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Development and validation of a liquid chromatography—tandem mass spectrometric method for the determination of the major metabolites of duloxetine in human plasma

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Abstract

A sensitive bioanalytical method for the measurement of two major circulating metabolites of duloxetine [4-hydroxy duloxetine glucuronide (LY550408) and 5-hydroxy-6-methoxy duloxetine sulfate (LY581920)] in plasma is reported. This method produced acceptable precision and accuracy over the validation range of 1–1000 ng/mL. Several issues had to be addressed in order to develop an LC/MS/MS assay for these metabolites. First, 4-hydroxy duloxetine glucuronide required chromatographic resolution from the 5-, and 6-hydroxy duloxetine glucuronide isomers. Second, the glucuronide conjugate is readily ionized under positive ESI conditions, while the sulfate conjugate required negative ESI conditions to obtain adequate sensitivity. Finally, the chromatographic conditions needed to separate the glucuronide isomers were not suitable for the analysis of the sulfate conjugate. The present method addressed these challenges, and was successfully applied to multiple human pharmacokinetic studies in which subjects received oral doses of duloxetine hydrochloride.

Keywords: Duloxetine metabolites; Validation; Plasma assay

1. Introduction

Cymbalta®, duloxetine hydrochloride [LY248686, (+)-(*S*)-*N*-methyl-γ-(1-naphthyloxy)-2-thiophenepropylamine hydrochloride] is a potent and balanced dual inhibitor of serotonin and norepinephrine (NE) uptake. It has demonstrated a relatively evenly balanced and potent inhibition of serotonin and NE reuptake at the transport sites in both *in vitro* and *in vivo* studies [1,2]. Duloxetine has no significant affinity for dopaminergic, adrenergic, cholinergic, histaminergic, opioid, glutamate, and GABA receptors *in vitro*, and does not inhibit monoamine oxidase (MAO). It is currently prescribed for the treatment of major depressive disorders (MDD), pain related to diabetic peripheral neuropathy, and stress urinary incontinence (SUI) in Europe (Yentreve®).

The safety and pharmacokinetics of duloxetine have been extensively evaluated in healthy subjects, and it has been shown to be safe and well-tolerated [3–6]. Duloxetine undergoes extensive metabolism, with only 3% of the total radioactivity in plasma represented by duloxetine after a single oral dose of [^{14}C]duloxetine in four healthy human subjects [7].

The two major metabolites identified in human plasma were a glucuronide conjugate of 4-hydroxy duloxetine (LY550408) and a sulfate conjugate of 5-hydroxy-6-methoxy duloxetine (LY581920) [7]. These metabolites have been shown to not contribute significantly to the pharmacological activity [8]. In order to evaluate the disposition of a larger portion of the administered dose, a selective and sensitive plasma assay is needed to assess exposure of these two metabolites in humans.

The present report describes an LC/MS/MS method for the quantification of the duloxetine metabolites LY550408 and LY581920 from a single aliquot of human plasma utilizing sequential SPE extraction, deuterated internal standards, positive ion ESI for the determination of LY550408, chromatographic resolution of LY550408 isomers, and negative ion ESI for the determination of LY581920. The method was successfully validated and applied to support multiple clinical studies.

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2. Experimental

2.1. Chemicals and reagents

Methanol (high purity) and acetonitrile (high purity) were obtained from EM Science (Gibbstown, NJ, USA). Ammonium formate, formic acid, sodium carbonate (analytical reagent grade) and hydroquinone (99+%) were obtained from Sigma–Aldrich (St. Louis, MO, USA). Phosphoric acid and glacial acetic acid (both analytical reagent grade) were obtained from Mallinckrodt (Paris, KY, USA). HPLC quality water (approximately 18 MΩ) was prepared using a Millipore Milli-Q water purification system (Millipore, Billerica, MA, USA). Heparinized human plasma was obtained from Biological Specialty Corporation (Colmar, PA, USA). A concentrated pH 3 buffer (water/5 M ammonium formate/formic acid (85/10/5, v/v/v) was diluted 1:5 with water before using in the SPE extraction procedure. The following compounds were synthesized at Eli Lilly and Company: LY550408; [$^2H_4$]LY550408 internal standard; LY581920; and [$^2H_4$]LY581920 internal standard. The synthesis of several major metabolites of duloxetine including LY550408 and LY581920 has been described previously by Kuo et al. [9,10]. Chemical structures of duloxetine and the conjugated metabolites of duloxetine monitored in this assay and their internal standards are shown in Fig. 1.

