Detection of atomoxetine and its metabolites in the urine by thin-layer chromatography and mass spectrometry

S. A. Karpushyna, S. V. Baiurka, T. O. Tomarovsky

Materials and methods. The volunteer’s urine samples after taking a single therapeutic dose of atomoxetine (2 capsules of 60 mg each of Strattera®) were studied. Sample preparation included diluting acid hydrolysis followed by the native compound and metabolites extraction with chloroform from the saturated solution of ammonium sulfate at pH of 11–12. Thin-layer chromatography studies of the extracts were carried out in 18 mobile phases including those proposed by The International Association of Forensic Toxicologists for general drug screening, and those widely used in forensic toxicological studies. The color reactions were carried out using a range of chromogenic reagents. A Varian 1200 L mass spectrometer (Netherlands) equipped with a dual quadrupole mass analyzer was applied for analysis of the eluates from chromatograms. Identification was undertaken at the direct introduction of the sample into the ion chamber, electron-impact ionization (70 eV), and full ion scanning mode.

Results. The spot of the native drug on the chromatogram was identified by the $R_f$ value. Two atomoxetine biotransformation products were identified by the molecular weights that correspond to the molecular ion peaks in the mass spectra.

Conclusions. Atomoxetine and its biotransformation products were detected in the urine under TLC screening conditions and identified using mass spectrometry method. Chromatographic mobility of the native compound, hydroxyatomoxetine, and dihydroxyatomoxetine in the TLC screening systems as well as the results of their visualization using chromogenic reagents applied for toxicological drug screening in the systematic toxicological analysis have been determined.

Key words: atomoxetine, hydroxyatomoxetine, dihydroxyatomoxetine, sample preparation, thin layer chromatography, mass spectrometry.

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Обнаружение атомоксетина и его метаболитов в моче методами тонкослойной хроматографии и масс-спектрометрии

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Цель работы – обнаружение атомоксетина и продуктов его биотрансформации в моче в условиях ТСХ-скрининга и идентификация метаболитов методом масс-спектрометрии.

Материалы и методы. Исследовали образцы мочи добровольца после приёма одноразовой терапевтической дозы атомоксетина (2 капсулы по 60 мг Strattera®). Пробоподготовка включала кислотный гидролиз с последующей экстракцией нативного соединения и метаболитов хлороформом из насыщенного раствора сульфата аммония при pH 11–12. Исследования экстрактов методом тонкослойной хроматографии проводили в 18 подвижных фазах, включавших рекомендованные Международной ассоциацией судебных токсикиологов для общего скрининга лекарственных веществ, а также фазы, которые широко используют при судебно-токсикиологических исследованиях. Цветные реакции производили с использованием ряда хромогенных реагентов. Для анализа элюатов с хроматограмм использовали масс-спектрометр Varian 1200 L (Нидерланды) с двойным квадрупольным масс-анализатором. Идентификацию проводили при прямом введении образца в ионную камеру, ионизации электронным ударом (70 eV) в режиме полного сканирования ионов.

Результаты. Пятно нативного препарата на хроматограмме идентифицировали по значению Rf. Два продукта биотрансформации атомоксетина были идентифицированы по молекулярным массам, соответствующим пикам молекулярных ионов в масс-спектрах.

Выводы. Атомоксетин и продукты его биотрансформации обнаружены в моче в условиях ТСХ и идентифицированы методом масс-спектрометрии. Установлена хроматографическая подвижность нативного соединения, гидроксиятомоксетина и дигидроксиатомоксетина в скрининговых ТСХ-системах, а также результаты их визуализации с помощью хромогенных реагентов, используемых для токсикиологического скрининга лекарственных средств в систематическом токсикиологическом анализе.

Ключевые слова: атомоксетин, гидроксиятомоксетин, дигидроксиатомоксетин, пробоподготовка, тонкослойная хроматография, масс-спектрометрия.


