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Determination of olanzapine in plasma by high-performance liquid chromatography using ultraviolet absorbance detection

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Abstract

A rapid method for the determination of olanzapine in plasma using high-performance liquid chromatography with ultra violet detection is described. Olanzapine was extracted from plasma with a mixture of hexane/dichloromethane (85:15), and then back extracted into phosphate buffer pH 2.8. Separation was achieved on a RP Select B C_{18} column and commonly administered drugs did not interfere with the assay. The limit of quantitation was 1.5 μg/l and the inter-day and intra-day relative standard deviations were less than 10%. Olanzapine was shown to be stable in plasma for up to 7 days when stored at 4 °C. Moreover, the addition of ascorbic acid was not necessary for the achievement of chemical stability during storage, or during the assay procedure. The method has been used to measure olanzapine concentrations in patients treated with various doses of the drug varying from 5 to 40 mg/day.

Keywords: Olanzapine

1. Introduction

Olanzapine is a novel antipsychotic drug that improves the positive and negative symptoms of schizophrenia [1,2]. It is structurally related to clozapine and is extensively metabolized in the liver by *N*-glucuronidation, allylic hydroxylation, *N*-oxidation and *N*-dealkylation to clinically inactive metabolites [3].

The steady state trough concentrations of olanzapine at usual daily doses of 5–20 mg, range from 5 to 50 μg/l [4]. Although no therapeutic range for plasma levels of olanzapine has been established, minimum effective therapeutic concentrations greater than 9 μg/l [5], or 23 μg/l [6] have been proposed as predictors of favorable treatment outcome in acutely ill patients with schizophrenia. In addition, recent studies have shown that olanzapine metabolism and hence plasma concentrations can be altered by drug interactions [7,8]. Hence, there is a need for simple robust methods of analysis that can be used for routine therapeutic drug monitoring.

Previous published methods have used high-performance liquid chromatography (HPLC) coupled with electrochemical detection [9–12]. Although these methods offer enhanced sensitivity in the low

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μg/l range, electrochemical detectors require optimal working conditions. Three HPLC methods using ultra violet detection have been published [13–15]. The two methods by Olesen add ascorbic acid to plasma samples to reduce oxidation of olanzapine during either solid-phase or a liquid–liquid extraction process, followed by normal (silica) phase chromatographic separation [13,14]. Both have the sensitivity required for monitoring patients on low dose olanzapine. By contrast, the method of Weigmann [15] does not add an antioxidant, and uses a solid-phase extraction followed by separation on a reversed-phase column. However the latter method lacks the required sensitivity to monitor plasma levels of patients on low doses of the drug.

Our method uses a robust reversed-phase HPLC separation with ultraviolet detection, is free from interferences, and has a limit of detection comparable to that achieved with electrochemical detection. Moreover, addition of ascorbic acid to stabilize olanzapine during storage and extraction is shown to be unnecessary.

2. Experimental

2.1. Specimens

Plasma specimens were obtained from routine specimens sent to the laboratory for therapeutic drug monitoring of olanzapine, or from drug-free volunteer subjects in the laboratory when blank plasma was required.

2.2. Chemicals and reagents

Authentic reference samples of olanzapine, 2 methyl-4-(4 methyl-1-piperazinyl)-10H thieno [2,3-b] [1,5] benzodiazepine and olanzapine N-oxide were donated by Eli Lilly Research Laboratories (Indianapolis, USA), while *N*-desmethylclozapine (used as the internal standard in the assay) was obtained from Novartis (Basel, Switzerland).

HPLC grade hexane, dichloromethane and acetonitrile were obtained from BDH Chemicals (Australia). All other chemicals were of analytical reagent grade.

2.3. Sample preparation

Plasma samples (1 ml) were aliquoted into 15 ml borosilicate glass test tubes to which 20 μl of *N*-desmethylclozapine internal standard (0.2 μg) and 500 μl of 0.1 *M* Na_2CO_3 were added. The tubes were shaken with 10 ml of a solvent mixture consisting of hexane/dichloromethane (85:15) for 5 min, then centrifuged at 1800 *g* for 5 min. The supernatant was transferred to clean 15 ml borosilicate glass tubes and 200 μl of 45 m*M* KH_2PO_4 (buffered to pH 2.8 with concentrated H_3PO_4) was added. The mixture was shaken for 30 s and then centrifuged as before. The upper organic layer was aspirated to waste and the tubes placed in a water bath at 50 °C under a stream of dry N_2 for approximately 30 s to evaporate traces of the solvent. An aliquot (80 μl) was injected into the HPLC system.

