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# A new high performance liquid chromatographic method for quantification of atomoxetine in human plasma and its application for pharmacokinetic study

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**Abstract**

Atomoxetine is the first, non-stimulant alternative to other stimulant medications used for the treatment of Attention-Deficit/Hyperactivity Disorder (ADHD). Reported methods for the determination of atomoxetine include expensive liquid chromatography tandem mass spectrometry (LCMS) and high performance liquid chromatography (HPLC) with liquid scintillation counting (LSC) detection. Till date, no method has been reported in literature to determine atomoxetine using HPLC with UV detection. In this paper, we describe a new HPLC method for the determination of atomoxetine using liquid–liquid extraction with tertiary butyl methyl ether and UV detector. This method was found to be linear over the concentration range of 0.05–3.0 μg/ml. The limit of quantification was 0.05 μg/ml. Intra- and inter-day precision was <15% and accuracy was in the range of 95.67–108.80%. Stability studies showed that atomoxetine was stable in human plasma for short- and long-term period for sample preparation and analysis. This method was used for sample analysis in a pharmacokinetic study of atomoxetine (25 mg) in five healthy adult female volunteers. The observed mean ± S.D. pharmacokinetic parameters $C_{max}$, $T_{max}$ and $AUC_{0-t}$ were 0.40 ± 0.06 μg/ml, 3.40 ± 0.42 h and 1.34 ± 0.52 μg h/ml, respectively.

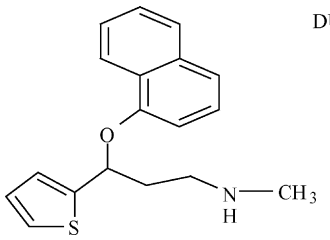

*Keywords:* Atomoxetine; Pharmacokinetics; Human plasma; UV detection; Indian females

## 1. Introduction

Atomoxetine hydrochloride [(−)-*N*-methyl-3-phenyl-3-(*o*-tolyloxy)-propylamine hydrochloride] is the first non-stimulant, orally administered, selective norepinephrine reuptake inhibitor. The US Food and Drug Administration approved it in November 2002 for the treatment of Attention-Deficit/Hyperactivity Disorder (ADHD) in patients of 6 years or older [1–3]. The precise mechanism by which atomoxetine produces its therapeutic effects in ADHD is unknown, but it is thought to be via the selective inhibition of the pre-synaptic norepinephrine transporter, as determined by the ex vivo uptake and neurotransmitter depletion studies. This keeps higher concentration of norepinephrine at work in the inter-neuron junctions in the brain [4–8].

High performance liquid chromatographic (HPLC) methods are widely used for the determination of drugs in biological matrix. The reported methods for the determination of atomoxetine include expensive liquid chromatography tandem mass spectrometry (LCMS) or HPLC with liquid scintillation counting (LSC) detection [9–11]. No method has been reported so far for determination of atomoxetine using HPLC with UV detection. We herewith report a new HPLC method for determination of atomoxetine in human plasma with UV detection having the limit of quantification of 0.05 μg/ml.

## 2. Experimental

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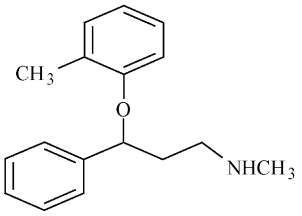
ATOMOXETINE




### 2.1. Materials and chemicals

Atomoxetine was obtained form Intas Pharmaceuticals Ltd., Ahmedabad, India and duloxetine (internal standard) from Torrent Pharmaceuticals Ltd., Ahmedabad, India. The atomoxetine (racemic mixture) and duloxetine used as reference standard were 99.9% pure base. All solvents used were of HPLC grade. Heptane sulphonic acid (analytical grade) was obtained from S.D. Fine-Chem. Ltd., Mumbai, India, and human blank plasma was obtained from Prathama Blood Bank, Ahmedabad, India.

