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Simultaneous quantification of atomoxetine as well as its primary oxidative and *O*-glucuronide metabolites in human plasma and urine using liquid chromatography tandem mass spectrometry (LC/MS/MS)

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Abstract

A sensitive and selective liquid chromatography tandem mass spectrometry (LC/MS/MS) method for the determination of atomoxetine and its metabolites (4-hydroxyatomoxetine, *N-des*-methylatomoxetine, and 4-hydroxyatomoxetine-*O*-glucuronide) has been developed for human plasma and urine. Using stable-labeled internal standards, the method proved to be accurate and precise for the analytes in all species, resulting in inter-batch accuracy (percent relative error, %RE) within 100 ± 13% and inter-batch precision (relative standard deviation, %RSD) within 11%. Stability was demonstrated for the analytes in neat solutions and the reconstitution solvent, as well as plasma and urine (with or without the deconjugation reagent). The method was simple, robust (utilized for the analysis of several hundred clinical study samples), and amenable to high sample throughput.

Keywords: Chromatography; Mass spectrometry; Quantification; Bioanalytical; ADHD; Clinical; Straterra™

1. Introduction

Atomoxetine hydrochloride (LY139603; formerly known as tomoxetine hydrochloride) is known chemically as (−)-*N*-methyl-γ-(2-methylphenoxy) benzenepropanamine hydrochloride. Atomoxetine is a potent inhibitor of the presynaptic norepinephrine transporter with minimal affinity for other monoamine transporters or receptors [1–2]. Atomoxetine hydrochloride (brand name: Straterra™, Eli Lilly and Company) has recently been approved in the United States for the treatment of attention-deficit/hyperactivity disorder (ADHD) in children, adolescents, and adults. ADHD is the most common neurobehavioral disorder of childhood. The incidence of ADHD is 5–10% of children, and the symptoms are known to persist in 10–60% of cases into adulthood [3–7]. Ninety percent of children diagnosed with ADHD in the United States were prescribed medication [8], the vast majority with a psychostimulant drug. However, atomoxetine is not a stimulant and does not have the abuse potential associated with methylphenidate and amphetamines [9–11].

In order to investigate the general disposition properties of atomoxetine, a robust bioanalytical method was needed for the measurement of atomoxetine (conjugated and unconjugated) and its metabolites in human plasma and urine. The physical and chemical characteristics of atomoxetine allows for its quantification by a variety of analytical techniques,

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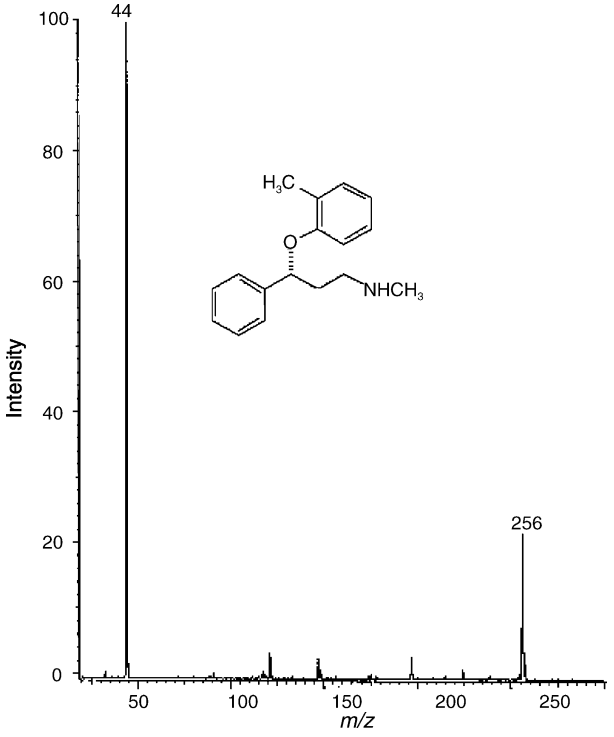

Fig. 1. The structure and product ion scan of atomoxetine.

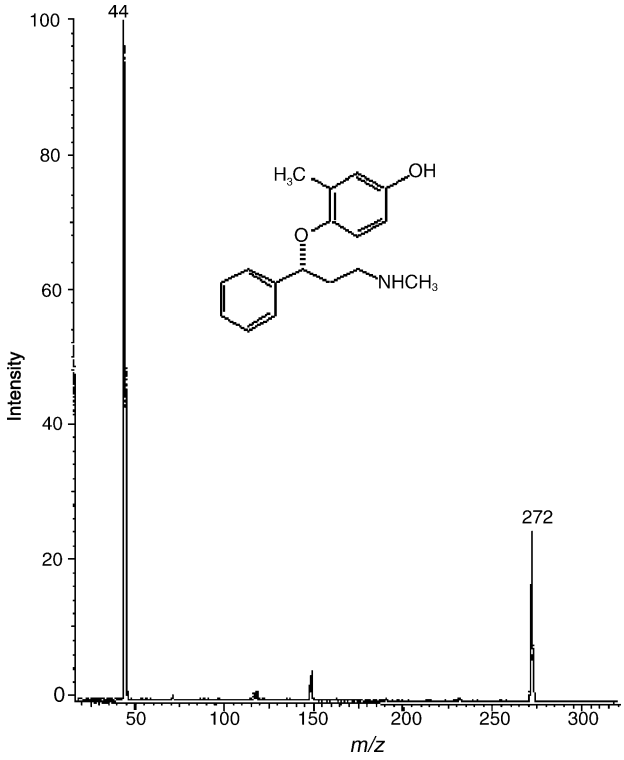

Fig. 2. The structure and product ion scan of 4-hydroxyatomoxetine.

including LC/UV, LC/florescence, LC/MS, and GC/MS. For our application, we utilized liquid chromatography tandem mass spectrometry (LC/MS/MS) for the quantification of atomoxetine and its metabolites (4-hydroxyatomoxetine, *N-des*-methylatomoxetine, and 4-hydroxyatomoxetine-*O*-glucuronide) since LC/MS/MS provided specificity, while minimizing analysis time. The method described in this manuscript was used to support a number of drug development activities in human subjects and patients.

2. Materials and methods

2.1. Chemicals and materials

Analyte compounds atomoxetine, *N-des*-methylatomoxetine, and 4-hydroxyatomoxetine (Figs. 1–3) were obtained from Eli Lilly and Company (Indianapolis, IN, USA). Internal standard compounds, [$^2H_7$]-*R*/*S*-atomoxetine (a racemic mixture of *R*- and *S*-atomoxetine) and [$^2H_5$]-4-hydroxyatomoxetine ([$^2H_5$]-4-hydroxyatomoxetine was not available at the time of the initial human plasma validation) were also obtained from Eli Lilly and Company (Figs. 4 and 5). Control human plasma was obtained from Biological Specialties Corporation (Colmay, PA, USA) and control human urine was obtained from Taylor Technology Inc. (Princeton, NJ, USA). High-performance liquid chromatographic (HPLC) optima-grade methanol and acetonitrile were obtained from Fisher Scientific (Fairlawn, NJ, USA). Trifluoroacetic acid (TFA, 99%, ∼13.28 M),

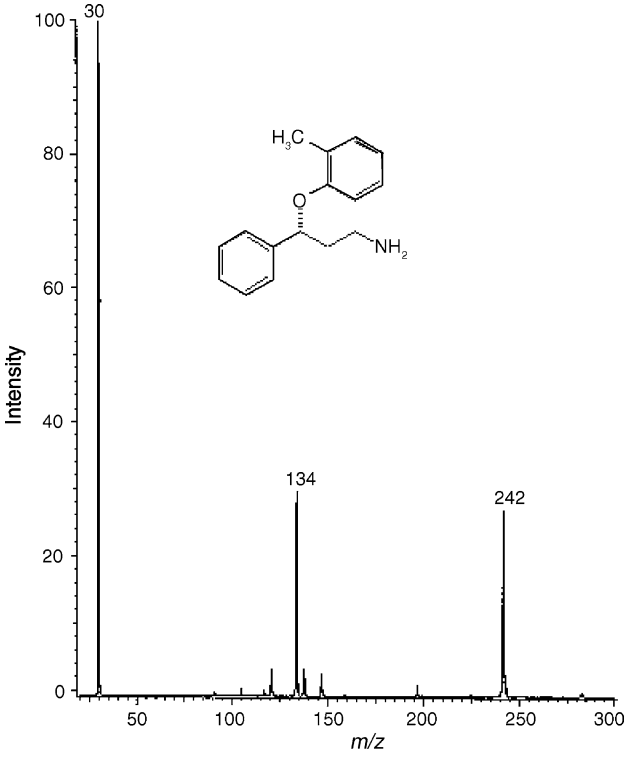

Fig. 3. The structure and product ion scan of *N-des*-methylatomoxetine; an adduct ion was monitored for the parent ion due to an approximate eight-fold increase in sensitivity for the adduct ion.

