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Quantitative determination of olanzapine in rat brain tissue by high-performance liquid chromatography with electrochemical detection

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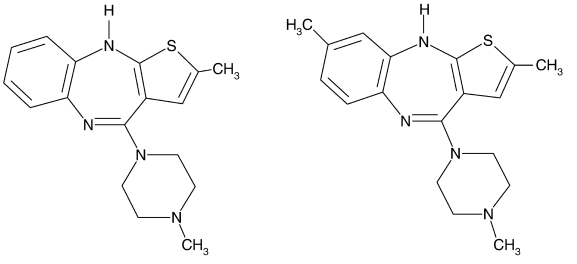
Abstract

A sensitive assay was developed for the measurement of olanzapine in rat brain tissue using HPLC with electrochemical detection. The assay has a lower limit of quantitation of 0.5 ng/ml in tissue homogenate and utilizes a liquid–liquid extraction followed by reversed-phase HPLC for the quantitative analysis of olanzapine. The method provided a linear response for olanzapine over a concentration range of 0.5–100 ng/ml with a coefficient of determination (r^2) greater than 0.9995. The extraction efficiencies of olanzapine and internal standard (LY170158) were greater than 82% in brain tissue. The intra-assay and inter-assay relative errors ranged from −5.38 to 17.60% and −3.25 to 10.53%, respectively. The intra-assay and inter-assay RSD values were in the range of 1.12 to 6.96% and 3.78 to 6.68%. Long-term stability studies showed that brain tissue homogenate samples spiked with olanzapine and internal standard are stable at −70°C for at least 110 days. However, a room temperature stability study showed that olanazapine was not stable in brain homogenate if the sample was exposed at 25°C longer than 2 h. This method has been used for the study of the disposition and pharmacokinetics of olanzapine in male Sprague–Dawley rats.

Keywords: Olanzapine

1. Introduction

Olanzapine {LY170053; 2-methy1-4-(4-methyl-1-piperazinyl)-10*H*-thieno[2,3-*b*][1,5]benzodiazepine, Fig. 1} is an atypical neuroleptic drug for the treatment of schizophrenia. Pharmacological research has demonstrated that olanzapine has nanomolar receptor affinity for dopamine D_1–D_5, serotonin $5HT_{2A/2B/2C}$, $5HT_3$ and $5HT_6$ receptors. In addition, olanzapine is a potent antagonist of α_1-adrenergic and histamine H_1 receptors. It also shows relatively weaker potency for five known subtypes of muscarinic receptors [1–3]. Animal studies indicated that



Olanzapine LY170158 (Internal standard)

Fig. 1. Chemical structures of olanzapine {2-methy1-4-(4-methyl-1-piperazinyl)-10*H*-thieno[2,3*b*][1,5]benzodiazepine} and the internal standard LY170158 {2,8-dimethyl-4-(4-methyl-1-piperazinyl)-10*H*-thieno[2,3*b*][1,5]benzodiazepine}.

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olanzapine has similar atypical pharmacological profiles to clozapine [1]. It was effective in the treatment of both positive and negative symptoms of schizophrenia with low incidence of extrapyramidal motor symptoms [4,5]. Bioanalytical methods for the quantitation of olanzapine were developed to meet the requirements of drug disposition studies and therapeutic drug monitoring. The first published quantitation method for olanzapine was developed by Goodwin et al. using gas chromatography–mass spectrometry (GC–MS). Although this assay had a high sensitivity of 0.1 ng/ml in human plasma, it involved several extraction steps and derivatization [6]. Several high-performance liquid chromatography (HPLC) and liquid chromatography (LC)–MS methods have subsequently been reported for the quantitative determination of olanzapine in plasma or serum using solid-phase extraction [7–10]. Additionally, a few papers reported the determination of olanzapine in human plasma and serum using liquid–liquid extraction for therapeutic drug monitoring [11,12]. To the best of our knowledge, no assays have been published for the quantitation of olanzapine in tissue using HPLC. In order to determine the concentration of olanzapine in animal brain tissue and study its pharmacokinetic properties in the central nervous system, a sensitive analytical assay was developed utilizing a one-step liquid–liquid extraction. A structural analog, LY170158 (Fig. 1), was used as the internal standard. This assay was validated over the range of 0.5–100 ng/ml and used to study the pharmacokinetics of olanzapine in the brain of male Sprague–Dawley rats.