2.4. HPLC instrumentation and chromatographic conditions

The HPLC system consisted of a Waters Associates (Milford, MA, USA) solvent delivery pump Model 515 and Model 486 ultraviolet detector operated at 270 nm. An automatic sample injector LC1650 (GBC Scientific Equipment Pty Ltd, Victoria, Australia) was used. Separations were achieved on a Merck LiChrospher 60 RP Select B 5 μ column (25×0.46 cm) using a mobile phase of 14% acetonitrile in water (containing 0.25% H_3PO_4 and 0.05% triethylamine), and pumped at a flow-rate of 1 ml/min.

2.5. Validation tests

2.5.1. Standard curve

Stock solutions of olanzapine and the internal standard (*N*-desmethylclozapine) were prepared at a concentration of 1 mg/ml in ethanol. Working standards were prepared by diluting the olanzapine stock standard solution in ethanol and the internal standard stock solution in water. All standards were stable for 3 months when stored at 4 °C. A five-point calibration curve was made by spiking blank plasma with the working olanzapine standard to cover a range of concentrations from 2 to 150 μg/l.

2.5.2. Precision and accuracy

The precision of the method was established by determining the inter-day and intra-day relative standard deviation (RSD) for the assay. The intra-assay variance was determined by analysing five 1 ml aliquots of plasma spiked at 5, 25 and 125 μg/l of olanzapine. The concentrations of olanzapine in these samples were calculated from a standard curve analysed on the same day. The inter-day RSD was determined by analysing 1 ml aliquots of the same spiked plasma on 5 different days. The concentrations of olanzapine in these samples were determined from the standard curve prepared on each of the respective days. Accuracy was determined from the mean of the concentrations obtained.

2.5.3. Limits of quantification and detection

The lower limit of quantification (LOQ) was defined as the concentration for which the RSD and accuracy were ≤20% while limits of detection (LOD) was defined as the concentration that produced 2.5–3 times the background noise.

2.5.4. Specificity

The interference by endogenous constituents in plasma was assessed by analysis of blank plasma samples while interference from a range of likely co-medications and their metabolites was investigated by direct injection of standards onto the HPLC column and measurement of their respective retention times.

2.5.5. Storage stability

The storage stability of olanzapine in plasma was assessed because of a previous report that suggested olanzapine was readily oxidized, and that lower levels were obtained on plasma samples stored at 4 °C for 7 and 14 days [14]. In our study, plasma samples from patients taking olanzapine were assayed within 24 h of collection, and then reassayed after storage at 4 °C for various times up to 7 days. In addition blank plasma spiked with olanzapine at concentrations of 33 or 90 μg/l were also analysed immediately after preparation and then again after storage at 4 °C for up to 14 days.

Because Olesen [14] had also reported that the addition of ascorbic acid could be used to increase the recovery of olanzapine in plasma samples where olanzapine had been oxidized, an additional experiment was performed. Plasma samples from patients taking the drug were split into two aliquots on arrival in the laboratory. The first aliquot was analysed immediately (without ascorbic acid). Ascorbic acid (10 μl of a 25% w/v/ml plasma) was added to the second aliquot and this sample was reanalysed 24–48 h later (with ascorbic acid). We reasoned that this experiment would show whether oxidative degradation that occurred after collection could be reversed. On both occasions (with or without ascorbic acid), samples were analysed in a single run, and olanzapine concentrations were determined from a standard curve prepared with and without the addition of ascorbic acid as appropriate.

The stability of olanzapine in the final extract (phosphate buffer pH 2.8) was also examined by injecting representative extracts onto the HPLC column immediately after extraction and 4 and 8 h later.

2.6. Data analysis

Differences between plasma samples containing olanzapine with or without ascorbic acid as an antioxidant were compared using the Wilcoxon Signed Rank test provided in SigmaStat Ver 2.0 (SPSS Inc, Chicago, IL, USA).