2.2. Preparation of stock solutions

Primary stock solutions of LY550408 and LY581920 for preparation of standards and quality controls (QC) were prepared from separate weighings. The primary stock solutions for LY550408 and [$^2H_4$]LY550408 were prepared at a concentration of 0.10 mg/mL in methanol/acetic acid/1 mg/mL hydroquinone solution (97/2/1, v/v/v). The primary stock solutions for LY581920 and [$^2H_4$]LY581920 were prepared at a concentration of 0.10 mg/mL in methanol/water (50/50, v/v), containing 2 mM sodium carbonate. Additional stock solutions were prepared separately at a concentration of 0.50 mg/mL in the solvents described previously. These stock solutions were only

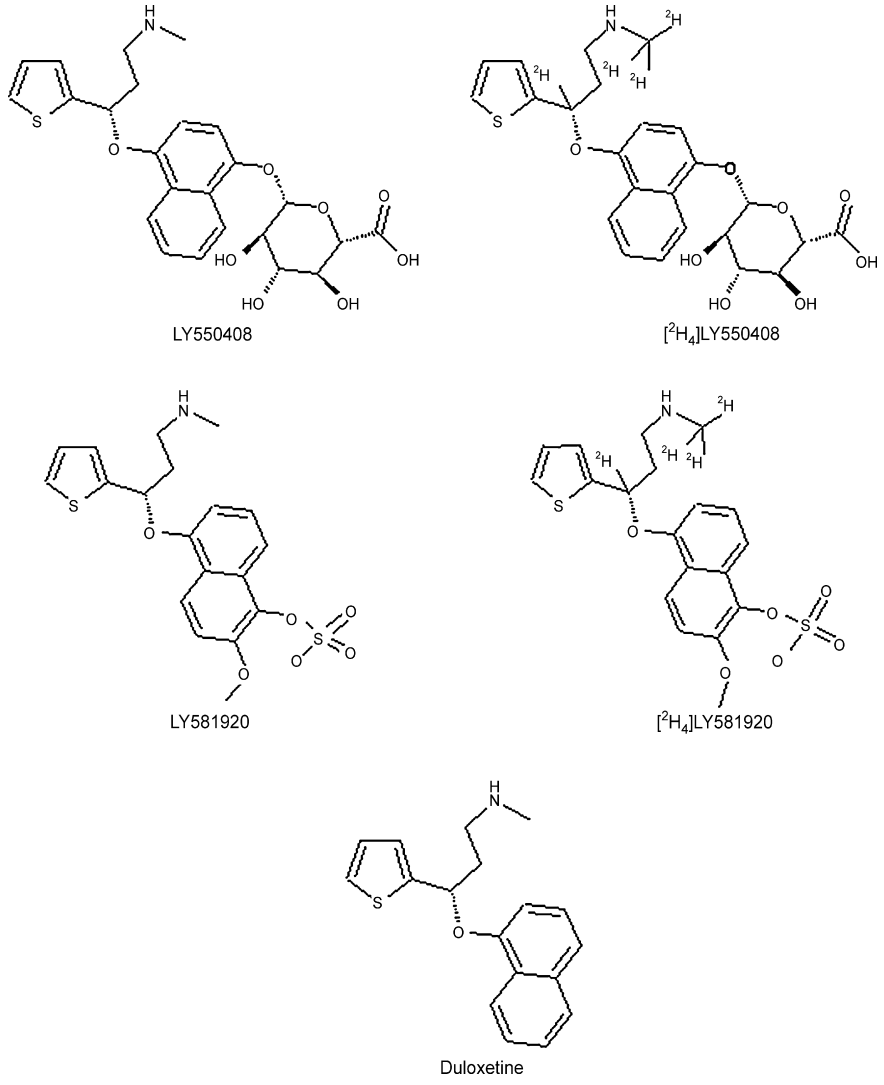

Fig. 1. Structures of LY550408, LY581920, their stable isotope internal standards and duloxetine.

utilized during the validation in order to prepare the dilution validation samples.

A working internal standard (WIS) solution was prepared by diluting 40 μL of each of the IS stock solutions in 9.92 mL of methanol/acetic acid/1 mg/mL hydroquinone solution (97/2/1, v/v/v). All of the stock and working solutions were stored at 2–8 °C and were stable for at least 27 days.

2.3. Calibration curves, quality control samples, and validation samples

Calibration curves were prepared by preparing a combined plasma standard containing 2000 ng/mL of each analyte LY550408 and LY581920. Serial dilutions with plasma were made to obtain the standard curve points of 1, 2, 10, 50, 100, 500, 650, 800, and 1000 ng/mL.

Quality control samples were prepared in plasma at the levels of 5, 480, and 900 ng/mL. Validation samples were prepared at the levels of 1, 50, 500, and 1000 ng/mL in human plasma. In addition, a dilution validation sample was prepared at a concentration of 8000 ng/mL.