2. Experimental

2.1. Reagents

Olanzapine (LY170053) and internal standard, LY170158 (Fig. 1), were synthesized at Eli Lilly Research Laboratories. Rat brains were collected from F-344 male rats after perfusion with 0.9% saline. Rat brain homogenate was prepared by adding 4 ml distilled water per gram of brain and blending with a Polytron homogenizer (Kinematica, Switzerland). HPLC-grade methanol, acetonitrile and dichloromethane were purchased from Burdick & Jackson (Muskegon, MI, USA). Cyclohexane (ACS reagent grade), was obtained from J.T. Baker (Phillipsburg, NJ, USA). Sodium carbonate anhydrous, *n*-propanol and 85% phosphoric acid (analytical-reagent grade), were obtained from Mallinckrodt (Paris, KY, USA). Deionized water was prepared with a Milli-Q water purification system. All glassware that contacted olanzapine were silylated according to a modified silylation method [13].

2.2. Preparation of standard curve solutions and quality control/validation solutions

Stock solutions of olanzapine and internal standard were prepared by dissolving 2 mg of pure substances into 20 ml of *n*-propanol by sonication. Two olanzapine stock solutions were prepared; one for the purpose of spiking standard curve samples and a second for spiking quality control (QC) and method validation samples. The olanzapine stock solutions were diluted with *n*-propanol to obtain 10, 1, 0.1 and 0.01 μg/ml working solutions. The internal standard stock solution was diluted with *n*-propanol to obtain a 0.5 μg/ml working solution. Both stock and working solutions of olanzapine and internal standard were stored at 4°C and were stable for at least 3 months.

2.3. Chromatographic apparatus

The chromatographic analysis was performed on a HPLC system consisting of a Hitachi L6200A solvent delivery pump (Tokyo, Japan), a Waters 717 autosampler (Milford, MA, USA) and an ESA 5100A Coulochem detector (Chelmsford, MA, USA). Separation of analytes was accomplished using a YMC Basic HPLC column (5 μm, 150×4.6 mm) which was maintained at 40°C using an Eppendorf column heater (Model CH-30, Westbury, NY, USA). The HPLC mobile phase consisted of 75 m*M* phosphate buffer (pH 7.0)–methanol–acetonitrile (48:26:26, v/v/v) with a flow-rate of 1.2 ml/min. The chromatographic peaks were detected by an ESA Coulochem II electrochemical detector (Model 5100A) with a dual analytical cell (Model 5011). The potentials of cell 1 and cell 2 in the dual analytical cell were set at −0.2 V and 0.2 V, respectively. A Model 5010 guard cell was installed

before the autosampler and its potential set to −0.3 V. The detector signal output range for cell 2 was monitored at 200 nA. The peak height integration for olanzapine and internal standard were performed using a Perkin-Elmer Nelson Access*Chrom data acquisition system (Cupertino, CA, USA).

2.4. Extraction procedures

A liquid–liquid extraction method was applied to isolate olanzapine and the internal standard from brain tissue samples. Brain tissue homogenate (0.5 ml per sample) was transferred to a 125×16 mm silylated screw cap glass tube to which 1 ml of 0.5% sodium carbonate and 30 μl working internal standard solution were added. A 5-ml aliquot of organic solvent (15% dichloromethane in cyclohexane) was added to the tube. The samples were sealed with PTFE lined caps and extracted by inversion (approximately 40 cycles per min) for 15 min. The extracted samples were centrifuged for 15 min at 1784 *g* for separation of the organic and aqueous phases. The organic phase was transferred into a 100×16 mm silylated glass tube and evaporated to dryness under nitrogen at 50°C. Following drying, the residue was dissolved in 200 μl of mobile phase by vortexing then sonicating for approximately 1 min. The samples were transferred to polypropylene autosampler vials for injection. Care should be taken to dry the samples for the minimum time necessary and to reconstitute them immediately. Extended drying times and/or extended delays before reconstitution have been seen to cause degradation of the samples. Previous studies have shown this degradation to be due to oxidation of the olanzapine molecule [10].

2.5. Calibration curve

A standard curve with eight points was prepared for each HPLC analysis during the validation and analysis of unknown samples. Standard curve samples were prepared by spiking blank brain homogenate with olanzapine working solutions (0.5 to 100 ng/ml) to obtain concentrations of 0.5, 1, 2.5, 5, 10, 25, 50 and 100 ng/ml. A quadratic least-square regression with a weighting factor of $1/y$ was used for standard curve calibration.

2.6. Assay validation procedures

A 3-day assay validation was performed to evaluate the selectivity, extraction efficiency, stability, inter-day and intra-day accuracy and precision of olanzapine in brain tissue homogenate.