3. Results and discussion

3.1. Chromatographic separation of analytes

Chromatograms obtained using the method outlined are shown in Fig. 1. Olanzapine and the internal standard *N*-desmethylclozapine eluted with retention times of approximately 5.4 and 11.2 min, respectively. No interferences were observed either from blank plasma, or for other drugs likely to be co-administered with olanzapine (Table 1). Olanzapine–*N*-oxide, an inactive metabolite also was separated from olanzapine.

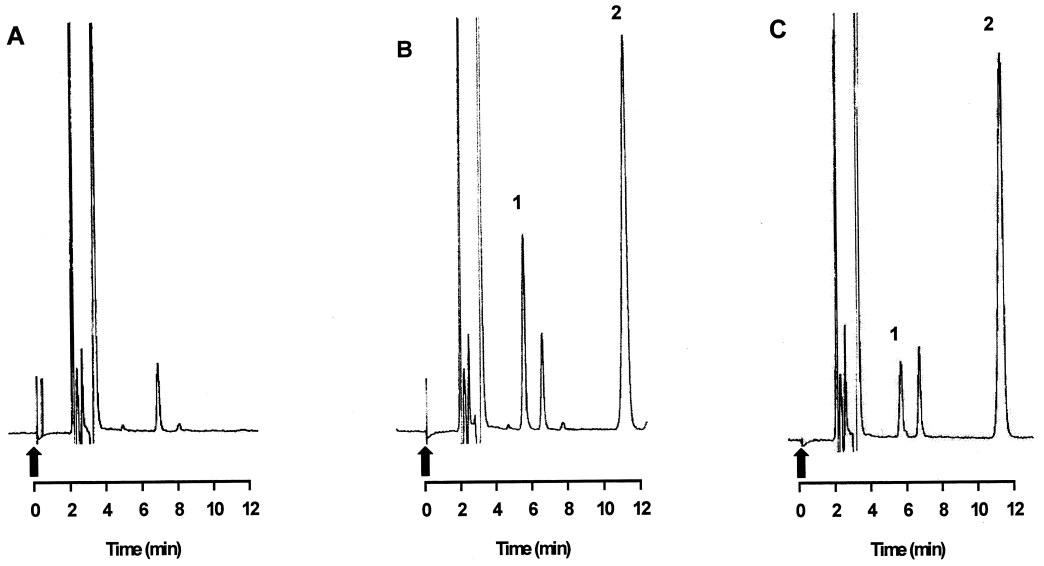

Fig. 1. (A) Chromatogram for an extract of blank plasma, (B) chromatogram for an extract of blank plasma spiked with olanzapine at a concentration of 25 μg/l and (C) chromatogram for a patient sample containing a measured concentration of 12 μg/l of olanzapine. The arrow indicates the point of injection on to the column, peak 1 = olanzapine (retention time 5.4 min), and peak 2 = *N*-desmethylclozapine (retention time 11.2 min).

Table 1
Retention times of drugs tested for interference in the assay[a]

Drug	Retention time (min)
Caffeine	7.6
Carbamazepine	>20
Citalopram	>20
Clozapine	13.2
Fluoxetine	>20
Haloperidol	>20
Lamotrigine	15.0
N-desmethylclozapine	11.2
Mexilitine	19.4
Mirtazapine	4.6
Olanzapine	5.4
Olanzapine–*N*-oxide	7.4
Quetiapine	17.4
Risperidone	18.0
Trazodone	>20
Tricyclic antidepressants[b]	>20
Venlafaxine	>20

[a] Retention times for olanzapine, olanzapine–*N*-oxide and *N*-desmethylclozapine are also shown.

[b] Amitriptyline, clomipramine, desipramine, dothiepin, doxepin, imipramine, nortriptyline.

3.2. Linearity

The standard curve for olanzapine in plasma was linear over the concentration range 2–150 μg/l with a correlation coefficient of ≥0.999.

3.3. Precision and accuracy

Precision was assessed by determining intra- and inter-day RSD (Table 2). Values were less than 7% within the concentration range found in patients treated with olanzapine. Accuracy, assessed over the same range varied from 97 to 102% of the spiked concentrations. Our method has a similar inter-day RSD to the recent normal-phase HPLC–UV method of Olesen et al. [13] and similar inter- and intra-day RSD's to those obtained by Weigmann et al. using a reversed-phase HPLC–UV separation [15].

