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Short communication

Determination of olanzapine in human blood by liquid chromatography–tandem mass spectrometry

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Abstract

A liquid chromatographic–tandem mass spectrometric (LC–MS–MS) assay was developed and validated to quantitatively determine olanzapine (OLZ) concentrations in human blood. Liquid–liquid extraction, using *n*-butanol:cyclohexane (3:47, v/v), was used to isolate OLZ and its internal standard, LY170158, from the biological matrix. Chromatographic resolution of OLZ from endogenous interferences and known metabolites was accomplished with a MetaChem Monochrom HPLC column (4.6×150 mm, d_p 5 μm). Detection occurred using a Perkin-Elmer Sciex API III Plus triple quadrupole mass spectrometer using positive ion APCI and multiple reaction monitoring (MRM). The linear dynamic range was from 5 to 500 ng ml^{-1} based on a 0.25-ml aliquot of human blood. The inter-day precision (%RSD) and accuracy (%RE) ranged from 3.65 to 10.64 and from −2.14 to 3.07, respectively. Modifications to an existing assay for the determination of OLZ in human plasma were necessary. A different structural analog was used as the internal standard due to instability observed for the original analog when using human blood as the matrix. A second modification was the addition of the anti-oxidant sodium ascorbate to inhibit degradation of OLZ in human blood, as has been noted by other investigators. Upon fortification of human blood with sodium ascorbate (final concentration, 0.33 m*M*), OLZ was found to be stable for at least 1 week at −70°C as well as through two freeze–thaw cycles. This assay, which will be used to investigate the distribution of OLZ in human blood, grants insight into the proper sample handling conditions needed to perform valid determinations of OLZ in human blood.

Keywords: Olanzapine

1. Introduction

Olanzapine (Fig. 1) is a novel, atypical antipsychotic drug used to treat schizophrenia and related disorders [1]. It is a member of a class of compounds known as thienobenzodiazepines, and has affinity for serotonin (5-HT_{2A}, 5-HT_{2C}), dopamine (D_1–D_4),

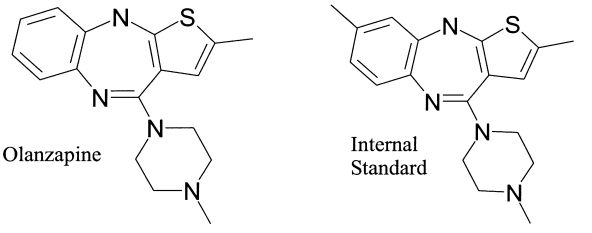

Fig. 1. Chemical structures of olanzapine and internal standard (LY170158).

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muscarinic (M_1–M_5), α_1-adrenergic and histaminergic (H_1) receptors [2,3]. Clinical studies have shown olanzapine (OLZ) to be effective in the treatment of schizophrenia, to have a lower incidence of treatment-emergent extrapyramidal symptoms and greater decreases in total psychopathology relative to haloperidol, and to have a favorable safety profile [4].

Liquid chromatography–tandem mass spectrometry has proven to be a powerful tool for determining drug concentrations in biological matrices [5–7]. In this paper, an LC–MS–MS assay is described for the quantitative determination of OLZ in human blood. This assay is a modified version of an LC–MS–MS assay used to determine OLZ in human plasma and serum [8]. Owing to the difficulties in extracting drugs from whole blood, a liquid–liquid extraction was used in place of the solid-phase extraction described in the aforementioned paper. Degradation in blood proved to be a significant problem for the previous internal standard, LY170222. Therefore, a different structural analog, LY170158, was chosen as the internal standard (Fig. 1). Finally, because OLZ was found to oxidize in human blood, the antioxidant ascorbic acid was added to the samples upon collection.

A search of the literature for alternative analytical methodology uncovered a paper by Robertson and McMullin [9] that discussed olanzapine concentrations in clinical serum and postmortem blood specimens. The authors used solid-phase extraction and capillary GC–NPD to measure sample concentrations; however, issues of olanzapine oxidation in blood and serum were not investigated, and the impact on the assay of circulating metabolites was not addressed.

This assay will be used to measure OLZ in human blood to determine its pharmacokinetics and distribution.

