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A liquid chromatography/tandem mass spectrometry assay for the analysis of atomoxetine in human plasma and *in vitro* cellular samples

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Abstract

A simple, rapid and sensitive method for quantification of atomoxetine by liquid chromatography-tandem mass spectrometry (LC-MS/MS) was developed. This assay represents the first LC-MS/MS quantification method for atomoxetine utilizing electrospray ionization. Deuterated atomoxetine (d3-atomoxetine) was adopted as the internal standard. Direct protein precipitation was utilized for sample preparation. This method was validated for both human plasma and *in vitro* cellular samples. The lower limit of quantification was 3 ng/ml and 10 nM for human plasma and cellular samples, respectively. The calibration curves were linear within the ranges of 3 ng/ml to 900 ng/ml and 10 nM to 10 μ M for human plasma and cellular samples, respectively ($r^2 > 0.999$). The intra- and inter-day assay accuracy and precision were evaluated using quality control samples at 3 different concentrations in both human plasma and cellular lysate. Sample run stability, assay selectivity, matrix effect, and recovery were also successfully demonstrated. The present assay is superior to previously published LC-MS and LC-MS/MS methods in terms of sensitivity or the simplicity of sample preparation. This assay is applicable to the analysis of atomoxetine in both human plasma and *in vitro* cellular samples.

Keywords

Atomoxetine; LC-MS/MS; human plasma; *in vitro*; protein precipitation

Introduction

Atomoxetine ((*R*)-*N*-Methyl- γ -(2-methylphenoxy)-benzenepropanamine) is a potent and selective inhibitor of the presynaptic norepinephrine transporter (Wong *et al.*, 1982), and the first nonstimulant drug approved by the FDA for the treatment of attention deficit hyperactivity disorder (ADHD) in children and adults (Christman *et al.*, 2004). ADHD is a highly prevalent neurobehavioral disorder which affects up to 12% of children worldwide and presents significant life impairments which often persist into adulthood (Biederman and Faraone, 2005). Children with ADHD have increased rates of academic underachievement, conduct problems and compromised family and peer interactions, and are at heightened risk

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for antisocial behavior, delinquency and substance abuse in adolescence and adulthood (Barkley, 1997). Furthermore, youth and adults with ADHD have a greater propensity for developing other psychiatric syndromes, including mood, anxiety, and personality disorders (Biederman and Faraone, 2005). The psychostimulants methylphenidate and amphetamine have long been considered the drugs of choice for ADHD, and have a somewhat larger effect size than atomoxetine (Newcorn *et al.*, 2008). However, the latter medication may be particularly well-suited to subgroups of individuals with ADHD, such as those with anxiety (Geller *et al.*, 2007) or substance use (Jasinski *et al.*, 2008) disorders (Jasinski *et al.*, 2008), or those who do not respond to or tolerate stimulants (Kratochvil *et al.*, 2002; Christman *et al.*, 2004; Yildiz *et al.*, 2011).

The cytochrome P450 isoenzyme, CYP2D6, is the primary enzyme responsible for the oxidative metabolism of atomoxetine (Ring *et al.*, 2002). CYP2D6 mediates the formation of the 4-hydroxyatomoxetine metabolite, which is equipotent to atomoxetine as an inhibitor of the norepinephrine transporter but found in substantially lower concentrations relative to the parent compound (0.1–1%). CYP2D6 is known to be highly polymorphic, with approximately 80 reported allelic variants, which may partially contribute to significant interindividual variability of both pharmacokinetics and pharmacodynamics of atomoxetine observed in clinical studies. Compared to extensive metabolizers, CYP2D6 poor metabolizers (approximately 7% of the population) may have up to a 10-fold higher area under the plasma concentration versus time curve (AUC) and 5-fold higher peak plasma concentrations after oral doses of atomoxetine (De Gregori *et al.*, 2010). Further, the half-life of atomoxetine in poor metabolizers is approximately 19 hours, compared to 4.5 hours in extensive metabolizers. In addition to metabolic enzymes, drug transporters also have a potential role in the disposition of atomoxetine. Although the significant interindividual variability of therapeutic response and tolerability of atomoxetine is likely not exclusively attributable to differences in drug metabolism and pharmacokinetics (Newcorn *et al.*, 2009), the considerable variability in these parameters across individuals necessitates the establishment of a sensitive and reliable quantification assay for atomoxetine for therapeutic drug monitoring.