2.4. Extraction procedure

Validation, QC, calibration curve, and clinical plasma samples were extracted employing a solid phase extraction technique (SPE) utilizing a high performance mixed reversed phase and cation exchange 96-well extraction plate from 3 M Empore (MPC-SD, part #6030). A 200 μL aliquot of each plasma sample was combined with 50 μL of 400 ng/mL WIS solution then mixed with 700 μL of a 1% phosphoric acid solution. The SPE plate was conditioned with 200 μL of methanol, followed by 200 μL of 1% phosphoric acid. The plasma samples were transferred to the SPE plate and drawn through with vacuum. The plate was washed with 500 μL of water and dried for approximately 30 s. The LY581920 analyte and IS were eluted with 250 μL of methanol/water (50/50, v/v) into a 96-well collection plate. The SPE plate was further washed with 250 μL of acetonitrile, followed by 250 μL of a pH 3 buffer. The LY550408 analyte and IS were eluted from the plate into a second 96-well collection plate using 200 μL of methanol/pH 3 buffer (80/20, v/v). The extracts in both collection plates were evaporated to dryness and reconstituted in 300 μL of an aqueous solution containing 20 μg/mL hydroquinone with 1% acetic acid. Separate injections were required for analysis of the LY550408 extract by positive ESI and the LY581920 extract by negative ESI. An injection volume of 50 μL was utilized for both extracts. The extraction procedure was either performed manually or with a Tomtec Quadra 96 workstation.

2.5. Instrumentation and operating conditions

The HPLC system consisted of a Shimadzu solvent delivery system (Kyoto, Japan) and a Gilson 215 autosampler (Middleton, WI, USA). HPLC separation for both analytes was achieved with an Aquasil C18 column (150 mm × 2 mm, 5 μm, Thermo Electron, Bellefonte, PA, USA). The solvent system for the LY550408 analysis consisted of an isocratic flow with a mobile phase of water/acetonitrile/formic acid (80/20/0.1, v/v/v). For the analysis of LY581920, the mobile phase consisted of water/acetonitrile/acetic acid (70/30/0.05, v/v/v). For both analytes the flow rate was 0.3 mL/min and the column temperature was approximately 22 °C.

Mass spectrometric detection was carried out using an Applied Biosystems/MDS Sciex API 3000 triple quadrupole mass spectrometer (Foster City, CA, USA) equipped with a TurboIonSpray™ interface. A Dell Dimension XPS R400 personal workstation (Round Rock, TX, USA) equipped with Applied Biosystems/MDS Sciex Analyst software (version 1.2) was used to collect and process the data.

For the LY550408 analyte and IS, selected reaction monitoring $(M+H)^+$ transitions m/z 490 → 154 and m/z 494 → 158 were monitored, respectively. The TurboIonSpray™ temperature was maintained at 500 °C, with nebulizing gas (nitrogen) and auxiliary gas (nitrogen) flow rates at 14 and 7 L/min, respectively. The curtain gas (nitrogen) flow rate was 8 L/min, and the ion spray voltage was set to 5000 V. The collision gas (nitrogen) flow setting was 5 units, which resulted in an analyzer pressure of 2.8×10^{-5} torr. For the LY581920 analyte and IS, selected reaction monitoring $(M-H)$- transitions m/z 422 → 268 and m/z 426 → 268 were monitored, respectively. All other settings were the same as for the previous analyte, with the exception of the source temperature which was increased to 550 °C, and the ion spray voltage which was set to −5000 V. The resulting product ion spectra for the metabolites are shown in Fig. 2 along with the proposed fragmentation schemes. As illustrated in the figure, the positive product ion spectrum for LY550408 produced a strong product ion at m/z 154 corresponding to the *N*-methyl-3-thiophenepropanamine portion of the molecule. For LY581920, the negative product ion spectrum shows a major ion at m/z 268 corresponding to the loss of the *N*-methyl-3-thiophenepropanamine. Other product ions were observed, but these transitions were chosen because they displayed the greatest intensity.

The peak area ratios of analyte to IS were plotted versus nominal analyte concentration in order to generate calibration curves. For LY550408, the calibration curve was fitted to a $1/x^2$ linear regression. For LY581920, the calibration curve was fitted to a $1/x^2$ quadratic regression.

2.6. Assay validation and stability testing

The method validation assays were carried out according to the currently accepted US Food and Drug Administration (FDA) bioanalytical method validation guidance [11]. The method's specificity was tested by screening six different batches of human blank plasma. The extraction efficiency and matrix suppression were investigated at the low (4 ng/mL) and high concentrations (800 ng/mL) using human plasma, with 3 replicates at each concentration. Precision and accuracy were determined by analyzing validation samples prepared at five concentrations in three separate runs. Six replicates were prepared and analyzed at each concentration.

