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Determination of Olanzapine in Human Plasma and Serum by Liquid Chromatography/Tandem Mass Spectrometry

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A liquid chromatographic/tandem mass spectrometric (LC/MS/MS) assay was developed for the quantitative determination of olanzapine (LY170053, OLZ) in human plasma and serum. Bond Elut C_2 solid-phase extraction cartridges (single cartridge or 96-well format), in conjunction with a positive pressure manifold, were used to extract OLZ and its internal standard, LY170222, from the biological matrix. Chromatographic resolution of OLZ from endogenous plasma interferences and its metabolites was accomplished with a MetaChem monochrom HPLC (4.6 × 150 mm, d_p 5 μm). Detection was effected with a Perkin–Elmer SCIEX API III Plus mass spectrometer using positive ion atmospheric pressure chemical ionization and a multiple reaction monitoring protocol. The linear dynamic range was from 250 pg ml^{-1} to 50 ng ml^{-1} of human plasma/serum using a 0.5 ml aliquot. The inter-day precision (relative standard deviation) and accuracy (relative error) in plasma ranged from 6.26 to 7.66% and from −3.54 to 7.52%, respectively. The intra-day precision and accuracy in serum ranged from 3.46 to 8.76% and from −8.06 to 12.46%, respectively. This assay is sensitive and selective, and will be used to support both human clinical and toxicological analyses. Furthermore, using the 96-well solid-phase extraction format, sample preparation can be easily automated. © **1998 John Wiley & Sons, Ltd.**

KEYWORDS: olanzapine; antipsychotic drug; liquid chromatography/tandem mass spectrometry; atmospheric pressure chemical ionization; multiple reaction monitoring

INTRODUCTION

Olanzapine (OLZ) (Fig. 1) is a novel, atypical antipsychotic drug used for the treatment of schizophrenia and related psychotic disorders.[1] It is a member of the thienobenzodiazepine class of chemicals and has high affinity for serotonin (5-HT_{2A}, 5-HT_{2C}), dopamine (D_1–D_4), muscarinic (M_1–M_5), α_1-adrenergic and histaminergic (H_1) receptors.[2,3] Clinical studies have shown olanzapine to be more effective than placebo in the treatment of psychotic disorders, to cause fewer extrapyramidal symptoms and greater decreases in total psychopathology relative to haloperidol, and to have tolerable side effects.[4]

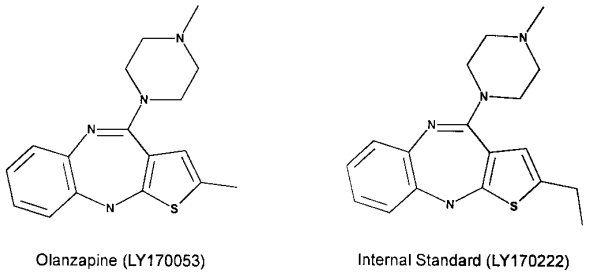

Figure 1. Structures of olanzapine (OLZ) (LY170053) and the internal standard (LY170222).

Previous methodology used for the quantitative determination of OLZ included a high-performance liquid chromatographic (HPLC) assay developed by Aravagiri *et al.*[5] that used electrochemical detection and a liquid–liquid extraction. This assay was validated using quality control (QC) samples over the range 1.17–25.67 ng ml^{-1}, using 1 ml plasma aliquots, and demonstrated good accuracy and precision. The instrumental run time was ~16 min per sample. Potential interferences from OLZ metabolites were not systematically investigated; however, the authors addressed the potential of several commonly co-administered drugs to interfere with OLZ quantitation. A second HPLC/electrochemical assay presented by Catlow *et al.*[6] used a solid-phase extraction (SPE) procedure and was validated using QC samples over the range 0.25–50 ng ml^{-1}, using 1 ml plasma aliquots. This assay had good accuracy and precision and reduced the chemical background through the use of SPE. The instrumental run time was ~12 min per sample. A negative chemical ionization (CI) gas chromatographic/mass spectrometric (GC/MS) assay presented by Goodwin *et al.*[7] used a liquid–liquid extraction and selected ion monitoring; however, this method was complicated and labor intensive.