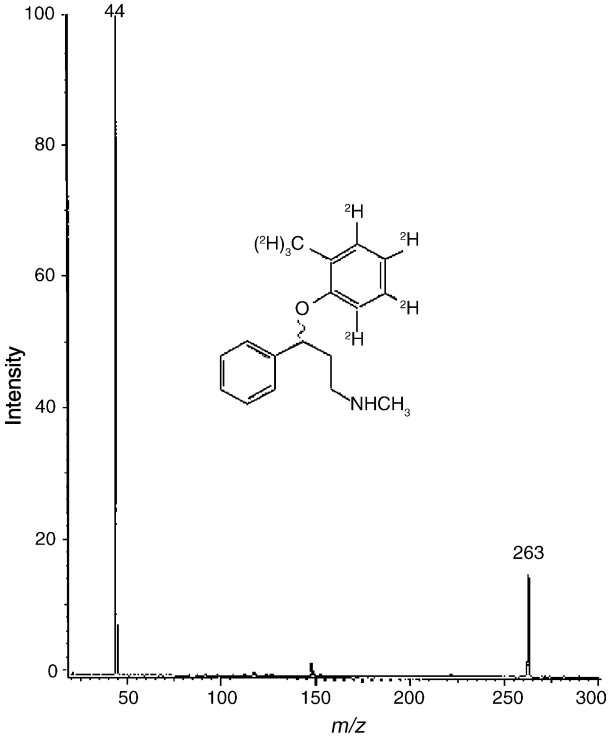

Fig. 4. The structure and product ion scan of *R,S*-[$^2H_7$]-atomoxetine.

sodium acetate trihydrate, ammonium acetate (analytical reagent grade), and β-glucuronidase (type B-1) were acquired from Sigma–Aldrich (St. Louis, MO, USA); and formic acid (88%, ∼23.6 M) was obtained from EM Science (Gibbstown, NJ, USA). High-performance liquid chromatographic optima-grade water was obtained from Fisher Scientific (Fairlawn, NJ, USA). Empore (3M, Minneapolis, MN, USA) 3 mL/7 mm polystyrenedivinylbenzene (SDB-XC) solid-phase extraction (SPE) cartridges and positive pressure extraction manifolds were obtained from Varian (Lake Forest, CA, USA). Brownlee Spheri-5 C18 polyfunctional columns (4.6 mm × 100 mm; d_p, 5 μm) were obtained from Perkin-Elmer (Norwalk, CT, USA).

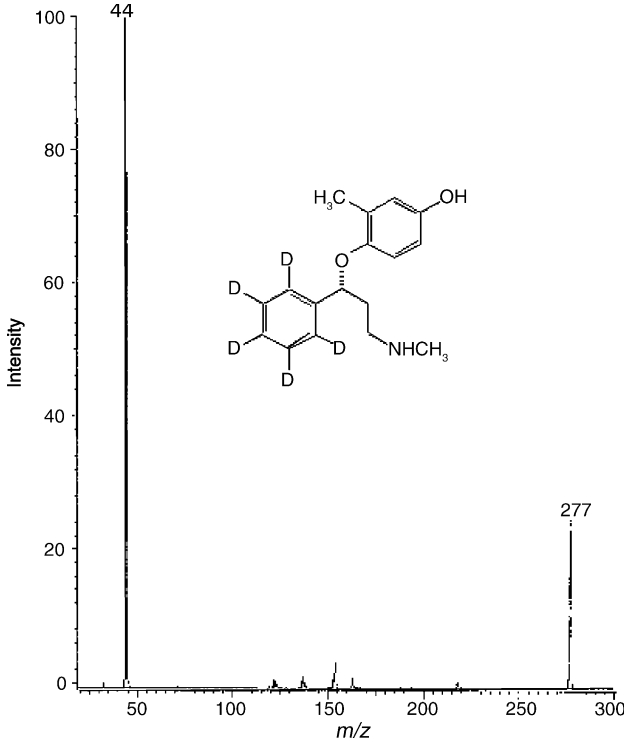

Fig. 5. The structure and product ion scan of [$^2H_5$]-4-hydroxyatomoxetine.

2.2. LC/MS equipment and analytical conditions

A Hewlett-Packard Series II 1090L Liquid Chromatograph system (Hewlett-Packard Company, Rockville, MD, USA), or a Hewlett-Packard Series II 1090L Liquid Chromatograph coupled with a CTC Analytics HTS Pal autosampler (Leap Technologies, Carrboro, NC, USA) was used for HPLC analyses. The chromatographic system consisted of a Brownlee Spheri-5 C18 polyfunctional column (4.6 mm × 100 mm; d_p, 5 μm) with the use of a binary gradient (mobile phase A: water, mobile phase B: 5 mM ammonium acetate, 47.2 mM formic acid, 4 mM trifluoroacetic acid in acetonitrile–water (85:15, v/v)). The formic acid, trifluoroacetic acid and ammonium acetate combination was utilized in obtaining optimal chromatographic analyte peak separation through ion pairing and pH optimization. Moreover, the mixture resulted in optimal sensitivity (ionization) in conjunction with the Finnigan MAT TSQ-700 or TSQ-7000 mass spectrometer compared to any mixture lacking one or more of these constituents. The gradient utilized the following profile: 0 min/75% B, 0.8 min/75% B, 1.0 min/100% B, 2.5 min/100% B, 3.0 min/75% B. The mobile phase was delivered at 1 mL/min directly into the APCI source. The autosampler was used to make 30–55 μL injections for human plasma extracts, or 25–50 μL injections for human urine extracts. The cycle time was ∼5 min. Mass spectrometric detection was performed using a Finnigan MAT TSQ-700 or TSQ-7000 (Finnigan Corporation, San Jose, CA, USA), operating in positive ion APCI mode with the vaporizer temperature set at ∼500 °C. The heated capillary temperature was set to ∼190 °C. The sheath gas flow and the argon collision gas pressure were ∼30 psig and $\sim 1.2 \times 10^{-5}$ Torr, respectively. Collision energy was set to −25 eV. Quantification was performed using selected reaction monitoring (SRM) with the following transitions: atomoxetine *m/z* 256 → *m/z* 44, 4-hydroxyatomoxetine *m/z* 272 → *m/z* 44, *N-des*-methylatomoxetine *m/z* 283 (an adduct ion) → *m/z* 30, [$^2H_7$]-atomoxetine *m/z* 263 → *m/z* 44, and [$^2H_5$]-4-hydroxyatomoxetine *m/z* 277 → *m/z* 44 with a scan time of 0.75 s/scan. The product ion scans for the analytes and internal standards are shown as Figs. 1–5. Mass calibration, data acquisition, chromatographic and mass spectral graphical representation and post-acquisition quantitative analyses were performed using Finnigan ICIS software. Regression analyses were performed using an application developed and validated in-house by Taylor Technology Inc.

2.3. Standard solutions

A stock solution of atomoxetine was prepared by dissolving approximately 3 mg of the compound in ~3 mL of 100% MeOH so that the final concentration was 1 mg/mL (calculations were corrected for potency). The weighing was prepared in duplicate: one for the standard curve and another for the validation or quality control (QC) samples. This approach was repeated for *N-des*-methylatomoxetine and 4-hydroxyatomoxetine. These solutions were diluted in methanol–water (10:90, v/v) to result in an intermediate solution with the concentration of 2 μg/mL atomoxetine, 8 μg/mL *N-des*-methylatomoxetine and 8 μg/mL 4-hydroxyatomoxetine. Stock solutions of [$^2H_7$]-atomoxetine and [$^2H_5$]-4-hydroxyatomoxetine were prepared at 100 μg/mL by dissolving 1 mg of the compound into ~10 mL of methanol–water (10:90, v/v). An internal standard working solution was prepared by bringing 75 μL of the 100 μg/mL [$^2H_7$]-atomoxetine solution and 300 μL of the 100 μg/mL [$^2H_5$]-4-hydroxyatomoxetine solution (when available) to 100 mL volume with water. This resulted in an internal standard working solution of 75 ng/mL [$^2H_7$]-atomoxetine and 300 ng/mL [$^2H_5$]-4-hydroxyatomoxetine. The stock, intermediate, and working solutions were stored at ~4 °C when not in use.

2.4. Sample preparation

The concentrations shown in this section represent the analyte concentrations used in the human plasma assay. The human urine assay used different analyte concentrations. See the Calibration and Assay precision and accuracy sections for more information. The same general sample preparation and extraction were used for both of the assays.

The intermediate calibration plasma pool (2 mL pool) was prepared by adding 200 μL of the intermediate solution (2 μg/mL atomoxetine, 8 μg/mL *N-des*-methylatomoxetine, and 8 μg/mL 4-hydroxyatomoxetine) to 1.8 mL of control plasma. The calibration sample pools (2 mL pools) were prepared by further diluting the intermediate pool in plasma, followed by serial dilutions in plasma. The calibration samples were prepared by placing 500 μL of the sample in a 12 mm × 75 mm polypropylene tube and adding 100 μL of the internal standard working solution. Duplicate standards were typically prepared for each analysis at atomoxetine concentrations of 25, 10, 5, 2.5, 1, 0.5, and 0.25 ng/mL (*N-des*-methylatomoxetine and 4-hydroxyatomoxetine were also contained in the samples with analyte concentrations four times greater than atomoxetine concentrations).