Rat brain tissues from five animals were used to evaluate selectivity. Blank samples were extracted and analyzed by HPLC for potential interfering peaks within the range of the retention time of olanzapine and the internal standard.

The extraction efficiency was determined by spiking olanzapine at concentrations of 25 and 100 ng/ml into blank brain tissue homogenate and comparing peak heights of extracted and neat standards prepared at the same concentrations.

The stability of olanzapine in brain tissue was studied under conditions based on different storage and processing procedures. Long-term frozen storage stability was conducted by spiking olanzapine in blank tissue homogenate at concentrations of 25 and 100 ng/ml. One set of samples was analyzed on day 0 before freezing while the remaining samples were aliquoted, stored at −70°C and analyzed on days 7, 14, 29 and 110. Room temperature stability was studied by incubating 25 and 100 ng/ml samples at 25°C and analyzing them at 0, 0.5, 1, 2 and 4 h. Extract stability was performed to evaluate the analyte stability in reconstitution solvent (mobile phase) following extraction. Triplicate samples were prepared at concentrations of 25 and 100 ng/ml. After extraction, samples were maintained at room temperature and injected at 0, 24 and 48 h following extraction. Stability of the analyte after one and two freeze–thaw cycles (from −70°C to 25°C) was investigated at concentrations of 25 and 100 ng/ml.

The effects of sample dilution were studied by mixing blank brain homogenate with spiked samples. Triplicate samples at 100 ng/ml were diluted 1-, 5-, 10- and 20-fold and analyzed.

2.7. Rat brain tissue pharmacokinetic study

Male Sprague–Dawley rats (body mass approximately 250 g) received from Harlan Sprague–Dawley were acclimated for 3–5 days before initiation of the tissue pharmacokinetic study. Olanzapine was administered subcutaneously at a dose of 3 mg/kg.

Animals were anesthetized using carbon dioxide and brain tissue was collected at the time points of 0.25, 0.5, 0.75, 1, 2, 3, 5, 8, 16, 24 and 48 h (three animals per time point). Before excision, the brains were perfused with saline to remove the blood in the tissue. A 4-ml volume of distilled water per gram of brain was added and the brain homogenized for approximately 20 s. The brain homogenate was frozen immediately on dry ice and stored at −70°C before analysis.

3. Results and discussion

3.1. Chromatography and selectivity

Representative HPLC chromatograms of olanzapine in rat brain homogenate extracts are shown in Fig. 2. Olanzapine and internal standard were detected at 7.5 and 10.5 min, respectively. No interfering peaks were observed in the range of 7 to 11 min for blank brain samples from five animals. Metabolites of olanzapine have been observed in plasma and urine from animals dosed with olanzapine [9,14]. These metabolites were more polar than olanzapine and eluted before olanzapine under HPLC conditions very similar to this assay [8]. Using this assay, no metabolites were observed in brain samples from animals dosed with olanzapine.

3.2. Linearity and sensitivity

Calibration curves for olanzapine (0.5–100 ng/ml) were obtained using quadratic least-squares regression of the peak height ratio (analyte peak height/internal standard peak height) versus concentrations. A weighting factor of $1/y$ was applied to each standard curve. Excellent linearity was achieved in 3 validation days within the specified concentration range. The correlation coefficients of the calibration regression lines were greater than 0.9995. The lower limit of quantification (LLQ) is defined as the lowest concentration on the calibration curve for which the relative error and relative standard deviation (RSD) are <20%. The current assay has an LLQ of 0.5 ng/ml of brain tissue homogenate. The inter-assay accuracy and precision values were less than 11% at the 0.5 ng/ml level (Table 1). Due to the dilution of the unknown brain samples with distilled water before homogenization, a 5× dilution factor was applied to yield results with units of ng olanzapine per gram of brain. Therefore, the effective lower limit of quantitation is 2.5 ng/g of tissue.