Stability samples containing LY550408 and LY581920 in frozen human plasma were periodically assayed along with fresh

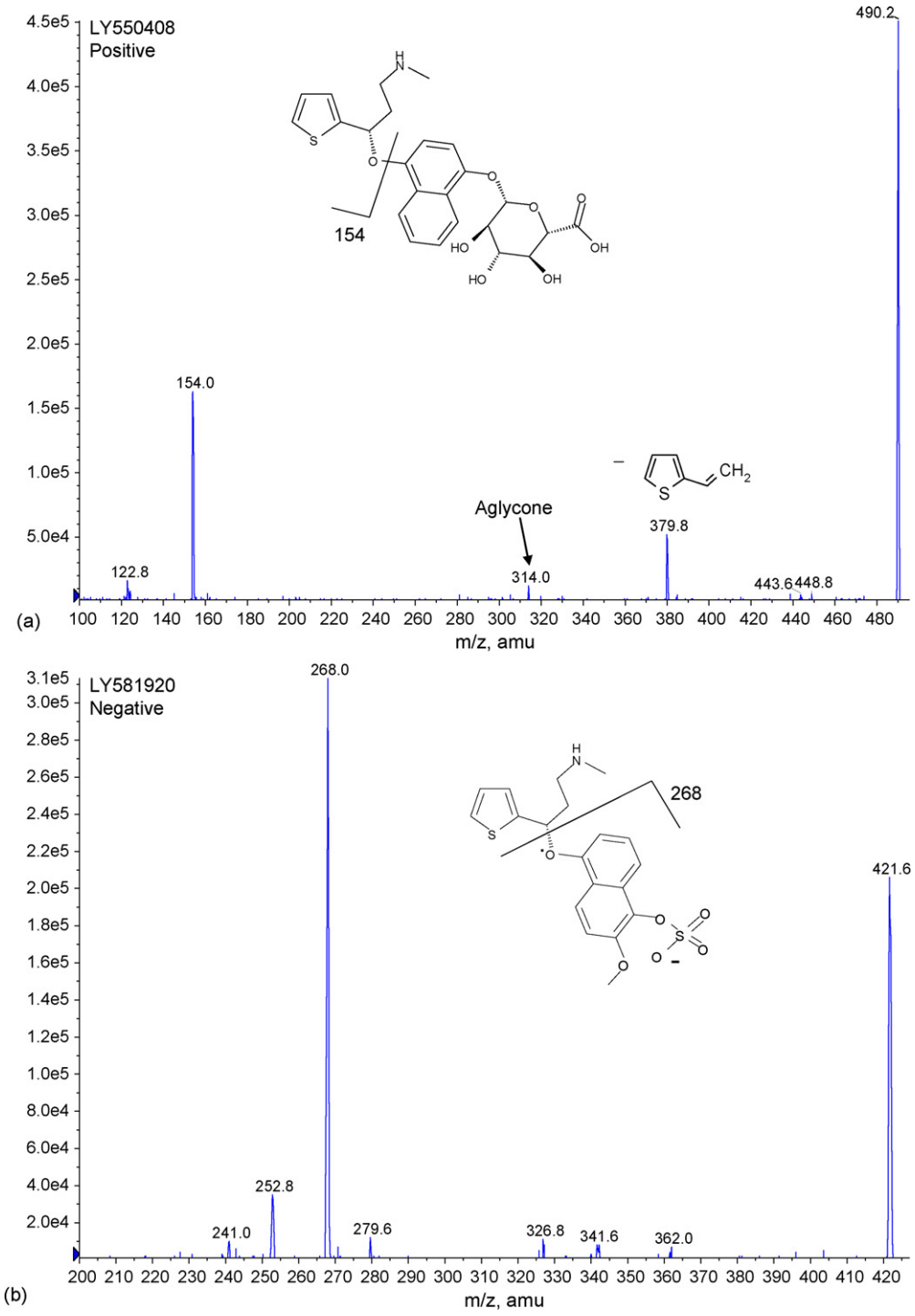

Fig. 2. Product ion mass spectra of (a) LY550408 and (b) LY581920 with potential fragmentation schemes for each analyte.

standards to determine frozen storage stability. Room temperature stability was determined by allowing stability samples to remain on the bench top for approximately 4 and 24 h prior to analysis with fresh standards.

2.7. Application

Twelve overtly healthy subjects were enrolled in a clinical trial where the pharmacokinetics of duloxetine and its two major metabolites were evaluated after a single 60 mg dose and at steady state during 60 mg once daily (QD) and 60 mg twice daily (BID) administration of duloxetine. The subjects ranged in age from 23 to 61 years, and provided written informed consent before study participation. The study protocol was approved by the Ohio Valley Institutional Review Board of Evansville, IN, USA. The study was conducted at West Pharmaceutical Services, Evansville, IN, USA, in accordance with applicable laws and regulations, good clinical practices, and the ethical princi-

ples that have their origin in the Declaration of Helsinki. The bioanalytical method described herein was utilized to analyze metabolite plasma samples collected during the study.

3. Results and discussion

3.1. Development and validation of the method

The analytes LY550408 and LY581920 were extracted utilizing a single SPE plate and plasma aliquot. The two analytes were eluted separately and analyzed with different LC and MS conditions. The normal LC/MS procedure was to assay the LY550408 extracts first, allow for column equilibration to the LY581920 mobile phase and MS conditions, and then assay the LY581920 extracts. With an analysis time of approximately 9 and 4 min for LY550408 and LY581920, respectively, a full 96-well plate run required approximately 21 h of MS analysis time. Figs. 3 and 4 depict representative mass chromatograms for the lowest standard curve point standards (1 ng/mL) extracted from human plasma for LY550408 and LY581920, respectively along with chromatograms for blank human plasma extracts.