To date, several analytical methods suitable for atomoxetine determinations have been reported including liquid chromatography- mass spectrometry (LC-MS), high performance liquid chromatography-fluorescence detection (HPLC-FL) and HPLC-UV assays. A sensitive HPLC-FL assay for atomoxetine plasma samples was previously developed in our laboratory (Zhu *et al.*, 2007). This assay used a liquid-liquid extraction followed by fluorescent derivatization. Time-consuming liquid-liquid extraction and derivatization process were among the major limitations of this method. Two HPLC-UV assays employing liquid-liquid extraction from human plasma were also reported recently (Guo *et al.*, 2007; Patel *et al.*, 2007). However, the sensitivity and selectivity of those UV-based HPLC assays are well recognized to be generally inferior to other detections such as FL and MS. Recently, a LC- tandem mass spectrometry (LC-MS/MS) method coupled with an atmospheric-pressure chemical ionization (APCI) source and a LC- single quadrupole MS method utilizing electrospray ionization (ESI) in the positive mode were developed (Mullen *et al.*, 2005; Choong *et al.*, 2009). These two methods require time-consuming solid phase extraction or liquid-liquid extraction for sample preparation, which limits the methods for the use in high-throughput analysis. Additionally, mass spectrometry-based assays utilizing MS/MS rather than single quadrupole MS detection, and ESI rather than APCI are currently viewed as the most robust and widely used approaches for quantitative analysis of drug concentrations in biological matrix in both research and clinical settings. Thus, a development of a LC-ESI-MS/MS assay with simplified sample preparation process is warranted for atomoxetine analysis.

The current study introduces a rapid and sensitive method for quantification of atomoxetine in human plasma and *in vitro* cellular samples, and represents the first atomoxetine quantification method utilizing tandem MS coupled with ESI.

Experimental

Chemicals

Atomoxetine hydrochloride and the deuterated internal standard (IS), d3-atomoxetine hydrochloride, were purchased from Toronto Research Chemicals Inc (Toronto, Canada) (Fig. 1). All other chemicals were of analytical grade and commercially available.

Instrumentation

A Shimadzu LC system consisting of a SCL-10Avp system controller, SIL-10AD autosampler, LC-10AT VP solvent delivery module and DGU-14A degasser was employed for the present study. This LC system was coupled to an Applied Biosystems API 3000 triple quadrupole mass spectrometer. Ionization was achieved via ESI in the positive mode and ions were monitored by multiple reaction monitoring. Atomoxetine and d3-atomoxetine were monitored via the transition m/z 256 > 44 and m/z 259 > 47, respectively. The following parameters were optimized for MS analysis of atomoxetine: curtain gas, 8 psi; nebulizer gas, 12 psi; collision gas, 4 psi; TurboIonSpray voltage, 4500 V; source temperature, 350 °C; declustering potential, 26 V; focusing potential, 130 V; collision energy, 35 eV; and collision cell exit potential, 8 V. For monitoring of d3-atomoxetine, all MS parameters were the same with the exception of declustering potential, focusing potential, and collision energy, which were optimized at 31 V, 120 V, and 31 eV, respectively. A 20 μ l sample volume was injected into the system for each analysis. Samples were run through a C18 guard column (20.0 $\times$ 4.0 mm, SupelGuard™, Part# 59564, Bellefonte, PA). The mobile phase was methanol containing 0.025% trifluoroacetic acid (v/v) and 0.025% ammonium acetate (w/v) at a flow rate of 200 μ l/min. Both atomoxetine and d3-atomoxetine were eluted between 4.0 and 4.5 min under the described experimental conditions. The running time for each sample was 9 min. Data were acquired and analyzed by AB Sciex Analyst Software, version 1.4.2 (AB Sciex, Toronto, Canada).

