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Analysis and pharmacokinetics of olanzapine (LY170053) and two metabolites in rat plasma using reversed-phase HPLC with electrochemical detection

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Abstract

A sensitive HPLC assay for measurement of the antipsychotic drug, olanzapine, in plasma has been developed. The assay has a limit of quantitation of 1 ng ml^{-1} in plasma and utilizes solid-phase extraction and electrochemical detection. The method provides a linear response for olanzapine over a concentration range of 1–100 ng ml^{-1} with coefficients of determination greater than 0.9912. The inter-assay precision was 15.9% at the limit of detection and ranged from 7.33% to 8.47% over the range of 5–100 ng ml^{-1}. The intra-assay precision was in the range 0.97%–26.0%. The inter-assay accuracy ranged from 98.9 to 118% and the intra-assay accuracy ranged from 92.5% to 125% of the theoretical value. In addition, the assay was extended to measure the plasma levels of two metabolites of olanzapine, namely the *N*-desmethyl- and the 2-hydroxymethyl analogs. The utility of the assay was demonstrated following the administration of a single oral dose of ^{14}C-olanzapine to rats where, at several time-points after dosing, the plasma was assayed for total radioactivity, levels of olanzapine, and the two metabolites. Olanzapine and two of its metabolites accounted for less than 50% of the total plasma radiocarbon; olanzapine accounting for approximately 39% at the C_{max}, *N*-desmethyl for 5% and 2-hydroxymethyl for 8% respectively. The plasma elimination half-times for olanzapine and the two metabolites were approximately the same, ranging from 3.3 to 4.4 h.

Keywords: Olanzapine; Metabolites; Plasma; HPLC; Electrochemical detection

1. Introduction

Olanzapine (LY170053; 2-methyl-4-(4-methyl-1-piperazinyl)-10*H*-thieno[2,3*b*][1,5]benzodiazepine; Fig. 1) is a novel "atypical" antipsychotic agent for the treatment of psychoses. It has affinity for serotonin 5-HT_2, dopamine D_2 and D_1 and cholinergic muscarinic receptors [1]. In order to study the pharmacokinetics of olanzapine, a sensitive assay with a 1 ng ml^{-1} limit of detection in rat plasma has been developed. The assay utilized

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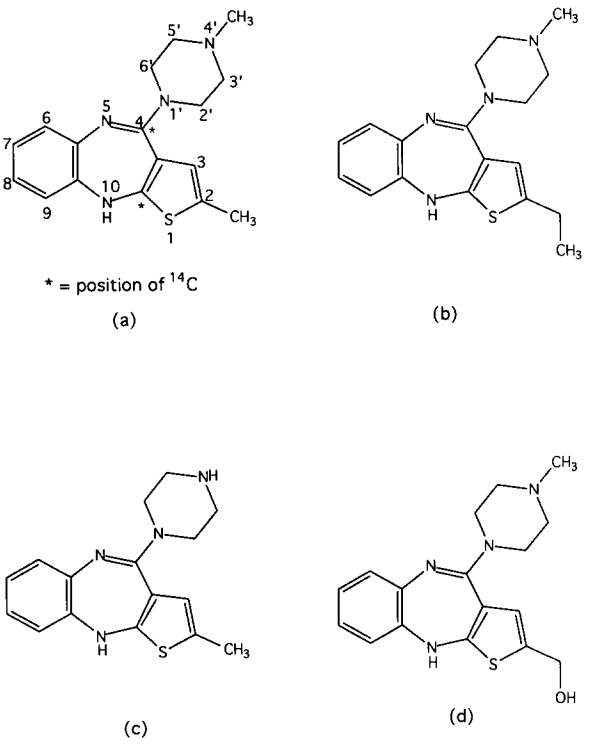

Fig. 1. Chemical structures of (a) olanzapine, (b) LY170222 (internal standard), (c) *N*-desmethyl- and (d) 2-hydroxymethyl-metabolites of olanzapine.

solid phase extraction followed by reversed-phase HPLC and electrochemical detection. A preliminary description of this assay has been reported previously [2]. In addition to olanzapine, this paper also describes the quantitation of two metabolites found in the plasma, namely the 2-hydroxymethyl-(LY290411) and the *N*-desmethyl-(LY170055; *N*-desmethyl) analogs (Fig. 1).