2. Experimental

2.1. Chemicals and materials

Olanzapine and compound LY170158 (internal standard) were obtained from Eli Lilly and Company (Indianapolis, IN, USA). Control human blood was obtained from human health services at Eli Lilly and Company. HPLC grade methanol, *n*-butanol, *n*-propanol, and cyclohexane were obtained from Burdick and Jackson (Muskegon, MI, USA). Analytical reagent grade trifluoroacetic acid (TFA), ammonium acetate, and sodium carbonate were obtained from Mallinckrodt (Paris, KY, USA), and water was from a Millipore Milli-Q system (Bedford, MA, USA). L-Ascorbic acid, 99+%, A.C.S. reagent, was from Aldrich (Milwaukee, WI, USA). Sodium hydroxide (5 N) solution was from Red Bird Service (Osgood, IN, USA).

2.2. Equipment and analytical conditions

The HPLC system consisted of the following components: two Shimadzu LC-10AD pumps, SCL-10A system controller, GT-104 solvent degasser, and a SIL-10AXL autosampler (Kyoto, Japan). The chromatographic system consisted of a MetaChem Monochrom HPLC column (4.6×150 mm, d_p 5 μm; Torrance, CA, USA) and used a mobile phase, delivered by a linear gradient at 1 ml min^{-1}, that consisted of the following components: (A) 100 m*M* ammonium acetate, and (B) methanol: isopropanol:water (15:4:1, v/v/v). The gradient profile was as follows (min/%B): 0.0/20, 0.1/20, 0.2/45, 5.0/70, 6.0/85, 6.1/20, 9.0/20. The autosampler was programmed to inject 50-μl sample aliquots every 9 min.

Mass spectrometric detection was performed on a Sciex API III Plus (Toronto, Canada) operating in the positive ion APCI mode. The heated nebulizer was set at 550°C with the discharge ionization current at 5 μA. The orifice potential was set at 55 V, and the collision gas thickness (90/10 Ar/N_2) was at ~275×10^{12} atoms cm^2. Nitrogen was used as the auxiliary and nebulizer gas and was set at 1.5 l min^{-1} and 80 p.s.i. (552 kPa), respectively. Quantitation was performed using multiple reaction monitoring of the following transitions: OLZ (m/z 313.4→256.2), and internal standard (m/z 327.3→270.1), with a dwell time of 350 ms. (The product ion spectrum of OLZ and the proposed structure of its product ion were previously reported [8].) Calibration was performed by plotting the peak area ratio of OLZ to the internal standard versus OLZ concentration using a linear regression with $1/x^2$ weighting. Finally, mass calibration, data acqui-

sition, chromatographic and mass spectral representation as well as post-acquisition quantitative analyses were performed using a suite of PE Sciex software applications: Tune 2.5, RAD 2.6, MacSpec 3.3, and MacQuan 1.3.

2.3. Standard solutions

A standard stock solution of OLZ was prepared by dissolving ~5 mg of OLZ in *n*-propanol so that the final concentration was 0.1 mg ml^{-1}. This solution was diluted with *n*-propanol to give a series of working solutions with concentrations of 5000, 2000, 1000, 500, 200, 100, and 50 ng ml^{-1}. The standard stock solutions were prepared in duplicate from separate weighings (standards vs. validation samples). A standard stock solution of LY170158 (internal standard) was also prepared by dissolving ~1 mg in *n*-propanol so that the final concentration was 20 μg ml^{-1}. This solution was diluted with *n*-propanol to give a working internal standard solution of 500 ng ml^{-1}. The stock solutions were placed in polypropylene screw-cap volumetric flasks and stored at approximately −20°C when they were not in use. Under these conditions, the standard solutions were stable for at least 90 days.

2.4. Sample preparation

The blood used to prepare the standard curve, validation samples and dilutions was pretreated with a 3.33 m*M* solution of sodium ascorbate by adding 100 μl per ml of whole blood (final concentration of ~333 μ*M*). The standard curve samples were prepared by placing 250 μl of the pretreated blood into a polypropylene centrifuge tube and spiking 25 μl of the appropriate standard working solution followed by 25 μl of the internal standard working solution. Standards were prepared for each analysis during the validation at the following concentrations: 500, 200, 100, 50, 20, 10, and 5 ng ml^{-1}.

The validation samples, used to evaluate accuracy and precision during the validation, were prepared in the same fashion as the standard curve samples. During the validation, five replicates at each of the following concentrations were prepared and analyzed on each of 3 days: 500, 50, and 5 ng ml^{-1}.