### 2.2. Apparatus and conditions

The HPLC system consisting of pump-PU-980 (Jasco Hachioji, Tokyo, Japan), detector-UV-975 (Jasco Hachioji) and auto sampler-AS-950-10 (Jasco Hachioji) was used. Chromatographic separation was carried out at UV wavelength 272 nm, by using Agilent SB-C18 (4.6 mm × 150 mm, 5 μm) column. The mobile phase consisted of acetonitrile: 5 mM heptane sulphonic acid buffer with 1% (v/v) of triethylamine, pH adjusted to 4.8 by glacial acetic acid (40:60, v/v). Flow rate of the mobile phase was maintained at 1 ml/min. Stock solution of atomoxetine 100 μg/ml and dilutions of 10 and 1 μg/ml were prepared in methanol and stored at 4 °C. Stock solution of duloxetine (internal standard) 10 μg/ml was prepared in methanol and stored at 4 °C.

### 2.3. Extraction procedure

Fifty microlitres of duloxetine (internal standard) (10 μg/ml) was added to 1 ml of human plasma, and vortex for 30 s. To this, 50 μl of saturated solution of sodium bicarbonate was added followed by 5 ml of tertiary butyl methyl ether (TBME). The mixture was vortexed for 2 min and centrifuged at 1800 × *g* for 10 min. The supernatant organic layer was transferred into a 10 ml conical glass tube and evaporated under the gentle stream of nitrogen gas. The residue was reconstituted in 100 μl of mobile phase, which was then injected to the HPLC column.

## 3. Validation

### 3.1. Calibration curve

Calibration curve was prepared in the concentration range of 0.05–3.0 μg/ml. The stock solution (100 μg/ml) in plasma was prepared and further serially diluted in plasma to get 3, 2, 1, 0.5, 0.25, 0.1, 0.05 μg of atomoxetine in 1 ml of human blank plasma which were then used for calibration curve. Fifty microlitres of internal standard (10 μg/ml) was added to each sample. Extraction was carried out as per the procedure described above. Standard curves were constructed by plotting ratio of the peak areas of atomoxetine and internal standard versus concentration. The calibration curves were obtained by least square linear regression analysis using weight scheme as $1/c$ ($c$ = concentration) using Borwin software Ver. 1.3.

### 3.2. Preparation of quality control (QC) samples to evaluate precision and accuracy of the assay method

The concentrations of atomoxetine were 2.40, 0.76, 0.076 μg/ml in human plasma to represent high, mid and low quality control samples, respectively. To prepare quality control samples, appropriate volumes of atomoxetine from the stock solution were transferred to 50 ml volumetric flask and diluted with human blank plasma to the mark and mixed. These quality control samples were stored at −80 °C and were removed at specific time for determining the accuracy and precision of the method.

### 3.3. Precision and accuracy

For the calculation of intra-day precision and accuracy, five replicates of quality control samples with atomoxetine (2.40, 0.76, 0.076 and 0.050 μg/ml) were extracted as described above and analyzed through HPLC. Their concentrations were calculated from the calibration curve. Inter-day accuracy and precision were calculated by taking four replicates of concentrations 2.40, 0.76, 0.076 and 0.050 μg/ml from QC samples for 3 consecutive days along with the standard calibration curve.

### 3.4. Recovery

Recovery of the extracted samples was calculated by analyzing four extracted samples of concentration 2.40, 0.76 and 0.076 μg/ml and then comparing the peak area ratio of these samples with those of unextracted atomoxetine samples.

### 3.5. Stability studies

Stability of atomoxetine in human plasma, during storage and processing was checked using quality control samples. For checking the auto sampler stability, three replicates of high and low controls were analyzed at 0 and 12 h. For freeze thaw stability, three replicates of high and low controls were frozen at −80 °C and analyzed after first and second freeze thaw cycle, while for bench top stability, three replicates of high controls were analyzed after 0 and 4 h at room temperature. Long-term stability of atomoxetine was checked for 30 days at −80 °C.

## 4. Pharmacokinetic study

The volunteers selected for the study were females of age group 23–45 years. The volunteers were instructed to be at

the study site 12 h prior to the study and were restricted from performing any strenuous physical and mental activity. One capsule of 25 mg atomoxetine was given to each volunteer in an overnight fasting condition. Three millilitres of blood samples were collected from each volunteer, using heparinized syringes from indwelling venous cannula at 0.0 h (predose) and 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0, 8.0, 10.0, 12.0 h postdose. Twenty-four and 48 h samples were collected by vein puncture using disposable needles and syringes. Blood samples were immediately transferred into clean glass blood collection vials labeled properly with volunteer number and sample number. These vials were then transferred to the plasma separation room where these vials were centrifuged at 3800 × *g* for 7 min at 4 °C. Plasma was separated from these samples and was transferred to properly labeled plasma collection vials which were stored at −80 °C till analysis.