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Liquid chromatography coupled with tandem mass spectrometry (LC/MS/MS) has become a powerful tool for the quantitative determination of drugs in biological matrices.[8–10] In this paper, an LC/MS/MS assay is presented for the quantitative determination of OLZ in human plasma and serum. A structural analog, LY170222, was used as the internal standard (Fig. 1). This method was developed to serve as a confirmatory assay for toxicological analyses and to support clinical studies. Therefore, the assay was developed to maximize selectivity without sacrificing the instrumental run time, which is <10 min per sample.

The metabolism of OLZ was investigated by Kassahun *et al.*,[11] who showed several OLZ metabolites were presented in human plasma: *N*-glucuronide, 2-hydroxy, *N*-oxide, *N*-desmethyl and 2-carboxylic acid derivatives. These metabolites, especially the *N*-oxide, have the potential to interfere mass spectrally with OLZ and the internal standard. The potential for mass spectral interference was systematically investigated, and chromatographic resolution of OLZ and the internal standard from OLZ metabolites that demonstrated mass spectral interference was obtained.

This assay has proven to be as sensitive as previous OLZ assays, using only 500 μl of plasma or serum, while decreasing the instrument run time. In addition, it offers enhanced selectivity through the use of MS/MS, chromatographic resolution, and an SPE procedure that, relative to the procedures referenced, is less cumbersome than the SPE procedure and more selective than the liquid–liquid extractions. Finally, a 96-well SPE extraction procedure was developed that greatly reduces the sample preparation time and is readily amenable to automation.

EXPERIMENTAL

Chemicals materials

Compound LY170053 (olanzapine, OLZ), compound LY170222 (internal standard), compound LY170055 (OLZ *N*-desmethyl metabolite), compound LY290411 (OLZ 2-hydroxy metabolite), compound OLZ *N*-oxide and compound OLZ 10-*N*-glucuronide were obtained from Eli Lilly (Indianapolis, IN, USA). Control human plasma and serum were obtained from human health services at Eli Lilly. HPLC-grade methanol and acetonitrile were obtained from Burdick and Jackson (Muskegon, MI, USA). Analytical-reagent grade propan-1-ol, trifluoroacetic acid (TFA) and ammonium acetate were obtained from Mallinckrodt (Paris, KY, USA), and high-purity water was produced using a Milli-Q system (Millipore, Redford, MA, USA). Bond Elut C_2 SPE cartridges (1 ml per 100 mg), Microlute C_2 96-well cartridge plates (1 ml extenders, 100 mg) and positive pressure extraction manifolds were obtained from Varian (Sugar Land, TX, USA).

LC/MS equipment and analytical conditions

The HPLC system consisted of the following components: two Shimadzu LC-10AD pumps, an SCL-10A system controller, a GT-104 solvent degasser and a Waters Model 717 Plus autosampler. The chromatographic system consisted of a MetaChem Monochrom HPLC column (4.6 × 150 mm, d_p 5 μm) and used a mobile phase, delivered by a linear gradient at 1 ml min^{-1}, that consisted of (A) 100 mM ammonium acetate and (B) 20% propan-1-ol in methanol. The gradient profile was as follows (minutes/%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 set up to inject 100 μl sample aliquots every 9 min.

Mass spectrometric detection was performed on a Perkin-Elmer SCIEX API III Plus instrument operating in the positive ion atmospheric pressure chemical ionization (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 $\sim 275 \times 10^{12}$ atoms cm^{-2}. Nitrogen was used as the auxiliary and nebulizer gas and was set at 1.5 l min^{-1} and 80 psi, respectively. Quantitation was performed using multiple reaction monitoring (MRM) of the transitions OLZ *m/z* 313.4 → 256.2 and internal standard *m/z* 327.6 → 270.0; the optional OLZ metabolite transitions were OLZ *N*-desmethyl *m/z* 299.4 → 198.2, OLZ 2-hydroxy *m/z* 329.4 → 272.2, OLZ *N*-oxide *m/z* 329.4 → 213.2 and OLZ 10-*N*-glucuronide *m/z* 489.0 → 355.2. A dwell time of 330 ms was used when monitoring OLZ and the internal standard and a dwell time of 200 ms was used when OLZ metabolites were monitored. The product ion spectra of OLZ and its internal standard, LY170222, are presented in Fig. 2. Mass calibration, data acquisition, chromatographic and mass spectral representation and post-acquisition quantitative analyses were performed using a suite of Perkin-Elmer SCIEX software applications: Tune 2.5, RAD 2.6, MasSpec 3.3 and MacQuan 1.3.

