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Short communication

# Sensitive quantification of atomoxetine in human plasma by HPLC with fluorescence detection using 4-(4,5-diphenyl-1H-imidazole-2-yl) benzoyl chloride derivatization

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**Abstract**

The first HPLC-fluorescence method for the determination of atomoxetine in human plasma was developed and validated. Atomoxetine was derivatized with 4-(4,5-diphenyl-1H-imidazol-2-yl) benzoyl chloride (DIB-Cl) under mild conditions, and separated isocratically on a C18 column using a HPLC system with fluorescence detection (λex: 318 nm, λem: 448 nm). A linear calibration curve was obtained over the concentration range 1–1000 ng/mL ($r = 0.999$). The limit of detection (S/N = 3) was 0.3 ng/mL. The relative standard deviations of intra-day and inter-day variations were ≤8.30% and 7.47%, respectively. This method is rapid, sensitive, and suitable for both basic and clinical studies of atomoxetine.


*Keywords:* Atomoxetine; 4-(4,5-Diphenyl-1H-imidazol-2-yl) benzoyl chloride

## 1. Introduction

Atomoxetine, a potent and selective norepinepherine transport inhibitor, is the first nonstimulant drug approved by the FDA for the treatment of attention-deficit hyperactivity disorder (ADHD) in children, adolescents and adults. ADHD is the most common neurobehavioral disorder among children with an estimated worldwide prevalence of 8–12% [1]. The pharmacological treatments of ADHD include the classic psychostimulant agents methylphenidate and amphetamine as well as the nonstimulant agent atomoxetine. Unlike traditional psychostimulants that are thought to produce therapeutic effects by increasing availability of dopamine and norepinephrine in the synaptic cleft by inhibiting presynaptic transporters [2], atomoxetine exerts its pharmacological effect by selectively inhibiting the presynaptic reuptake of norepinephrine [3].

Atomoxetine is well absorbed in gastrointestinal tract and predominantly metabolized by cytochrome P4502D6 (CYP2D6). After oral administration of 20 mg twice daily in adults, maximum plasma concentrations have been observed in the range of 160–184 ng/mL in CYP2D6 extensive metabolizers and up to 925 ng/mL in genetically deficient CYP2D6 poor metabolizers. The mean plasma concentrations of atomoxetine in adults reportedly range from 3 to 500 ng/ml in 24 h after a single dose of 10 mg [4–6]. These data suggest that therapeutic drug monitoring (TDM) may be of value given the broad range of concentrations possible in individuals according to their CYP2D6 genotype.

In order to investigate the pharmacokinetic properties of atomoxetine, a sensitive, but simplified analytical method is needed that could be applied easily to situations of TDM. A number of analytical methods based on liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS) have been developed for the determination of atomoxetine in human plasma and urine with lower limits of quantification of 0.25 and 1 ng/ml, respectively [6–9]. These methods appear reliable but are expensive and not always practical to apply in TDM due to both the associated costs and time constraints of "send out" specimens. In the present study, a sensitive and reliable HPLC-fluorescence detection method was developed for the

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determination of atomoxetine in human plasma by derivatization with 4-(4,5-diphenyl-1H-imidazole-2-yl) benzoyl chloride (DIB-Cl). This method, to our knowledge, represents the first description of analysis of atomoxetine that does not require MS detection.

## 2. Experimental

### 2.1. Chemicals

Atomoxetine was a gift from Eli Lilly & Company (Indianapolis, IN). The derivatizing agent 4-(4,5-diphenyl-1H-imidazol-2-yl) benzoyl chloride (DIB-Cl) was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). 1-Methyl-3-phenylpropylamine (MPPA) which served as the internal standard (IS), was obtained from Sigma–Aldrich (St. Louis, MO). All other agents were of the highest analytical grade and obtained through Fisher Scientific Co. (Fairlawn, NJ).

### 2.2. Preparation of reagents and atomoxetine standard solution

The derivatization reagent DIB-Cl was prepared at a concentration of 0.1 mmol/L in acetonitrile and stored at 4 °C. Atomoxetine stock solution (10 mg/mL) was prepared in water. This stock solution was determined to be stable for at least 3 weeks when stored at 4 °C. This working solution was found to be sable for at least 5 days at 4 °C. Human plasma spiked with atomoxetine was prepared by evaporating 10 μL of each working standard to dryness under nitrogen and adding 100 μL of blank human plasma to the residue resulting in a concentration range from 1 to 1000 ng/mL.