2. Experimental

2.1. Materials

LY170222 (2-ethyl-4-(4-methyl-1-piperazinyl)-10*H*-thieno[2,3-*b*][1,5]benzodiazepine; Fig. 1) was used as an internal standard. Olanzapine (LY170053), internal standard (LY170222) and the metabolite standards (LY290411, LY170055) were synthesized at Eli Lilly and Company. High purity acetonitrile, methanol, ethyl acetate and *n*-propyl alcohol were purchased from Burdick & Jackson (Muskegon, MI). Ammonium hydroxide and glacial acetic acid were obtained from Mallinckrodt (Paris, KY). Potassium phosphate monobasic–sodium hydroxide buffer (50 mM, pH 6) and potassium carbonate–potassium borate–potassium hydroxide buffer (50 mM, pH 10) were from Fisher Scientific (Fair Lawn, NJ). HPLC-grade water was prepared with a Milli-Q water purification system. Bond Elute Certify LRC cartridges were purchased from Varian (Harbor City, CA).

2.2. Preparation of reagents and solutions

Stock solutions of olanzapine and internal standard were prepared by dissolving 2.5 mg of each compound in *n*-propyl alcohol using sonication and diluting to 25 ml to give a concentration of 100 ng μl^{-1}. The olanzapine stock solution was diluted with n–propyl alcohol to 1, 0.1 and 00.1 ng μl^{-1} working solutions. This internal standard stock solution was further diluted with *n*-propyl alcohol to a concentration of 0.5 ng μl^{-1}

Plasma standards for establishing the standard curves were prepared by adding the appropriate aliquots (50, 100 μl) of the olanzapine working solutions (1, 0.1, 0.01 ng μl^{-1}) to 1 ml of control Fischer F344 rat plasma to give final concentrations of 1, 5, 10, 50 and 100 ng ml^{-1} of olanzapine. To each plasma standard solution, 10 μl of internal standard (equal to 5 ng) was added. The spiking solutions and calibration standards were prepared on a daily basis.

The mobile phase consisted of a mixture of 75 mM phosphate buffer (adjusted to pH 7 with 5 M sodium hydroxide), acetonitrile and methanol (48:26:26, v/v/v). The mobile phase was filtered through a 0.22 μm GV membrane filter (Millipore Corp., Milford, MA) and degassed with helium prior to use.

2.3. Instrumentation

The LC system consisted of a Model 9010 Varian solvent delivery system, a Shimadzu SCL-10A system controller, a SIL-10A Shimadzu au-

toinjector, a CTO-10A Shimadzu column oven, a YMC basic column (4.6 × 150 mm i.d., 5 μm), a Coulochem II electrochemical detector (Model 5200) from ESA with a Model 5010 analytical cell and a Model 5020 guard cell. The potentials of the screening cell, analytical cell, and guard cell were set to −0.2 V, 0.2 V and −0.3 V, respectively. The full scale gain range was set at 200 nA. The LC system was operated isocratically at 40°C with a flow rate of 1.2 ml min^{-1}. Peak height ratios of drug to internal standard were measured and processed using an Access*Chrom data acquisition system (Perkin Elmer-Nelson).

3. Procedures

3.1. Rat pharmacokinetics

Eighteen male Fischer 344 rats (about 200 g body weight) were obtained from Harlan Sprague-Dawley, IN. All animals were acclimatised for 3–5 days before a single oral dose of ^{14}C-olanzapine (specific activity: 26.2 μCi mg^{-1}) at 8 mg kg^{-1} was administered. The dosing solution was prepared by dissolving ^{14}C-olanzapine and non-radioactive olanzapine in 1 N HCl and adjusting the pH to 6 with 0.1 N NaOH. The typical administered volume was 0.4 ml. Animals were anesthetized by carbon dioxide and blood samples were collected by cardiac puncture using heparinized tubes at 0.5, 1, 3, 6, 12 and 24 h after dosing (3 rats per time point). Plasma was obtained by centrifugation. Radioactivity in plasma was obtained by counting aliquots (100 μl) of plasma samples in a liquid scintillation counter. Triplicate samples were prepared by the addition of 10 ml scintillation cocktail (Aquassure; Du Pont-NEN Research Products) to each aliquot, vortexed and counted for radioactivity. All plasma samples were stored at −70°C prior to analysis. Plasma concentrations of olanzapine were measured using the HPLC assay as described below.