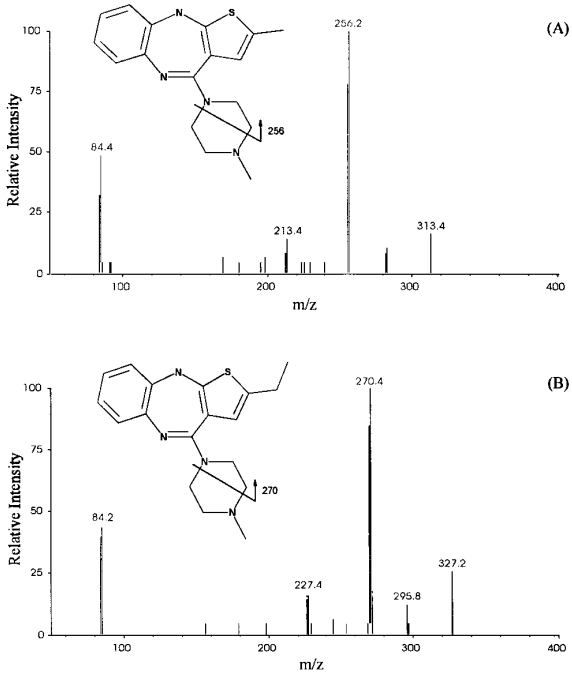

Figure 2. Product ion mass spectra obtained by collision-induced dissociation of (A) olanzapine and (B) internal standard. The proposed structures of the base product ions of olanzapine and the internal standard are indicated.

Standard solutions

A stock standard solution of OLZ was prepared by dissolving ~2.5 mg of OLZ in propan-1-ol so that the final concentration was 1 mg ml^{-1} (adjusted for potency, i.e. the actual mass of drug, adjusted for salt content). This solution was diluted to give a series of working standard solutions with concentrations of 500, 400, 250, 100, 50, 25, 10, 5 and 2.5 ng ml^{-1}. The stock standard solutions were prepared in duplicate from separate weighings; one set was used to prepare standard samples and the other to prepare either validation or QC samples. A stock standard solution of LY170222 (internal standard) was also prepared by dissolving ~2.5 mg of LY170222 in propan-1-ol so that the final concentration was 1 mg ml^{-1}. This solution was diluted to give a working internal standard solution of 140 ng ml^{-1}. The stock standard solutions were placed in polypropylene vials and stored at ~4 °C when they were not in use. Under these conditions, the standard solutions were stable for at least 35 days.

Sample preparation

Calibration samples were prepared by placing 500 µl of plasma in a polypropylene centrifuge tube and spiking 50 µl of the appropriate working standard solution followed by 50 µl of the working internal standard solution. Duplicate standards were prepared for each analysis during the validation and study sample analysis at concentrations of 50, 25, 5, 1, 0.5 and 0.25 ng ml^{-1}.

The validation samples, used to evaluate accuracy and precision during the validation, and the QC samples, used during the analysis of study samples, were prepared in the same fashion as the calibration samples. During the validation, five replicates at each of 50, 5 and 0.25 ng ml^{-1} were prepared and analyzed on each of three days. During the analysis of study samples, QC samples were prepared in duplicate (or more) at concentrations of 40, 5 and 0.5 ng ml^{-1} and run with each analysis.

A blank sample (no internal standard or standard) was prepared and run with each analysis and was included in both validation and study sample analyses. The blank was prepared by spiking 100 µl of propan-1-ol (to substitute for the spike solutions) into 500 µl of control plasma.

The study samples were prepared by adding 500 µl of the sample, 50 µl of propan-1-ol and 50 µl of the working internal standard solution to a polypropylene centrifuge tube.

The sample preparation procedures outlined above were also used to prepare the human serum samples; control human serum was substituted for the control plasma.