### 2.3. Optimization of the derivatization conditions of atomoxetine with DIB-Cl

To optimize the reaction conditions for the derivatization of atomoxetine with DIB-Cl, the effects of reaction time (5–120 min), pH (8.0–10.0), and the molar ratio of DIB-Cl to atomoxetine (10–200) on the derivative yields were investigated at room temperature while the concentration of atomoxitine was set at 20 ng/mL. Details of the derivatization procedure are described in the section of *sample pre-treatment and derivatization*.

### 2.4. HPLC system

The HPLC system consisted of a Waters 2690 Separations module (Waters, Milford, MA), a Phenomenex Luna C18 (2) 250 mm × 4.6 mm, 5 μm reversed-phase column preceded by a 4 mm × 3 mm C18 guard column (Phenomenex, Torrance, CA) and a Waters 474 scanning fluorescence detector (Waters, Milford, MA). The separation was performed at room temperature using acetonitrile: water (75:25, v:v) with the flow rate set at 1.0 mL/min. The excitation and emission wavelengths were set at 318 nm (λex), 448 nm (λem).

### 2.5. Plasma sample pretreatment and derivatization with DIB-Cl

Atomoxetine was extracted from human plasma by a liquid–liquid extraction method as follows: to a 100 μL aliquot of plasma, 50 μL of the IS MPPA (2 μmol/L) were added and vortexed briefly, followed by the addition of 500 μL of 0.05 M borate buffer (pH 10.0) and 1.5 mL of ethyl acetate. Samples were vortexed for 5 min and then centrifuged for 10 min at 2000 × *g*. The organic layer was transferred to fresh tubes and evaporated to dryness under nitrogen. The residues were derivatized by adding 10 μL of carbonate buffer (10 mmol/L, pH 9.0) and 180 μL of 0.1 mmol/L DIB-Cl. The mixture was vortexed briefly and the derivatization was allowed to proceed at room temperature for 30 min. The reaction was terminated by the addition of 10 μL of 28% ammonia solution. Lastly, 10 μL of the resultant mixture was subjected to HPLC analysis with fluorescence detection.

### 2.6. Method validation

Calibration curves of atomoxetine were prepared in the concentration range of 1–1000 ng/mL in human plasma. Intra- and inter-assay variations were assessed by spiking plasma with atomoxetine at concentrations of 1, 50, and 1000 ng/mL. The assay precision was calculated as the relative standard deviation (%RSD). The accuracy of the method was determined by calculating the relative error (%RE). The lower limit of detection was also evaluated. The stability of atomoxetine derivative was determined by comparing the peak areas of the derivative 48 h after termination of the reaction to those right after completion of the reaction. The extraction recovery of atomoxetine (1, 50, and 1000 ng/mL) and IS (MPPA, 30 ng/mL) were determined by comparing atomoxetine and IS peak areas of extracted samples to those extracted blank plasma spiked with atomoxetine and IS.

## 3. Results and discussion

### 3.1. Reaction mechanism

DIB-Cl, a derivative of lophine (2,4,5-triphenyl imidazole), was originally synthesized by Nakashima and co-workers as a fluorescence labeling reagent for primary and secondary amines intended for analysis [10]. DIB-Cl has proven to be a useful derivatization reagent for the HPLC analysis of amphetamine, methamphetamine, other sympathomimetic amines, bisphenol A, and various other compounds [11–15]. The merits of the DIB-Cl derivatization assay include high selectivity, high fluorescence quantum yield, and mild derivatization reaction conditions. Since atomoxetine possesses a secondary amine with the potential for labeling *via* N-acylation by DIB-Cl, the reaction scheme shown in Fig. 1 was predicted.