The validation samples, used to evaluate the percent relative error (%RE or accuracy) and percent relative standard deviation (%RSD or precision) of the assay, and the QC samples, used when analyzing study samples, were prepared in the same fashion as the calibration samples. During the validation, at least five replicates at atomoxetine concentrations of 25, 10, and 0.25 ng/mL were prepared and analyzed on three separate days (*N-des*-methylatomoxetine and 4-hydroxyatomoxetine were also contained in the samples with analyte concentrations four times that of atomoxetine concentrations). During study sample analysis, QC samples were prepared in at least duplicate at atomoxetine concentrations of 20, 10, and 0.5 ng/mL (*N-des*-methylatomoxetine and 4-hydroxyatomoxetine were also contained in the samples with analyte concentrations four times that of atomoxetine concentrations) with each batch of samples analyzed.

A concentrated sample was also used as a validation sample. At least five replicates at 100 ng/mL atomoxetine (*N-des*-methylatomoxetine and 4-hydroxyatomoxetine were also contained in the samples with analyte concentrations four times that of atomoxetine concentrations) were prepared without internal standard, diluted 100 times (resulting in an atomoxetine concentration of 1 ng/mL) and extracted. The dilution factor varied, depending upon the matrix. The 100 times dilution factor was used in the human plasma validation. A 10 times dilution factor was used in the human urine validation.

Blank matrix samples and blank matrix spiked with internal standards were both prepared in duplicate and analyzed with each sample batch. The blank matrix spiked with internal standards was prepared by adding 100 μL of the internal standard working solution to 500 μL of matrix. The blank matrix sample was prepared by adding 100 μL of water (to replace the internal standard addition) to 500 μL of matrix.

Study samples were prepared by adding 500 μL of study sample matrix to a 12 mm × 75 mm polypropylene tube (sample preparation varied, dependent upon the use of the conjugated or unconjugated assay[4]). All samples (except blank plasma samples) were prepared by adding 100 μL of the internal standard working solution and 1 mL of 200 mm TFA in water to each sample followed by vortex mixing. The samples were extracted using 3 mL/7 mm polystyrenedivinylbenzene solid-phase extraction cartridges. The cartridges were preconditioned with MeOH (0.5 mL) followed by 200 mM TFA in water (1 mL). A Cerex (Varian, Lake Forest, CA, USA) positive pressure manifold was used to force the wash and elution solvents through the extraction cartridges. The samples were added and pushed through the cartridges. The samples were washed with 1 mL of MeOH–water (15:85, v/v) and eluted with 1 mL of 26 mM TFA in ACN. Using a TurboVap (Zymark Inc., Hopkinton, MA, USA), the eluents were concentrated to dryness under nitrogen at ~45 °C. The dry residues were reconstituted with 100 μL of ACN, vortex-mixed, and centrifuged at ~2500 rpm for approximately 5 min. The reconstituted samples were transferred

[4] If the unconjugated assay was employed, internal standard and TFA were added to the samples and the samples were extracted. If the conjugated assay was needed, 500 μL of 10,000 units/mL of β-glucuronidase (in 0.1 M sodium acetate buffer, pH 5.0) was added to every sample. The tubes were capped, vortex-mixed, and incubated at 37 °C for at least 2 h. Once the incubation was complete, the internal standard and TFA were added to the samples, followed by extraction.

into autosampler vials containing 100 μL of water. The samples were briefly vortex-mixed and centrifuged to remove air-bubbles. This process was also completed in a 96-well SPE plate format, utilizing a Tomtec Quadra 96 (Tomtec, Hamden, CT, USA).

Since the glucuronide standard was not available, the conjugated and unconjugated assays were conducted similarly, with the addition or omission of the enzyme and incubation.

2.5. Calibration

For the plasma assay, two standard curves were prepared in the range of 0.25–25 ng/mL for atomoxetine at concentrations of 25, 10, 5, 2.5, 1, 0.5, and 0.25 ng/mL. In the same standard curve samples, *N-des*-methylatomoxetine and 4-hydroxyatomoxetine were present in a range of 1–100 ng/mL at concentrations of 100, 40, 20, 10, 4, 2, and 1 ng/mL. The urine assay utilized two standard curves in the range of 1–200 ng/mL for atomoxetine and *N-des*-methylatomoxetine at concentrations of 200, 100, 50, 25, 10, 5, 2, and 1 ng/mL, while 4-hydroxyatomoxetine was present in the range of 10–2000 ng/mL at concentrations of 2000, 1000, 500, 250, 100, 50, 20, and 10 ng/mL. One set of the standard curve samples was analyzed at the beginning of each sample batch and the second set was analyzed at the end of each sample batch (for all batches). The peak area ratios of atomoxetine and *N-des*-methylatomoxetine to the [$^2H_7$]-atomoxetine internal standard were related to concentration using a linear regression with $1/x^2$ weighting. The peak area ratio of 4-hydroxyatomoxetine to the [$^2H_5$]-4-hydroxyatomoxetine internal standard ([$^2H_7$]-atomoxetine was used when the [$^2H_5$]-4-hydroxyatomoxetine internal standard was not available) was related to concentration using a linear regression with $1/x^2$ weighting.

The quantification of the 4-hydroxyatomoxetine-*O*-glucuronide was conducted indirectly by comparing the concentration of 4-hydroxyatomoxetine in the conjugated assay versus the concentration of 4-hydroxyatomoxetine in the unconjugated assay.

3. Results and discussion

3.1. Linearity

Typical calibration curve data for atomoxetine, *N-des*-methylatomoxetine, and 4-hydroxyatomoxetine are shown as Table 1 (human plasma and urine). The atomoxetine and *N-des*-methylatomoxetine calibration curves were constructed by plotting the peak area ratio of the corresponding analyte to the [$^2H_7$]-atomoxetine internal standard. For the human plasma assay, the 4-hydroxyatomoxetine calibration curve was constructed by plotting the peak area ratio of 4-hydroxyatomoxetine to the [$^2H_7$]-atomoxetine internal standard (the [$^2H_5$]-4-hydroxyatomoxetine internal standard was not available when the initial validation was completed). For the urine assay, the 4-hydroxyatomoxetine-calibration curve was constructed by plotting the peak area ratio of 4-hydroxyatomoxetine to the [$^2H_5$]-4-hydroxyatomoxetine internal standard.

Table 1
Calibration data for atomoxetine, 4-hydroxyatomoxetine, and *N-des*-methylatomoxetine from the human plasma and urine accuracy and precision batches

Compound	K0 (*Y*-intercept)	K1 (slope)	Coefficient of determination (R^2)
Human plasma			
Aromoxetine			
Batch 1	1.568×10^{-2}	1.29×10^{-1}	0.9915
Batch 2	1.366×10^{-2}	1.06×10^{-1}	0.9969
Batch 3	2.047×10^{-2}	1.66×10^{-1}	0.9985
4-hydroxyatomoxetine			
Batch 1	2.146×10^{-3}	2.95×10^{-2}	0.9939
Batch 2	-4.820×10^{-3}	3.56×10^{-2}	0.9964
Batch 3	-1.332×10^{-2}	2.79×10^{-2}	0.9893
N-des-methylatomoxetine			
Batch 1	7.710×10^{-4}	5.87×10^{-3}	0.9988
Batch 2	7.210×10^{-4}	7.34×10^{-3}	0.9928
Batch 3	6.810×10^{-4}	8.56×10^{-3}	0.9969
Human urine			
LY404363			
Batch 1	-1.25×10^{-3}	6.74×10^{-3}	0.9972
Batch 2	-1.53×10^{-3}	6.83×10^{-3}	0.9983
Batch 3	-1.62×10^{-3}	6.91×10^{-3}	0.9978
424478			
Batch 1	-2.21×10^{-2}	4.79×10^{-2}	0.9987
Batch 2	-5.13×10^{-2}	4.91×10^{-2}	0.9987
Batch 3	-4.70×10^{-2}	5.10×10^{-2}	0.9991
137877			
Batch 1	-3.35×10^{-3}	3.15×10^{-2}	0.9967
Batch 2	-2.08×10^{-3}	3.57×10^{-2}	0.9959
Batch 3	-2.71×10^{-3}	3.49×10^{-2}	0.9903

3.2. Assay precision and accuracy

Validation samples were prepared and analyzed to evaluate the intra-day and inter-day accuracy and precision of the analytical method in human plasma and urine. At least five replicates of each validation concentration were analyzed along with two standard curves on each of the 3 days for the primary assays. The data for the intra-day and inter-day precision and accuracy of the method determined by analyzing five replicates at 25, 10, and 0.25 ng/mL of atomoxetine (100, 40, and 1 ng/mL of *N-des*-methylatomoxetine and 4-hydroxyatomoxetine) on each of 3 days for human plasma are reported as Table 2. Table 3 depicts the intra-day and inter-day precision and accuracy data for the human urine assay (utilizing five replicates at 200, 150, 15, and 1 ng/mL of atomoxetine and *N-des*-methylatomoxetine, and 2000, 1500, 150, and 10 ng/mL of 4-hydroxyatomoxetine). The data for the intra-day precision and accuracy of the method were determined by analyzing five replicates at the same concentrations on a single day. The accuracy of the method was determined by calculating the relative error (%RE) and the precision by

Table 2
Intra-day and inter-day accuracy and precision of atomoxetine, 4-hydroxyatomoxetine, and *N-des*-methylatomoxetine in human plasma