All samples were extracted by adding 600 µl of water to each sample tube prior to loading the samples in preconditioned SPE cartridges. (The same extraction procedure was used for both the SPE cartridges and the 96-well cartridge plate.) The SPE cartridges were preconditioned with 500 µl of methanol followed by 500 µl of water. The positive pressure manifold was adjusted such that the flow rate was ~2 ml min^{-1}. After loading the samples, the cartridges were washed with 1 ml of water and eluted with 500 µl of 5% water in methanol containing 0.1% TFA. The samples were concentrated to dryness in 1.5 ml polypropylene microcentrifuge tubes using a Savant Speed Vac. The dried residues were reconstituted with 125 µl of reconstitution solution (90% 100 mM ammonium acetate–8% methanol–2% propan-1-ol). Finally, the samples were transferred to autosampler vials and centrifuged at ~5000*g* for 5 min, and 100 µl aliquots were injected into the mass spectrometer for analysis.

Calibration

Two sets of standard samples were prepared at concentrations of 50, 25, 5, 1, 0.5 and 0.25 ng ml^{-1}. One set of standard samples was run at the beginning of each analysis and the other set at the end of each analysis. The peak area ratio of OLZ to the internal standard was related to concentration using a linear regression with $1/y^2$ weighting.

Validation procedures

Validation samples were prepared and analyzed to evaluate the intra-day accuracy and precision of the analytical method in both human plasma and serum and the inter-day accuracy and precision in human plasma. Five replicates of each of the validation concentrations (50, 5 and 2.5 ng ml^{-1}) were analyzed along with two sets of standard samples on each of three 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. This was performed by analyzing three replicates at each of two concentrations, 25 and 0.5 ng ml^{-1}. In addition, the effect of the matrix on the detection of the analytes (matrix effect) 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 plasma or serum; the blanks were surveyed for interfering peaks. The selectivity was tested in both human plasma and serum. Finally, four common OLZ metabolites were obtained: 2-hydroxy, *N*-oxide, 10-*N*-glucuronide and *N*-desmethyl. The metabolites (at 50 ng ml^{-1}) were analyzed individually, and simultaneously in the presence of OLZ and the internal standard to verify the chromatographic resolution and determine the extent of mass spectral interference.

The stability of OLZ in human plasma and serum was studied under a variety of storage and process conditions. The freezer stability (−70 °C) was evaluated by preparing three validation samples at 0.5, 25 and 1000 ng ml^{-1} and analyzing them after being stored at −70 °C for 1, 2 and 4 weeks. The stability of the analytes in the injection solvent (extract stability) was studied to verify that the compounds would not

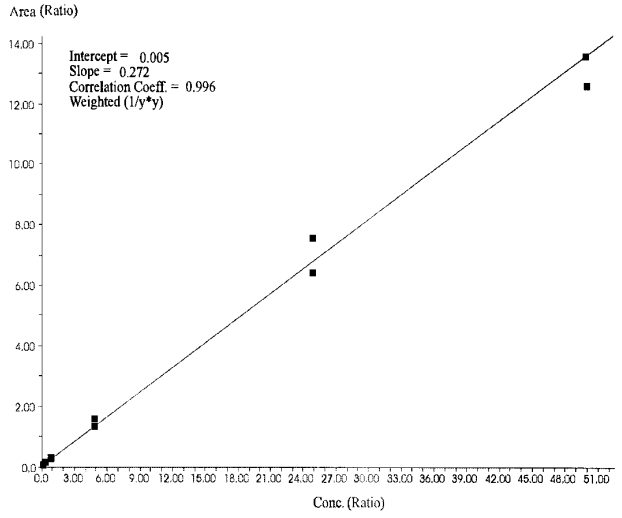

Figure 3. Representative calibration graph for olanzapine in human plasma.

degrade over the course of an analysis. This was accomplished in two ways: first, by performing identical calibration runs at the beginning and end of each analysis throughout the validation and during study sample analysis and, second, by extracting samples and storing them overnight at room temperature and injecting them into the LC/MS/MS system the following day with a fresh calibration graph. The back-calculated standard sample concentrations were used to evaluate the analysis stability. The effect of three freeze–thaw cycles on the stability of the analytes was determined by analyzed triplicate validation samples at 0.5, 25 and 1000 ng ml^{-1} after one, two and three freeze–thaw cycles. Room temperature stability was studied by analyzing triplicate validation samples at 0.5, 25 and 1000 ng ml^{-1} after being incubated in the biological matrix, prior to extraction, for 4 and 24 h. Finally, sample dilutions were tested by analyzing triplicate 50 ng ml^{-1} samples that were diluted with control plasma from a 1000 ng ml^{-1} (dilution factor 1:20) concentrated plasma sample. The stability of the spiking stock standard solutions was tracked by analyzing a neat system suitability sample prior to each analysis at 40 ng ml^{-1}. The same stock standard solutions were sampled for over 1 month to ensure instrumental accuracy and sensitivity for the day, and also stock solution stability.