Preparation of stock solutions, calibration curve and quality control samples

A stock solution of 10 mM atomoxetine was prepared in deionized water while a stock solution of the IS, d3-atomoxetine, was prepared in acetonitrile at 5 mg/ml. The IS was diluted to 400 nM in acetonitrile as the working solution for both plasma and cellular samples. All stock solutions were stored at -70 °C. The calibration curve was established by diluting atomoxetine in human plasma at concentrations of 3 ng/ml, 9 ng/ml, 30 ng/ml, 90 ng/ml, 300 ng/ml, 900 ng/ml. The concentrations chosen for quality control (QC) of human plasma were 9 ng/ml, 90 ng/ml, and 300 ng/ml. The calibration curve for *in vitro* samples was established by diluting atomoxetine in 1% Triton X-100 at the concentrations of 10 μ M, 2 μ M, 1 μ M, 200 nM, 100 nM, 20 nM and 10 nM. Triton X-100, a commonly used reagent to solubilize cells for *in vitro* experiments, was utilized in the present atomoxetine cellular uptake studies at a concentration of 1%. The concentrations chosen for the QCs of the *in vitro* experiment were 20 nM, 1 μ M and 6 μ M.

In vitro atomoxetine cellular uptake study

The developed assay was applied to an *in vitro* study investigating the uptake of atomoxetine in human embryonic kidney 293 (HEK293) cells. HEK293 cells were cultured in 24-well cell culture plates using the previously published method (Zhu *et al.*, 2010). After reaching confluence, cells were incubated with various concentrations of atomoxetine ranging from 3 to 100 μ M at 37°C for 10 min. Following incubation, the cells were washed

twice in ice cold Dulbecco's phosphate buffered saline and then solubilized in 200 μ l 1% Triton X-100 for the analysis of atomoxetine. The final atomoxetine concentrations were normalized with cellular protein concentrations determined by a Pierce BCA protein assay.

Sample preparation

The samples were prepared by mixing 60 μ l of atomoxetine plasma or *in vitro* cellular samples with 120 μ l of acetonitrile containing the IS d3-atomoxetine (final IS concentration: 400 nM). The samples were then centrifuged for 30 min at 16,000 $\times g$ at 4°C to remove proteins. The resulting supernatant was collected for LC-MS/MS analysis.

Results and Discussion

Linearity

Calibration curves of atomoxetine in plasma and in cellular samples were constructed by plotting the concentration versus analyte-to-IS peak area ratio. Linearity was assessed using weighted ($1/x^2$) regression analysis. In plasma samples, atomoxetine was determined to be linear between 3 ng/ml and 900 ng/ml while atomoxetine in cellular samples was determined to be linear between 10 nM and 10 μ M. For each matrix, the correlation coefficient was found to be more than 0.999 in 3 independent experiments.

Accuracy and Precision

The intra-day and inter-day accuracy and precision were assessed at concentrations of 9 ng/ml, 90 ng/ml, and 300 ng/ml in plasma samples, and 20 nM, 400 nM and 6 μ M in *in vitro* cellular samples. Five replicates of each concentration were used to validate the accuracy and precision. The results presented in Table 1 indicate that accuracy and precision were within the acceptable range in both plasma and cellular samples (FDA, 2001). For plasma samples, intra-day and inter-day accuracy ranged from 98.7% to 102.0 % and 98.8% and 101.2% respectively. Intra-day and inter-day precision ranged from 1.1% to 3.3% and 2.5% to 3.3% respectively. For cellular samples, intra-day and inter-day accuracy ranged from 96.0% to 102.3% and 96.0% and 100.8% respectively while intra-day and inter-day precision ranged from 0.9% to 3.2% and 2.3% and 3.5%, respectively.

