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Determination of atropine in biological specimens by high-performance liquid chromatography

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ABSTRACT

A sensitive and selective method for the determination of atropine in biological specimens has been developed. Samples alkalinized with sodium hydroxide were extracted with dichloromethane, and the organic phase was evaporated in a water-bath at 50°C for *ca.* 10 min. The residue was dissolved in the mobile phase and injected into a reversed-phase column (TSK gel ODS-120A). The retention time for atropine could be varied by changing either the acetonitrile–water ratio in the mobile phase or the pH of the mobile phase. Acetonitrile–water (2:8, v/v) containing 6 m*M* phosphoric acid was used as mobile phase. Samples of 200 μl or less were injected into the chromatograph and measured at 215 nm. The recoveries of atropine added to drug-free specimens were satisfactory with coefficients of variation of 4% or less. Ninety-two compounds tested did not interfere with the assay of atropine. The method has been applied for monitoring atropine concentrations in cases of organophosphate and drug poisoning.

INTRODUCTION

Atropine and related alkaloids have been used for the treatment of gastrointestinal diseases, cardiopathy, parkinsonism and so on. Atropine is a potent anticholinergic agent, and paralyses the parasympathetic nervous system by blocking the action on effector cells of the acetylcholine released at nerve ending. Atropine is an antidote to poisoning by cholinesterase inhibitors because of this action. It also stimulates the central nervous system. Since atropine is eliminated

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almost entirely by the kidneys, abnormalities of kidney function may lead to toxic reactions in patients receiving atropine [1].

Atropine has been analysed in biological specimens by bioassay [2,3], radioimmunoassay [4–6], thin-layer chromatography (TLC) [7], gas chromatography (GC) [8,9], and gas chromatography–mass spectrometry (GC–MS) [10,11]. Previous high-performance liquid chromatographic (HPLC) methods [12–16] are not very sensitive or not suitable for atropine analysis in biological specimens.

The aim of this study was to develop a sensitive and selective method for the determination of atropine in biological samples by extraction of atropine with dichloromethane [17–19] and HPLC analysis.

EXPERIMENTAL

Apparatus

The method was developed using a Waters liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.), equipped with a Model 6000A high-pressure pump, a U6K loop injector, a Model S-310A UV detector, a Chromatopac C-R2AX integrator (Shimadzu, Kyoto, Japan) and a column oven. The pH was measured on a Model HM-15A digital pH meter (TOA Electronics, Tokyo, Japan).

Reagents

HPLC-grade acetonitrile was from Wako (Osaka, Japan). All other reagents were of reagent grade. Aqueous stock standard solutions (1 mg/ml) of atropine sulphate (Wako) containing 10% urea were used to prepare all working standards.

Procedure

Sample preparation. A 400-μl volume of standard or sample was placed into a 10 × 100 mm tapered glass centrifuge tube. To the tube were added 200 μl of 0.4 *M* sodium hydroxide and 1.5 ml of dichloromethane. The mixture was vortex-mixed for 1 min, then centrifuged at 1400 *g* for 5 min. The bottom layer was transferred to another 10 × 100 mm tube. This tube was immersed in a water-bath at 50°C, and the dichloromethane was evaporated with an air stream. The residue was dissolved in 400 μl of the mobile phase, and 200 μl or less were injected into the chromatograph.

Liquid chromatography. A 250 mm × 4.6 mm I.D. column packed with TSK gel ODS-120A (particle size 5 μm) (Tosoh, Tokyo, Japan) was used for the determination of atropine. The mobile phase was acetonitrile–water (2:8, v/v) containing 6 m*M* phosphoric acid. The pH of the solution was 2.7. The flow-rate of the mobile phase was 1.0 ml/min at 50°C. The absorbance was measured at 215 nm. Serum fenitrothion and urinary *p*-nitro-*m*-cresol (the metabolite) were measured by our HPLC method [20]. Peak areas were calculated by an on-line computing integrator. Atropine was quantitated by use of the external standard method [21].

Clinical application

Case 1. A 56-year-old male ingested *ca.* 100 ml of an unknown liquid. He vomited a white fluid twice. He consulted our hospital *ca.* 1.5 h after ingestion, and had diarrhoea and mild abdominal pain. His pupils were isocoric with light miosis (diameter *ca.* 2.5 mm), and his conscious level was clear. Gastric lavage was performed, followed by forced diuresis by fluid therapy with Lactec® G and with Lasix® (furosemide). Subsequently, Camag® (magnesium oxide), Adsorbin® (natural aluminum silicate) and Magcorol® (magnesium citrate) were administered into the gastric tube. Hemodialysis and hemoperfusion were performed for 6 h. Abnormal values were obtained, showing leukocytosis (16 600/mm^3), hypercreatine phosphokinasemia (1247 I.U./l) and hypocholinesterasemia (0.02 $\triangle$pH). Metabolic acidosis was also found.