Table 1. Intra-day validation statistics for OLZ in human plasma

Day	Parameter	Validation sample level (ng ml^{-1}) 0.25	5	50
1	Average (ng ml^{-1})	0.26	5.32	45.86
	SD (ng ml^{-1})	0.02	0.16	1.12
	Accuracy (RE) (%)	4.00	6.40	−8.28
	Precision (RSD) (%)	7.69	3.00	2.44
	n	5	5	5
2	Average (ng ml^{-1})	0.23	5.62	53.48
	SD (ng ml^{-1})	0.01	0.32	1.04
	Accuracy (RE) (%)	−8.00	12.40	6.96
	Precision (RSD) (%)	4.35	5.69	1.94
	n	5	5	5
3	Average (ng ml^{-1})	0.24	5.19	50.51
	SD (ng ml^{-1})	0.02	0.39	2.46
	Accuracy (RE) (%)	−4.00	3.80	1.02
	Precision (RSD) (%)	8.33	7.51	4.87
	n	5	5	5

Table 2. Intra-day validation statistics for OLZ in human serum

Day	Parameter	Validation sample level (ng ml^{-1}) 0.25	5	50
1	Average (ng ml^{-1})	0.25	5.62	45.97
	SD (ng ml^{-1})	0.02	0.19	4.03
	Accuracy (RE) (%)	0.00	12.40	−8.06
	Precision (RSD) (%)	8.00	3.38	8.77
	n	5	5	5

Table 3. Intra-day validation statistics for OLZ in human plasma using the 96-well SPE format

Day	Parameter	Validation sample level (ng ml^{-1}) 0.25	5	50
1	Average (ng ml^{-1})	0.26	5.11	44.47
	SD (ng ml^{-1})	0.01	0.14	1.03
	Accuracy (RE) (%)	4.00	2.20	−11.06
	Precision (RSD) (%)	3.85	2.74	2.32
	n	5	5	5

RESULTS AND DISCUSSION

Linearity

A typical calibration graph for OLZ is shown in Fig. 3. The calibration graph was derived by plotting the peak area ratio of OLZ to the internal standard *vs.* the plasma concentration of OLZ. Over a 15 day period during the validation of OLZ in the two biological matrices, a total of 18 calibration graphs were analyzed. The correlation coefficients obtained were 0.9859 or higher.

Assay precision and accuracy

The data for the intra-day assay precision and accuracy for human plasma, determined by analyzing five replicates at 0.25, 5 and 50 ng ml^{-1} on each of three days, are reported in Table 1. The intra-day assay precision and accuracy for human serum and in human plasma using the 96-well dish format are reported in Tables 2 and 3, respectively. The inter-day assay precision and accuracy data for human plasma are reported in Table

Table 4. Inter-day validation statistics for OLZ in human plasma

Day	Parameter	Validation sample level (ng ml^{-1}) 0.25	5	50
1	Average (ng ml^{-1})	0.24	5.38	49.95
	SD (ng ml^{-1})	0.02	0.34	3.60
	Accuracy (RE) (%)	−4.00	7.60	−0.10
	Precision (RSD) (%)	8.33	6.32	7.21
	n	15	15	15

Table 5. OLZ stability in human plasma and serum

Stability test	Minimum time stable	
Stock solution stability (−20 °C)	35 days	
Stability test	**Minimum time or cycles stable**	
	Plasma	Serum
Room temperature	24 h	24 h
Freeze–thaw	3 cycles	3 cycles
Extract stability	24 h	24 h
Storage stability (−70 °C)	59 days	16 days

4. The accuracy of the method was determined by calculating the relative error (RE), and the precision was determined by calculating the relative standard deviation (RSD).

In human plasma, the inter-day precision (RSD) ranged from 6.26 to 7.66% and the inter-day accuracy (RE) ranged from −3.54 to 7.52%, over the three concentrations evaluated. Similarly, in human serum, the intra-day precision (RSD) ranged from 3.46 to 8.76% and the intra-day accuracy (RE) ranged from −8.06 to 12.46%.

