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A simple and sensitive GC/MS method for the determination of atropine during therapy of anticholinesterase poisoning in serum samples

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Atropine is used in the daily clinical practice for the treatment of poisonings caused by anticholinesterase pesticides, due to its sympathomimetic action. The investigation of the cause of the adverse effects that appear during atropine administration showed the necessity for the development and validation of a simple, rapid, sensitive, and specific method for the determination of atropine levels in serum samples. The developed method includes liquid-liquid extraction with ethyl acetate: dichloromethane (3:1, *v/v*) and derivatization using N,O-bis(trimethylsilyl)-trifluoracetamide (BSTFA) with 1% trimethylchlorsilane (TMCS) in acetonitrile environment. The method was found to be selective, linear, accurate, and precise according to international guidelines. The recovery was higher than 85.9%, the limit of quantification was 2.00 ng/ml, and the calibration curve was linear within the range of 2.00–500 ng/ml ($R^2 \geq 0.992$). Accuracy and precision were also calculated and were found to be less than 5.2 and 8.7%, respectively. The developed method was applied in a real case of accidental poisoning with chlorpyrifos in order to determine the atropine serum levels of the patient. The proposed method proved to be useful for the investigation of adverse effects that appear during atropine treatment of patients poisoned by anticholinesterase pesticides and it can also be used for the investigation of poisonings caused after consumption of atropine containing plants. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: atropine; anticholinesterase poisoning; GC/MS

Introduction

Anticholinesterase pesticides includes organophosphates and carbamates and are used mainly in agriculture and gardening.[1,2] The number of poisonings with anticholinesterase pesticides is estimated worldwide at some millions per year, causing thousands of deaths.[1,3] In developing countries, the most common cause of pesticide poisonings is due to occupational exposure, while high mortality rates are usually the result of suicide attempts.[3] Accidents can also happen due to misuse of these pesticides.

Anticholinesterase poisoning is a serious condition; respiratory failure is the major reason for mortality. Anticholinesterase pesticides share a common mode of action, the inhibition of acetylcholinesterase (AChE) which is only reversible in the case of carbamates.[1,4,5] The toxic effects are due to the hyperactivity of the cholinergic system, as acetylcholine accumulates at central and peripheral sites.[6] Exposure to anticholinesterase pesticides causes signs and symptoms of an acute cholinergic syndrome due to hyperstimulation of parasympathetic muscarinic (bradycardia, bronchoconstriction, bronchorrhoea, hypotension, abdominal cramps, and other gastrointestinal symptoms, miosis, sweating, hypersalivation) and nicotinic receptors (hypertension, tachycardia, fibrillation, fasciculation, necrosis of striated muscles), as well as of central muscarinic and nicotinic receptors (tremor, movement in coordination, seizures, central depression of respiration, coma, death).[1,3]

Anticholinesterase poisoning needs a rapid diagnosis and appropriate treatment, artificial respiration, and careful monitoring. Early recognition of pesticide may decrease the mortality rate among these patients. Treatment for acute organophosphate poisoning involves administration of atropine, pralidoxime (counter AChE inhibition), and diazepam (for CNS protection).[3] On the other hand, in case of carbamate poisoning, the use of oximes is controversial and considered contraindicated,[7] so the recommended treatment is the combination of atropine and diazepam.[1]

Atropine is an anticholinergic drug that acts pharmacologically via blocking acetylcholine receptors of the muscarine subtype.[3] Atropine is the initial drug of choice in acute organophosphate poisoning and the only antidotal therapy in carbamate poisoning, due to its efficacy for their parasympathomimetic effects.[1] Atropine, as an antimuscarinic drug, blocks the muscarinic receptors, but it is not capable of countering effects provoked by nicotinic hyperstimulation; so atropine can relieve only the respective symptoms in an anticholinesterase poisoning. The blockage of these receptors causes symptoms like tachycardia, dilated pupils, decreased gastrointestinal motility, dry hot skin, and dry mouth due to reduced sweat and saliva production. Apart from these peripheral effects, atropine also affects the central nervous system and causes agitation, disorientation, and hallucinations.[8]

