboratory Co., Ltd., Shanghai, China). The rats were housed in temperature controlled rooms with access to food and water *ad libitum* prior to surgery. All animal experiments were performed in strict accordance with the protocol approved by the Institutional Animal Care and Use Committee of Central South University. The rats were anesthetized by an intraperitoneal injection of chloral hydrate solution (400 mg/kg). Microwaveable heating pad was used to maintain the animals' body temperature. Heads of the rats were shaved before placing them in a stereotaxic apparatus. An incision was made in the scalp, the bregma was located and used as the reference point for positioning the microdialysis probe. An intracerebral guide cannula was stereotaxically inserted through a cranial burr hole made by a skull drill, using the following coordinates, in relation to the bregma: −3.2 mm anterior, −0.8 mm lateral and −6.0 mm ventral, according to the rat stereotaxic atlas [24]. When experimenting, the guide cannula was replaced by the brain microdialysis probes (CMA/12) with 4 mm membranes which were purchased from CMA/Microdialysis AB Inc. (CMA, Stockholm, Sweden).

2.6.2. Recovery of olanzapine by microdialysis sampling

The retrodialysis method was utilized to obtain the *in vivo* recovery for probe calibration. The brain microdialysis probe was inserted into the prefrontal cortex. Ringer's solution with olanzapine (100 ng/ml) was perfused through the probe at a constant flow rate of 1.5 μl/min using an infusing pump (CMA/402). One hour after the probe implantation, the perfusate (C_{perf}) and dialysate (C_{dial}) of olanzapine were determined by LC–MS/MS. The relative *in vivo* recovery (R_{dial}) of olanzapine across the dialysis membrane was defined as $R_{dial} = (C_{perf} - C_{dial})/C_{perf}$. The concentrations ($C_m$) of olanzapine in microdialysate were converted to the actual unbound concentrations in rat brain (C_u) as follows: $C_u = C_m/R_{dial}$.

2.6.3. Dosing and sampling

For dosing purpose, 16 mg olanzapine was dissolved in 20 ml deionized water acidified with citric acid (pH 5) which make the final concentration to be 0.8 mg/ml. 100 mg verapamil and 120 mg alcohol extract of *S. sphenanthera* Rehd. et Wils were dissolved in

20 ml 0.5% CMC-Na solution respectively which make the final concentration to be 5 mg/ml and 6 mg/ml. Rats in the verapamil group and schisandra group were given daily doses of verapamil 25 mg/kg or the alcohol extract of *S. sphenanthera* Rehd. et Wils 30 mg/kg by oral gavages for 7 consecutive days in the morning (09:00–11:00 h). The control group was received 0.5% CMC-Na solution without drugs instead for 7 days. On the eighth day, after probes implantations, the probes were perfused with Ringer's solution at a flow rate of 1.5 μl/min and allowed to equilibrate for 1 h. Subsequently, rats in different groups received drug administrations as the same as the passed 7 days. Twenty minutes after the first administration, rats were given an oral bolus dose of 4 mg/kg of olanzapine. Six rats were used in each group. The sampling interval was 30 min in the first 6 h and then turned to be 60 min in the next 14 h. Samples were kept at −70 °C until analysis.

2.7. Statistics

The results were represented as mean ± standard deviation. Statistical analysis was performed by one-way ANOVA followed by SNK-q test to compare the control group with the verapamil group and schisandra group. The level of statistical significance was set at $p \leq 0.05$.

3. Results and discussion

3.1. Method development

To the best of our knowledge, to date there are no publications or reports which focus on the development and validation of analytical method using HPLC–MS/MS for the quantitative determination of olanzapine in microdialysates.

During the initial stage of method development, different mobile phases were investigated. Ammonium acetate added in the mobile phase was helpful to get a better peak shape. Some studies had reported that olanzapine was astable and oxidated easily. But in our study, we found that the absorbability of olanzapine induced great problem for its accurate determination especially when the concentration was low. That is why high concentration of Vit C was used in this experiment. Experiment proved that high concentration of Vit C can solve the absorbability problem of olanzapine well.