### 3.2. Optimization of derivatization of atomoxetine with DIB-Cl

An investigation into what constituted a sufficient reaction time was undertaken. Reaction time periods ranging from 5

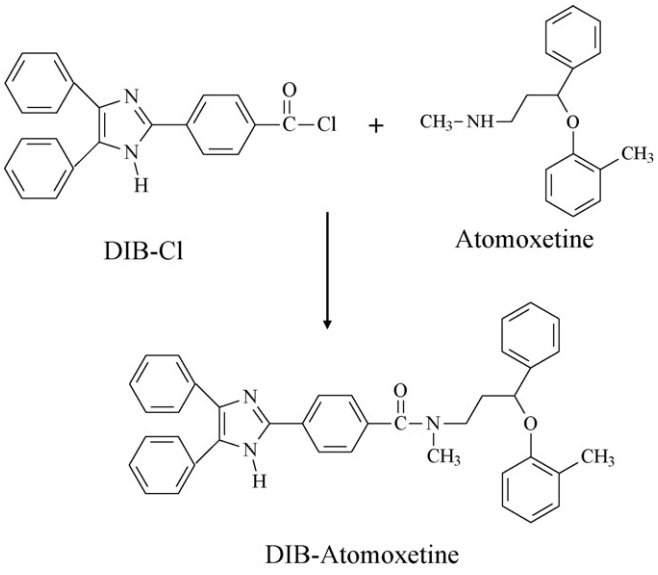


Fig. 1. The reaction scheme for labeling of atomoxetine with DIB-Cl.

to 120 min were assessed relative to their respective derivative yields. The results indicated that the yields increased during first 20 min following the initiation of the reaction and then reached a relative plateau. Adjustment of the pH of the reaction buffer ranging from 8.0 to 10.0 had no significant effect on DIB-Cl derivative yields. Additionally, the effect of varying the molar ratio of DIB-Cl to atomoxetine in the range of 10–200 was also explored by utilizing different concentrations of DIB-Cl. The results indicated that the derivative yields reached a maximum level when the molar ratio was >50. Accordingly, in the described experiments, the derivatization reaction was completed after 30 min of incubation at room temperature with the molar ratio of DIB-Cl to atomoxetine set at >50 and the pH of the carbonate buffer maintained at 9.0.

### 3.3. Calibration curve and detection limit

Calibration curves in spiked human plasma were obtained by plotting the concentration of atomoxetine *vs.* the peak area ratio of atomoxetine to IS. Calibration curves of atomoxetine were linear over the concentrations range 1–1000 ng/mL with a correlation coefficient ($r$) of 0.999. A lower limit of detection of 0.3 ng/mL (1.2 pg on column) was obtained at a signal-to-noise ratio of 3.

### 3.4. Validation

The intra- and inter-day variations were assessed using human plasma spiked with atomoxetine to produce concentrations of 1, 50, and 1000 ng/mL. The results in Table 1 indicate that the %RSD of intra-day and inter-day precision studies were less than 8.30% and 7.47%, respectively. The intra-day and inter-day bias ranged from −5.15% to 5.01%. The peak area ratios of the re-injection of the atomoxetine derivative 48 h after termination of the reaction to those of control were within 0.99–1.06 indicating that the derivative was stable for at least 48 h. The recovery of the extraction for atomoxetine at 1, 50, and 1000 ng/mL as well as IS at 30 ng/mL in human plasma was 69.82%, 75.44%, 71.24%, and 77.76%, respectively. Fig. 2a and b show typical chromatograms obtained from blank human plasma and human plasma spiked with 1 ng/mL of atomoxetine, respectively. No interferences were observed at the retention times of atomoxetine (13.6 min) and IS (6.8 min).

Table 1
Intra- and inter-day precision and accuracy of atomoxetine in human plasma

| | Spiked concentration (ng/ml) | | |
|---|---|---|---|
| | 1 | 50 | 1000 |
| Batch 1 ($n = 5$) | | | |
| Observed intra-day mean (ng/mL) | 0.95 ± 0.05 | 49.38 ± 1.07 | 991.59 ± 82.28 |
| Intra-day precision (%) | 5.19 | 2.17 | 8.30 |
| Intra-day accuracy (%) | −4.70 | −1.24 | −0.84 |
| Batch 2 ($n = 5$) | | | |
| Observed intra-day mean (ng/mL) | 0.95 ± 0.03 | 49.36 ± 2.63 | 1026.45 ± 59.80 |
| Intra-day precision (%) | 3.03 | 5.34 | 5.83 |
| Intra-day accuracy (%) | −5.15 | −1.27 | 2.65 |
| Batch 3 ($n = 5$) | | | |
| Observed intra-day mean (ng/mL) | 1.02 ± 0.08 | 51.21 ± 2.37 | 1050.12 ± 81.56 |
| Intra-day precision (%) | 7.75 | 4.62 | 7.77 |
| Intra-day accuracy (%) | 2.09 | 2.43 | 5.01 |
| Inter-day ($n = 15$) | | | |
| Observed inter-day mean (ng/mL) | 0.98 ± 0.06 | 49.70 ± 2.32 | 1029.16 ± 76.88 |
| Inter-day precision (%) | 6.17 | 4.67 | 7.47 |
| Inter-day accuracy (%) | −2.46 | −0.60 | 2.92 |