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As atropine use may cause side effects because the upper atropine therapeutic serum levels (2–25 ng/ml) are close to toxic levels (30–100 ng/ml),[9] it should be closely monitored during treatment.[10] The necessity for the investigation of the cause of the adverse effects that appear during atropine administration led to the development of analytical methods for atropine determination in biological fluids. Atropine has been determined in blood, plasma, or serum samples by gas[11–13] or liquid[6,14–17] chromatography. The drawback of gas chromatography (GC) methods for the determination of atropine is its heat instability,[12] so a derivatization step is necessary in order to convert atropine to a less unstable derivative. The aim of our study was the development, optimization, and validation of a simple, rapid, sensitive, and specific method for the determination of atropine, commonly used during therapy of anticholinesterase poisonings, in serum samples. The developed method proved useful in the management of the adverse effects that appear during atropine administration due to its toxicity. It can also be used for the investigation of clinical or forensic cases related to the consumption of atropine-containing plants.

Experimental

Chemicals and solutions

Reference standard stock solutions of atropine and codeine-d3 (internal standard) at a concentration of 1.0 mg/ml (>99.9 % pure) in methanol were purchased from LGC Promochem (Molsheim, France). Analytical or high performance liquid chromatography (HPLC) grade solvents (hexane, ethyl acetate, dichloromethane, acetonitrile, isopropanol) were purchased from Merck (Darmstadt, Germany). Analytical reagents were purchased as follows: pentafluoropropionic anhydride (PFPA) 99%, heptafluorobutyric anhydride (HFBA) 99%, N-Methyl-N-(Tert-Butyldimethylsilyl)trifluoroacetamide (MTBSTFA), and N,O-bis(trimethylsilyl)-trifluoracetamide (BSTFA) with 1% trimethylchlorsilane (TMCS) from Fluka (Steinheim, Germany), acetic anhydride 97% from Mallinckrodt (St Louis, MO, USA) and pyridine 99.5% from Ferak (Berlin, Germany). Human drug-free serum was obtained after informed consent, from healthy donors, was pooled and before its use was screened by GC/MS for the presence of atropine or other drugs.

The stock solutions of atropine and codeine-d3 were diluted with methanol to give a series of working standard solutions. The working solutions for the preparation of calibrators and quality control (QC) samples contained atropine at a concentration of 0.10, 0.25, 0.30, 0.50, 2.00, 5.00, 10.0, 20.0, and 25.0 μg/ml. The working internal standard solution contained codeine-d3 at a concentration of 5.00 μg/ml. The stock solutions were kept according to the instructions of their commercial certificates and all working solutions were kept at 4 °C. An aliquot of 20 μl from the appropriate working standard solution of atropine was added to 980 μl aliquot of human drug free serum. Calibrators were freshly prepared daily at the following final concentrations of atropine: 2.00, 5.00, 10.0, 40.0, 100, 200, and 500 ng/ml. QC samples were also prepared daily with human serum at three levels: 6.00, 100, and 400 ng/ml.

GC/MS analysis and apparatus

In this study, an Agilent GC/MS model 6890 N/5975, equipped with a DB-5MS column (30 m x 0.25 mm i.d. x 0.25 μm film thickness) using helium as carrier gas at a flow rate of 1.0 ml/min was used. A volume of 1 μl was injected in the splitless mode using an Agilent 7683B Series autosampler system at 280 °C. The gas chromatograph oven temperature was raised from initial temperature 100 °C (1 min hold time) to 300 °C at a rate of 20 °C/min and the final temperature was held for 4 min. The interface and ion source temperatures were 300 and 200 °C, respectively. The GC run lasted 15 min and the retention time of silylated atropine was 8.51 min. The mass spectrometer was operated in electron ionization (70 eV) and selective ion monitoring (SIM) mode. The mass fragments used for the identification of analytes were: **124**, 140, and 361 for atropine, and **374**, 346, and 359 for the codeine-d3 (internal standard), whereas the bold marked ions were used for the quantification.

A 691 digital pH-meter (Metrohm, Zofingen, Switzerland) with a glass electrode was used for the adjustment of pH of the samples. An MT 19 vortex (Chiltren, London, UK), a Reacti-Vap Model 18780 (Pierce, Rochford, IL, USA) evaporator using nitrogen, and a cooling centrifuge (Sigma 4 K10, Germany) were also used.