Atomoxetine ((3R)-N-methyl-3-(2-methylphenoxy)-3-phenylpropan-1-amine hydrochloride) is a modern antidepressant drug relating to a class of selective norepinephrine reuptake inhibitors (SNRI). Atomoxetine was the first non-stimulant drug approved by the FDA (USA) in late 2002 year for the treatment of ADHD in both children and adults [1,2]. Unlike traditional psychostimulants, atomoxetine does not have a potential for abuse; it is not classified as a controlled substance. The therapeutic daily dose is 40 mg daily increased to 80 mg after 3 days and up to 100 mg after 2 to 4 weeks [3]. Atomoxetine has also been shown to be effective for treating therapeutically resistant depression [4,5]. It can cause a range of side effects [6–8], and episodes of acute and lethal atomoxetine poisoning also were registered [3,9–11].

Atomoxetine is actively metabolized in the body via hydroxylation, N-desmethylation, and benzyl oxidation. Less than 3 % of atomoxetine dose is excreted as an unchanged drug [2,3,12]. The postmortem atomoxetine concentration in the urine was 0.1 mg/L. Half-life is 5.2 h and 21.6 h depending on the type of metabolizers: an extensive and a poor type respectively [3].

TLC screening is the most accessible type of screening procedure that is used in forensic toxicology. The International Association of Forensic Toxicologists (TIAFT) has proposed unified TLC systems for which a database of Rf values for more than 16.000 toxicologically important drugs has been created [3]. Studies on the determination of the chromatographic mobility parameters for atomoxetine in the TLC systems recommended by TIAFT have not been carried out.

Aim

The aim of the study was the detection of atomoxetine and its biotransformation products in the urine under TLC screening conditions and identification of the metabolites using mass spectrometry method.

Materials and methods

The pure substance of atomoxetine was isolated from the medicine “Strattera®” (7 capsules, 60 mg; “Lilly” (serial number C406168), Czech Republic) according to the method described in the article [13]. Atomoxetine-base was obtained by extraction of the analyte with chloroform from the aqueous solution at pH of 11 saturated with ammonium sulphate. The volunteer’s urine samples after taking a single therapeutic dose of atomoxetine (2 capsules of 60 mg each of Strattera®) were studied. Urine was collected in separate portions of 50 mL for 14 hours, starting after the 5th hour of taking the antidepressant. All chemicals were of analytical grade or better.

Sample preparation. The concentrated hydrochloric acid in the volume of 1 ml (it was based on the ratio of 0.10 mL of the acid for every 2.0 mL of the biological fluid) was added to 20 mL of the urine and the mixture was heated in a boiling water bath for 30 min. The obtained hydrolysate was cooled, placed in a separatory funnel, and shaken with 7 mL of diethyl ether three times for 5 min each time. The organic layer was separated and discarded. The aqueous layer was placed in a separatory funnel again, alkaliﬁed with the sodium hydroxide 20 % solution to pH of 11–12, ammonium sulphate
was added until the saturated solution was obtained, and atomoxetine-base was extracted with 10 mL of chloroform three times each time. The extracts obtained were combined, filtered through a pleated blue band paper filter containing 0.2 g of anhydrous sodium sulphate, and evaporated in a water bath at 40 °C to remove chloroform. The dry residue was dissolved in 5 mL of chloroform, mixed thoroughly, transferred to a volumetric flask with a capacity of 25.00 mL adjusting to the specified volume with the same solvent.

Thin-layer chromatography studies of the extracts. The Merk chromatography plates were used. Aliquot of the 1/10 part of the obtained biological extract, aliquot of the 1/10 part of the blank biological extract were concentrated to the minimum volume of ~0.05 mL and applied on four TLC plates as spots. Then 10 μL of the standard solution of atomoxetine in methanol (1.0 mg/mL) was spotted next using microsyringe. The remaining biological extract evaporated to the minimum volume of ~0.05 mL was applied as a band only on the chromatography plate, which then was developed in the mobile phase of chloroform – acetone – 25 % ammonia solution (25:5:0.3). Then the zone in the chromatogram corresponding to this band was not treated by the location reagent.