	Atomoxetine concentration (ng/mL)			4-Hydroxyatomoxetine concentration (ng/mL)			*N-des*-Methylatomoxetine concentration (ng/mL)		
	0.25	10	25	1	40	100	1	40	100
Batch 1									
Intra-day mean	0.22	9.64	24.22	1.17	41.91	110.11	1.02	41.30	104.47
Intra-day accuracy	−12.00	−3.60	−3.12	17.00	4.77	10.11	2.00	3.25	4.47
Intra-day precision	9.09	1.97	2.89	2.56	6.68	4.67	5.88	4.75	3.00
n	5	5	5	5	5	5	5	5	5
Batch 2									
Intra-day mean	0.24	9.81	24.95	0.99	37.48	101.11	1.07	38.31	98.06
Intra-day accuracy	−4.00	−1.90	−0.20	−1.00	−6.30	1.11	7.00	−4.23	−1.94
Intra-day precision	8.33	2.75	1.80	12.12	7.02	6.47	2.80	3.21	2.55
n	5	5	5	5	5	5	5	5	5
Batch 3									
Intra-day mean	0.23	10.07	24.94	119.00	41.37	93.96	0.99	40.86	101.93
Intra-day accuracy	−8.00	0.70	−0.24	19.00	3.42	−6.04	−1.00	2.15	1.93
Intra-day precision	4.35	2.18	1.80	6.72	7.78	7.79	3.03	4.97	4.60
n	5	5	5	5	5	5	5	5	5
Inter-day mean	0.23	9.84	24.70	1.12	40.25	101.73	1.02	40.16	101.49
Inter-day accuracy	−8.00	−1.60	−1.20	12.00	0.63	1.73	2.00	0.40	1.49
Inter-day precision	8.70	2.85	2.51	10.71	8.37	8.90	4.90	5.33	4.22
n	15	15	15	15	15	15	15	15	15

Table 3
Intra-day and inter-day accuracy and precision of atomoxetine, 4-hydroxyatomoxetine, and *N-des*-methylatomoxetine in human urine

	Atomoxetine concentration (ng/mL)				4-Hydroxyatomoxetine concentration (ng/mL)				*N-des*-Methylatomoxetine concentration			
	1	15	150	200	10	150	1500	2000	1	15	150	200
Batch 1												
Intra-day mean	0.96	15.68	144.31	192.19	10.09	160.30	1465.78	1952.65	1.03	16.80	152.61	200.63
Intra-day accuracy	−4.00	4.53	−3.79	−3.91	0.90	6.87	−2.28	−2.37	3.00	12.00	1.74	0.31
Intra-day precision	7.29	6.44	5.16	6.15	4.66	2.86	1.80	1.25	10.68	8.99	6.50	6.90
n	5	5	5	5	5	5	5	5	5	5	5	5
Batch 2												
Intra-day mean	1.10	15.16	146.57	200.02	10.61	154.40	1492.69	2008.43	0.99	14.90	144.34	195.74
Intra-day accuracy	10.00	1.07	−2.29	0.01	6.10	2.93	−0.49	0.42	−1.00	−0.67	−3.77	−2.13
Intra-day precision	5.45	1.32	2.85	3.54	3.11	2.71	3.15	2.67	10.10	4.30	4.00	6.48
n	5	5	5	5	5	5	5	5	5	5	5	5
Batch 3												
Intra-day mean	0.99	14.99	145.07	191.99	10.35	152.90	1458.11	1929.99	0.94	15.17	142.59	185.87
Intra-day accuracy	−1.00	−0.07	−3.29	−4.01	3.50	1.93	−2.79	−3.50	−6.00	1.13	−4.94	−7.07
Intra-day precision	11.11	2.47	2.59	2.70	2.42	3.57	2.16	2.47	5.32	6.66	4.03	3.16
n	5	5	5	5	5	5	5	5	5	5	5	5
Inter-day mean	1.02	15.28	145.31	194.73	10.35	155.87	1472.19	1963.69	0.99	15.62	146.51	194.08
Inter-day accuracy	2.00	1.87	−3.13	−2.64	3.50	3.91	−1.85	−1.82	−1.00	4.13	−2.33	−2.96
Inter-day precision	9.80	4.32	3.50	4.50	3.86	3.54	2.49	2.70	9.09	8.64	5.61	6.33
n	15	15	15	15	15	15	15	15	15	15	15	15

Table 4
Summary of atomoxetine, 4-hydroxyatomoxetine, and *N-des*-methylatomoxetine stability in human plasma and urine

(Human) matrix	Room temperature stability (in matrix)	Freeze-thaw stability (in matrix)	Extract stability[a] (in extract)	Storage stability (in matrix)
Plasma	24 h	3 cycles (−70 °C and −20 °C)	24 h	83 days (−70 °C and −20 °C)
Urine	24 h	4 cycles (−70 °C)	111 h	365 days (−70 °C)

[a] Extract stability was conducted at room temperature.

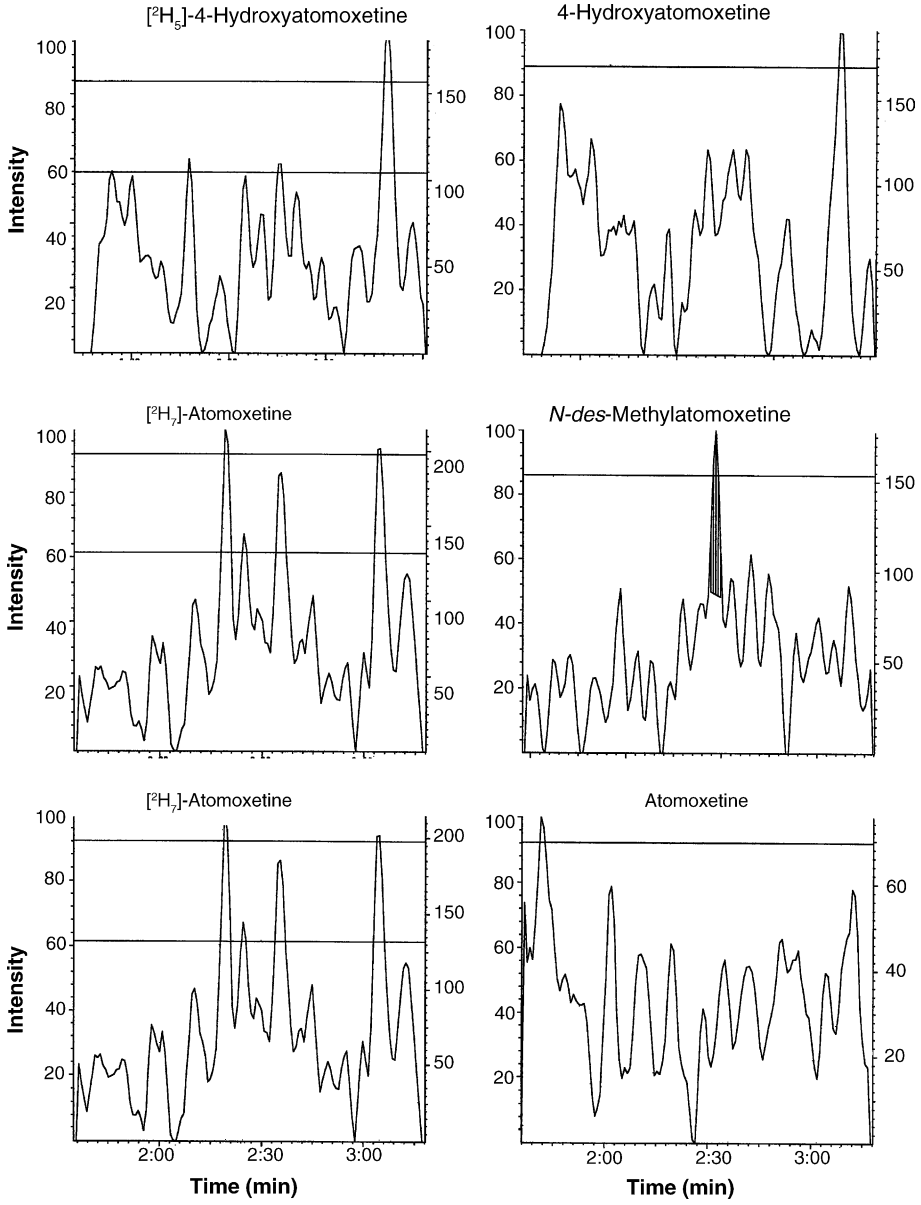

Fig. 6. Mass chromatograms of a typical blank human plasma extract.

calculating the relative standard deviation (% RSD). For the atomoxetine analysis in human plasma, the inter-batch precision ranged from 2.51% to 8.70% and the inter-batch accuracy ranged from −8.00% to −1.20%. The inter-batch precision for atomoxetine in the human urine assay ranged from 3.50% to 9.80% and the inter-batch accuracy ranged from −3.13% to 2.00%. For the 4-hydroxyatomoxetine analysis in human plasma, the inter-batch precision ranged from 8.37% to 10.71% and the inter-batch accuracy ranged from 0.63% to 12.00%. The inter-batch precision for 4-hydroxyatomoxetine in the human urine assay ranged from 2.49% to 3.86% and the inter-batch accuracy ranged from −1.85% to 3.91%. For the *N-des*-methylatomoxetine analysis in human plasma, the inter-batch precision ranged from 4.22% to 5.33% and the inter-batch accuracy ranged from 0.40% to 2.00%. The inter-batch precision for the *N-des*-methylatomoxetine in human urine ranged from 5.61% to 9.09% and the inter-batch accuracy ranged from −2.96% to 4.13%. Assay accuracy and precision was also completed when standard curve and validation samples were incubated with β-glucuronidase. The accuracy and precision of the assay, when samples were incubated with β-glucuronidase, were similar to the data shown as Table 2.