Stability

The stability of atomoxetine in human plasma and cellular samples under various storage conditions was evaluated. Freeze-thaw stability was evaluated after 3 freeze (–70°C)/thaw cycles. As indicated in Table 2, the concentrations of QC samples after 3 freeze-thaw cycles ranged from 98.7% to 100.4% and 100.3% to 102.3% of those from freshly prepared samples for human plasma and *in vitro* cellular samples, respectively. To determine the stability of samples awaiting analysis in the autosampler, QC samples were prepared and maintained in the autosampler at 4°C for 3 h before injection. The remaining concentrations after autosampler storage were 98.7% to 101.0 % and 97.5% to 104.9% for plasma and cellular samples, respectively, relative to controls. Short-term stability of plasma samples was assessed by leaving the QC samples at room temperature for 4 h before analysis. This time was chosen based on the expected duration that the samples could be maintained at room temperature during the sample preparation. The ratios of the concentrations of atomoxetine determined after 4 h exposure to ambient room temperature to that measured immediately after preparation ranged from 96.3% to 101.2% for human plasma samples, and 95.6 % to 99.9% for *in vitro* samples.

Sensitivity and Selectivity

The lower limit of quantification (LLOQ) of atomoxetine in both matrices was established using the criteria outlined in the Guidance for Industry Bioanalytical Method Evaluation published by the Center for Drug Evaluation and Research (FDA, 2001). The LLOQ in plasma and *in vitro* cellular samples were determined to be 3 ng/ml and 10 nm, respectively, with a signal to noise ratio of 5. The accuracy of plasma and cellular samples was within 99.2% – 103.4% and 102.3% and 107.4%, respectively. The %RSD of precision was less than 2.9% and 4.3% for plasma and cellular samples, respectively.

Beyond the present MS/MS assay, to our knowledge there is only one other published tandem MS method applicable to determining plasma atomoxetine concentrations which was reported by Mullen and colleagues (2005). In that report, the LLOD was demonstrated to be 0.25 ng/ml when 0.5 ml plasma samples were prepared via solid phase extraction. The ionization methods utilized in our assay and Mullen's method were ESI and APCI, respectively. Due to much smaller sample volume (60 µl) requirement and simplified sample preparation (direct protein precipitation) utilized in the present study, the LLOQ of this assay is higher than that reported by Mullen and associates. However, the on-column detection limit of our assay was calculated to be 19.3 pg, essentially the same as that reported in the LC-APCI-MS/MS assay (i.e. 18.7 pg on column).

Previously, we reported a sensitive HPLC-FL assay for atomoxetine plasma analysis with a LLOQ of 1 ng/ml (Zhu et al., 2007). However, while offering the advantage of analysis which did not require MS instrumentation, the major limitation of this assay is that sample preparation involved time-consuming liquid-liquid extraction and fluorescent derivatization steps. Additionally, a relatively longer run-time (16 min) was required to separate atomoxetine from other interfering peaks. The sensitivity of the LC/MS assay reported by Choong and colleagues (2009) is comparable to that of our assay. However, Choong's method required a larger sample volume (0.5 ml) and solid phase extraction to achieve the reported sensitivity. In addition to the methods discussed above, two HPLC-UV methods have also been developed for quantification of atomoxetine in human plasma (Guo et al., 2007; Patel et al., 2007). Both assays demanded much larger plasma sample volumes (1 ml) and time-consuming liquid-liquid extraction for sample preparation, which may be challenging in some pediatric sample collections but generally impractical for small animal research purposes. Furthermore, the potential for a lack of selectivity is an intrinsic limitation of UV detection-based HPLC assay. Such limitations often require carefully selected mobile phase, analytical columns and other chromatographic conditions in order to avoid interference peaks. Additionally, in the case of clinical application, additional interfering chromatographic peaks as a result of concurrent medication use cannot be anticipated.