3.4. Limits of quantitation and detection

The LOQ for the determination of olanzapine in plasma was 1.5 μg/l and the limit of detection (LOD) was 1 μg/l. The LOQ value was 2-fold lower than that reported by Raggi et al. [12], but about

Table 2
Intra- and inter-day assay relative standard deviation and accuracy for olanzapine in plasma ($n=5$)

Olanzapine (μg/l)	Intra-day		Inter-day	
	RSD (%)	Accuracy (%)	RSD (%)	Accuracy (%)
5	3.5	102	6.4	100
25	2.6	98	4.9	100
125	0.7	98	2.4	97

4-fold higher than that reported by Catlow et al. [11], both of whom used electrochemical detection. By comparison with other HPLC methods using UV detection, our LOQ was similar to that reported by Olesen et al. [14], but 2- or 4-fold lower than those reported by Olesen et al. [13] and Weigmann et al. [15], respectively.

3.5. Storage stability

Olesen et al. [14] reported recoveries of 45 and 13% for spiked serum samples containing olanzapine when stored at 4 °C for 1 and 2 weeks, respectively, and a 40% decrease in concentration of plasma samples stored at room temperature for 24 h. Oxidation was suggested as the reason for the low recoveries, and ascorbic acid was used successfully to reduce degradation during storage and extraction. By contrast, our experiment (Table 3), using a wide range of relevant concentrations, clearly showed that both spiked control plasma samples and samples from patients taking olanzapine (no ascorbic acid added) could be stored at 4 °C for up to 7 days with no significant loss of drug. In addition control plasma samples have been stable when stored frozen at −20 °C for up to 3 months (data not shown).

The use of ascorbic acid to reverse olanzapine already oxidised as suggested by Olesen [14], also was tested using both spiked control plasma samples, and patient samples. Samples were assayed with and without ascorbic acid and the olanzapine concentrations calculated from their respective calibration curves (Table 4). There were no significant differences between conditions (Wilcoxon Signed Rank test = −22, $P=0.148$), and assay calibration curves prepared with and without ascorbic acid gave similar ratios for olanzapine–*N*-desmethylclozapine. Thus, in our hands, olanzapine proved to be stable in plasma with no measurable degradation occurring

Table 3
Concentration of olanzapine in plasma (μg/l) stored at 4 °C for various times up to 14 days

Sample identification	Storage time (days)					
	0	1	2	5	7	14
Control (33 μg/l)	33		34		34	31
Control (90 μg/l)	90	92	87			
Patient 1	29	27				
Patient 2	63	60				
Patient 3	15	15	18			
Patient 4	41	37	38			
Patient 5	26	25	27			
Patient 6	40	39	40			
Patient 7	31			30		
Patient 8	13			15		
Patient 9	2.5		2			
Patient 10	43		41	39	36	
Patient 11	16				15	
Patient 12	14		13		10	
Patient 13	19			18		
Patient 14	13			14		
Patient 15	6			7		

Table 4
Olanzapine plasma concentrations in samples, with or without the addition of ascorbic acid (10 μl of a 25% w/v solution/ml plasma)

Sample identification	Plasma olanzapine (μg/l)	
	With ascorbic acid[a]	Without ascorbic acid
Control (25 μg/l)	25	24
Control (33 μg/l)	31	27
Patient 1	41	39
Patient 2	8	8
Patient 3	16	16
Patient 4	21	22
Patient 5	25	24
Patient 6	13	13
Patient 7	45	44
Patient 8	35	36
Patient 9	29	28

[a] Wilcoxon Signed Rank test = −22, $P=0.148$, not significantly different.

during extraction or storage as outlined above. It is interesting to note that Lasko has recently reported that olanzapine is highly unstable (98% decomposition after 3 days at room temperature) when calf serum was used to prepare quality control samples but did not find any losses when human serum was used [16]. While this does not explain the earlier observations of instability in human plasma, it does highlight the importance of matrix composition for this analyte.