A double blank sample (no internal standard or standard) was prepared by spiking 50 μl of *n*-propanol (to substitute for the standard solutions) into 250 μl of control pretreated blood, and run with each analysis. A blank sample (no standard) was prepared by spiking 25 μl of *n*-propanol and 25 μl of internal standard working solution into 250 μl of control pretreated blood, and run with each analysis.

All samples were extracted by adding 500 μl of 2% sodium carbonate (w/v) and 3 ml *n*-butanol:cyclohexane (3:47, v/v) to each sample tube. Next, the samples were placed on a bed shaker for 15 min followed by centrifugation at ~3000 *g* for 10 min. Using a pipette, 2 ml of each organic layer was transferred to a second polypropylene tube and concentrated to dryness under a stream of nitrogen (~40°C). The dried residues were reconstituted with 500 μl of reconstitution solution (100 m*M* ammonium acetate aq.:methanol:isopropanol (45:4:1, v/v/v), transferred to autosampler vials, and injected (50 μl) onto the LC–MS–MS for analysis.

2.5. Validation procedures

Validation samples were prepared and analyzed to evaluate the intra-day and inter-day accuracy and precision of the analytical method in human blood. Five replicates of each of the validation concentrations (500, 50 and 5 ng ml^{-1}) were analyzed along with one set of standard samples on each of 3 days using the same instrument.

The extraction efficiency of OLZ and the internal standard were determined by comparing the peak areas of extracted samples to the peak areas of extracted blanks spiked with standard and internal standard. In addition, the effect of the matrix on the detection of the analytes (matrix suppression) was evaluated by comparing the extracted blanks spiked with standard and internal standard to neat standards at the same concentrations. The selectivity of the assay was investigated by processing and analyzing blanks prepared from six independent lots of control blood; the blanks were surveyed for interfering peaks at the transitions monitored. The metabolism of OLZ was investigated by Kassahun et al. who showed several OLZ metabolites were present in human plasma [10]. The selectivity of the assay with respect to known OLZ metabolites was demonstrated [8].

The stability of OLZ in human blood was studied

under a variety of storage and process conditions. The freezer stability (−70°C), the stability of the analytes in the injection solvent (extract stability), the freeze–thaw stability (two cycles), and room temperature stability (24 h in blood) were studied by analyzing triplicate validation samples at 50 and 500 ng ml^{-1} under the described conditions. Finally, sample dilutions were tested by analyzing duplicate 50 ng ml^{-1} samples that were diluted with control pretreated whole human blood from a 2500 ng ml^{-1} blood sample (dilution factor of 1:50).

3. Results

3.1. Linearity and calibration

Good linearity was observed over the concentration range of 5–500 ng ml^{-1} of OLZ: $y = 0.02243x - 0.01308$ (average of 13 separate analysis days). The calibration graph was derived by plotting the peak area ratio of OLZ to the internal standard versus plasma concentration of OLZ; a linear regression with $1/x^2$ weighting was used. During the validation, the correlation coefficients obtained were 0.9983 or higher. The residual error of the calibration graph, as determined by the percent error of the back-calculated standard samples, was less than ±15% in all cases (13 separate analyses).

3.2. Precision and accuracy

The data for the intra-day and inter-day assay precision and accuracy, determined by analyzing five replicates at 5, 50, and 500 ng ml^{-1} on each of 3 days, are reported in Tables 1 and 2, respectively. The accuracy of the method was determined by calculating the percent relative error (%RE), and the precision was determined by calculating the percent relative standard deviation (%RSD). In human blood, the inter-day precision (%RSD) ranged from 3.65 to 10.64, and the inter-day accuracy (%RE) ranged from −2.14 to 3.07, over the three concentrations evaluated.

3.3. Stability

Standard stock solutions of OLZ and the internal standard were found to be stable for at least 35 days when prepared in *n*-propanol and stored in polypropylene vials at approximately −20°C. Also, OLZ was found to be stable at approximately −70°C (in human blood) for at least 7 days. No degradation of OLZ or the internal standard was observed in the reconstitution solvent during the period the samples were on the autosampler waiting to be injected (up to 48 h), during two freeze–thaw cycles, or during incubation at room temperature (up to 24 h) in human blood.