Chromatograms were developed in chloroform, and then in one of the mobile phases which are listed in Table 1 using rectangular glass chambers (25 × 25 × 12 cm). UV-light, ninhydrin solution in acetone and Dragendorff–Munier reagent were used for the visualization. Then atomoxetine was eluted from the chromatogram band untreated by the location reagents with 4 mL of methanol (the elution yield was 99.2 %), the eluate was filtered through a blue band paper filter.

The color reactions were carried out with 0.5 mL of the obtained eluates using pieces of chromatographic plates and a range of the chromogenic reagents listed in Table 2.

Mass Spectrometry studies of the eluates. A Varian 1200 L mass spectrometer (Netherlands) equipped with a dual quadrupole mass analyzer was applied for analysis. Detection was undertaken at the direct introduction of the sample into the ion chamber, electron-impact ionization (70 eV), and full ion scanning mode.

Results

Three spots were detected on the chromatograms of the biological extracts which were obtained after sample preparation of the volunteer’s urine after taking a single therapeutic dose of atomoxetine. Blank biological extracts did not give corresponding spots. The spot of the native compound was identified by \( R_f \), the value of which coincided with the specified retention parameter for atomoxetine in the standard solution.

Figure 1 shows atomoxetine-base mas spectrum (EI, 70 eV), \( m/z \) (Irel,%): 255 [M]+(4), 176 (4), 148 (16), 117 (4), 108 (6), 91 (6), 72 (7), 65 (3), 44 (100.00). Found, \( m/z \): 255.15 [M]+, C17H21NO. Calculated, \( m/z \): 255.4 [3]. The compliance with the database of NIST 17 mass spectrum library was of 85–87 %.

Figures 2, 3 show mass spectra of the eluates from chromatograms corresponding to the other two spots that did not match the native drug. Suspected products of atomoxetine biotransformation were identified by the molecular weights that correspond to the molecular ion peaks in the mass spectra.


Dihydroxyatomoxetine mas spectrum (EI, 70 eV), \( m/z \) (Irel,%): 271 [M]+(1), 254 [M-OH]+(2), 237 [M-OH-OH]+(1). Found, \( m/z \): 271.4 [M]+, C17H21NO3. Calculated, \( m/z \): 271.5.

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Fig. 1. Mass spectrum of atomoxetine-base obtained from the substance of the drug.

Fig. 2. Mass spectrum of hydroxyatomoxetine extracted from the tested urine samples.

Fig. 3. Mass spectrum of dihydroxyatomoxetine extracted from the tested urine samples.

Table 2. Colour of the interaction products of atomoxetine and its metabolites with the chromogenic reagents

<table>
<thead>
<tr>
<th>No.</th>
<th>Reagent</th>
<th>Atomoxetine (sensitivity, µg in spot) [13]</th>
<th>Hydroxy-atomoxetine</th>
<th>Dihydroxy-atomoxetine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>UV light 254 nm/365 nm</td>
<td>fluorescence purple (0.3)/light yellow (0.5)</td>
<td>fluorescence purple/light yellow</td>
<td>fluorescence pink/light yellow</td>
</tr>
<tr>
<td>2</td>
<td>Dragendorff–Munier</td>
<td>orange (0.5)</td>
<td>orange</td>
<td>orange</td>
</tr>
<tr>
<td>3</td>
<td>Ninhydrin solution</td>
<td>pink-violet (2.0)</td>
<td>pink-violet</td>
<td>pink-violet</td>
</tr>
<tr>
<td>4</td>
<td>Mandelin</td>
<td>light-pink (5.0)</td>
<td>yellow</td>
<td>yellow</td>
</tr>
<tr>
<td>5</td>
<td>Mandelin followed by formaldehyde vapour</td>
<td>yellow (5.0)</td>
<td>yellow</td>
<td>yellow</td>
</tr>
<tr>
<td>6</td>
<td>Marquis</td>
<td>pink → light – pink (2.0)</td>
<td>pink → yellow</td>
<td>red</td>
</tr>
<tr>
<td>7</td>
<td>Froehde</td>
<td>blue (1.0)</td>
<td>blue → brown</td>
<td>green</td>
</tr>
<tr>
<td>8</td>
<td>Liebermann</td>
<td>Light – pink (10.0)</td>
<td>orange → pink</td>
<td>red → yellow</td>
</tr>
<tr>
<td>9</td>
<td>Erdmann</td>
<td>Light – yellow (10.0)</td>
<td>yellow → orange</td>
<td>orange</td>
</tr>
<tr>
<td>10</td>
<td>Sulphuric acid concentrated</td>
<td>pink → light – yellow (10.0)</td>
<td>pink</td>
<td>red</td>
</tr>
</tbody>
</table>
Table 2. Colour of the interaction products of atomoxetine and its metabolites with the range of chromogenic reagents presented in Table 2.

