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Determination of olanzapine in human breast milk by high-performance liquid chromatography with electrochemical detection

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Abstract

A reversed-phase high-performance liquid chromatographic–electrochemical assay was developed and validated for the quantification of olanzapine in human breast milk. The assay involved a solid-phase extraction (SPE) of olanzapine and its internal standard on a Bond Elut Certify LRC mixed-mode cartridge. After conditioning of the SPE cartridge, human milk (1 ml) was passed through the cartridge. The cartridge was washed with five separate washing steps to remove endogenous compounds, and the analytes were eluted with ethyl acetate–ammonium hydroxide (98:2, v/v) solution. The eluate was evaporated to dryness (gentle stream of nitrogen at 40°C), and the residue was dissolved in mobile phase. The extract was injected onto a YMC basic column (150 mm×4.6 mm I.D., 5 μm particle size) at a flow-rate of 1 ml/min. A mixture of 75 m*M* phosphate buffer, pH 7.0–acetonitrile–methanol (48:26:26, v/v/v) was used as the mobile phase. Standard curves with a lower limit of quantitation of 0.25 ng/ml of olanzapine were linear ($r^2 \geq 0.9992$) over a range of 0.25–100 ng/ml. Based on the analysis of quality control (QC) samples, the average inter-day accuracy (RE) was 99.0% with an average precision (CV) of 6.64% over the entire range. The stability of olanzapine in human milk was established after three freeze–thaw–heat cycles and storage at −70°C for 10 months. The validated method was used to measure olanzapine concentrations in human milk during a clinical trial. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Olanzapine, a thienobenzodiazepine sold under the tradename, Zyprexa (Fig. 1), is a new atypical antipsychotic therapy with 5-hydroxytryptamine, dopamine $D_1/D_2/D_4$ antagonist activity and anticholinergic properties [1,2]. Since its introduction in 1997, Zyprexa has been approved in over 50 countries for treating patients with schizophrenia. Schizophrenia is a neurological disorder that strikes about 1% of the world's population, or around 50 million people. Typical neuroleptic drugs treat the positive symptoms of psychosis (hallucinations, paranoia and delusions) but are largely ineffective in treating

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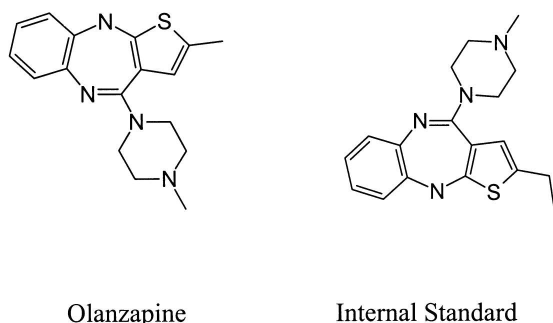

Fig. 1. Structures of olanzapine and internal standard.

many of the negative symptoms (low levels of interest, lack of motivation, social withdrawal and poverty of speech). Approximately 30% of acutely ill schizophrenics respond poorly to treatment with conventional neuroleptics; therefore, a new class of anti-psychotic agents are clearly needed [3]. One new promising anti-psychotic is olanzapine which has shown to be effective in treating both positive and negative symptoms of schizophrenia [4].

In order to study the clinical pharmacokinetics of a drug it is necessary to have a reliable, accurate and sensitive analytical method for the determination of the compound in various body fluids. Determining drug levels in human breast milk is of importance because the excreted drug is a potential source of unwanted exposure and resulting pharmacological effects in the nursing infant. Assaying for drugs in milk can present unique bioanalytical challenges due to its high and variable lipid, carbohydrate and protein content. A limited number of assays for measuring various neuroleptic drugs in human milk have been reported for haloperidol [5,6], diazepam [7], chlorpromazine [8], chloroprothiexene, zuclopenthixol and flupentixol [9] however, no specific assay has been reported for olanzapine.

A sensitive assay for measuring olanzapine in human plasma was developed by Goodwin et al. [10] using a gas chromatography–mass spectrometry method with liquid–liquid extraction and negative chemical ionization mass spectrometry with selective ion monitoring. Recently, two high-performance liquid chromatography (HPLC) methods have been published for measuring olanzapine in human and rat plasma using electrochemical detection [11,12]. The aim of this work was to develop a sensitive and robust method for measuring olanzapine in human milk that could be used to support clinical studies.