Since we were also concerned about the stability of olanzapine in the extraction procedure, we undertook investigations of the back-extraction step utilised in our assay procedure. In the initial assay development, we tried back-extraction using 0.05 *M* HCl since this had been used successfully for other assays in the laboratory. Recoveries of both olanzapine and the internal standard were high, but olanzapine was unstable in this medium (pH=1.75). Acidic extracts from both patient plasma samples and spiked plasma samples showed a 15% decrease in olanzapine concentrations after 3 h. However, 45 m*M* KH_2PO_4 buffered to pH 2.8 proved to be a satisfactory back-extraction medium, and olanzapine was stable in this solution for at least 8 h, enabling assays to be run overnight using an automatic injector.

3.6. Application of the method to routine therapeutic drug monitoring

To demonstrate the utility of the method we show data from assays of routine samples from patients taking olanzapine at doses ranging from 5 to 40 mg/day. Fig. 2A shows a scatter plot of olanzapine concentration vs. dose for 131 patients. The mean plasma olanzapine was 28 μg/l with a range from 3 to 122 μg/l. The dotted regression line ($y = 7.35 + 1.257x$; $r^2 = 0.19$) shows that plasma concentration increases with dose, but the regression explains only 19% of the inter-patient variability in the data. Abnormally low concentrations observed in two patients taking 35 and 40 mg, respectively were not attributable to co-therapy with inducing drugs, and are most likely to be the result of poor compliance. Fig. 2B shows concentration–dose plots for four individual patients. These data suggest that dose and steady-state concentration are linearly related for individual patients, and support the use of target concentration monitoring for olanzapine.

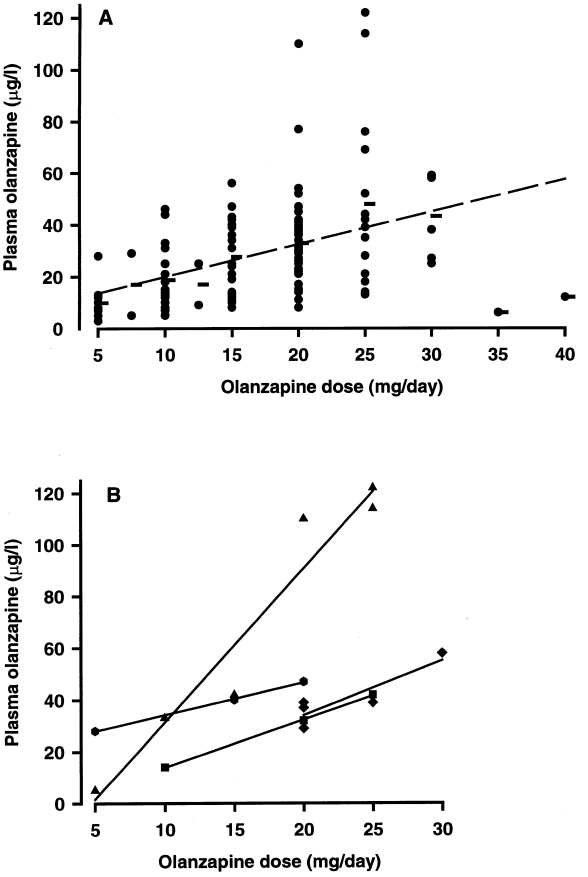

Fig. 2. (A) Scatter plot of steady-state plasma olanzapine vs. dose for 131 patients taking therapeutic doses of olanzapine. The dotted line shows the regression of concentration and dose ($y = 7.35 + 1.257x$; $r^2 = 0.19$) for the group as a whole. The horizontal lines show means at individual dose levels ranging from 5 to 40 mg/day. (B) Concentration–dose plots for four patients where assays were available at three or more dose rates.

4. Conclusions

This method reported has the required sensitivity, accuracy and precision both for routine monitoring of drug concentrations in patients taking between 5 and 40 mg of olanzapine each day and also for use in specialised pharmacokinetic studies. It uses a simple solvent extraction, followed by separation on a reversed-phase HPLC column with detection of

peaks by UV absorbance at 270 nm. The addition of ascorbic acid as an antioxidant was not necessary for preservation of olanzapine in plasma samples. The method has been used routinely in our laboratory for a number of years and has proved to be robust and reliable.

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