3.2. Matrix effects results

Although the protein- and cell-free microdialysates are clearer than other biologic matrix, they are not suitable for direct injection in LC–MS/MS analysis as there are a large amount of salts. Using the column switching technique, most of the salts in microdialysates can be efficiently washed to the waste, so the matrix effect can be reduced or avoided. Matrix effects were assessed by comparison of standards in Ringer's solution and in water at three concentrations (0.17, 1.7 and 17 ng/ml). The comparison of standards prepared in Ringer's solution and in water showed no significant difference in the peak areas, so there was no significant matrix interference existed.

3.3. Method validation

3.3.1. Selectivity

The selectivity of the method was tested by analyzing drug-free brain microdialysate from rats proposed to ensure no interference with olanzapine and IS. No other endogenous peaks were observed. Representative chromatograms for a blank sample, a LLOQ and a study sample are presented in Fig. 3.

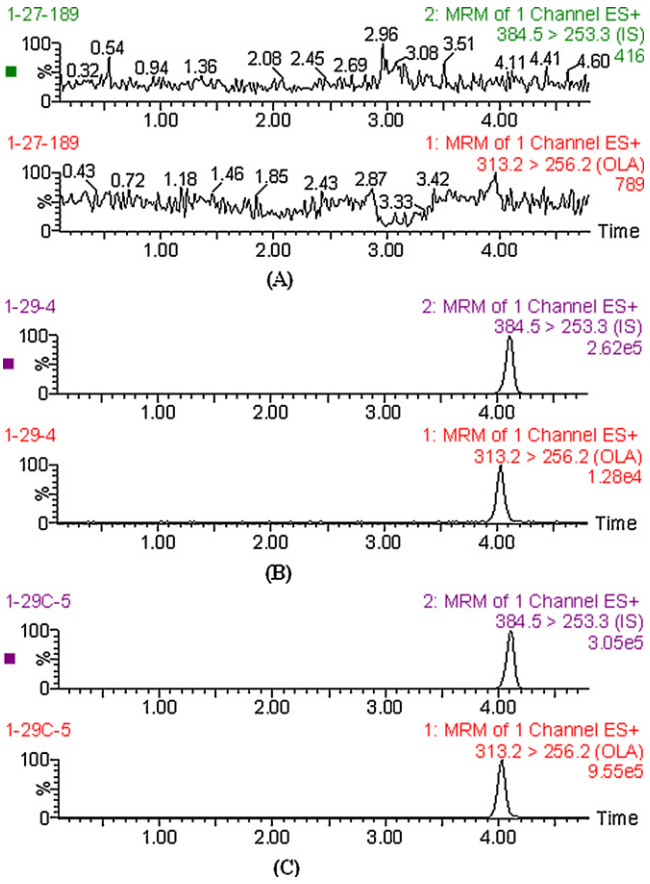

Fig. 3. Representative chromatograms: (A) blank brain microdialysate; (B) the lowest limit of qauntitation: blank brain microdialysate spiked with 0.085 ng/ml olanzapine and (C) brain microdialysate in rat 2.5 h after intragastric administration with olanzapine which concentration is 5.04 ng/ml.

3.3.2. Linearity, accuracy and precision

The calibration curve was constructed by plotting the peak area ratio of olanzapine to the IS *versus* the concentration of olanzapine. The regression equation was $y = 0.6183x - 0.0087$ ($r^2 = 0.9983$). The calibration data revealed good linear correlations. The lowest limit of detection (LLOD) or quantitation (LLOQ) were estimated as the amount of olanzapine that resulted in a signal three times or ten times than the noise (S/N ≥ 3 or S/N ≥ 10). The LLOD and LLOQ were calculated to be 0.017 ng/ml and 0.085 ng/ml, respectively. The LLOQ values suggest that the LC–MS/MS procedure established is far more sensitive than the method reported previously. The results of intra-day and inter-day accuracy and precision are summarized in Table 1. For all the samples evaluated, the variability was less than 13.24% (R.S.D)

3.3.3. Stability

Stability data are shown in Table 2. The results showed that olanzapine was stable under ambient temperature for 12 h, and −70 °C for 45 days. It has good freeze-thaw stability. All the samples evaluated displayed variability of less than 9.66% (R.S.D).

3.3.4. Microdialysis probe relative recovery

Based on the redialysis experiments, the relative recovery was determined to be 16.59 ± 2.38% for brain probes. The concentrations of olanzapine determined in microdialysates were corrected by the relative recovery of the probe used.