3.2. Assay validation

The assay validation was performed with spiked rat plasma samples over 3 days at concentrations of olanzapine of 1, 5, 10, 50 and 100 ng ml^{-1} containing 10 μl of internal standard. The precision and accuracy of the assay were evaluated at the above concentrations over 3 days. The intra-assay precision was determined by analyzing four spiked plasma samples at each concentration on the same day. The inter-assay precision was calculated as the RSD of the daily averages. The extraction efficiency was determined by comparing the peak height from extracted samples to those of unextracted standards.

Plasma samples from the rats were added to test tubes (13 × 100 mm) followed by addition of 10 μl of the internal standard solutions (0.5 ng μl^{-1} of LY170222) and 3 ml of phosphate buffer (50 mM, pH 6). The samples were vortexed for 5 s and loaded onto Bond Elute LRC Certify cartridges (130 mg of sorbent) which were conditioned by passing 3 ml of methanol and 3 ml of phosphate buffer (50 mM, pH 6) through them before use. 5 ml of phosphate buffer (50 mM, pH 6), 1 ml of phosphate buffer (50 mM, pH 10)/methanol (70:30, v/v), and 1 ml of 1.0 M acetic acid were applied sequentially. The residual acid was removed by applying vacuum (15 mm Hg) for 5 min followed by washing with 6 ml of methanol. The cartridges were dried under vacuum (15 mm Hg) for 2 min before eluting them with 3 ml of 2% ammonium hydroxide in ethyl acetate (v/v). The eluate was evaporated to dryness at 50°C under nitrogen. To each residue, 100 μl of HPLC mobile phase was added and vortexed for 30 s. The final solutions were analyzed by HPLC.

The measurement of 2-hydroxymethyl and *N*-desmethyl metabolites in plasma samples was determined by separate metabolite standard curves for each compound over a 1 day analysis. Stock solutions of 100 ng μl^{-1} were prepared for each olanzapine metabolite. A mixed stock solution with a concentration of 10 ng μl^{-1} for each metabolite was prepared by adding 1 ml of 100 ng μl^{-1} 2-hydroxymethyl and *N*-desmethyl analogs to a 10 ml volumetric flask and diluting to 10 ml with *n*-propyl alcohol. A serial dilution was then made to give the mixed metabolite working solutions at concentrations of 1, 0.1 and 0.01 ng μl^{-1}. The standard curves were prepared at concentrations of 1, 5, 10, 50 and 100 ng ml^{-1} as described

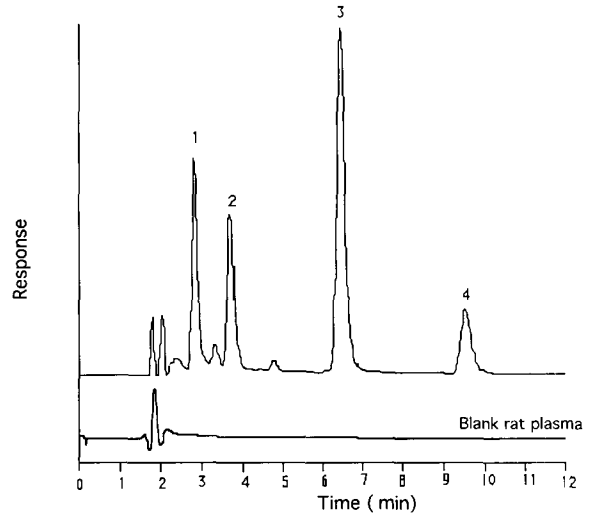

Fig. 2. Chromatogram of extracted, control rat plasma spiked with 10 ng ml^{-1} each of olanzapine, 2-hydroxymethyl and *N*-desmethyl-analogs (top) and blank plasma (bottom). 1 = 2-hydroxymethyl, 2 = *N*-desmethyl, 3 = olanzapine and 4 = internal standard.

above. Triplicate plasma validation samples were prepared at concentrations of 1, 10 and 100 ng ml^{-1} for intra-assay precision purposes.