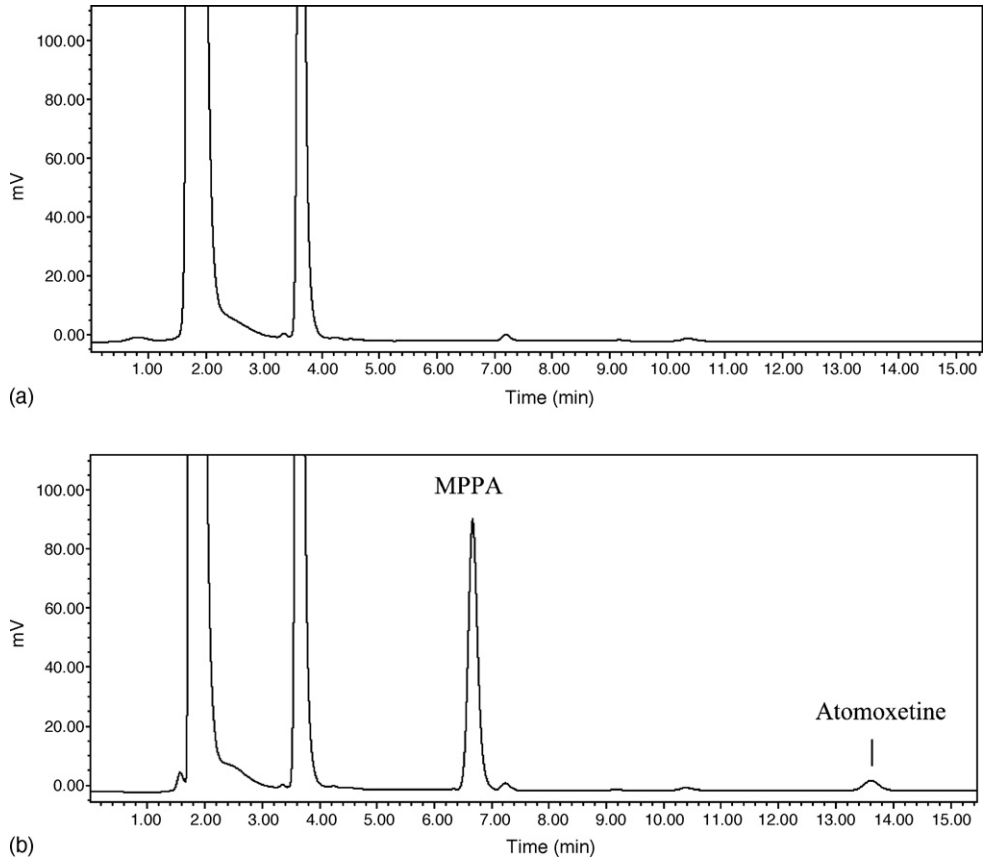


Fig. 2. Typical chromatograms of blank human plasma (a) and human plasma spiked with 1 ng/mL atomoxetine (b).

## 4. Conclusions

A rapid and sensitive HPLC method with fluorescence detection for atomoxetine measurement in human plasma was developed. The method is based on derivatization of atomoxetine with DIB-Cl to form the fluorescent derivative DIB-atomoxetine. The derivatization conditions are both simple and mild (i.e., 30 min at room temperature, pH 9.0) and the derivative is stable. Using this method, a well-defined peak of the DIB-atomoxetine derivative was obtained by the separation on a C18 column without any interfering peaks. A linear calibration curve was obtained from 1 to 1000 ng/mL ($r = 0.999$) with a lower limit of detection of 0.3 ng/mL. This sensitive and reliable method does not require mass spectrometry for detection and provides an opportunity for laboratories not equipped with MS instrumentation a means to measure atomoxetine concentrations in support of clinical and pre-clinical research. Given the increasing use of both psychostimulant and non-psychostimulant medication for the treatment of ADHD in children and adults, a reliable and easily performed analytical method for atomoxetine determinations in plasma should promote increased research and pharmacokinetic study and understanding of this novel pharmacological agent.

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