Sample preparation

An aliquot of 20 μl from the working internal standard solution (codeine-d3: 5.00 μg/ml) were added to all samples (1.0 ml). The pH of the samples was adjusted to 9.00 with the addition of 0.1 M KOH. Then, the samples were liquid-liquid extracted with 3 ml of mixture of ethyl acetate: dichloromethane (3:1, *v/v*) and they were centrifuged at 2000 rpm for 10 min. The supernatant organic phase was collected and evaporated to dryness under a gentle stream of N_2 at 40 °C. The analytes were derivatized by adding to the residue 50 μl of acetonitrile and 50 μl of BSTFA with 1% TMCS subsequently, vortex mixing and heating at 70 °C for 30 min. After cooling, the samples were injected (1 μl) into the GC/MS system (splitless mode).

Results and discussion

Method development and optimization

The proposed method includes the combination of a liquid-liquid extraction procedure with derivatization using BSTFA with 1% TMCS in acetonitrile environment followed by GC-MS analysis, for the determination of atropine in serum samples. Atropine is thermal sensitive and during its GC analysis there was a problem of atropine degradation to two artifacts (atropine -CH_2O and atropine -H_2O), due to its heat instability in the injector. The mass spectra of atropine and its two artifacts are shown in Figure 1. During the development of the method, different temperatures of the injector (200, 220, 240, 260, and 280 °C) were tested but the problem of atropine's heat instability remained. Therefore, the derivatization was necessary in order to convert atropine to a more heat-stable derivative, and different derivatization reagents (HFBA, PFPA, MTBSTFA, BSTFA with 1% TMCS, and acetic anhydride in pyridine) were tried. Poor derivative stability was observed when MTBSTFA was used, as peaks of the two artifacts (atropine -CH_2O and atropine -H_2O) were also observed, probably due to heat instability of the derivative. When PFPA, HFBA, or acetic anhydride in pyridine was used, there was only one more artifact peak of atropine -H_2O. Only BSTFA with 1% TMCS gave no artifacts. The peak areas of atropine and its artifacts with and without derivatization with the different reagents tried, as well as the % ratios of artifact to atropine are presented in Table 1. An overall review of the results

