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Simple validated LC–MS/MS method for the determination of atropine and scopolamine in plasma for clinical and forensic toxicological purposes

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ABSTRACT

A liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the determination of atropine and scopolamine in 100 μL human plasma was developed and validated. Sample pretreatment consisted of protein precipitation with acetonitrile followed by a concentration step. Analytes and levobupivacaine (internal standard) were separated on a Zorbax XDB-CN column (75 mm × 4.6 mm i.d., 3.5 μm) with gradient elution (purified water, acetonitrile, formic acid). The triple quadrupole MS was operated in ESI positive mode. Matrix effect was estimated for deproteinised plasma samples. Selected reaction monitoring (SRM) was used for quantification in the range of 0.10–50.00 ng/mL. Interday precision for both tropanes and intraday precision for atropine was <10%, intraday precision for scopolamine was <14% and <18% at lower limit of quantification (LLOQ). Mean interday and intraday accuracies for atropine were within ±7% and for scopolamine within ±11%. The method can be used for determination of therapeutic and toxic levels of both compounds and has been successfully applied to a study of pharmacodynamic and pharmacokinetic properties of tropanes, where plasma samples of volunteers were collected at fixed time intervals after ingestion of a buckwheat meal, spiked with five low doses of tropanes.

1. Introduction

Atropine, defined as a racemic mixture of (*S*)-hyoscyamine and (*R*)-hyoscyamine, and scopolamine have an important role among natural alkaloids used in medicine and are also dealt with in toxicological studies. During the last fifty years diverse techniques have been used for their determination in various matrices. The majority of publications deal with plant tissues and biosynthesis, pharmaceutical drug development and subsequent quality control. Unfortunately, both compounds are also intentionally or unintentionally abused (dilution of illegal drugs, control of victims), consumed with contaminated food products, or parts of plants are accidentally ingested (mostly by children). They can also be minor constituents of otherwise harmless herb preparations, and they can be also absorbed through the skin while handling plants [1–13]. Atropine and scopolamine are health and life threatening alkaloids and thus the possibility of their identification and quantitation is important in clinical and forensic toxicology. Such analyses represent quite a challenge for the analyst due to low concentrations in biological samples as well as because of compounds' pronounced polar properties. It is evident from the reviews covering analysis of tropane alkaloids in biological matrices [4–6] that chromatographic methods are predominant among those used for their determination. The sample preparation is dependent on physico-chemical properties and biological matrix of interest. Atropine is a basic compound with pK_a 9.9 and K_{ow} of 1.8, while scopolamine is slightly basic with pK_a 7.6 and octanol/buffer pH 7.4 partition coefficient of 1.2 [14]. Both are soluble in water, especially scopolamine, their salts show excellent water solubility, and their free base form is more soluble in apolar organic solvents [4,6,14]. Considering their properties, different procedures are used for the preparation of liquid biological samples: dilution/filtration [4,10,15,16], protein precipitation [4,17–19], liquid–liquid extraction (LLE) [4,20–24] and solid-phase extraction (SPE) [4,18,25–33]. While the simple

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methods usually require small volume of sample from 50 to 200 μL, one to two mL are used for LLE and SPE.

Chromatographic procedures are used for clinical and forensic identification of atropine and scopolamine [34] or for metabolism studies [18,35,36]. Different quantitative chromatographic methods for their determination in blood (serum, plasma) and urine have been developed [15–17,19,20,23,24,26–29,32,33,37–43], most of them are using mass-spectrometric detection. GC–MS methods have not found wide spread use as trimethylsilyl [28,32,41] or pentafluoropropyl [39] derivatives of thermally labile atropine and scopolamine should be prepared before chromatography to achieve successful mass spectrometric quantification based on electron (EI) [28,32,38,41] or chemical ionisation (CI) [39]. Lower limit of quantification (LLOQ) is reported from 10 to 100 ng/mL [28,38,39], and as low as 0.2 ng/mL for scopolamine when using tandem instrument [32]. Better sensitivity and selectivity have been achieved by using LC–MS [27,33] with atmospheric pressure chemical ionisation (APCI) or LC–MS/MS instruments [15,17,19,20,23,24,29,33,37,40,42,43] with electrospray ionisation (ESI). Here reported LLOQs are ranging from 0.02 to 10 ng/mL, depending on type of sample, preparation procedure and volume of injection. As far as known, after simple deproteinisation the lowest LLOQ of 0.05 ng/mL was reported for rabbit plasma [17] and 0.25 ng/mL for human plasma [19]. To the best of our knowledge there was no previously published method for the determination of scopolamine after simple deproteinisation of human plasma (blood).