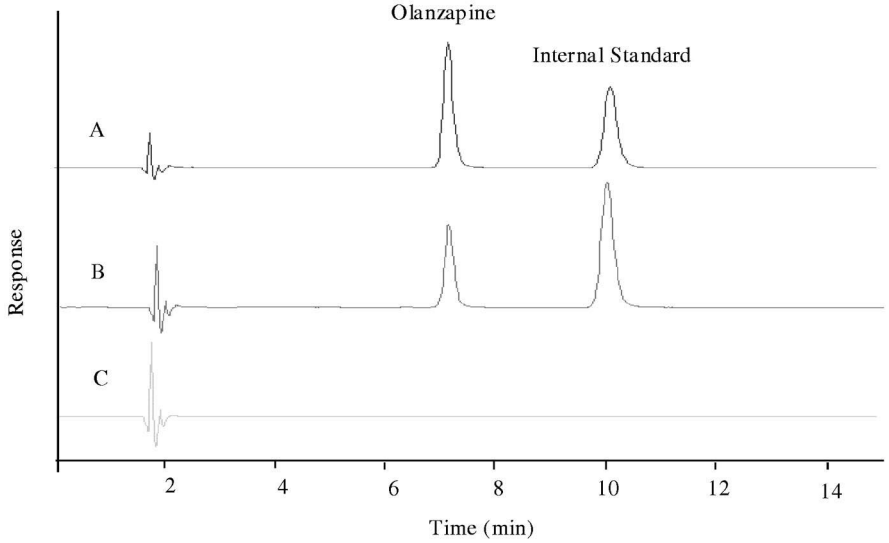

Fig. 2. Representative HPLC chromatograms of (A) olanzapine in rat brain homogenate following a subcutaneous dose of olanzapine (3 mg/kg, 2 h post dose); (B) control brain homogenate spiked with 10 ng/ml olanzapine and (C) blank brain homogenate.

Table 1
Intra- and inter-day assay precision and accuracy for olanzapine in rat brain tissue homogenate (n=5 for intra-batch and n=15 for inter-batch)

Day		Validation sample concentration (ng/ml)		
		0.5	50	100
1	Intra-batch mean	0.50	52.3	100.2
	Intra-batch accuracy (RE, %)	7.20	4.56	0.16
	Intra-batch precision (RSD, %)	3.39	1.12	6.56
2	Intra-batch mean	0.50	48.0	95.5
	Intra-batch accuracy (RE, %)	6.80	−4.04	−4.52
	Intra-batch precision (RSD, %)	6.96	1.26	2.61
3	Intra-batch mean	0.60	49.8	94.6
	Intra-batch accuracy (RE, %)	17.6	−0.48	−5.38
	Intra-batch precision (RSD, %)	4.56	0.76	1.31
3-day inter-assay statistics	Inter-batch mean	0.60	50.0	96.8
	Inter-batch accuracy (RE, %)	10.53	0.01	−3.25
	Inter-batch precision (RSD, %)	6.68	3.78	4.73

RE: Relative error.
RSD: Relative standard deviation.

3.3. Extraction efficiency, accuracy and precision

The extraction efficiencies of olanzapine from brain tissue homogenate were 82.0 and 87.0% at the concentrations of 25 and 100 ng/ml, respectively. The internal standard (LY170158) had an extraction efficiency of 93.4% at the concentration of 30 ng/ml. Five replicates of spiked brain tissue homogenate at three concentrations (0.5, 50 and 100 ng/ml) were prepared and assayed on 3 days to determine intra-day and inter-day accuracy and precision. The intra-day and inter-day relative errors ranged from −5.38 to 17.60% and −3.25 to 10.53%, respectively (Table 1). The intra-day and inter-day assay RSD values were in the range of 0.76 to 6.96% and 3.78 to 6.68%, respectively.

3.4. Stability study and sample dilution

A stability study conducted at room temperature suggested limited stability of olanzapine in rat brain homogenate under these conditions. Although olanzapine concentrations were relatively stable for 2 h at room temperature, the percentage of olanzapine spiked in brain homogenate dropped significantly after incubation for 4 h (Fig. 3). It has been previously reported that olanzapine is stable in human plasma and serum for at least 24 h at 25°C [9]. This result suggests that olanzapine stability may vary in different biological matrices. Therefore, brain tissue samples containing olanzapine should be extracted within 2 h after thawing. The results of freeze–thaw stability, extract stability and long term frozen storage stability, are shown in Tables 2–4. Data from two freeze–thaw cycles demonstrated that olanzapine remains stable following the freezing at −70°C and thawing at 25°C (Table 2). Olanzapine was also stable for 48 h in the sample extract (Table 3). There was no sign of degradation of olanzapine in brain tissue homogenate in over 100 days of storage at −70°C (Table 4). Also, sample dilutions up to 20-fold were demonstrated to yield concentrations with acceptable accuracy (<15% relative standard error).

3.5. Subcutaneous administration

This method has been successfully applied in an animal tissue pharmacokinetic study. Following a single subcutaneous administration (3 mg/kg), olanzapine concentrations peaked rapidly (0.5 h), at a C_{max} of approximately 3.4 μg/g in brain. At 24 h after dosing, a mean concentration of 2 ng/g of olanzapine was found in brain tissue (Fig. 4).