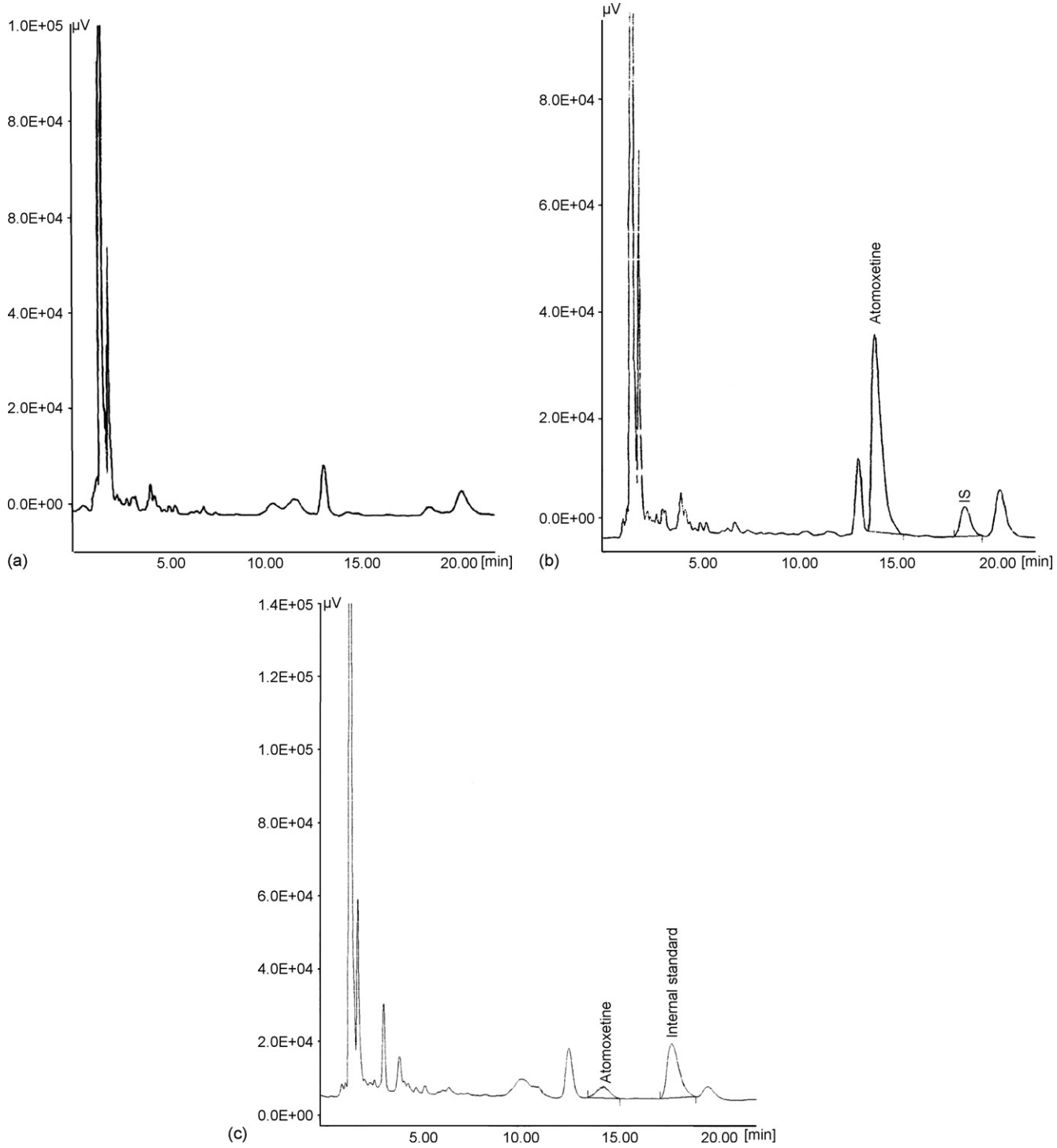



Fig. 1. (a) Chromatogram of blank plasma, (b) chromatogram of plasma containing 2.5 μg/ml atomoxetine and (c) chromatogram of plasma containing 0.05 μg/ml atomoxetine.