The sensitivity (LLOQ:10 nM or 3 ng/ml) demonstrated in the present study is more than adequate for the therapeutic drug monitoring of atomoxetine as the minimum (trough) steady-state concentrations of the parent drug in plasma are generally above 30 ng/ml in patients treated with typical clinical doses (Sauer et al., 2003; Sauer et al., 2005; Matsui et al., 2011). Additionally, since there is no recognized "therapeutic window" or threshold plasma concentration for response to atomoxetine, the primary interest in determining atomoxetine concentrations in the clinic is in the confirmed or suspected presence of a genetic polymorphism of CYP2D6, which is well documented to result in significantly higher systemic concentrations of atomoxetine.

Selectivity was assessed by analyzing blank plasma and cellular samples. For plasma samples, six samples of blank plasma from different sources were prepared utilizing direct protein precipitation, and submitted for LC-MS/MS analysis. The chromatograms were

compared with those chromatograms generated from the blank samples spiked with 3 ng/ml (LLOQ) of atomoxetine. No peaks were observed in blank human plasma and blank cellular samples, while atomoxetine and the IS d3-atomoxetine are the only peaks observed in the spiked samples. Representative chromatograms of blank human plasma and blank cellular samples as well as the blank matrices spiked with atomoxetine at the concentrations of LLOQ were presented in Figure 2.

Matrix effect and recovery

The effect of matrix on the ionization of atomoxetine and d3-atomoxetine was assessed by comparing the absolute peak area of the analytes dissolved in the supernatant of processed blank human plasma and blank cellular samples to that of the analytes prepared in the injection solvent (67% acetonitrile). The preparation of the supernatant of human plasma and cellular lysates was detailed in *Sample Preparation* section. Observed values were within 95.1% – 108.6% and 88.2% – 96.2% for plasma and cellular samples, respectively, indicating that the matrix effect was insignificant (Table 3). The direct protein precipitation resulted in excellent recovery of atomoxetine and d3-atomoxetine ranging from 93.8% to 113.2% for both plasma and cellular samples (Table 3).

Application

This method was successfully utilized to measure the uptake of atomoxetine in HEK293 cells. Atomoxetine concentrations in cellular samples were determined utilizing a one-step direct protein precipitation procedure. The intracellular concentrations of atomoxetine increased proportionally when cells were incubated with atomoxetine at concentrations below 30 μ M. Atomoxetine cellular uptake was noted to reach a plateau when the drug concentration was increased to 100 μ M (Fig 3). This observation suggests that the uptake of atomoxetine may be mediated by a saturable drug transporter(s). The LC-MS/MS assay described in the present study proved to be straightforward, and displayed the selectivity and sensitivity required for measurement of intracellular concentrations of atomoxetine in cultured cells. A representative chromatogram of the samples derived from cells incubated with 3 μ M of atomoxetine is provided in Figure 2e.

Conclusion

This fully validated method for quantification of atomoxetine in both human plasma and *in vitro* cellular samples is the first LC-MS/MS method coupled with ESI to be reported. This method is unique in its simplicity as it does not require time consuming extraction or derivatization steps. The present study represents the first direct protein precipitation sample preparation for atomoxetine analysis, which proved to be more time- and cost-effective relative to other reported sample preparation methods (Mullen et al., 2005; Guo et al., 2007; Patel et al., 2007; Choong et al., 2009). This assay may prove more applicable to atomoxetine analysis than previously reported MS methods, such as the LC-MS and LC-APCI-MS/MS assays (Mullen et al., 2005; Choong et al., 2009), since LC-ESI-MS/MS method is currently viewed as the mainstay of drug analysis and clinical drug monitoring. Furthermore, a small sample volume (60 μ l) proved adequate for analysis, which makes the assay particularly suitable for the pharmacokinetic studies in small animals such as mice and rats where the volumes of collected plasma are typically limited. This method was successfully applied to an *in vitro* study measuring intracellular uptake of atomoxetine, and would be suitable for human and animal atomoxetine pharmacokinetic studies as well.

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Figure 1.
Chemical structures of atomoxetine (a) and the internal standard d3-atomoxetine (b).

Figure 2.