The three positional isomers, 4-, 5-, and 6-hydroxy duloxetine all form glucuronide conjugates in humans [7]. This assay quantifies the most abundant of these, the 4-hydroxy duloxetine glucuronide (LY550408). All of the glucuronide conjugate standards were not synthetically prepared, but the unconjugated isomers had been synthesized for pharmacological testing [9,10]. Thus, liver slice incubations with 4-, 5-, and 6-hydroxy duloxetine which produced the desired glucuronide conjugates were utilized during method development. As the isomers have the same ion transition, acceptable chromatographic separation was required for quantification of LY550408. Several LC columns were evaluated during method development. Since

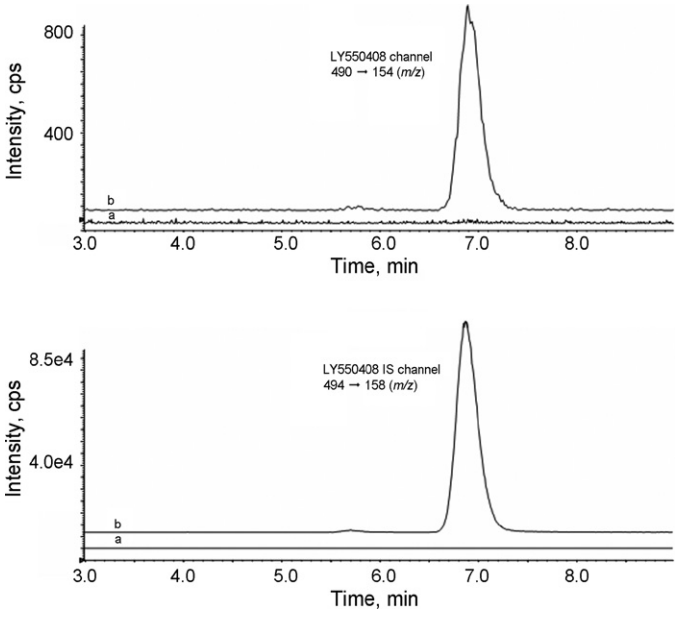

Fig. 3. Representative mass chromatograms for (a) blank human plasma, and (b) 1 ng/mL plasma standard (LLOQ) for LY550408 and IS, [$^2H_4$]LY550408 (chromatograms were offset for illustrative purposes).

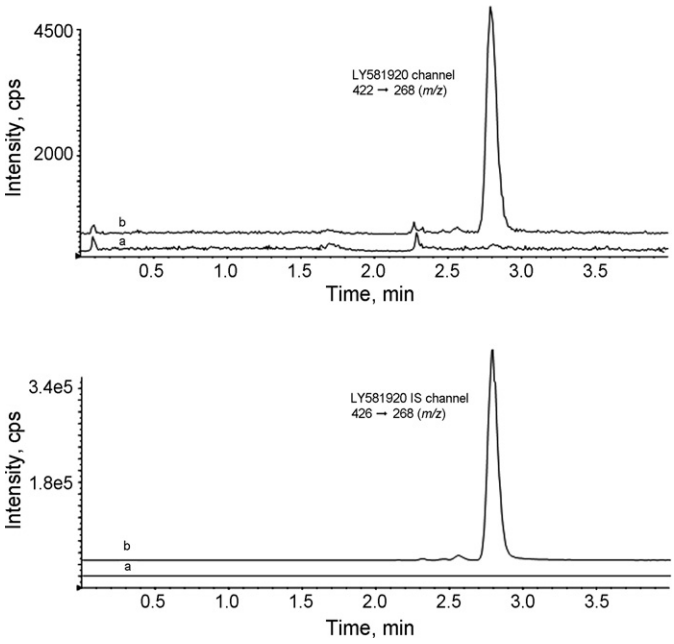

Fig. 4. Representative mass chromatograms for (a) blank human plasma and (b) 1 ng/mL plasma standard (LLOQ) for LY581920 and IS, [$^2H_4$] LY581920 (chromatograms were offset for illustrative purposes).

glucuronide conjugates typically demonstrate poor retention with reversed phase systems, and MS sensitivity is generally increased with higher concentrations of organic solvent in the mobile phase, the retention time of LY550408 was evaluated with columns possessing high retention properties for polar molecules. A column switching system was utilized to evaluate 12 reversed phase columns in an automated fashion, and identified the Aquasil C18 column as having the best retention of LY550408. Following this assessment, the resolution of the positional isomers, produced by the liver slice preparations, was evaluated on additional reversed phase columns including Chromegabond Fluorosep RP-Phenyl (ES Industries), Keystone Prism RP and RPN (Thermo Scientific), and Waters Symmetry Shield RP. The Aquasil C18 column again proved to be the best column to achieve adequate resolution of the 3 isomeric glucuronides. Fig. 5 shows a representative chromatogram illustrating the separation of the three isomeric glucuronides.