Discussion
Sample preparation conditions were optimized on the basis of early obtained results of extraction yield of atomoxetine from aqueous solutions with organic solvents depending on pH and nature of salting-out agent [14]. The pre-treatment of the biological fluid using dilute acid hydrolysis was carried out because the drug is excreted mostly urinary as the major oxidative metabolite of 4-hydroxyatomoxetine-O-glucuronide (80% of the administered dose) [2,3,12]. Urine samples were collected according to the literature half-life values of atomoxetine [3].

The obtained results of the identification of atomoxetine metabolites in the studied urine samples are consistent with the literature data: 4-hydroxyatomoxetine was found as the major atomoxetine oxidation product in the urine of persons with both an extensive and poor type of metabolism [15,16] and dihydroxyatomoxetine, namely, 2-hydroxymethyl-4-hydroxyatomoxetine was found for poor metabolizers [16].

The chromatographic mobility of atomoxetine and its metabolites was studied in 18 mobile phases including those proposed by TIAFT (No. 1–9) for general drug screening, and those widely used in forensic toxicological studies (No. 10–18) [3]. According to the TIAFT recommendations, using several chromatographic systems, a minimum of three, preferably with the low correlation of \( R_f \) values, significantly increases the reliability of substance identification by TLC [3]. As can be seen from Table 1, atomoxetine and its metabolites had low \( R_f \) values in mobile phases No. 1–9, only the phase of ethyl acetate–methanol–25% ammonia (85:10:5) (No. 4) allowed to achieve a satisfactory separation of the native drug and metabolites. The chloroform–acetone–25% ammonia solution (25:5:0.3) mobile phase (No. 15) showed higher values of the chromatographic mobility of atomoxetine and its metabolites and a satisfactory separation. So, it was chosen for the TLC-purification in the current study. The mobile phase of chloroform–dioxane–acetone–25% ammonia solution (47.5:45.5:2.5) (No. 11) also can be recommended for TLC study of the biological extracts containing atomoxetine and its metabolites as a low correlated one with the mobile phases No. 4 and No. 5.

The colors selective with respect to biogenic matrix components were obtained with ninhydrin, Froehde, Liebermann, Erdman’s, Marquies reagents, and concentrated sulphuric acid (Table 2). The sensitivity of the color reactions for the atomoxetine standard methanol solution was in the range of 0.5–10.0 μg in the spot [13]. This work is a contribution to the toxicological screening of atomoxetine using TLC.

Obtained data can be used in the practice of forensic and clinical toxicology.

Conclusions
1. Atomoxetine and its biotransformation products were detected in the urine under TLC screening conditions and identified using mass spectrometry method.
2. Chromatographic mobility of the native compound, hydroxyatomoxetine and dihydroxyatomoxetine in the TLC screening systems as well as the results of their visualization using chromogenic reagents applied for toxicological drug screening in the systematic toxicological analysis have been determined.

Prospects for further research. The developed conditions for detection of atomoxetine and its metabolites in the urine under the conditions of TLC screening will be used for further creation of toxicological examination algorithm of biological samples for the presence of atomoxetine in the cases of lethal intoxications by antidepressants.

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