Representative LC-MS/MS chromatograms of blank human plasma (a), blank cellular lysate (b), blank human plasma spiked with 3 ng/ml atomoxetine (c), blank cellular lysate spiked with 10 nM atomoxetine (d), cellular lysate samples collected from HEK293 cells after incubation with 3 μ M of atomoxetine (e).

Figure 3.

Intracellular accumulation of atomoxetine in HEK293 cells. Intracellular concentrations of atomoxetine were determined after HEK293 cells were incubated with atomoxetine (3 to 100 μM) at 37°C for 10 min. Atomoxetine concentrations were normalized to cellular protein concentrations. Data are expressed as mean $\pm$ SEM from 3 independent experiments.

Intra- and inter-day precision and accuracy of LC-MS/MS assay for the determination of atomoxetine in human plasma and *in vitro* cellular samples

Table 1

	human plasma (ng/ml)				cellular samples (nM)			
	9	90	300		20	400	6000	
Spiked concentration								
Batch 1 (n=5)								
Intra-day accuracy (%)	102.0	98.4	100.5		102.2	96.1	98.8	
Intra-day precision (%)	2.9	3.3	3.3		1.8	3.0	1.7	
Batch 2 (n=5)								
Intra-day accuracy (%)	99.7	99.3	101.4		96.0	96.3	102.3	
Intra-day precision (%)	3.2	2.9	1.1		0.9	2.0	3.2	
Batch 3 (n=5)								
Intra-day accuracy (%)	99.7	98.7	101.6		96.5	95.7	101.4	
Intra-day precision (%)	4.0	3.2	2.9		3.0	2.7	1.8	
Inter-day (n=15)								
Intra-day accuracy (%)	100.5	98.8	101.2		98.2	96.0	100.8	
Inter-day precision (%)	3.3	2.9	2.5		3.5	2.3	2.6	

Table 2

Stability study of atomoxetine in human plasma and cellular matrix. The data are presented as the means of 3 independent experiments.

Human plasma			Cellular samples			
Spiked conc (ng/ml)	Measured conc (ng/ml)		Spiked conc (nM)	Measured conc (nM)		Ratio (%)
	Control	After storage		Control	After storage	
Freeze/thaw cycles						
9	9.2	9.1	20	19.5	19.6	100.5
90	85.4	85.1	400	408.5	417.7	102.3
300	293.8	294.9	6000	6210.0	6233.3	100.3
Autosampler						
9	9.0	8.9	20	19.1	20.1	104.9
90	85.6	86.4	400	398.5	388.4	97.5
300	294.9	294.9	6000	6113.3	6156.7	100.7
Short term						
9	9.0	8.7	20	19.5	18.7	95.6
90	84.9	84.7	400	406.2	405.7	99.9
300	294.0	297.5	6000	6213.3	6163.3	99.2

Table 3

Matrix effect (n=6) and recovery (n=3) of atomoxetine and d3-atomoxetine in human plasma and in vitro cellular matrix

Human plasma				Cellular samples		
Spiked conc	Matrix effect (% , mean $\pm$ SD)	Recovery (% , mean $\pm$ SD)	Spiked conc	Matrix effect (% , mean $\pm$ SD)	Recovery (% , mean $\pm$ SD)	
Atomoxetine						
9 ng/ml	95.1 $\pm$ 6.2	101.7 $\pm$ 10.2	20 nM	96.2 $\pm$ 4.6	98.3 $\pm$ 6.3	
90 ng/ml	95.3 $\pm$ 9.8	108.0 $\pm$ 4.4	400 nM	88.2 $\pm$ 3.7	97.4 $\pm$ 8.5	
300 ng/ml	108.6 $\pm$ 10.9	97.9 $\pm$ 5.3	6000 nM	91.9 $\pm$ 4.8	113.2 $\pm$ 11.3	
d3-atomoxetine						
400 nM	97.6 $\pm$ 5.9	93.8 $\pm$ 5.4	400 nM	89.3 $\pm$ 6.6	102.1 $\pm$ 2.6	