Case 2. A 31-year-old male was found unconscious in his hospital bed. He was in hyperthermia (37.5°C), his blood pressure was up (systolic 150 mmHg, diastolic 120 mmHg), and he had a rapid pulse (120 beats per min). His pupils were dilated (right diameter 6.0 mm, left diameter 6.5 mm), and he showed no light reflex. He had deep tendon reflex and Babinski's reflex. He had a dry mouth and urinary retention. Other laboratory tests did not show abnormal findings.

RESULTS AND DISCUSSION

Optimization of mobile phase

The retention time (t_R) for atropine could be varied by changing either the acetonitrile–water ratio (A/W) in the mobile phase or the pH of the mobile phase. When the A/W was 5:5, 4:6, 3:7 and 2:8, the t_R was 3.8, 3.9, 4.7 and 8.1 min, respectively, at a mobile phase pH of 2.7 and a column temperature of 50°C. When the A/W was 1:9, atropine was not detected. When the pH of the mobile phase of A/W 5:5 was 2.7, 3.1 and 3.2, the t_R was 3.8, 6.2 and 7.9 min, respectively. When the column temperature was 40 or 50°C, the t_R was 3.8 min in a mobile phase of A/W 5:5 with pH 2.7. The mobile phase of A/W 5:5 with pH 3.2 gave a similar chromatogram. For the determination of patients' specimens, we chose to use the HPLC conditions decribed in *Procedure*. Fig. 1. shows chromatograms of atropine.

Linearity and limit of detection

Calibration graphs from spiked samples were found to be linear over the concentration range 0–500 μg/ml for atropine ($r = 0.998$). The limit of detection at a signal-to-noise ratio of 5:1 was 8.5 ng/ml for atropine, when a 200-μl sample was injected into the column.

Repeatability and recovery

Coefficients of variation (C.V.) and the recoveries determined by assaying six samples of serum or urine containing different concentrations of atropine are

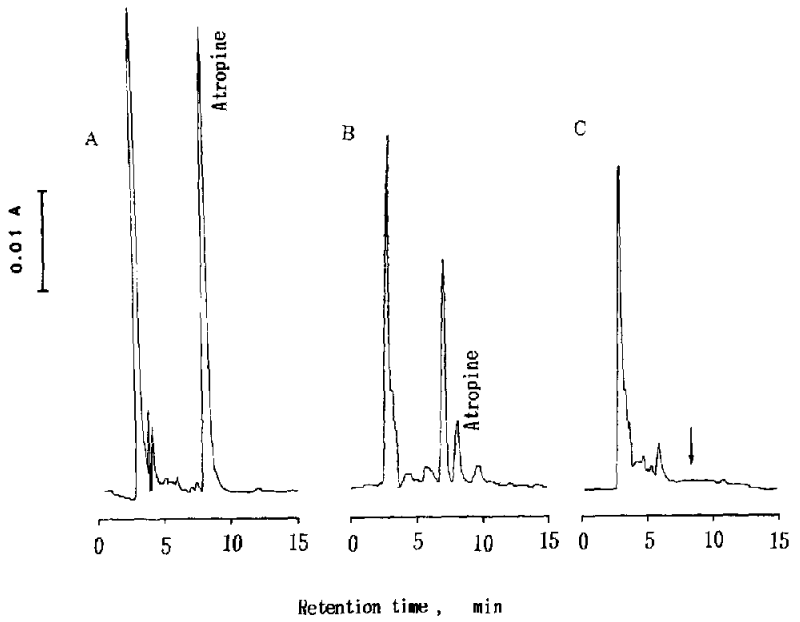

Fig. 1. Chromatograms of atropine (A) from a standard atropine solution (10 μg/ml), (B) from a patient's urine sample (concentration of atropine, 1.3 μg/ml) and (C) from a control urine sample.

presented in Table I. As shown, the C.V. were 1.1–3.4% in serum and 0.4–2.6% in urine.

Recoveries from serum to which 0.1–100 μg/ml atropine were added ranged from 98 to 103%. Recoveries from urine samples were 96–102%.