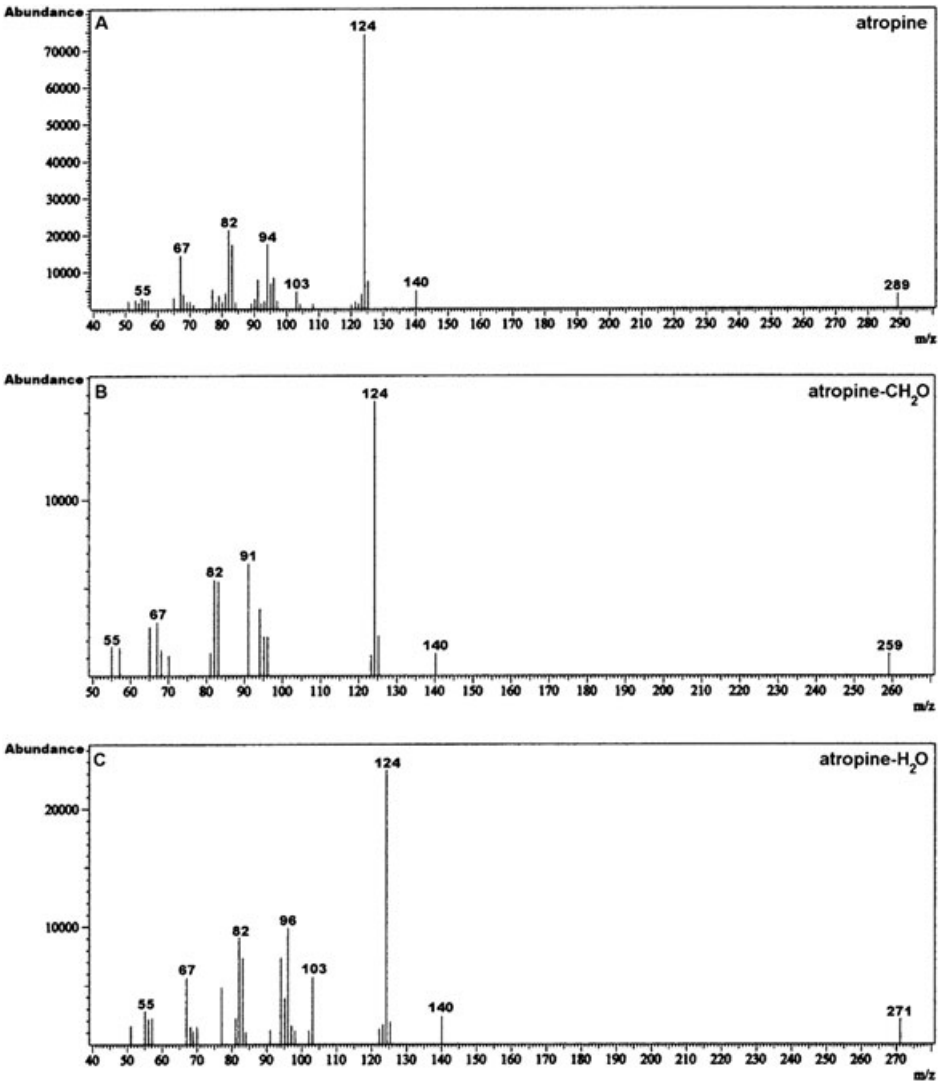

Figure 1. Mass spectrum (electron ionization/scan mode from 50 to 500 amu) of (A) atropine, (B) atropine -CH_2O and (C) atropine -H_2O.

Table 1. Mean peak area and % ratio of artifact to atropine using different derivatization reagents.

Derivatization reagent	Mean peak area (n = 3)			% Ratio of artifact to atropine	
	Atropine	Atropine -CH_2O	Atropine -H_2O	Atropine -CH_2O	Atropine -H_2O
Underivatized	431432	53042	77193	12.3	17.9
PFPA	556181	6071	448054	1.09	80.6
HFBA	663092	6110	562111	0.92	84.8
Acetic anhydride in pyridine	1125421	5914	232014	0.53	20.6
MTBSTFA	985023	337012	43431	34.2	4.41
BSTFA with 1% TMCS	2263007	4702	9008	0.21	0.40

indicated that BSTFA with 1% TMCS should be the first choice as a derivatizing reagent for atropine. The conditions of this derivatization were optimized in order to obtain the highest sensitivity. Different values of temperature (50, **70**, and 90 °C) and duration of the reaction (15, **30**, 45, and 60 min) were tried. The optimal conditions are in bold. The mass spectrum of silylated atropine is presented in Figure 2.

Chromatographic conditions like injector temperature (240, 260, **280**, and 300 °C), ion source temperature (180, **200**, 220, and 240 °C), interface temperature (280, 290, **300**, and 310 °C), initial (60, 80, **100**, and 120 °C) and final (280, 290, **300**, and 310 °C) column temperature, as well as the column temperature rate (10, **20**, 30, and 40 °C/min), the initial temperature hold time (0.0, 0.5, **1.0**, and 1.5 min), and the carrier gas flow rate (0.6, 0.8, **1.0**, and 1.2 ml/min) were also optimized. The optimal conditions are in bold.

Optimization of the extraction procedure was also performed and the liquid-liquid extraction was chosen as a more rapid technique

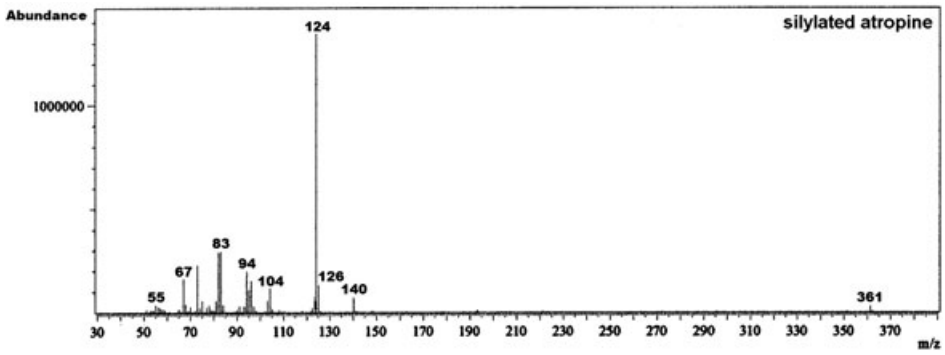

Figure 2. Mass spectrum (electron ionization/scan mode from 50 to 500 amu) of silylated atropine.