3.3. Stability

The stability of atomoxetine, *N-des*-methylatomoxetine, and 4-hydroxyatomoxetine in human plasma and urine was studied under a variety of storage and process conditions. The freezer storage stability was performed at −70 °C in

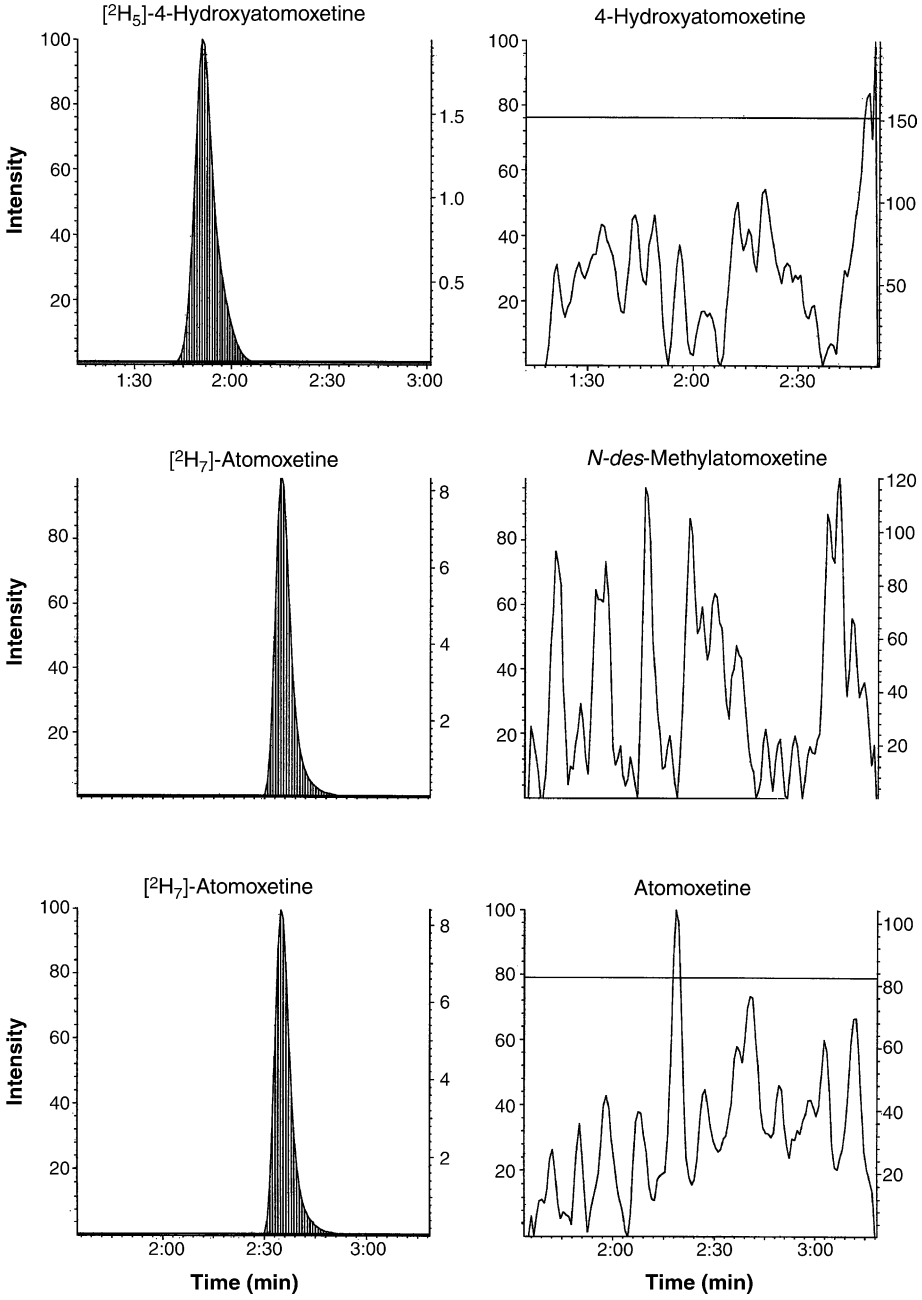

Fig. 7. Mass chromatograms of a typical blank plus internal standard human plasma extract (injected immediately after a ULOQ standard).

both matrices and −20 °C in plasma. Human plasma storage stability was established for at least 83 days by preparing at least triplicate stability samples at 0.5, 20, and 1000 ng/mL of atomoxetine (2, 80, and 4000 ng/mL of *N-des*-methylatomoxetine and 4-hydroxyatomoxetine). Human urine storage stability was evaluated by preparing at least triplicate stability samples at 3 and 150 ng/mL of atomoxetine and *N-des*-methylatomoxetine (30 and 1500 ng/mL of 4-hydroxyatomoxetine). The other plasma and urine stability experiments explained below were completed using the same analyte concentrations as the freezer storage stability experiments. Freeze-thaw stability (−70 °C in both matrices and −20 °C in plasma) was demonstrated through at least three cycles. Analyte stability was verified in plasma and urine when stored at room temperature for at least 24 h. The stability of the three analytes in plasma and urine extracts was demonstrated when stored at room temperature for at least 24 h. The stability of the analytes in the injection solvent was established to verify that the compounds would not degrade over the course of an analysis. This was accomplished by extracting samples, storing them overnight at room temperature and injecting them into the LC/MS/MS system the following day with fresh standard curve(s) and QC samples. Analytes were considered

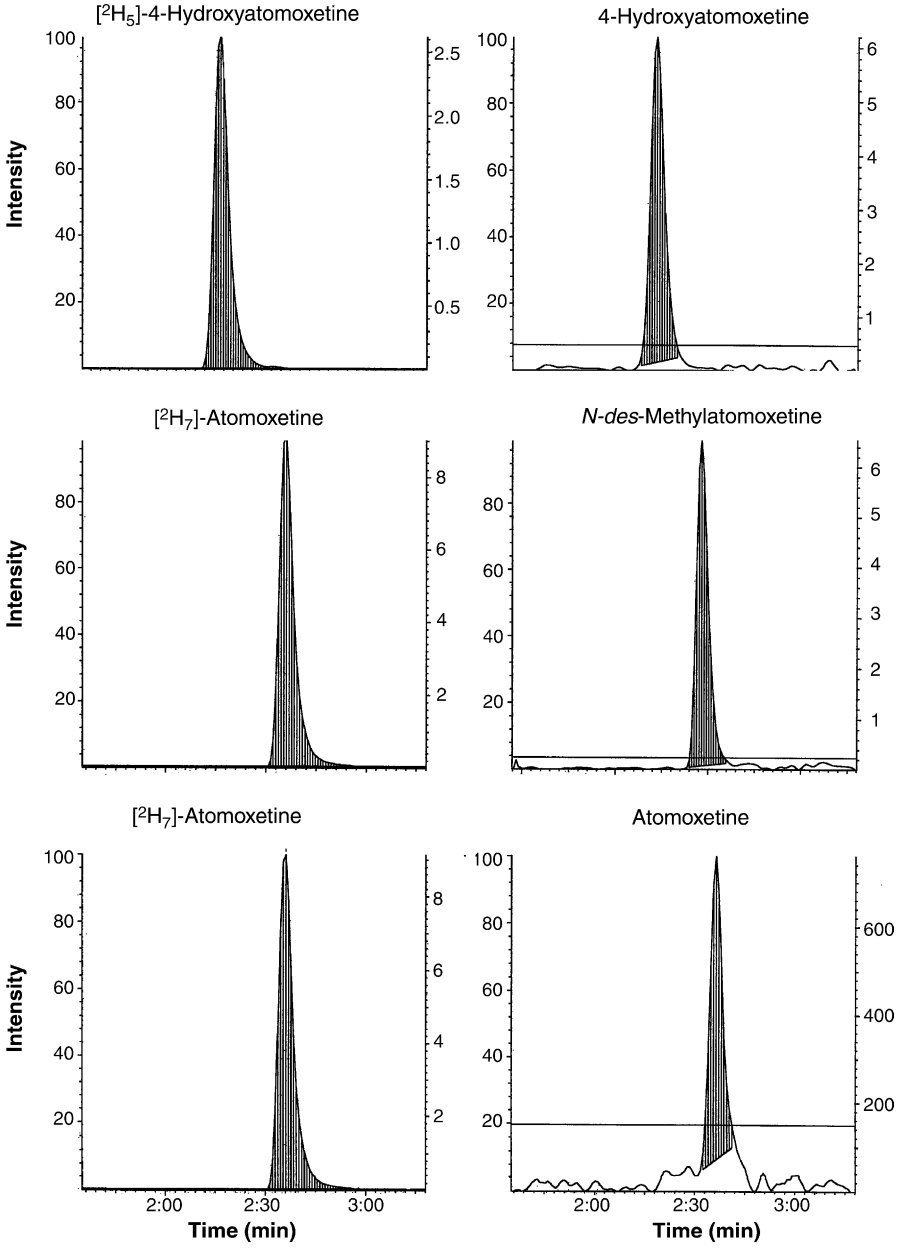

Fig. 8. Mass chromatograms of a typical LLOQ human plasma extract.