The study was performed according to the guidelines of the World Medical Assembly of Helsinki concerning the ethical consideration in human experiments. Protocol was approved by the Institute Ethics Committee.

Pharmacokinetic parameters maximum plasma concentration ($C_{max}$) and time to reach maximum plasma concentration ($T_{max}$) were obtained directly from the plasma concentration time curve while area under the curve ($AUC_{0-t}$) was calculated by trapezoidal rule [10].

## 5. Results and discussion

A new analytical method for estimation of atomoxetine in human plasma was developed and validated. Duloxetine was used as an internal standard as it shows similar chromatographic behavior and structural properties. Solid phase extraction procedures for extraction of atomoxetine from human plasma have been used in reported methods [9], however, the current method uses liquid–liquid extraction procedure using TBME. The method is more stable, reproducible and specific in comparison to other reported methods. The TBME and sodium bicarbonate were used to improve specificity of analytes and to reduce endogenous peaks. The analytes were more stable at alkaline pH and therefore sodium bicarbonate solution was added. Retention time of atomoxetine and internal standard (duloxetine) were 13.9 and 18.4 min, respectively. Total HPLC run time was 25 min.

### 5.1. Chromatograms

Representative chromatograms of blank plasma and plasma with atomoxetine are shown in Fig. 1a–c. Though the retention time of the drug is more compared to the reported LC–MS/MS methods, the extraction procedure is inexpensive and easy to perform. The glacial acetic acid, triethyl amine and heptane sulphonic acid combination (used for the chromatographic system) was utilized in obtaining separation through ion pairing and pH optimization.

### 5.2. Linearity and lower limit of quantification

Standard curves were constructed by plotting ratio of peak areas of atomoxetine and internal standard versus concentration and were linear in the range of 0.05–3.0 μg/ml. The correlation coefficient was found to be more than 0.998 ($n = 3$) for extracted samples and 0.997 ($n = 3$) for unextracted samples. The mean ± standard deviation of slope and intercept of regression curve were 0.807 ± 0.139 and 0.034 ± 0.016, respectively. The limit of quantification was 0.05 μg/ml.

### 5.3. Precision and accuracy

Intra-day and inter-day (Table 1) precision and accuracy were determined by taking five replicates of high, mid and low quality control samples. The intra-day precision (%CV) of quality control samples prepared to yield concentrations of 2.40, 0.76, 0.076 and 0.050 μg/ml were 12.28, 13.37, 4.89 and 6.44%, respectively, and inter-day precision (%CV) of quality controls prepared to yield concentrations of 2.40, 0.76, 0.076 and 0.050 μg/ml were 8.71, 6.86, 7.53 and 4.99%, respectively. Accuracy ranged between 95.67 and 108.80%.

### 5.4. Recovery from plasma

Recovery of atomoxetine was calculated by taking four replicates of high, mid and low quality controls and was found to be 60.11, 81.55, and 61.48, respectively, i.e. recovery was in the range of 60.11–81.55%. The recovery of duloxetine (internal standard) was 81.08%.

### 5.5. Stability

The results of freeze thaw stability, bench top stability, auto sampler stability (Table 2) showed that atomoxetine was stable for a short period of time. The results of long-term stability (Table 2) suggested that atomoxetine was stable on storage for a longer period of time.

### 5.6. Pharmacokinetic study

The method mentioned above was applied to a pharmacokinetic study of atomoxetine in healthy, adult, female volunteers. The mean plasma concentration versus time profiles of volunteers are shown in Fig. 2. The pharmacokinetic parameters (mean ± S.D.) $C_{max}$, $T_{max}$ and $AUC_{0-t}$ of atomoxetine in female volunteers after a single oral dose of 25 mg were found to

Table 1
Intra-day and inter-day precision and accuracy for atomoxetine

| Nominal concentration (μg/ml) | Estimated concentration (μg/ml) | Precision (%CV) | Accuracy (%) |
|---|---|---|---|
| Intra-day (data is expressed as mean ($n = 5$)) | | | |
| 2.40 | 2.30 | 12.28 | 95.67 |
| 0.76 | 0.74 | 13.37 | 97.11 |
| 0.076 | 0.074 | 4.89 | 97.63 |
| 0.050 | 0.054 | 6.44 | 108.80 |
| Inter-day (data is expressed as mean ($n = 15$)) | | | |
| 2.40 | 2.55 | 8.71 | 106.11 |
| 0.76 | 0.80 | 6.86 | 105.70 |
| 0.076 | 0.076 | 7.53 | 100.86 |
| 0.050 | 0.053 | 4.99 | 106.96 |