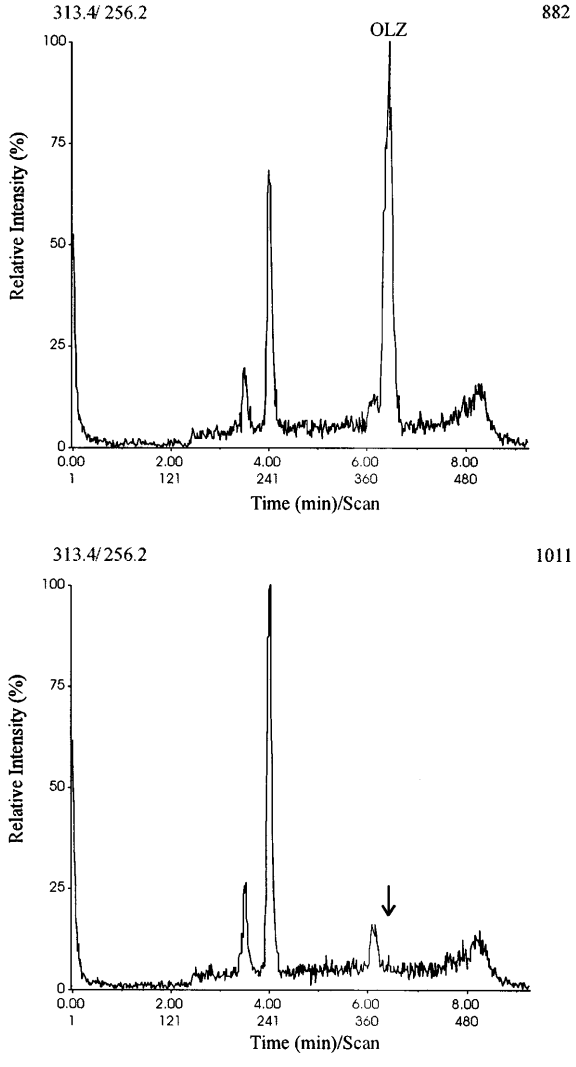

Figure 4. Representative ion chromatograms of a 250 pg ml^{-1} standard sample (top) and blank sample (bottom) extracted from human plasma. The arrow indicates the retention time of OLZ in the blank sample.

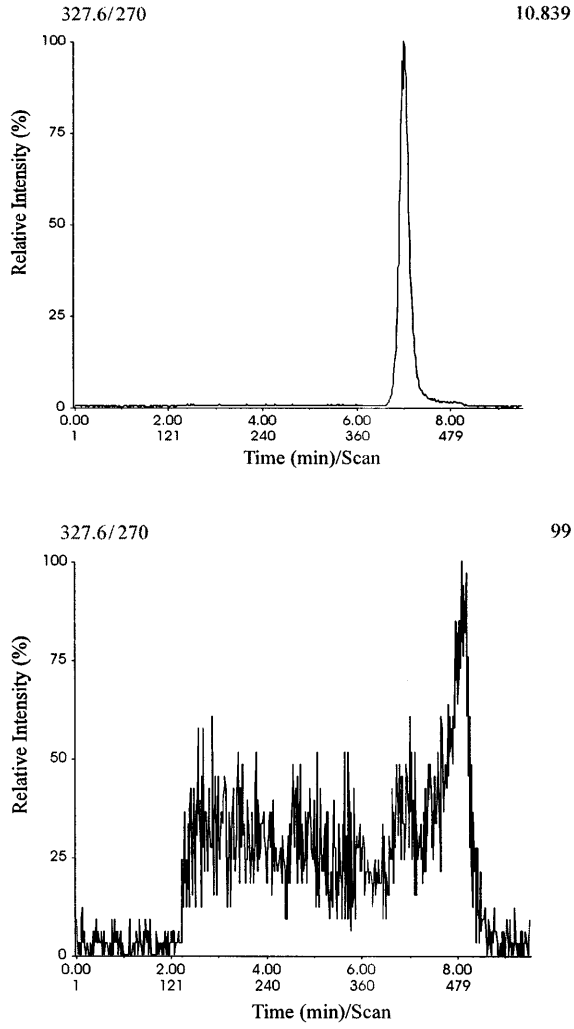

Figure 5. Representative ion chromatograms of the internal standard (top) and blank sample (bottom) extracted from human plasma.