stable in plasma and reconstitution solvent if the calculated concentration was within or equal to 100 ± 20% of the theoretical concentration. The stability of the analytes was the same in plasma when incubated with β-glucuronidase. Stock and spiking solution stability were demonstrated at 406 days for the stock and 293 days for the spiking solution (incubating the solutions at ∼4 °C) by injecting fresh and aged solutions in the same batch and comparing the peak areas/heights (data not shown). Stored stock and spiking solutions were considered stable if the analyte peak areas/heights were within or equal to ± 7% of the fresh analyte solution(s) peak area/height. Two system suitability samples were injected before each human plasma batch at 0.5 ng/mL of atomoxetine (2 ng/mL of 4-hydroxyatomoxetine and *N-des*-methylatomoxetine) and 25 ng/mL of atomoxetine (100 ng/mL of 4-hydroxyatomoxetine and *N-des*-methylatomoxetine). The LLOQ concentration was also used for the system suitability samples in the urine assay. LC/MS/MS system performance and sensitivity were tested using these system suitability samples. A summary of the plasma and urine stability data is shown as Table 4.

3.4. Assay selectivity

The selectivity of the assay was investigated in both matrices by processing and analyzing blanks prepared from at least six independent lots of control human plasma or urine (±β-glucuronidase). The blanks were investigated for interference

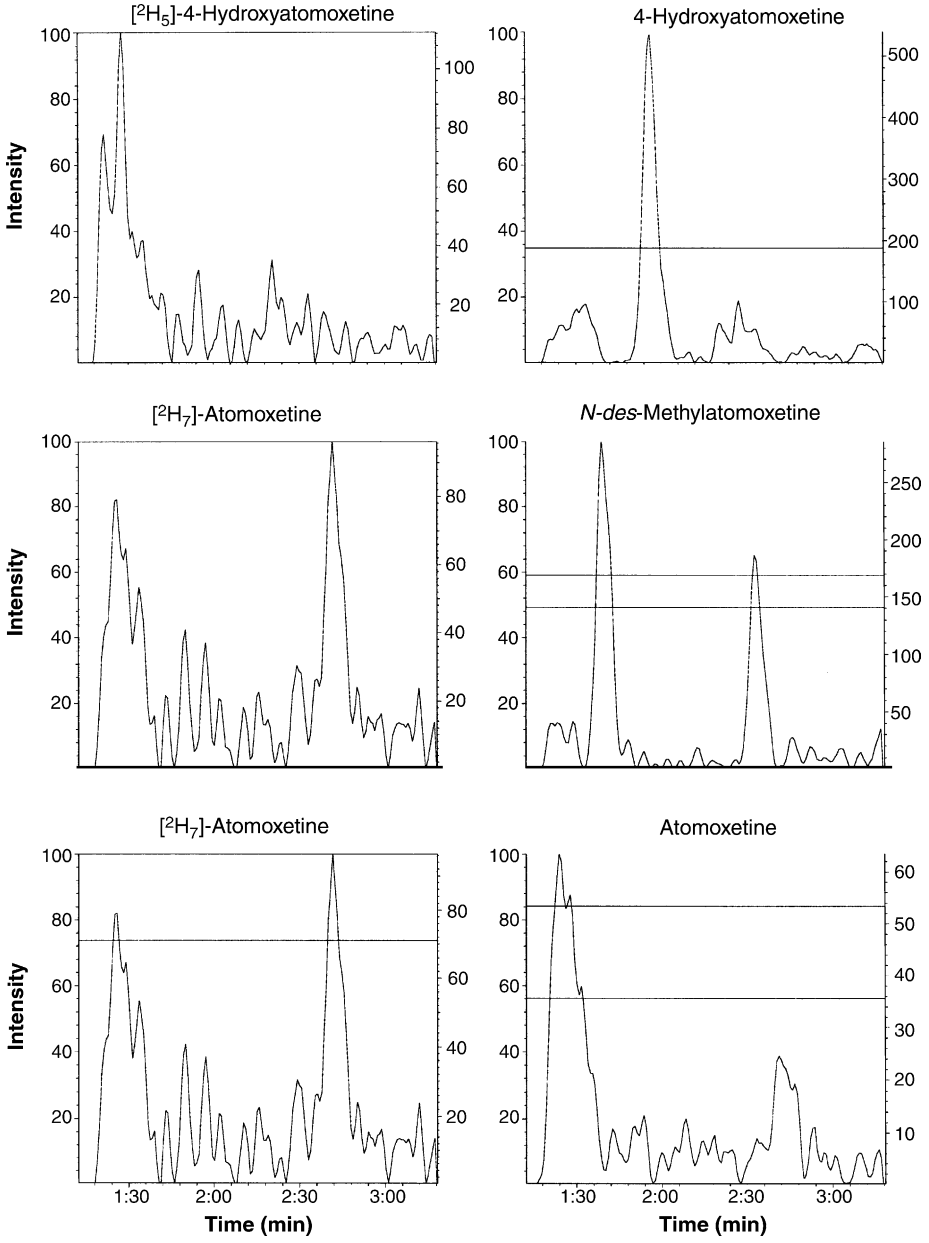

Fig. 9. Mass chromatograms of a typical blank human urine extract.

peaks. The selectivity of the assay was demonstrated by the absence of endogenous substances, in the drug-free matrices, that could interfere with the quantification of atomoxetine, *N-des*-methylatomoxetine, and 4-hydroxyatomoxetine. Potential interferences were minimized by combining solid-phase extraction with the separation power of HPLC and the selectivity of selected reaction monitoring mass spectrometric detection. Representative mass chromatograms of a blank sample (no drug or internal standard), blank plus internal standard sample (no drug), and LLOQ standards in human plasma (Figs. 6–8, respectively) and human urine (Figs. 9–11, respectively) indicated that analyte(s) and internal standard(s) interferences were minimal (data not shown).

3.5. Limits of quantification, extraction efficiency, matrix effects, dilutions, and carryover

The lower and upper limits of quantification (LLOQ/ULOQ) are defined as the lowest and highest concentrations, respectively, on the calibration graph at which an acceptable accuracy within or equal to $100 \pm 20\%$ [(mean assay concentration/theoretical concentration) $\times$ 100)] and precision within or equal to 20% (%RSD) were obtained. The LLOQ and ULOQ of the human plasma assay are 0.25 and 25 ng/mL for atomoxetine, respectively, and 1 and 100 ng/mL for 4-hydroxyatomoxetine and *N-des*-methylatomoxetine, respectively. The LLOQ and ULOQ of the human urine assay are 1 and 200 ng/mL for atomoxetine and *N-des*-