The stability of olanzapine in rat plasma was studied by spiking three concentrations (8, 64 and 200 ng ml^{-1}) of olanzapine into rat plasma (n = 5). The resulting samples were stored at approximately −70°C for up to 19 weeks.

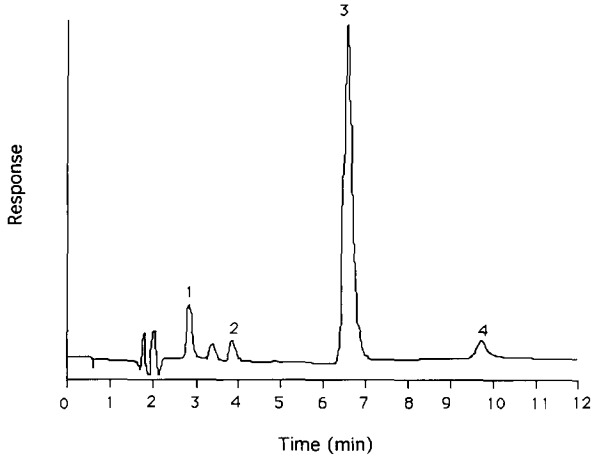

Fig. 3. Chromatogram of rat plasma extract at 3 h following an oral dose of 8 mg kg^{-1} ^{14}C-olanzapine, 1 = 2-hydroxymethyl, 2 = *N*-desmethyl, 3 = olanzapine and 4 = internal standard.

Table 1
Validation of olanzapine analysis in rat plasma. Experimentally determined values for the validation samples

	Concentration (ng ml^{-1})				
Value	1	5	10	50	100
Day 1					
Intra-assay average	1.23	4.99	9.60	46.2	95.7
Intra-assay accuracy (%)	123	99.9	96.0	92.5	95.7
Intra-assay SD	0.06	0.05	0.57	3.98	7.66
Intra-assay precision (%)	4.90	0.97	5.96	8.60	8.00
Day 2					
Intra-assay average	1.06	4.88	10.5	52.0	107
Intra-assay accuracy (%)	106	97.6	105	104	107
Intra-assay SD	0.04	0.22	0.30	3.41	10.5
Intra-assay precision (%)	3.30	4.42	2.81	6.56	9.80
Day 3					
Intra-assay average	1.25	5.33	11.3	50	102
Intra-assay accuracy(%)	125	107	113	100	102
Intra-assay SD	0.32	0.58	0.44	1.89	5.74
Intra-assay precision (%)	26.0	10.9	3.90	3.77	5.62
Intra-assay statistics					
Inter-assay average	1.18	5.07	10.5	49.5	102
Inter-assay accuracy (%)	118	101	105	98.9	102
Intra-assay SD	0.19	0.37	0.82	3.78	8.60
Intra-assay precision (%)	15.9	7.33	7.88	7.64	8.47

4. Results

Fig. 2 shows a chromatogram of extracted blank rat plasma and a chromatogram obtained from an extract of blank plasma spiked with 10 ng ml^{-1} of olanzapine, 2-hydroxymethyl and *N*-desmethyl metabolites. The chromatogram demonstrated base-line separation of olanzapine from the two metabolites. Under the isocratic

conditions, all compounds eluted within 15 min. The retention times were 2.9 min for 2-hydroxymethyl, 3.9 min for *N*-desmethyl, 6.6 min for olanzapine and 9.6 min for the internal standard. Fig. 3 shows the chromatogram of a rat plasma extract 3 h after receiving olanzapine at a dose of 8 mg kg^{-1}.