Previous experience with the hydroxy duloxetine metabolites had shown instability in non-matrix based preparations (internal communication). As a precaution, acetic acid was added to lower the solution pH, and hydroquinone was added as an antioxidant. This reagent (methanol/acetic acid/1 mg/mL hydroquinone, 97/2/1, v/v/v) was also utilized as the diluent for the working IS solution, and proved to be adequate for both of the internal standards.

During method development, the synthetic preparation of the LY550408 standard and internal standard was achieved first and method development/validation proceeded with this molecule. Following the synthesis of the LY581920 standard/IS, it was discovered that this metabolite was eluted prior to the final SPE step. Examination of the various SPE washes showed that LY581920 and IS were eluted by the 50/50 methanol/water wash step. This

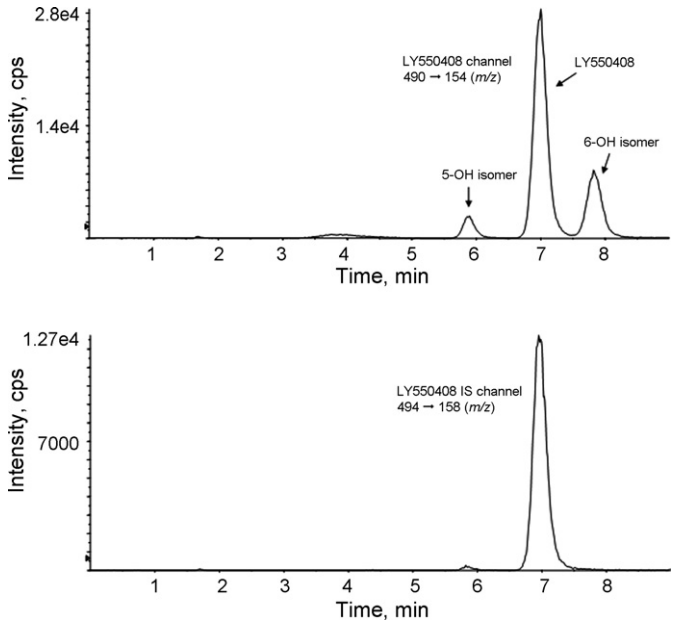

Fig. 5. Mass chromatograms for LY550408 and the respective IS from the analysis of a plasma sample from a subject dosed with duloxetine.

elution of the two analytes in separate elution steps proved fortuitous in clinical studies where expedited sample analysis was desired, as separate LC/MS/MS systems could be utilized for the positive and negative conditions, thus decreasing the sample turn-around time.

Calibration curves were constructed by plotting the analyte/IS peak area ratios for the standards against the known concentrations. Once plotted, the calibration curves were used to determine the analyte concentrations in validation, QC, stability and study samples. During method development, calibration curves were calculated using linear or quadratic regression without weighting, and using $1/x$ and $1/x^2$ as weighting factors. In order to establish the best fit and weighting, back-calculated calibration concentrations were determined. The calibration curve with the best accuracy and precision throughout the standard curve range was considered the best fit. For LY550408, the calibration curves were fit over the concentration range (1–1000 ng/mL) using a $1/x^2$ weighted linear fit. For LY581920, the calibration curves were fit over the same concentration range using a $1/x^2$ weighted quadratic fit. The correlation coefficient (r) was greater than 0.996 for LY550408 and greater than 0.998 for LY581920 during the analysis of the three validation batches. Duplicate standard curves were analyzed for each validation batch, one each at the beginning and end of the run.

Stability samples ($n = 3$) containing LY550408 and LY581920 (4, 800, and 8000 ng/mL) in frozen human plasma were periodically assayed along with fresh standards to determine storage stability. Room temperature stability was determined by allowing stability samples to remain on the bench top for approximately 4 and 24 h prior to analysis with fresh standards. Stock solutions were stored refrigerated (approximately 2–8 °C) and compared to fresh stocks in order to determine stability of the metabolites in neat solutions.

Table 1
Assay parameters and stability of LY550408 and LY581920 in human plasma

Experiment	LY550408	LY581920
Extraction efficiency (N, SD)	85% (12, 5.6%)	97% (12, 4.6%)
Matrix suppression (N, SD)	3% (12, 5.2%)	19% (12, 1.7%)
Extract stability	3 days RT	3 days RT
Long-term frozen stability at −20 and −70 °C	97 days	66 days
Bench top stability	24 h	24 h
Freeze-thaw	3 cycles	3 cycles

RT = room temperature, N = number of replicates, SD = standard deviation.