TABLE I

COEFFICIENTS OF VARIATION AND RECOVERIES IN THE ANALYSIS OF ATROPINE, CALCULATED FROM SIX PREPARATIONS WITHIN A RUN

Atropine added (μg/ml)	Serum			Urine		
	Found (μg/ml)	C.V. (%)	Recovery (%)	Found (μg/ml)	C.V. (%)	Recovery (%)
0.1	0.098	3.4	98	0.096	1.9	96
1	1.03	1.9	103	1.02	0.4	102
10	10.1	1.1	101	9.8	1.4	98
100	99.0	2.9	99	101.2	2.6	101

TABLE II

RETENTION TIMES OF COMPOUNDS TESTED FOR INTERFERENCE

HPLC conditions: TSK gel ODS-120A (250 mm × 4.6 mm I.D.); mobile phase, acetonitrile–water (2:8, pH 2.7); flow-rate, 1 ml/min; temperature. 50°C; UV detection wavelenght, 215 nm.

Compound[a]	Retention time (min)	Compound[a]	Retention time (min)
Cloxazolam	2.9	Primidone	9.1
Uric acid	3.0	Lidocaine	9.2
Urea	3.0	Dimethoate	13.2
Creatine	3.0	Salicylic acid	16.3
Creatinine	3.0	Acetylsalicylic acid	16.3
Lactic acid	3.3	Phenobarbital	16.4
Pralidoxime iodide	3.3	Disopyramide	16.8
Procainamide	4.0	Bromvalerylurea	17.3
Acephate	4.1	Chlorpheniramine	18.7
Sulpiride	4.4	Phenacetin	19.3
Theophylline	4.5	*m*-Cresol	20.7
Acetaminophen	4.7	*p*-Cresol	21.1
Methylephedrine	5.7	*o*-Cresol	22.3
Hipurric acid	6.3	*p*-Nitro-*m*-cresol	27.6
Caffeine	6.5	Dichlorvos	37.2
Methomyl	7.2	Furosemide	38.6
Tropic acid	7.9	Pentobarbital	41.3
Atropine	8.1	Phenytoin	45.6
Piperacillin	8.6	Nitrazepam	52.1
Sulthiam	8.7	Carbamazepine	56.3

[a] Following compounds did not interfere: amitriptyline, aprindine, biperiden, brotizolam, bromazepam, chlordiazepoxide, chlorpromazine, clonazepam, clotiazepam, diazepam, diltiazem, diphenhydramine, estazolam, etizolam, flunitrazepam, haloperidol, hydroxyzine, imipramine, isopropylantipyrine, levomepromazine, maprotiline, medazepam, nalidixic acid, nicardipine, promethazine, setiptiline, thiamylal, thiopental, thioridazine, triazolam, valproic acid, warfarin, baycarb, chlorobenzilate, chlorpyrifos, cyanophos, diazinon, ethylthiometon, fenitrothion, glufosinate, glyphosate, kelthane, malathion, parathion, trichlorfon, vamidothion, *o*-dichlorobenzene, *p*-dichlorobenzene, naphthalene, α-naphthol, β-naphthol, nicotine, toluene (53 compounds).

Selectivity

Fifty-seven drugs and 35 other substances, such as organophosphorus compounds, were tested for possible interference. The t_R values of the main compounds are shown in Table II. None of the compounds tested interfered with the assay of atropine.