Linear calibration curves were obtained in the concentration range 1–100 ng ml^{-1} with coefficients of determination greater than 0.9912 for olanzapine, 0.9963 for 2-hydroxymethyl and 0.9948 for *N*-desmethyl. The validated limit of quantitation (LOQ) was 1 ng ml^{-1} for all three compounds

The validation results for olanzapine and the two metabolites are shown in Tables 1 and 2 respectively. The intra-assay precision for olanzapine ranged from 0.97% to 26.0%; the inter-assay precision was 15.9% at the limit of quantitation and 7.33%–8.47% over the range 5–100 ng ml^{-1}. The precision for the metabolite validation samples ranged from 6.57% to 26.0% for 2-hydroxymethyl olanzapine and 4.56% to 6.74% for *N*-desmethyl olanzapine.

4.1. Extraction efficiency and stability

The extraction efficiency was expressed as the ratio of the peak height of the analytes from extracted plasma standards to those obtained from chromatographic standard solutions prepared in the mobile phase at equivalent concentrations. The averaged extraction efficiency of olanzapine, 2-hydroxymethyl olanzapine, *N*-desmethyl olanzapine and internal standard from rat plasma was 56.9%, 69.1%, 58.6% and 78% respectively, at 1, 10 and 100 ng ml^{-1}

The recovery percentages of olanzapine for the stability samples were in the range 84.5%–95.3% for three concentrations (8, 64 and 200 ng ml^{-1}) over a period of 19 weeks.

Table 2
HPLC assay for accuracy and precision of 2-hydroxymethyl olanzapine and *N*-desmethyl olanzapine in rat plasma ($N = 3$)

	Concentration (ng ml^{-1})		
Value	1	10	100
Metabolite: 2-hydroxymethyl olanzapine			
Average	1.01	10.2	92.9
Accuracy (%)	101	102	92.9
SD	0.262	1.01	6.10
Precision (%)	26.0	9.97	6.57
Metabolite: *N*-desmethyl olanzapine			
Average	1.21	8.62	99.4
Accuracy (%)	121	86.2	99.4
SD	0.055	0.581	5.88
Precision (%)	4.56	6.74	5.92

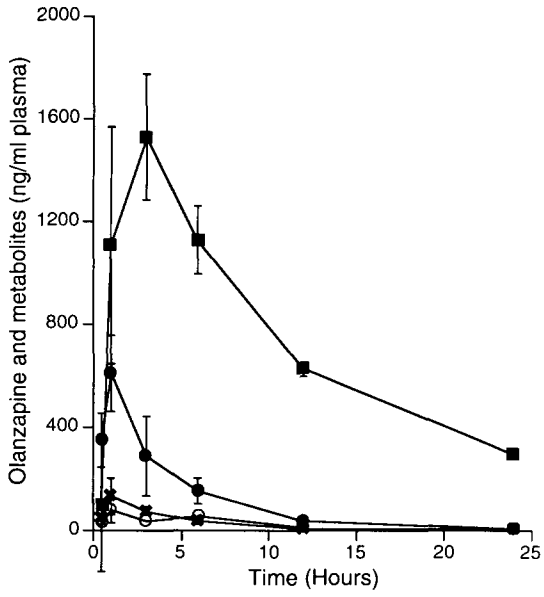

Fig. 4. The plasma concentration of total plasma radiocarbon (■), olanzapine (●), and two metabolites [2-hydroxymethyl olanzapine (x) and *N*-desmethyl-olanzapine (○)] following a single oral dose of ^{14}C-olanzapine at 8 mg kg^{-1}.

4.2. Rat pharmacokinetics

Fig. 4 shows the plasma concentration–time curves for olanzapine and two of its metabolites. The plasma drug concentration reached a maximum 1 h after the dose was administered. The area under the plasma concentration–time curves (AUC), C_{max}, and $T_{1/2}$ of olanzapine and its metabolites are presented in Table 3. Plasma radiocarbon C_{max} was 1530 ng equivalent ^{14}C-olanzapine ml^{-1}. The corresponding C_{max} for olanzapine was 609 ng ml^{-1} which accounted for

Table 3
Plasma concentration of olanzapine and two metabolites in Male Fischer 344 rats following the administration of a single, oral dose of ^{14}C-olanzapine at 8 mg kg^{-1}