Table 1
Inter-day and intra-day precision for the determination of olanzapine in Ringer's solution ($n = 5$).

Concentration (ng/ml)	Inter-day precision		Intra-day precision	
	Mean measured	R.S.D (%)	Mean measured	R.S.D (%)
0.17	0.163	11.8	0.175	13.14
1.7	1.757	7.4	1.783	8.81
17	16.461	7.25	16.769	7.34

Table 2
Stability of olanzapine under different conditions ($n = 3$).

Storage conditions	Concentration (ng/ml)		R.S.D (%)
	Nominal	Mean measured	
Freeze-thaw stability	0.17	0.167	6.59
	1.7	1.811	9.66
	17	17.119	6.47
Stability at room temperature (12 h)	0.17	0.179	7.82
	1.7	1.649	7.32
	17	17.934	9.05
Stability at −70 °C (45 days)	0.17	0.179	7.26
	1.7	1.791	8.71
	17	16.297	9.31

Table 3
Pharmacokinetic data of olanzapine (4 mg/kg, oral gavages) in rat brain in different treatments. Data are expressed as mean ± standard deviation means from six individual rats at each group: AUC, area under the curve; $t_{1/2}$, half-life; C_{max}: maximum concentration.

Parameters	Control group	Verapamil group	Schisandra group
$AUC_{0-20\,h}$ (ng h/ml)	294.14 ± 52.78	1377.13 ± 409.61*	289.62 ± 81.60
$AUC_{0-\infty}$ (ng h/ml)	300.94 ± 57.45	1431.91 ± 420.15*	296.78 ± 83.42
C_{max} (ng/ml)	93.91 ± 35.91	217.32 ± 82.55*	75.25 ± 34.74
t_{max} (h)	1.25 ± 0.32	1.50 ± 0.52	1.50 ± 0.42
$t_{1/2}$ (h)	2.64 ± 0.61	3.82 ± 0.79	3.25 ± 0.96

* $p < 0.05$; difference is significant compared to the control.

3.4. Application to brain microdialysis analysis of olanzapine

This method had been used successfully in the determination of olanzapine in microdialysates. Pharmacokinetic data of olanzapine in different treatments is shown in Table 3. The concentrations *versus* time curves in rat brain after olanzapine (4 mg/kg) administration are shown in Fig. 4. Compared with control group, verapamil increased the C_{max} and $AUC_{0-20\,h}$ of olanzapine in brain by 131.41% and 375.81% respectively, which difference had statistic significance. The concentration of olanzapine in rat brain had no significant changes when coadministrated with the clinical dose of alcohol extract of *S. sphenanthera* Rehd. et, which mean that the *S. sphenanthera* Rehd. et had no effect on the concentration of olanzapine in brain when co-administrating clinically.

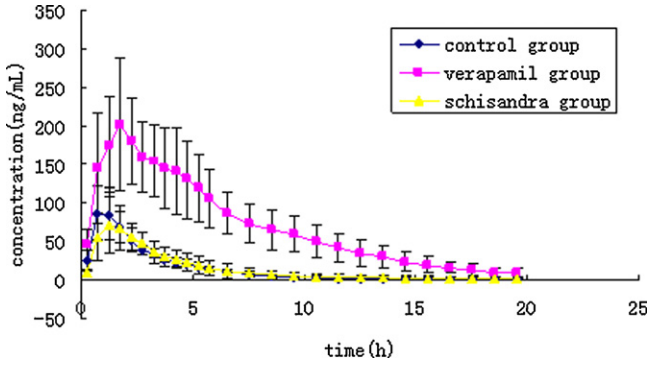

Fig. 4. Concentration–time profiles for olanzapine in brain microdialysates after oral gavages administration of olanzapine at the dosage of 4 mg/kg. The data are represented as mean ± standard deviation means from six individual microdialysis experiments for each group.

4. Conclusion

In conclusion, the methodology of HPLC–MS/MS coupled with column-switching technique for the determination of olanzapine in rat brain microdialysates was developed. This method was highly sensitive and rapid, which provided a new assistance for determination of olanzapine in complicated biologic matrix. Our study proved that clinical dose of alcohol extract of *S. sphenanthera* Rehd. et Wils had no effect on the concentration of olanzapine in brain, but verapamil could significantly increase olanzapine's concentration in rat brain.

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