Stability

Stock standard solutions of OLZ and the internal standard were found to be stable for 35 days when prepared in propan-1-ol and stored in polypropylene vials at ~ −20 °C when not being used. Also, OLZ was found to be stable at ~ −70 °C (in human plasma and serum) for at least 59 and 16 days, respectively.

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 24 h), during three freeze–thaw cycles, or during incubation at room temperature (up to 24 h) in each of the matrices. The stability data are presented in Table 5.

Assay specificity

The specificity of the assay was demonstrated by the absence of endogenous substances, in drug-free

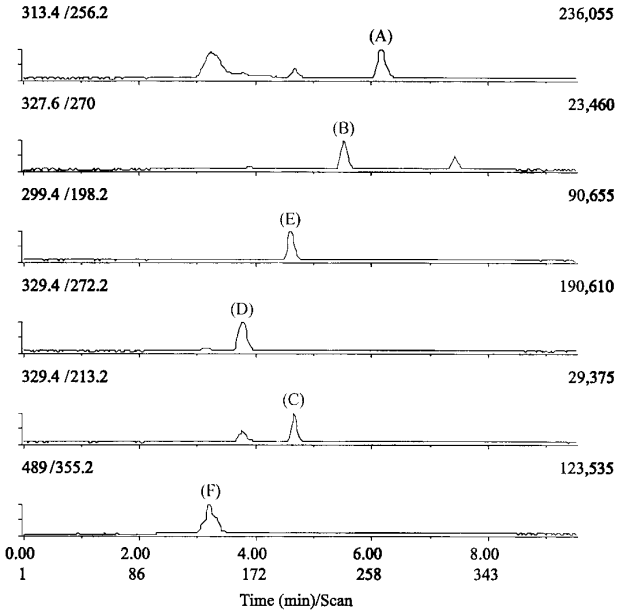

Figure 6. Representative ion chromatograms of 50 ng ml^{-1} of (A) OLZ, (B) internal standard, (C) OLZ *N*-oxide, (D) OLZ 2-hydroxy, (E) OLZ *N*-desmethyl and (F) OLZ 10-*N*-glucuronide. The peak shape was distorted by grouping multiple chromatograms.

matrices, that interfere with the quantitation of OLZ at the lower limit of quantitation (LLOQ). Potential interferences were minimized by combining a SPE procedure with the separation power of HPLC and the selectivity of MS/MS. A representative ion chromatogram of a 0.25 ng ml^{-1} standard with its associated blank sample is presented in Fig. 4. A representative ion chromatogram of the internal standard and its associated blank sample is presented in Fig. 5. Furthermore, owing to mass spectral interference between OLZ and its metabolites, chromatographic resolution was achieved to prevent a deleterious effect on analyte quantitation (Fig. 6).

Limits of quantitation, extraction efficiency and dilutions

The lower and upper limits of quantitation are defined as the lowest and highest concentrations on the calibration graph at which an acceptable accuracy of at least 100 ± 20% ((mean assay concentration/theoretical concentration) × 100) and precision of at least 20% (RSD) were obtained. The LLOQ and ULOQ of this assay were 0.25 and 50 ng ml^{-1}, respectively.

The extraction efficiency of OLZ was 71–103% and that of the internal standard, compound LY170222, was 90–97%. The biological matrices were found to have no effect on the signal of the analyte and internal standard.

Samples containing high concentrations of the analyte were diluted 1:20 in control human plasma or serum. It was demonstrated that the accuracy of the dilutions was no more than 12% from the theoretical value.

CONCLUSION

This paper has described a validated LC/MS/MS assay for the determination of OLZ that offers several advantages over previous methodologies. The extraction procedure is relatively simple and requires only 500 µl of plasma or serum. When the 96-well extraction format is used, the time necessary to prepare samples is significantly reduced. The method offers excellent sensitivity and selectivity, and it can be used to determine OLZ concentrations in both human plasma and serum. Since OLZ is chromatographically resolved from its common plasma metabolites, the assay could also be used to determine the metabolite concentrations with only slight modification of the extraction procedure. The freezer, freeze–thaw, analysis, and room temperature stability of OLZ was investigated, and no significant degradation was observed. Dilutions can be employed to represent the original sample accurately, and the extraction efficiency was determined to be ~88%.

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