Stability of LY550408 in plasma was demonstrated at −20 and −70 °C for at least 97 days and at room temperature for at least 24 h. Stability of LY581920 in plasma was demonstrated at −20 and −70 °C for at least 66 days and at room temperature for at least 24 h. Storage stability studies have been conducted for a longer period of time for LY550408 compared to LY581920 as the LY550408 reference standard was available first. Stock solutions at a concentration of 100 μg/mL were stable for at least 100 days for both analytes. The 500 μg/mL stock solutions of LY550408 and LY581920 were stable for at least 89 and 29 days, respectively when stored at 2–8 °C. All stability information is summarized in Table 1.

Both intra-day and inter-day accuracy and precision of the method were evaluated from six replicates of validation samples of known concentrations (1, 50, 500, 1000, and 8000 ng/mL). The 8000 ng/mL validation sample was analyzed after a 10-fold dilution with blank human plasma. The experiments were repeated on 3 different days and the data are displayed in Table 2. The accuracy of the method was defined as the relative error (%RE) of the mean of the replicate measurements from the theoretical values. The precision of the method was defined as the relative standard deviation (%RSD) calculated from replicate measurements. All intra- and inter-day precision and accuracy values were acceptable, and spanned the entire concentration range. The validated limit of quantification was 1 ng/mL for both analytes.

The extraction efficiencies of LY550408 and LY581920 from human plasma were assessed by comparisons of the peak areas from extracted samples to those from blank plasma sample extracts spiked with the appropriate concentrations of analytes.

Table 2
Validation accuracy and precision summary for LY550408 and LY581920 in human plasma ($n = 3$ days, six replicates per day)

	1	50	500	1000	8000[a]
LY550408 Concentration (ng/mL)					
Mean	1.03	51.94	508.75	1005.57	8234.94
Accuracy (%RE)	2.66	3.89	1.75	0.56	2.94
Precision (%RSD)	6.65	0.87	1.04	1.95	2.08
LY581920 Concentration (ng/mL)					
Mean	1.01	50.27	497.17	1000.59	8354.61
Accuracy (%RE)	0.80	0.53	−0.57	0.06	4.43
Precision (%RSD)	8.11	3.05	4.16	8.54	4.54

[a] Diluted 1:10 before analysis with blank human plasma.

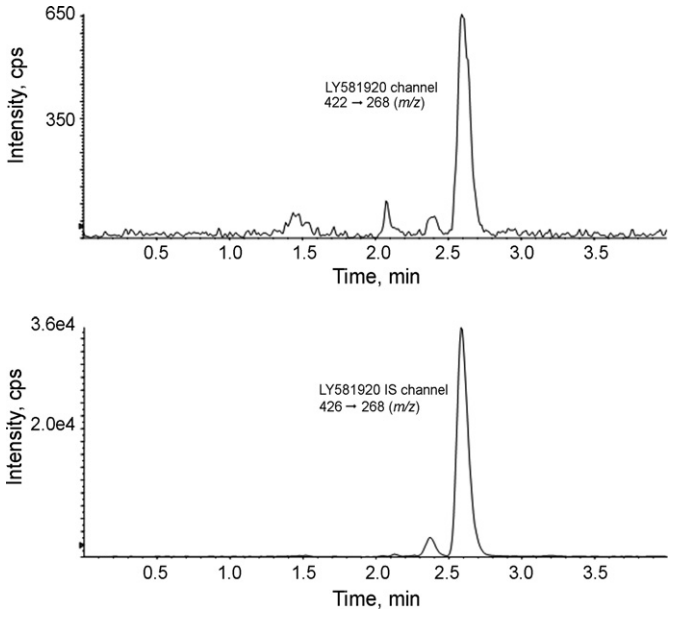

Fig. 6. Mass chromatograms for LY581920 and the respective IS from the analysis of a plasma sample from a subject dosed with duloxetine.

The average extraction efficiency of LY550408 and its stable isotope IS was approximately 85%. For LY581920 and its IS, the average extraction efficiency was 97 and 85%, respectively. Matrix suppression was determined by comparing the peak areas from blank plasma sample extracts spiked with analytes to neat standards at the same concentrations. Both the LY550408 and its IS were suppressed by approximately 3%. The matrix suppression for LY581920 and its IS was approximately 19 and 20%, respectively. The differences in matrix suppression values between the two analytes may be due to the chromatography conditions. For LY550408, the retention time is longer, and thus the analyte ionization is less likely to be influenced by matrix components. The shorter retention time for LY581920, and thus possible co-elution with matrix components, may lead to the higher suppression value observed. The extraction efficiency and matrix suppression data are summarized in Table 1, along with the number of replicates and standard deviations of these determinations.