Presented here is a sensitive and reproducible HPLC assay for olanzapine in breast milk. The HPLC method utilizes a mixed-mode solid-phase extraction (SPE) column for isolating olanzapine and a structurally related thienobenzodiazepine (Fig. 1), which is used as the internal standard. The analytes were separated by reversed-phase HPLC and detected using electrochemical detection. This method was used to measure olanzapine concentrations in breast milk samples from a clinical trial designed to assess the safety of breast feeding during maternal drug therapy.

2. Experimental

2.1. Chemicals and reagents

Olanzapine [2-methyl-4-(4-methyl-1-piperazinyl)-10H-thieno[2,3-*b*][1,5]benzodiazepine] and the internal standard [2-ethyl-4-(4-methyl-1-piperazinyl)-10H-thieno[2,3-*b*][1,5]benzodiazepine] were synthesized at Eli Lilly (Indianapolis, IN, USA). HPLC-quality water was double deionized and filtered through a Millipore Milli-Q System (Marlborough, MA, USA). HPLC-grade methanol, *n*-propanol, acetonitrile and methylene chloride were purchased from Mallinckrodt (Paris, KY, USA). Reagent-grade phosphoric acid (85%), glacial acetic acid, 5 *M* sodium hydroxide and 25% ammonium hydroxide were purchased from J.T. Baker (Phillipsburg, NJ, USA). Ethyl acetate was purchased from Burdick and Jackson (Muskegon, MI, USA). Potassium phosphate monobasic–sodium hydroxide buffer, 50 m*M*, pH 6.0 (PPMSH) and potassium carbonate–potassium borate–potassium hydroxide buffer, 50 m*M*, pH 10.0 (PCPBPH) were purchased from Fisher Scientific (Pittsburgh, PA, USA). Control (blank) breast milk was donated from healthy nursing mothers.

2.2. Sample preparation and extraction procedure

Milk was warmed in a 50°C water bath for approximately 5 min. The milk was removed from the water bath, vortex mixed, and a 1-ml aliquot was pipetted into a silylated 100 mm×16 mm glass

screw-cap tube followed by 3 ml of 50 m*M* phosphate buffer (pH 6.0) and 50 μl of internal standard (0.5 ng/μl). The sample was vortex mixed and placed back into the water bath for at least 5 min but no more than 15 min. The sample was removed from the water bath, vortex mixed and added to a Bond Elut Certify LRC ion-exchange/reversed-phase cartridge (Varian, Harbor City, CA, USA) which had been conditioned by sequential washing with 3 ml of methanol and 3 ml of 50 m*M* phosphate buffer, pH 6.0 (PPMSH). Vacuum was applied to the cartridge to allow the sample to pass through at a rate of approximately 1 drop/s. The cartridge was sequentially washed with 3 ml pH 6.0 buffer (PPMSH), 1 ml of washing buffer (PCPBPH–methanol, 70:30, v/v) and 1 ml of 1.0 *M* acetic acid. The cartridge was then dried under vacuum for approximately 5 min. The cartridge was further washed with 2 ml of methylene chloride followed by 3 ml of methanol and again dried under full vacuum for approximately 5 min. The analytes were eluted from the cartridge with 3 ml of 2% (v/v) ammonium hydroxide in ethyl acetate. The eluate was evaporated to dryness at approximately 40°C using a gentle stream of nitrogen. The residue was reconstituted with 100 μl of HPLC mobile phase. To prevent the formation of *N*-oxides, and to maintain maximum analyte recovery, the samples were removed from the drying apparatus within 5 min after complete evaporation of the eluting solution. Sample residues were reconstituted with mobile phase within 10 min after drying.