than solid-phase extraction. Different organic solvents (ethyl acetate, dichloromethane, hexane) or mixtures of solvents (ethyl acetate: dichloromethane (3:1, *v/v*), dichloromethane: isopropanol (9:1, *v/v*), hexane: ethyl acetate (3:1, *v/v*)) were tested during the development of the sample preparation. The highest value of recovery (≥85.9%) without base line interferences was achieved when a mixture of ethyl acetate: dichlomethane (3:1, *v/v*) was used. This liquid-liquid extraction system showed clear advantages over the others tested as it gives a very good clean-up of the serum samples, as well as high and reproducible absolute recovery.

The developed method is simple, as it uses solvents that are common in every laboratory, and rapid as it includes a single step of liquid-liquid extraction leading to clean extracts without the need of solid-phase equipment. In our study, we compared more derivatization techniques than previously reported[11,12] and we present more data about the heat stability of the atropine derivatives. When this method is compared with previously published methods,[12–16] it shows lower limit of quantification (LOQ) and wider linear dynamic range, that includes lower therapeutic, toxic, and lethal serum levels of atropine.

Method validation

The validation of the analytical method was based on FDA and ICH guidelines[18,19] for bioanalytical method validation and the evaluated parameters were: selectivity, specificity, sensitivity, linearity, absolute recovery, accuracy, and precision.

The lack of the interference from endogenous serum compounds (selectivity) at the respective retention times of atropine and codeine-d3 was checked by analyzing six different blank human serum samples. Specificity refers to the ability of analytical method to separate and quantify the analyte in the presence of other components. Specificity was examined by applying the extraction procedure to spiked serum samples (n = 6) with 40 substances (aldicarb, azinphos methyl, carbofuran, chlorpyrifos, dialifos, diazinon, malathion, methamidophos, methidathion, methomyl, mevinphos, terbufos, alprazolam, amitriptyline, atenolol, biperiden, bromazepam, carbamazepine, citalopram, clomipramine, clozapine, diazepam, diltiazem, 7-aminoflunitrazepam, haloperidol, levomepromazine, lidocaine, metoprolol, mirtazapine, nordiazepam, olanzapine, paracetamol, paroxetine, phenobarbital, phenytoin, sertraline, thioridazine, valproic acid, venlafaxine, and zolpidem) at a concentration of 10.0 μg/ml. There were no interfering peaks present in drug free (n = 6) and spiked (n = 6) serum samples at the retention times of either the analyte or the internal standard.

The LOQ was experimentally chosen as the atropine concentration in serum samples yielding signal-to-noise ratio of at least 10:1, with correct relative ions intensities and a retention time within ± 0.2 min of the average calibrator retention time. The LOQ concentration of atropine was found to be 2.00 ng/ml. A representative SIM chromatogram of spiked serum sample at atropine LOQ