Table 2
Stability data of atomoxetine in human plasma

| | Nominal concentration (μg/ml) | Mean estimated concentration (μg/ml) | S.D. | Mean estimated concentration (μg/ml) | S.D. |
|---|---|---|---|---|---|
| Bench top | | 0 h | | 4 h | |
| | 2.40 | 2.20 | 0.09 | 2.07 | 0.03 |
| | 0.076 | 0.074 | 0.00 | 0.079 | 0.01 |
| Freeze thaw | | 0 h (cycle 1) | | 24 h (cycle 2) | |
| | 2.40 | 2.73 | 0.03 | 2.64 | 0.10 |
| | 0.076 | 0.076 | 0.01 | 0.079 | 0.01 |
| Auto sampler | | 0 h | | 12 h | |
| | 2.40 | 2.73 | 0.03 | 2.75 | 0.02 |
| | 0.076 | 0.076 | 0.01 | 0.082 | 0.005 |
| Long term | | Day 1 | | Day 30 | |
| | 2.40 | 2.30 | 0.32 | 2.39 | 0.34 |
| | 0.076 | 0.074 | 0.00 | 0.081 | 0.01 |

Data is expressed as mean and standard deviation (S.D.) ($n = 3$).

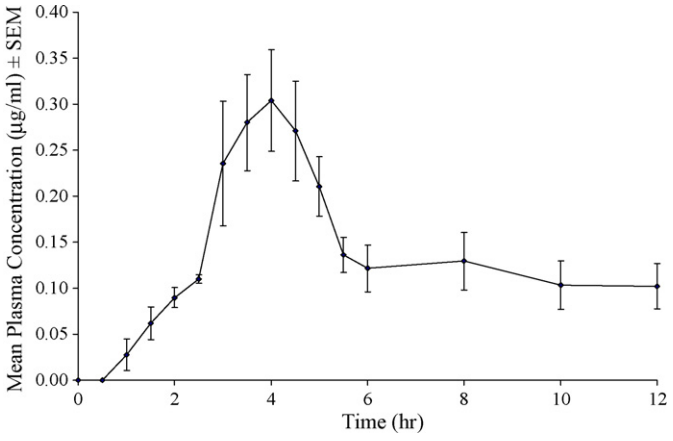



Fig. 2. Mean plasma profile of 25 mg oral dose of atomoxetine in healthy female volunteers.

be $0.40 \pm 0.06$ μg/ml, $3.40 \pm 0.42$ h and $1.34 \pm 0.52$ μg h/ml, respectively. No side effects were reported during the study. Sauer et al. have reported [12] that in extensive metabolizer individuals, the absorption maximum plasma concentration of atomoxetine is 533 ng/ml (coefficient of variance 32.0%) after the 1 mg/kg dose and occurs at a median time of 1–2 h following oral administration. In poor metabolizer group the mean $C_{max}$ after similar dose is approximately two-fold higher than that of extensive metabolizer. As the plasma pharmacokinetic parameters and biotransformation of atomoxetine are not affected by sex or race [1], it is justifiable to compare our result with the published results. The data reveal that maximum plasma concentrations in Asian Indian females are on the lower side compared to the published value, for the same in other populations.

## 6. Conclusion

A new, economical, easy to perform and sensitive method for determination of atomoxetine in human plasma was developed. This is the first reported HPLC method with UV detection for the determination of atomoxetine. Method of extraction used in our procedure is economical compared to the reported solid phase extraction methods. Moreover, stability studies showed that atomoxetine was stable for short- and long-term periods (30 days) of time. To improve the selectivity and specificity of analytes, chromatographic separation was carried out at UV wavelength 272 nm, by using C18 (4.6 mm × 150 mm, 5 μm) column. This analytical procedure was successfully applied to a pharmacokinetic study of atomoxetine in healthy adult female volunteers and can be used for bioequivalence/clinical study of atomoxetine.

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