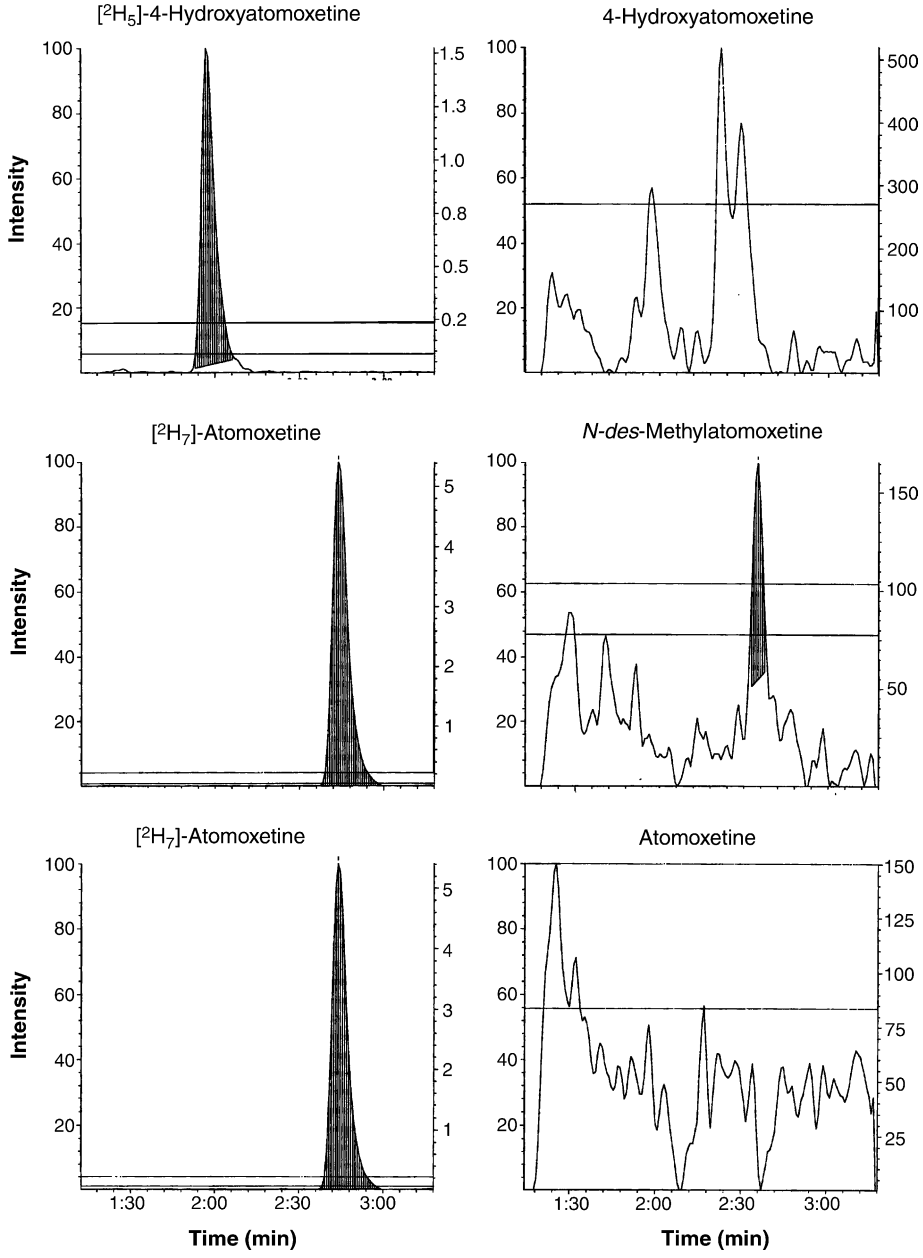

Fig. 10. Mass chromatograms of a typical blank plus internal standard human urine extract (injected immediately after a ULOQ standard).

methylatomoxetine, respectively, and 10 and 2000 ng/mL for 4-hydroxyatomoxetine, respectively.

The extraction efficiency of atomoxetine, *N-des*-methylatomoxetine, 4-hydroxyatomoxetine, [$^2H_7$]-atomoxetine, and [$^2H_5$]-4-hydroxyatomoxetine (when available) were determined by comparing analyte/IS peak areas of extracted samples to those extracted blanks spiked with analytes and internal standards. Three replicates were analyzed at each of two concentrations, 0.5 and 20 ng/mL of atomoxetine (2 and 80 ng/mL of *N-des*-methylatomoxetine and 4-hydroxyatomoxetine). Similar experiments, at different concentrations, were completed in the human urine assay. In addition, the effect of the matrix on the detection of the analytes and internal standards (matrix effect) was evaluated by comparing the extracted blanks spiked with analytes and internals standards with neat spikes of analytes and internal standards at the same concentrations. The extraction efficiency and matrix effect data for atomoxetine, *N-des*-methylatomoxetine, and 4-hydroxyatomoxetine in plasma and urine are shown as Table 5. The biological matrices were found to have minor effects on the signal of the analytes and internal standards (although the matrix suppression was 28% for the 4-hydroxyatomoxetine in human plasma, it was consistent from lot to lot, data not shown).

Human plasma sample dilutions were tested by analyzing a minimum of triplicate samples at 1 ng/mL

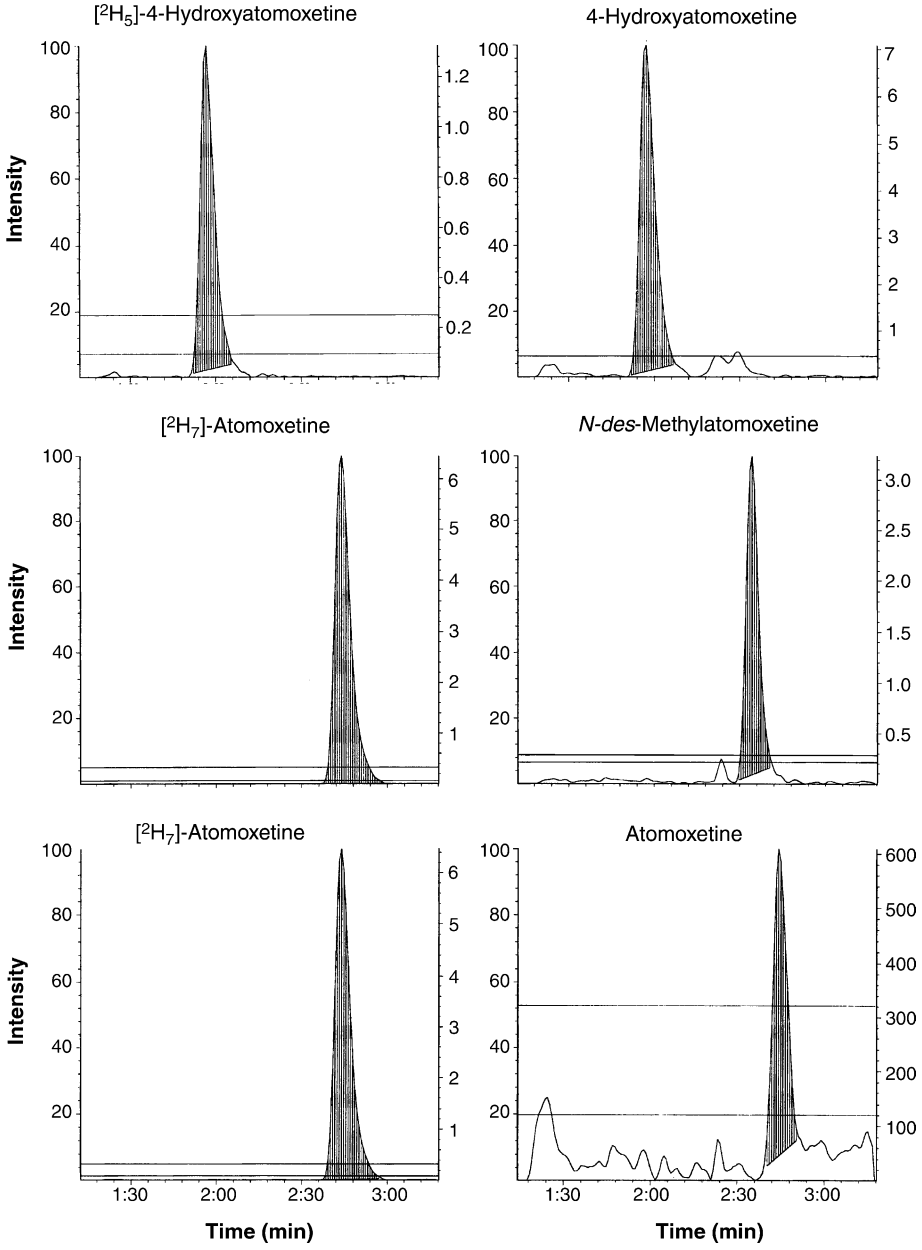

Fig. 11. Mass chromatograms of a typical LLOQ human urine extract.

of atomoxetine (4 ng/mL of *N-des*-methylatomoxetine and 4-hydroxyatomoxetine) that were diluted into blank matrix from 100 ng/mL of atomoxetine (400 ng/mL of *N-des*-methylatomoxetine and 4-hydroxyatomoxetine) concentrated samples with a dilution factor of 100. In the urine assay, the following validation sample concentrations were used: 1, 15, 150, and 200 ng/mL of atomoxetine and *N-des*-methylatomoxetine, and 10, 150, 1500 and 2000 ng/mL of 4-hydroxyatomoxetine. Sample dilutions were validated in human urine by analyzing triplicate samples at 190.4 ng/mL of atomoxetine and *N-des*-methylatomoxetine (1904 ng/mL of 4-hydroxyatomoxetine) that were diluted into blank matrix from 1904 ng/mL of atomoxetine and *N-des*-methylatomoxetine (19040 ng/mL of 4-hydroxyatomoxetine) concentrated samples with a dilution factor of 10. A dilution was considered accurate and precise if the %RSD was within or equal to 20% and the %RE was within or equal to $100 \pm 20\%$. It was demonstrated that the accuracy of the dilutions was acceptable in both matrices. Table 6 provides data regarding dilution accuracy for atomoxetine, 4-hydroxyatomoxetine, and *N-des*-methylatomoxetine in human plasma and urine.

Carryover was tested by injecting blank plasma samples or reconstitution solvent immediately after the high standard curve sample. If the area of the analytes was less than or equal to 20% of the low standard curve sample peak area, the

Table 5
Extraction efficiency and matrix effect of atomoxetine, 4-hydroxyatomoxetine, and *N-des*-methylatomoxetine in human plasma and urine

(Human) matrix	Compound	Mean extraction efficiency (%)	Mean matrix effect[a] (%)
Plasma	Atomoxetine	97	5
	4-Hydroxyatomoxetine	105	28
	N-des-Methylatomoxetine	87	7
	[$^2H_7$]-Atomoxetine[b]	99	4
	[$^2H_5$]-4-Hydroxyatomoxetine[b,c]	–	–
Urine	Atomoxetine	83	NC
	4-Hydroxyatomoxetine	83	NC
	N-des-Methylatomoxetine	91	NC
	[$^2H_7$]-Atomoxetine[b]	86	NC
	[$^2H_5$]-4-Hydroxyatomoxetine[b,c]	84	NC

NC = not calculated; all mean values were calculated from $n \geq 3$.
[a] A positive response indicates matrix suppression of the analyte and a negative result indicates enhancement.
[b] Internal standard concentration is not shown since it was constant in all samples (see Section 2.4).
[c] The extraction efficeincy for [$^2H_5$]-4-hydroxyatomoxetine was completed in human urine only since this internal standard was not available when the plasma assay was originally validated.