Table 1
Intra-day validation statistics for OLZ in human blood

Day	Parameter	Validation sample level (ng ml^{-1})		
		5	50	500
1	Average (ng ml^{-1})	5.60	50.24	512.35
	SD (ng ml^{-1})	0.31	2.64	21.43
	Accuracy (%RE)	12.01	0.48	2.47
	Precision (%RSD)	5.45	0.26	4.18
	n	5	5	5
2	Average (ng ml^{-1})	4.47	47.90	518.02
	SD (ng ml^{-1})	0.15	4.29	19.01
	Accuracy (%RE)	−10.65	−4.19	3.60
	Precision (%RSD)	3.31	8.96	3.67
	n	5	5	5
3	Average (ng ml^{-1})	4.84	48.65	515.75
	SD (ng ml^{-1})	0.18	1.99	19.93
	Accuracy (%RE)	−3.18	−2.69	3.15
	Precision (%RSD)	3.63	4.09	3.86
	n	5	5	5

3.4. Assay specificity

The specificity of the assay was demonstrated by the absence of endogenous substances, in drug free blood, that interfere with the quantitation of OLZ at the lower limit of quantitation (LLOQ). Combining a

Table 2
Inter-day validation statistics for OLZ in human blood

Day	Parameter	Validation sample level (ng ml^{-1})		
		5	50	500
1	Average (ng ml^{-1})	5.00	48.90	515.40
	SD (ng ml^{-1})	0.53	3.07	18.80
	Accuracy (RE%)	−0.61	−2.14	3.07
	Precision (RSD%)	10.64	6.27	3.65
	n	15	15	15

liquid–liquid extraction procedure with the separation power of HPLC and the selectivity of tandem mass spectrometry minimized potential interferences. Representative ion chromatograms of a 5 ng ml^{-1} standard with its associated blank sample are presented in Fig. 2. Representative ion chromatograms of the internal standard and its associated blank sample are presented in Fig. 3. Furthermore, due to mass spectral interference between OLZ and its

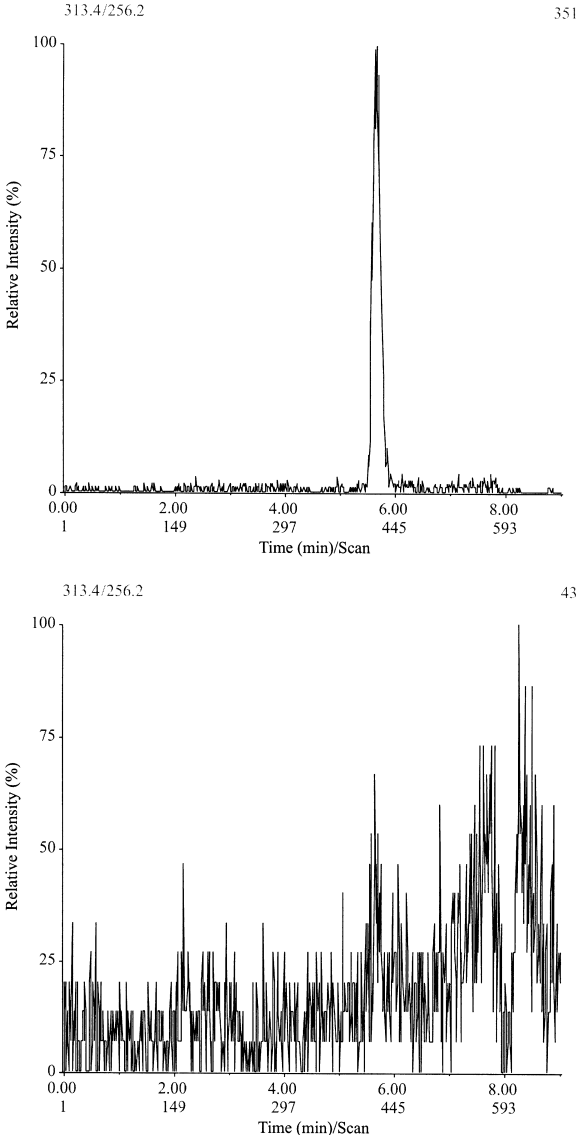

Fig. 2. Representative ion chromatograms of a 5 ng ml^{-1} olanzapine standard and an olanzapine blank extracted from human blood.