2.3. High-performance liquid chromatography

The HPLC system consisted of a Shimadzu LC-10AD pump, SCL-10A system controller, SIL-10AXL auto injector (Shimadzu, Kyoto, Japan), ESA Coulochem II Model 5200A electrochemical detector equipped with a Model 5011 dual analytical cell (electrodes 1 and 2 set at +200 mV and −200 mV, respectively) and a Model 5020 guard cell set at −300 mV (ESA, Chemstord, MA, USA). The analytical column was a YMC basic, 150 mm×4.6 mm I.D., 5 μm particle size (YMC, Wilmington, NC, USA) which was preceded by a YMC basic guard column, 23 mm×4.6 mm I.D., 5 μm particle size. The HPLC mobile phase was 75 m*M* phosphate buffer–methanol–acetonitrile (48:26:26, v/v/v). The 75 m*M* phosphate buffer was prepared by adding 5.1 ml of concentrated phosphoric acid to approximately 900 ml of water, adjusting the pH to 7.0 with 5 *M* sodium hydroxide and diluting with water to a final volume of 1 l. The flow-rate was 1.2 ml/min and the column was maintained at 40°C using a Model CH-30 column heater (FIAtron Systems, Oconomowol, WI, USA). Integration by peak heights was performed using a Perkin Elmer Access*Chrom data reduction system (PE Nelson, Cupertino, CA, USA).

2.4. Preparation of standard solutions

Two standard stocks of olanzapine were prepared in *n*-propanol at concentrations of 100 μg/ml. These stocks were prepared from different weighings of the compound; one stock was designated as the calibration curve stock and the other was the validation sample stock. Working standards were prepared in *n*-propanol from the stock standards at concentrations of 10, 1, 0.1 and 0.01 μg/ml.

2.5. Standard curves and validation samples

A nine-point standard curve ranging from 0.25 ng/ml to 100 ng/ml was prepared for each validation analysis. Validation samples were prepared by spiking control breast milk (1-ml aliquots) with 0.25 ng, 50 ng and 100 ng of olanzapine. Five replicates at each concentration were assayed on three separate days to determine the inter- and intra-assay precision and accuracy of the method.

2.6. Stability experiments

Ten months of freezer stability of olanzapine in human milk was determined at −70°C and after three freeze–thaw–heat conditions. The stability of reconstituted milk extracts was assessed as well as the stability of the working standard stocks of olanzapine.

2.7. Extraction recovery experiments

Two sets of standards containing olanzapine at 0.5 ng/ml and 100 ng/ml were prepared: one set in human milk and the other set in mobile phase. In addition, two sets of standards containing internal

standard (5 ng/ml) were prepared in a similar manner. Milk standards were processed and analyzed as described in Sections 2.2 and 2.3, while standards prepared in mobile phase were injected directly onto the column.

3. Results

3.1. HPLC electrochemical detection

Representative chromatograms of control human milk and standard curve samples at 0.25 ng/ml, 1 ng/ml and 5 ng/ml of olanzapine are shown in Fig. 2. The chromatographic separation was completed within 13 min with olanzapine and the internal standard eluting at approximately 7.5 and 11.5 min, respectively. There was no evidence of interferences or late eluting peaks. A representative chromatogram derived from milk obtained from a patient administered olanzapine is shown in Fig. 3.

3.2. Standard curve statistics and assay precision and accuracy

Mean back-calculated values for standards are shown in Table 1. The linearity of the assay during the validation as represented by the coefficient of determination ranged from 0.9992 to 1.0000. The average accuracy of the back-calculated values