Time	Total radiocarbon (ng ml^{-1}) Mean ± SD	Olanzapine (ng ml^{-1}) Mean ± SD	2-Hydroxymethyl (ng ml^{-1}) Mean ± SD	*N*-desmethyl (ng ml^{-1}) Mean ± SD
0.5	1106 ± 260	352 ± 105	48.7 ± 12.1	31.1 ± 11.8
1	1530 ± 463	609 ± 149	130.6 ± 72.1	80.2 ± 51.1
3	1129 ± 243	286 ± 154	72.7 ± 15.2	25.9 ± 20.3
6	626 ± 133	152 ± 47.7	31.8 ± 7.20	35.2 ± 28.3
12	296 ± 28.9	33.8 ± 6.55	5.80 ± 2.11	9.01 ± 3.29
24	97.8 ± 15.4	4.44 ± 2.92	3.44 ± 3.41	0.97 ± 0.056
Mean C_{max} (ng ml^{-1})	1530	609	131	80.2
Mean T_{max} (h)	1	1	1	1
Mean AUC (0–24 h)[a] (ng h ml^{-1})	11080	2580	574	418
$T_{1/2}$ (h) (1–24 h)[a]	5.86	3.31	4.37	3.84

[a] Numbers in parentheses represent the time points used for calculation.

39.8% of total plasma radiocarbon. The plasma AUC was 11 080, 2580, 574 and 418 ng eq. hour ml^{-1} for radiocarbon, olanzapine, 2-hydroxymethyl and *N*-desmethyl metabolites respectively. The AUC of olanzapine accounted for 23.3% of total radiocarbon AUC, while those of 2-hydroxymethyl and *N*-desmethyl accounted for 5.18% and 3.77% of total radiocarbon AUC respectively.

5. Discussion

A sensitive HPLC assay was developed and validated for the orally-active antipsychotic agent, olanzapine, in plasma. The limit of quantitation of the assay was 1 ng ml^{-1} and its utility has been demonstrated in an experiment following the administration of a single dose of 8 mg kg^{-1} of ^{14}C-olanzapine to rats. In addition to the measurement of olanzapine in the plasma, total plasma radioactivity was quantitated. The fact that total plasma radioactivity, whether at the C_{max} or measured in terms of plasma AUC, was always larger than that for olanzapine, was an indication of the extensive metabolism of olanzapine. At the C_{max} of both plasma olanzapine and radioactivity, olanzapine accounted for 39.8% of total plasma radiocarbon and in terms of plasma AUC, olanzapine accounted for 23.3% of the total plasma radiocarbon AUC.

The *N*-desmethyl- and 2-hydroxymethyl- metabolites have previously been reported as urinary metabolites of olanzapine in the rat [3]. This present report not only demonstrates their presence in the circulating rat plasma but also provides quantitative data. Plasma concentrations of the two metabolites were similar to one another, levels of the 2-hydroxymethyl being slightly higher than that of the *N*-dealkylated analog. The 2-hydroxymethyl metabolite accounted for 8.56% of total plasma radiocarbon at the C_{max} while the *N*-desmethyl metabolite accounted for 5.24%. The contribution of the metabolites to the total plasma radiocarbon AUC was 5.18% for the 2-hydroxymethyl and 3.77% for *N*-desmethyl. Clearly, olanzapine and the two metabolites quantitated herein do not account for the majority of the total plasma radiocarbon. Aromatic hydroxylated metabolites of olanzapine have been reported in the urine of rats given an oral dose of olanzapine [4] and, together with their glucuronidated forms, these metabolites may be present in the plasma. The assay described here was not able to detect these metabolites. The *N*-oxide (N4) of olanzapine was synthesized but could not be quantitated with the present assay due to erratic binding and

elution properties on the solid phase extraction cartridge.

A similar antipsychotic compound, clozapine, has also been shown to be partially metabolized to the *N*-desmethyl- and *N*-oxide analogs which have been quantitated in plasma [5, 6].

The plasma elimination half-time values for olanzapine and the two metabolites, over a period of 1–24 h, were similar, ranging from 3.3–4.4 h. The half life of plasma radiocarbon was somewhat longer (approximately 6 h).

The assay reported herein for olanzapine and two metabolites should prove useful in defining the pharmacokinetic parameters associated with the oral administration of olanzapine to the animal species used in the pharmacological and toxicological evaluation of the drug.

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