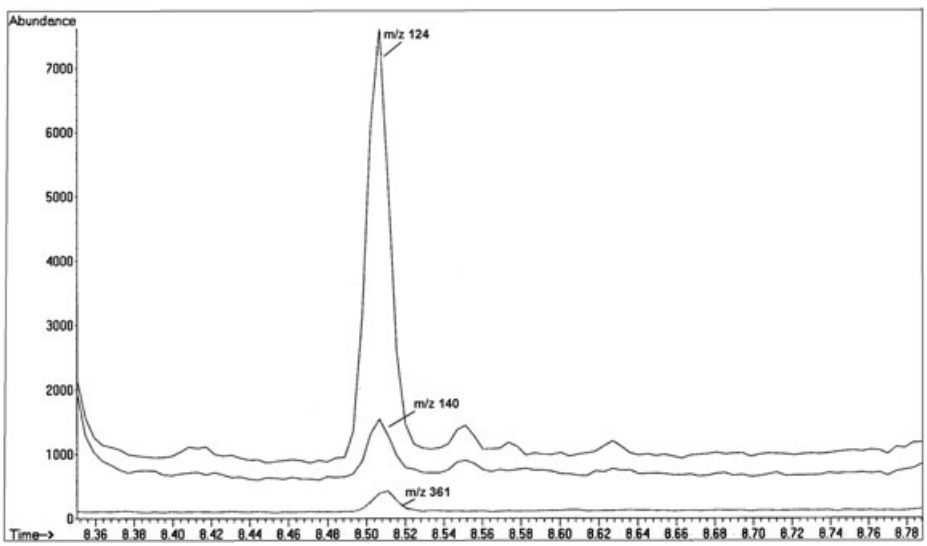

Figure 3. A representative SIM chromatogram of spiked serum sample at LOQ atropine concentration (2.00 ng/ml).

Table 2. Accuracy and precision of the developed method for the determination of atropine in serum samples at three QC levels

Atropine concentration added (ng/ml)	Intra-day (n = 6)			Inter-day (n = 36)		
	Concentration found (ng/ml)	% Er	% RSD	Concentration found (ng/ml)	% Er	% RSD
6.00	6.25 ± 0.41	4.2	6.6	6.31 ± 0.55	5.2	8.7
100.0	96.0 ± 4.2	−4.0	4.4	95.5 ± 3.8	−4.5	4.0
400	396 ± 17	−0.8	4.3	404 ± 14	1.0	3.5

concentration is shown in Figure 3. During linearity study, calibration samples were prepared and analyzed in triplicate in six runs, and the linear dynamic range was 2.00–500 ng/ml. Calibration curve was best fitted by least square linear regression using weighting factor $1/x^2$ (x = concentration). R^2 exceeded 0.992 and the % RSD of slopes during six consecutive days was found to be 4.2%.

Absolute recovery at QC levels was assessed by running six replicates at each concentration. The three QC levels were chosen to cover the range of calibration curve, and the serum concentrations of atropine were 6.00, 100, and 400 ng/ml. The QC samples were prepared, in the same way, with calibrators. Absolute recovery of the method was calculated as the percentage of the response of the analyte in the sample compared to that of a standard solution containing the analyte at the same concentration. The following equation was used:

$$\%\text{AbsoluteRecovery} = (\text{peakareaofspikedserumsample}) \times 100/(\text{meanpeakareaof6runsofstandardsolution}) \quad (1)$$

The absolute recovery (calculated at three QC levels) ranged from 85.9 to 96.2%. Precision was calculated as the % percentage of relative standard deviation (% RSD) within a single run (intra-day, n = 6) and between different runs (inter-day, n = 36) and the respective values of precision were less than 6.6 and 8.7%, respectively (Table 2). Accuracy was determined as the percentage of deviation between measured and nominal concentration. Intra- and inter-day accuracy was ranged from −4.0 to 4.2%, and from −4.5 to 5.2%, respectively (Table 2).

Method application

The developed method for the determination of atropine in serum samples was successfully applied to a clinical case of accidental poisoning with chlorpyrifos during atropine treatment. A 38-year-old male farmer, during a field chemical pest control started not to feel well; he showed symptoms of sedation, acute adnominal pain, and gradual loss of his consciousness. The patient was immediately admitted to hospital where it was found that he had depressed plasma pseudocholinesterase (butyrylcholinesterase) activity. Antidotal treatment (pralidoxime, atropine, and diazepam) was given. The daily intravenous administration of atropine was 4 mg (drop-wise). Serum samples were obtained from the patient 30 h and 35 h after the offset of the atropine administration. Atropine concentrations in the serum samples were found to be 32.5 and 26.8 ng/ml, respectively. The concentrations found were at the upper therapeutic levels.[9]

Conclusions

We present a simple, rapid, sensitive, and specific method for the determination of atropine in serum samples. The developed method is useful in clinical practice to monitor atropine levels during therapy of anticholinesterase poisonings for the investigation of possible adverse effects and the avoidance of atropinism. The proposed method can also be used for the investigation of clinical or forensic cases related to the consumption of atropine containing plants.

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