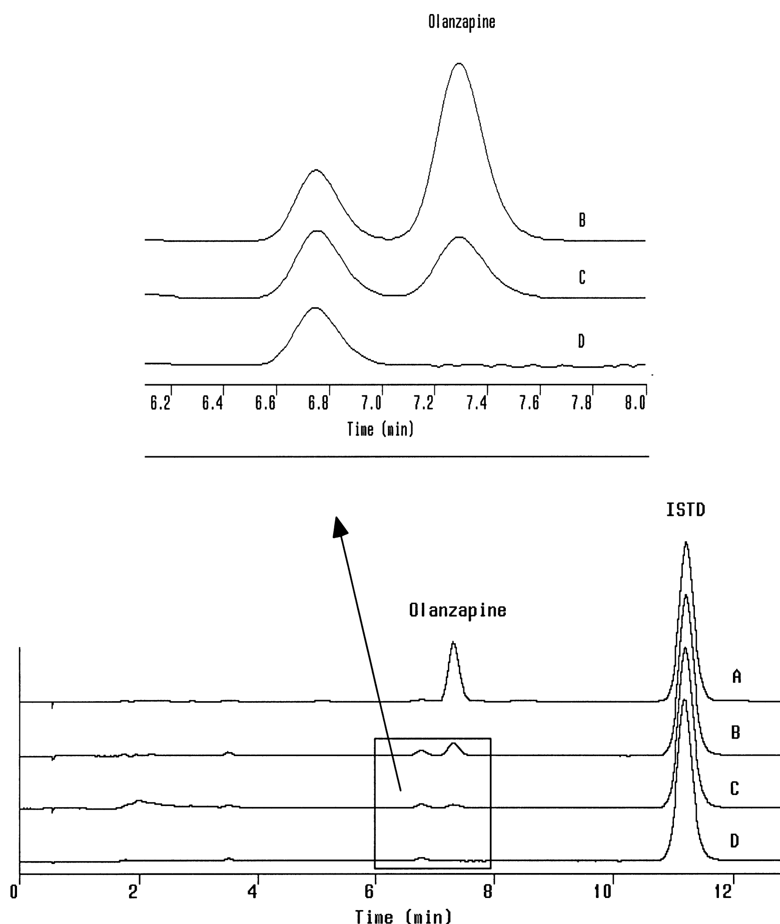

Fig. 2. Chromatogram of calibration samples at 5 ng/ml (A), 1.0 ng/ml (B), 0.25 ng/ml (C), and control human milk (D). ISTD=internal standard. Insert shows enlarged lower standards and control.

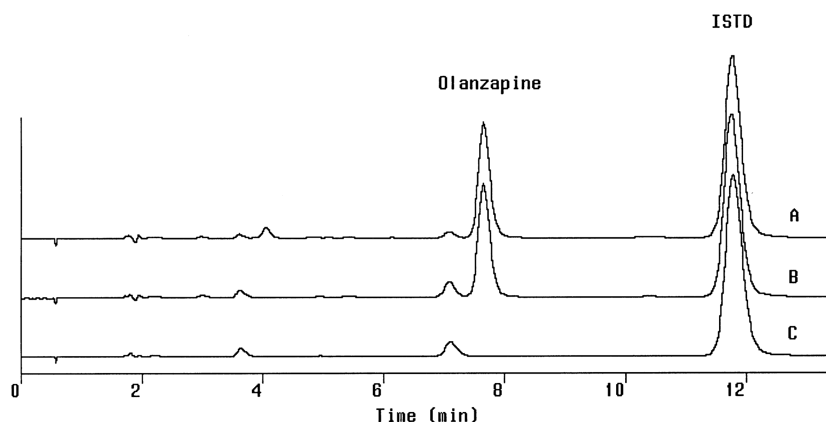

Fig. 3. Chromatogram of a human milk sample from a clinical trial (A), human milk spiked with olanzapine at 10 ng/ml (B), and control human milk (C).

Table 1
Mean back-calculated standard curve statistics of HPLC method[a]

Inter-assay statistics	0.25 ng/ml	0.5 ng/ml	1 ng/ml	5 ng/ml	10 ng/ml	25 ng/ml	50 ng/ml	100 ng/ml	Overall average
Mean (ng/ml)	0.271	0.521	1.01	4.82	9.69	24.6	49.7	100	–
Accuracy (% RE)	108	104	101	96.4	96.9	98.2	99.5	100	101
Precision (% CV)	12.6	3.29	5.42	6.16	5.22	3.74	2.25	1.06	4.96
Days	10	10	10	10	10	10	10	10	10
n (total)	10	10	10	10	10	10	10	10	80

[a] Data represents three days of validation and seven days of clinical trial sample analysis.

ranged from 96.4 to 108% with precision between 1.06 and 12.6%. The curves proved to be highly reproducible with slopes ranging from 0.0682 to 0.0895 and y-intercept values consistently near zero.

The precision and accuracy of the assay were determined from validation samples prepared at 0.25 ng/ml, 50 ng/ml and 100 ng/ml in replicates of five over three separate analyses (Table 2). In addition, Table 2 also contains quality control sample data obtained during patient sample analyses at concentrations of 5 ng/ml, 25 ng/ml and 75 ng/ml. Overall, the inter-day accuracy of the assay from 0.25 ng/ml to 100 ng/ml ranged from 94.8 to 102% with precision ranging from 2.49 to 10.6%.