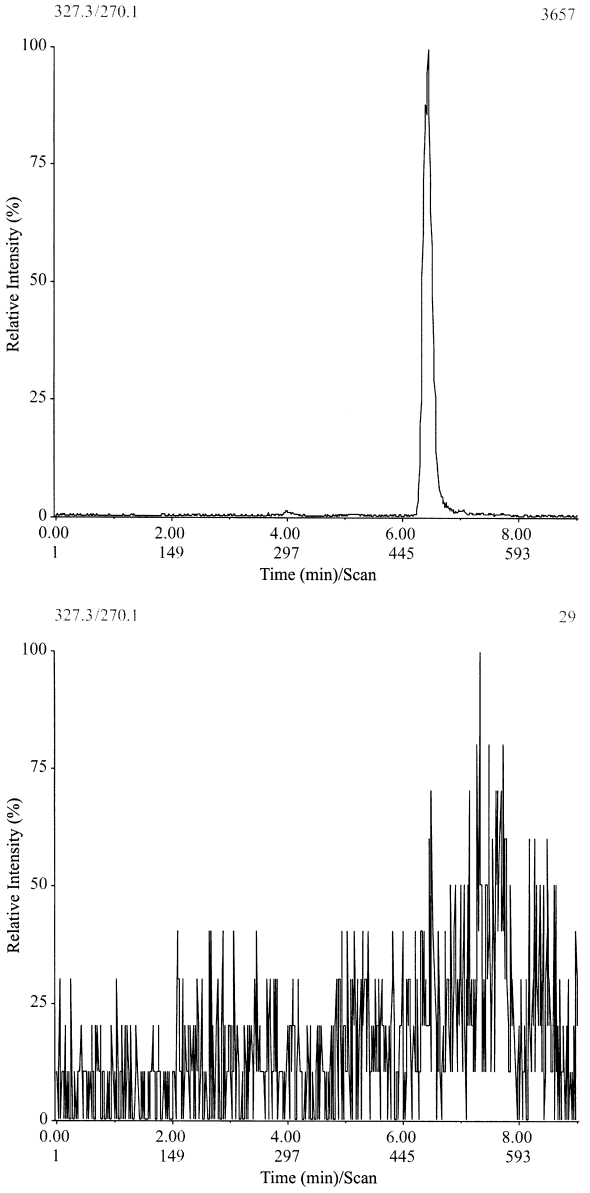

Fig. 3. Representative ion chromatograms of the internal standard (LY170158) and an LY170158 blank extracted from human blood.

metabolites, chromatographic resolution was achieved to prevent a deleterious effect on analyte quantitation [8].

3.5. Extraction efficiency and dilutions

The extraction efficiency of OLZ was 73.6–93.2%, and the extraction efficiency of the internal

standard, compound LY170158, was 78.1–83.9%. The biological matrix was found to suppress the signal of the analyte and internal standard by ~2.8%.

Samples containing high concentrations of the analyte were diluted up to1:50 in control human blood. It was demonstrated that the accuracy of the dilutions was off by no more than 10.2% from the theoretical value.

4. Discussion

In a previous communication, we described an assay for the determination of OLZ in human plasma using SPE [8]. This method has a linear dynamic range of 0.5–1000 ng ml^{-1}. Unfortunately, a number of problems were experienced when this extraction was applied to the analysis of OLZ in human blood. In addition to variable recovery, practical issues such as cartridge plugging were observed owing to the consistency of the matrix. Another important issue was matrix ion suppression, which both limited the response for OLZ and created significant variation in the assay. Using the approach first reported by Buhrman et al., matrix ion suppression was differentiated from recovery [11]. The high recovery and low matrix ion suppression reported, both for OLZ and its internal standard, are indications of a highly selective extraction.

Another major issue affecting the bioanalysis of OLZ in human blood is stability. The results presented demonstrate the usefulness of the anti-oxidant sodium ascorbate in maintaining stability allowing successful assay validation as well as short-term storage stability (1 week). Extended long-term stability is being investigated. Despite the high amount of sodium ascorbate used, no adverse affect was realized on the sensitivity of the assay indicating that this additive did not partition into the organic layer.

The assay presented allows for an accurate and precise determination of OLZ in human blood and will be used to assess the distribution of OLZ in blood. This method could also be applied to clinical analysis, provided necessary precautions are taken upon sample collection to inhibit OLZ degradation in the matrix. The instability of OLZ in human blood, which was further documented by the results present herein, calls into question the validity of OLZ concentrations reported for post-mortem cases. Obviously, post-mortem tissue redistribution presents another complicating factor. The selectivity of the present assay, along with the documented stability for sample handling and storage, validates this method as a legitimate tool for studies of OLZ where human blood is the matrix.

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