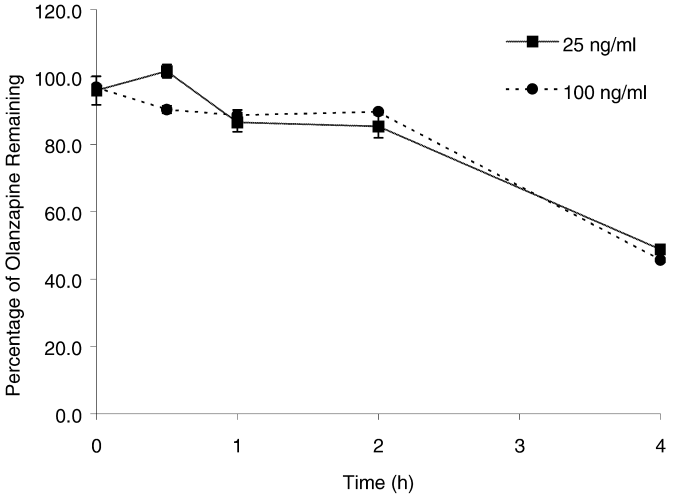

Fig. 3. Room temperature (25°C) stability of olanzapine in rat brain tissue homogenate (n=3, mean±SD).

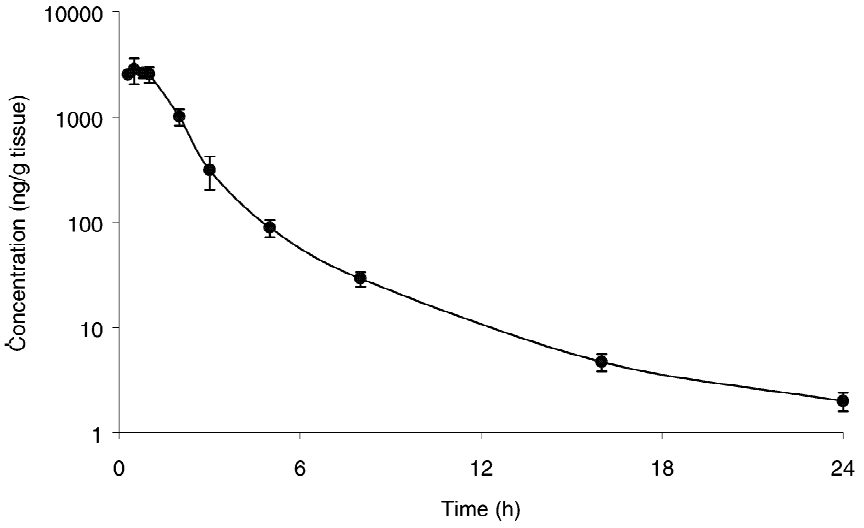

Fig. 4. Brain tissue concentration of olanzapine following subcutaneous administration of olanzapine (3 mg/kg) to male Sprague–Dawley rats (n=3, mean±SD).

Table 2
Stability of olanzapine after two freeze–thaw cycles[a] (n=3, mean±SD)

Freeze–thaw cycles	Validation sample concentration (ng/ml)	
	25	100
0	25.3±1.3	102.1±2.1
1	24.0±4.0	92.8±7.2
2	26.6±6.3	92.7±7.3

[a] Samples frozen at −70°C and thawed at 25°C.

Table 3
Stability of olanzapine reconstituted in mobile phase at 25°C after extraction (n=3, mean±SD)

Quantitation time (h)	Concentration (ng/ml)	
	25	100
0	24.8±0.9	104.6±4.6
24	23.2±7.3	92.9±7.1
48	26.0±3.9	94.8±5.2

Table 4
Long-term frozen storage stability of olanzapine at −70°C (n=3, mean±SD)

Quantitation time (days)	Concentration (ng/ml)	
	25	100
0	24.3±2.9	92.9±7.1
7	25.5±2.1	91.4±8.6
14	27.8±11.3	97.6±2.4
29	23.3±6.8	94.2±5.8
110	23.3±6.8	86.6±13.4

In summary, the present method is the first described for quantitative analysis of olanzapine in animal brain tissue. The sample treatment involves a simple and rapid one-step liquid–liquid extraction procedure. The extraction efficiency for olanzapine is ≥82% and the sensitivity allows quantitation down to 0.5 ng/ml homogenate. The assay can be utilized for tissue pharmacokinetic studies in animals with a high degree of precision and accuracy.

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