Table 2
Validation and quality control sample statistics for HPLC method

Inter-assay statistics	0.25 ng/ml[a]	5 ng/ml[b]	25 ng/ml[b]	50 ng/ml[a]	75 ng/ml[b]	100 ng/ml[a]	Overall average
Mean (ng/ml)	0.252	4.94	23.7	50.2	72.4	102	–
Accuracy (% RE)	101	98.8	94.8	101	96.6	102	99.0
Precision (% CV)	8.37	10.6	7.26	2.49	8.36	2.74	6.64
Days	3	7	7	3	7	3	5
n (total)	15	14	14	15	14	15	87

[a] Concentrations used during three-day validation.
[b] Concentrations used during the analysis of clinical trial samples.

Table 3
Stability of olanzapine after three freeze–thaw–heat cycles

Theoretical values (ng/ml)	Freeze–thaw–heat samples[a]			Positive control samples[b]		
	5	25	75	5	25	75
Mean	4.63	23.4	72.6	4.68	23.5	73.8
Accuracy (% RE)	92.6	93.6	96.8	93.6	94	98.4
Precision (% CV)	1.77	0.427	2.28	1.4	1.95	0.207
n	3	3	3	3	3	3

[a] Samples were frozen, thawed and heated three times before analysis.
[b] Samples prepared and not frozen before analysis.

3.3. Stability of olanzapine

Milk samples were spiked at 10 ng/ml and 75 ng/ml and stored frozen at −70°C for 10 months. After 10 months of storage, samples at each concentration were analyzed in replicates of five. The mean olanzapine values were 9.57 ng/ml and 74.8 ng/ml, respectively, with accuracy ranging from 95.7 to 99.7% and precision ranging from 1.50 to 3.20%.

To assess the effects of freezing, thawing and heating on olanzapine spiked milk, samples were subjected to three freeze (−70°C)–thaw–heat (50°C) cycles before analysis. The mean accuracy and precision of the freeze–thaw–heated samples (Table 3) were similar to those obtained for freshly prepared samples indicating that olanzapine remained stable for at least three freeze–thaw–heat cycles. In addition, sample extracts were shown to be stable for at least 48 h after extraction. Standard stock solutions of olanzapine in *n*-propanol were shown to remain stable for four months at 4°C.

3.4. Extraction recovery

The results of the comparison of neat standards versus milk extracted standards indicated that the extraction recovery of olanzapine in human milk was 89.1% at 0.5 ng/ml and 103.3% at 100 ng/ml. The extraction recovery of the internal standard from milk at 5 ng/ml was 86%.

4. Discussion

Human milk contains high amounts of fat which can vary within a single individual and between individuals from 7 to 100.5 mg/ml [13]. Once human milk is frozen and thawed, the solution becomes heterogeneous even after vigorous mixing. In this heterogeneous state, olanzapine distributes more in the fat than the skim milk phase which makes it difficult to obtain a homogeneous representative sample. Therefore, two techniques were investigated in an attempt to homogenize thawed milk prior to sampling. One technique involved sonicating the milk using either a typical bench-top sonicating bath or a high-frequency probe sonicator. The previous proved ineffective at homogenizing human milk, and the latter was extremely labor intensive. A second technique investigated involved heating the milk in a water bath at approximately 50°C. When heated, the lipid phase was solubilized and the milk returned to its original consistency. The milk samples remained in this state for several minutes which allowed enough time to pipette the samples. This warming step was shown not to affect the stability of olanzapine for at least three freeze–thaw–heat cycles. After the addition of the buffer and internal standard, the samples were placed back into a water bath for an additional 5 to 15 min. This step was necessary to ensure homogeneity and to permit the diluted milk samples to more easily flow through the SPE cartridges. Generally, milk samples flowed easily though the SPE cartridges with slight vacuum. Unlike the previously described plasma method, an additional washing step using methylene chloride was needed to help remove more lipophilic endogenous compounds in the milk. With the five different washing steps, the SPE procedure provided an extract which was free of any endogenous compounds that eluted at the retention times of olanzapine or its internal standard. In order to prevent the formation of *N*-oxides and maintain maximum ana-

lyte recovery drying temperature should not exceed 40°C. Additionally, the sample tubes should be removed from the drying apparatus within 5 min after the samples have dried and redissolved in the HPLC injection solvent within 10 min.

The HPLC method described has been validated and is currently in use to support the analysis of olanzapine in human breast milk after oral administration of olanzapine. The method has proven to be robust and sensitive with a limit of quantitation of 0.25 ng/ml. To date, the method has been shown to be very rugged as over 200 milk samples have been analyzed using a single HPLC analytical column without any apparent loss of sensitivity or resolution.

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