Table 6
Dilution accuracy of atomoxetine, 4-hydroxyatomoxetine, and *N-des*-methylatomoxetine in human plasma and urine

(Human) matrix	Dilution factor	Mean[a] % deviation from theoretical concentration		
		Atomoxetine	4-Hydroxyatomoxetine	*N-des*-Methylatomoxetine
Plasma	100	0.3	9.8	13.9
Urine	10	−8.6	0.1	−8.4

[a] Samples were diluted and analyzed, $n \geq 3$.

carryover was considered acceptable. Throughout the validation experiments and the study sample analysis, carryover was negligible and acceptable.

4. Conclusions

LC/MS/MS assays for the determination of atomoxetine, 4-hydroxyatomoxetine, *N-des*-methylatomoxetine, and 4-hydroxyatomoxetine-*O*-glucuronide in human plasma and urine have been developed and validated. The extraction procedure is relatively simple and requires only 500 μL of plasma or urine. The method offers excellent sensitivity and selectivity. The freezer, freeze-thaw, analysis, and room temperature stability of the analytes were investigated, and no significant degradation was observed for the duration evaluated. Dilutions can be employed to accurately quantify the

Table 7
Cumulative amounts of atomoxetine and metabolites excreted in urine from 0 to 24 h following a 90-mg dose

	Arithmetic mean	
	CYP2D6 extensive metabolizer subjects	(CV%) CYP2D6 poor metabolizer subjects
Atomoxetine		
μg	151 (73.8)	816 (78.4)
Percentage of dose	0.168 (73.8)	0.907 (78.4)
N-des-Methylatomoxetine		
μg	30.0 (97.8)	342 (89.9)
Percentage of dose	0.0352 (97.8)	0.402 (89.9)
4-Hydroxyatomoxetine		
μg	1150 (33.6)	346 (41.6)
Percentage of dose	1.20 (33.6)	0.361 (41.6)
4-Hydroxyatomoxetine-*O*-glucuronide		
μg	56500 (26.9)	8680 (54.2)
Percentage of dose	59.0 (26.9)	9.08 (54.2)
Total		
Percentage of dose	60.4 (26.6)	10.8 (52.8)

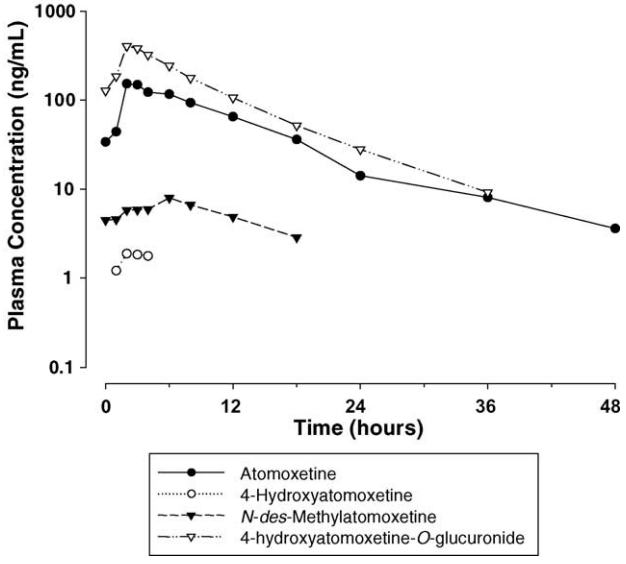

Fig. 12. Mean plasma concentration–time profile for CYP2D6 extensive metabolizer subjects ($n = 4$). Multiple 20-mg doses of atomoxetine were administered twice daily over 6 days. (●) Atomoxetine, (○) 4-hydroxyatomoxetine, (▼) *N-des*-methylatomoxetine and (▽) 4-hydroxyatomoxetine-*O*-glucuronide.

Table 8
Non-compartmental pharmacokinetic parameters for atomoxetine and its metabolites in CYP2D6 extensive metabolizer subjects following oral administration of atomoxetine[a]

Parameter	Arithmetic mean (CV %)			
	Atomoxetine	4-Hydroxyatomoxetine	*N-des*-Methylatomoxetine	4-Hydroxyatomoxetine-*O*-glucuronide
$C_{ss,max}$ (ng/mL)	159.70 (51.9)	2.03 (17.5)	7.02 (71.5)	413.88 (35.5)
T_{max}[b] (h)	2.00 (1.00–3.00)	2.50 (2.00–4.00)	3.50 (2.00–6.00)	2.00 (2.00–4.00)
Half-life[c] (h)	5.34 (3.67–9.09)	–	8.97 (2.11–21.9)	6.74 (5.90–8.30)
$AUC_{0-\tau}$ (μg h/mL)	1.08 (64.3)	–	0.0618 (86.4)	2.74 (13.6)
CL_{ss}/F (L/(h kg))	0.373 (75.1)	–	–	–
V_z/F (L/kg)	2.33 (51.0)	–	–	–

[a] Multiple 20-mg doses of atomoxetine were administered twice daily (BID) over 6 days.
[b] Median (range).
[c] Mean (range).

analytes within the standard curve range. The assays were robust, simple, and amenable to high sample throughput.

The described methods were successfully applied to the measurement of plasma concentrations of atomoxetine, 4-hydroxyatomoxetine, *N-des*-methylatomoxetine, and 4-hydroxyatomoxetine-*O*-glucuronide after administration of atomoxetine hydrochloride to humans. Furthermore, this method has been adapted for the measurement of these analytes in urine. The cumulative amounts of atomoxetine and its metabolites in urine were determined following oral administration and a summary of the derived values is shown as Table 7. The metabolite concentrations observed using bioanalysis were similar to those obtained in a study utilizing radiolabeled atomoxetine [12]. A graphical representation of the plasma versus time concentration curve is shown as Fig. 12. In humans, atomoxetine is predominantly metabolized by cytochrome P450 (CYP) 2D6 [13]; and therefore, its pharmacokinetics [14] and metabolism [12] are influenced by the polymorphic expression of this enzyme. The enzymatic activity of CYP2D6 is determined by a genetic polymorphism resulting in two major populations of individuals with either active metabolic capabilities (CYP2D6 extensive metabolizers, EM) or poor metabolic capabilities (CYP2D6 poor metabolizers, PM) [15,16]. The plasma pharmacokinetic profiles from individuals that have been genetically characterized as CYP2D6 extensive metabolizers are shown as Table 8. These results have been previously published, in part, elsewhere [12].

References

[1] D.T. Wong, P.G. Threlkeld, K.L. Best, F.P. Bymaster, J. Pharmacol. Exp. Ther. 222 (1982) 61–65.
[2] D.R. Gehlert, S.L. Gackenheimer, D.W. Robertson, Neurosci. Lett. 157 (1993) 203–206.
[3] T. Spencer, J. Biederman, T. Wilens, M. Harding, D. ODonnell, S. Griffin, Child Adolesc. Psych. 35 (1996) 409–432.
[4] J.M. Swanson, J.A. Sergeant, E.J.S. Sonuga-Barke, P.S. Jensen, D.P. Cantwell, Lancet 351 (1998) 429–433.
[5] G. Weiss, Psychopharmacol. Bull. 21 (1985) 169–177.
[6] R. Pary, S. Lewis, P.R. Matuschka, P. Rudzinskiy, M. Safi, S. Lippmann, Ann. Clin. Psychiatry 14 (2002) 105–111.
[7] L.A. Adler, H.C. Chua, J. Clin. Psychol. 63 (2002) 29–35.
[8] C. Samson, PSTT 2 (1999) 307–308.
[9] R. Kuczenski, DS. Segal, J. Neurochem. 68 (1997) 2032–2037.
[10] F.P. Bymaster, J.S. Katner, D.L. Nelson, S.K. Hemrick-Luecke, P.G. Threlkeld, J.H. Heiligenstein, S.M. Morin, D.R. Gehlert, K.W. Perry, Neuropsychopharmacology 27 (2002) 699–711.
[11] J.W. Tidey, J. Bergman, J. Pharmacol. Exp. Ther. 285 (1998) 1163–1174.
[12] J.M. Sauer, G.D. Ponsler, E.L. Mattiuz, A.J. Long, J.W. Witcher, H.R. Thomasson, K.A. Desante, Drug Metab. Dispos. 31 (2003) 98–107.
[13] B.J. Ring, J.S. Gillespie, J.A. Eckstein, S.A. Wrighton, Drug Metab. Dispos. 30 (2002) 319–323.
[14] N.A. Farid, R.F. Bergstrom, E.A. Ziege, C.J. Parli, L. Lemberger, J. Clin. Pharmacol. 25 (1985) 296–301.
[15] D.A.P. Evans, A. Maghoub, T.P. Sloan, J.R. Idle, R.L. Smith, J. Med. Genet. 17 (1980) 102–105.
[16] E. Steiner, L. Bertilsson, J. Sawe, I. Bertling, F. Sjoqvist, Clin. Pharmacol. Ther. 44 (1988) 431–435.