Table 3
Quality control sample summary for analysis of LY550408 and LY581920 in human plasma

Parameter	QC 5 ng/mL	QC 480 ng/mL	QC 900 ng/mL
LY550408			
N	18	18	18
Average	4.77	471.42	885.55
RE (%)	−4.69	−1.79	−1.61
RSD (%)	3.85	2.81	3.82
LY581920			
N	16	16	16
Average	4.90	480.05	888.01
RE (%)	−2.06	0.01	−1.33
RSD (%)	4.33	2.57	3.79

Table 4
Mean pharmacokinetic parameters of LY550408 and LY581920 in healthy subjects ($n = 12$) receiving 60-mg duloxetine as a single dose and a QD and BID dose at steady state

Parameter (units)	Single AM dose	QD AM dose	BID AM dose
LY550408			
T_{max} (h)	6.0	6.0	6.0
C_{max} or $C_{ss,max}$ (ng/mL)	428	483	656
$AUC_{0-\infty}$ or $AUC_{\tau,ss}$ (ng h/mL)	7525	6968	6466
$t_{1/2}$ (h)	11.8	NC	13.9
LY581920			
C_{max} or $C_{ss,max}$ (ng/mL)	6.0	6.0	6.0
$AUC_{0-\infty}$ or $AUC_{\tau,ss}$ (ng h/mL)	264	278	386
$AUC_{0-\infty}$ (ng h/mL)	3179	3169	3396
$t_{1/2}$ (h)	10.2	NC	12.7

Abbreviations: T_{max} = time to maximal plasma concentration, C_{max} = maximum plasma concentration, AUC = area under the plasma concentration versus time curve, $t_{1/2}$ = half-life, NC = not calculated.

3.2. Analysis of clinical samples

The method was successfully applied to the determination of plasma LY550408 and LY581920 concentrations in healthy

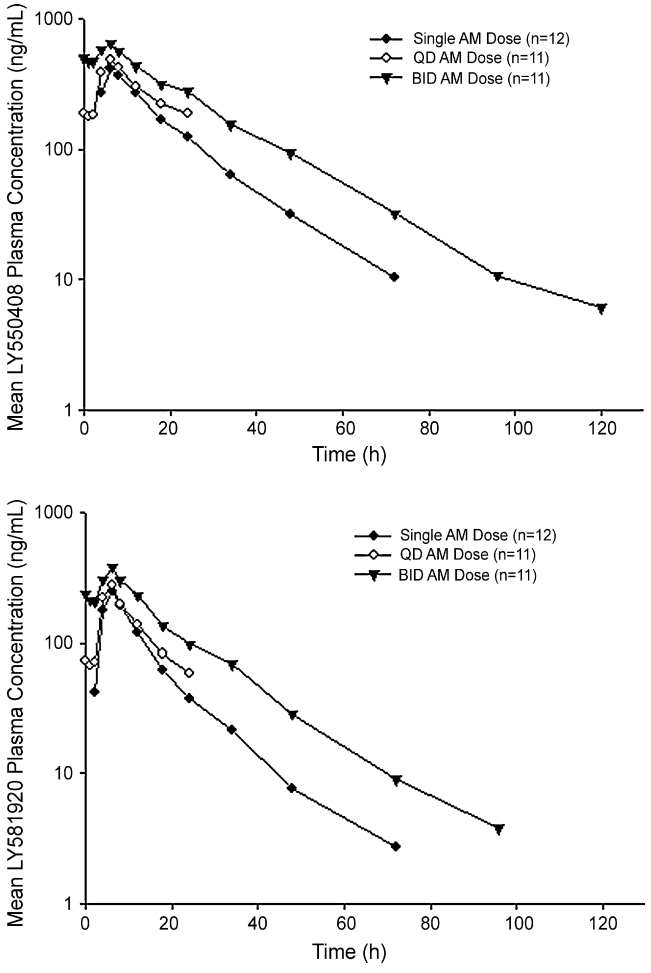

Fig. 7. Representative mean plasma concentration time curves of LY550408 and LY581920 in healthy subjects receiving 60-mg duloxetine in the morning as a single dose, and a QD and BID dose at steady state.

human volunteers. During the sample analysis for the study presented here, a total of 497 samples were analyzed in 4 batches for LY581920 and six batches for LY550408. The additional batches were needed for LY550408 to reanalyze samples that were above the quantification limit during the initial analysis. Representative mass chromatograms of plasma samples from subjects dosed with duloxetine are shown as Figs. 5 and 6 for LY550408 and LY581920, respectively. Quality control samples were analyzed with each batch, and the intra- and inter-day precision and accuracy results are shown as Table 3. Table 4 presents a summary of the mean pharmacokinetic parameters for LY550408 and LY581920 determined following a 60 mg oral dose of duloxetine given as a single dose and a QD and BID dose at steady state. Profiles of the mean plasma concentrations for the metabolites versus time after these doses are shown as Fig. 7.

4. Conclusions

A selective, robust, accurate, and precise LC/MS/MS method was developed for the analysis of the major circulating metabolites of duloxetine, LY550408 and LY581920 in human plasma. The procedure provides acceptable chromatographic resolution of LY550408 and its positional isomers, has excellent extraction efficiency and produces a minimum of matrix effects on ionization. The method was validated over the concentration range of 1 to 1000 ng/mL for both analytes. The method has been successfully applied to numerous clinical studies to derive pharmacokinetic parameters for LY550408 and LY581920 [12].

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