The presented study was initiated as a response to a mass food poisoning accident with the symptoms of a classic anticholinergic syndrome which occurred in Slovenia in September 2003. All intoxicated persons had consumed buckwheat (*Fagopyrum* sp., Polygonaceae) flour food products within the last few hours. After GC–MS analysis of samples of disputable buckwheat flour, the presence of tropane alkaloids atropine and scopolamine was confirmed. Further macroscopic examination of whole buckwheat grain revealed the presence of thorn-apple seeds (*Datura stramonium* L.). The National Institute of Public Health established an ad hoc self-reporting scheme and identified 73 cases with symptoms of tropane alkaloid toxicity. Risk characterization was carried out [1] and provisional maximum residue levels (MRLs) were proposed for atropine and scopolamine mixture in buckwheat food products as 4.0 μg/kg and 2.0 μg/kg, respectively, based on estimation of Acute Reference Doses (ARfD) from the lowest recommended paediatric therapeutic doses, as there were no available No Observed Adverse Effect Levels (NOAEL) for atropine and scopolamine at that time. A study with volunteers was realised to assess the possibility of less severe intoxication with mild transient and not recognized symptoms. The ingested atropine/scopolamine doses were low, from 0.12/0.10 to 12.10/9.50 μg/kg body mass (bm) [44,45]. As it was expected that the observed levels of alkaloids in blood would also be low, there was a need to develop and validate a simple and reliable chromatographic analytical method, capable to measure as low concentrations of target alkaloids as possible, with a high throughput.

Table 1
HPLC gradient profile.

Time (min)	Solvent A (%)	Solvent B (%)	Flow (mL/min)
0.00	90.0	10.0	0.500
1.00	90.0	10.0	0.500
2.00	50.0	50.0	0.500
4.00	50.0	50.0	0.500
4.20	58.0	42.0	0.700
5.00	90.0	10.0	0.700
6.90	90.0	10.0	0.700
7.00	90.0	10.0	0.500

2. Experimental

2.1. Chemicals and reagents

HPLC grade acetonitrile Chromasolv® gradient grade, for HPLC (>99.9%) was purchased from Sigma–Aldrich (Steinheim, Germany), formic acid (98–100%) from Kemika (Zagreb, Croatia) and pure water (18 MΩ) was prepared by using a system Sartorius Stedim Biotech, ARIUM pro UV/DI.

Scopolamine hydrochloride (>99%) and atropine base (USP testing specifications) were purchased from Sigma–Aldrich, reference standard of levobupivacaine hydrochloride (99.8%), kindly donated by ABBOTT (North Chicago, USA), was used as internal standard (IS).

2.2. LC–MS/MS quantitative method

2.2.1. Instrumentation

For chromatographic separation an AT 1100 Series HPLC system (Agilent Technologies, Waldbronn), consisting of a degasser (G13791), a binary pump (G1312A), a thermostatic column department (G1316A) and autosampler (G1313A) was used. The compounds were detected by Micromass Quattro Micro™ API triple quadrupole (Waters, UK) after positive ionisation with electrospray.

2.2.2. Chromatographic conditions

A Zorbax XDB-CN column (75 mm × 4.6 mm i.d., 3.5 μm) protected by 0.5 μm frit (Agilent) was used. The temperature of column department was set to 30 °C. The conditions of gradient elution are presented in Table 1. Solvent A was a mixture purified water–formic acid–acetonitrile (89.9:0.1:10, v/v/v). Acetonitrile solution containing 0.1% (v/v) of formic acid was used as a solvent B. The injection volume was 20 μL.

2.2.3. Optimisation of MS/MS conditions

Initial mass spectrometric conditions for both tropanes and the internal standard were acquired by direct infusion of the solution of each compound, at concentration 200 ng/mL in water–acetonitrile (50:50, v/v) mixture, into the source and were used for the development of the method. Afterwards they were re-optimised by using the mobile phase as a solvent, with the same composition as at the elution time for particular compound. The following

Table 2
SRM transitions and optimised MS conditions for the measurement of atropine, scopolamine and internal standard levobupivacaine (quantitation trace in bold).

Compound	Precursor ion (*m/z*)	Product ion (*m/z*)	Cone voltage (V)	Collision energy (eV)	Time window (min)
Scopolamine	304.3	**138.1**	26	20	0.0–3.0
		156.1	26	20	
Atropine	290.2	**124.2**	38	28	3.0–4.5
		93.1	38	28	
Levobupivacaine (IS)	289.2	**140.1**	28	20	4.5–7.0
		98.1	28	20	