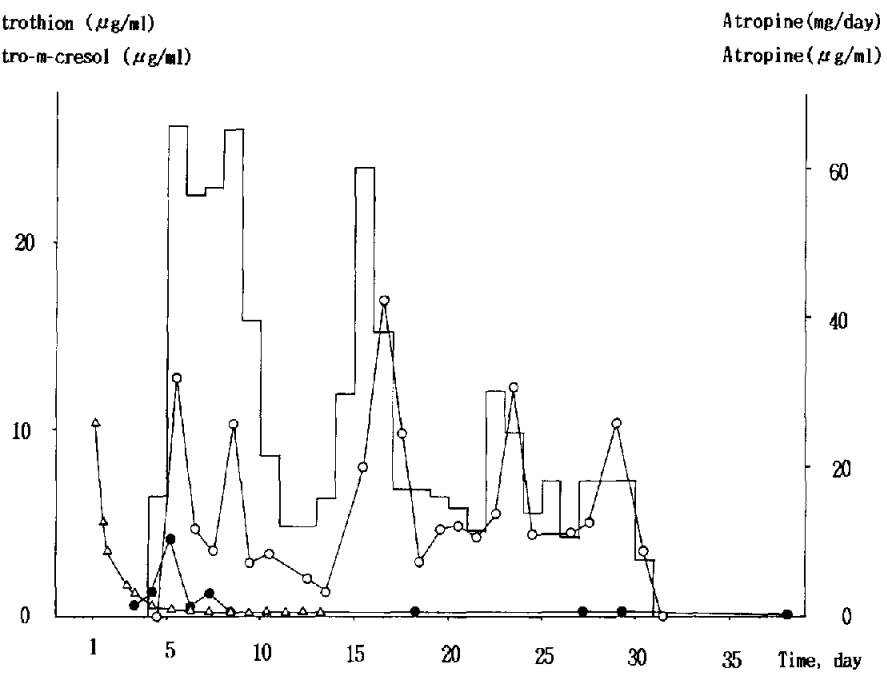

Fig. 2. Concentration change and atropine dose: (○) urinary atropine; (△) serum fenitrothion; (●) urinary *p*-nitro-*m*-cresol.

Clinical application

Case 1. We suspected organophosphate poisoning on the basis of clinical findings. It was revealed 3.5 h after hospitalization, from the results of analysis of serum and gastric juice by our 1C2MP HPLC[a] [22], that the substance that had caused the poisoning was fenitrothion (Sumithion®). Fig. 2 shows the concentration change of serum fenitrothion, urinary *p*-nitro-*m*-cresol, urinary atropine and the dose. Serum atropine concentrations were below the limit of detection (8.5 ng/ml) at the dose that we administered. This was considered to be due to a very rapid decline in the level of atropine [3,23,24]. Consequently, a great deal of atropine is required for the management of organophosphate poisoning [25,26]. We were able to detect atropine in the urine, and the urinary concentrations correlated well with doses of atropine (Fig. 2). The manifestations of organophosphate poisoning (miosis and salivation) began on the fourth hospital day. The occurrence of fenitrothion poisoning was identical with the detection of urinary *p*-nitro-*m*-cresol. The patient left an ICU room after 26 days.

[a] 1C2MP is an abbreviation of one column and two mobile phases. The column was a TSK gel ODS-120A, and the two mobile phases were acetonitrile–water (1:1, v/v) and acetonitrile–water (1:1, pH 2.7). We were able to identify many compounds by this method.

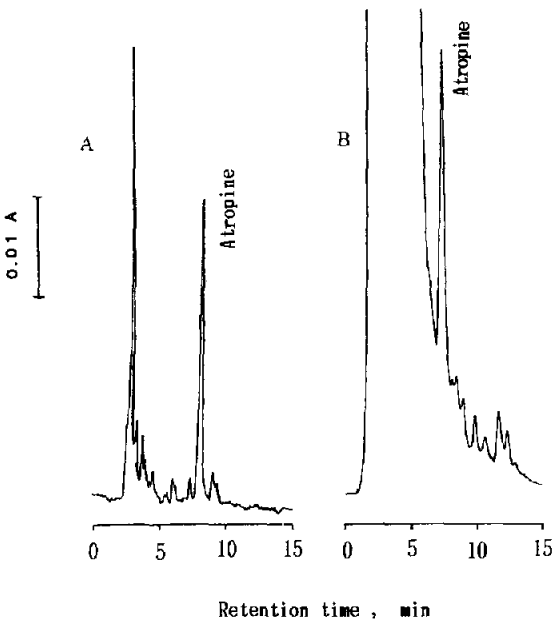

Fig. 3. Chromatograms obtained from a patient's urine sample: (A) by this method (concentration of atropine, 136 μg/ml); (B) by the acetonitrile deproteinization method (concentration of atropine, 139 μg/ml).

Case 2. We suspected atropine poisoning on the basis of the clinical findings, and identified and determined atropine in the urine by our 1C2MP HPLC method [22]. Fig. 3 shows the chromatograms: Fig. 3A shows a chromatogram obtained by this method (dichloromethane extraction) and Fig. 3B a chromatogram obtained by the acetonitile deproteinization method [27]. The urinary atropine concentration was found to be 136 μg/ml by the former and 139 μg/ml by the latter method.

We have thus developed a sensitive and selective method for the determination of atropine in biological samples by a combination of dichloromethane extraction of atropine and HPLC analysis. Samples alkalinized with sodium hydroxide were extracted with dichloromethane, the organic phase was evaporated in a water-bath at 50°C for *ca.* 10 min, and the residue dissolved in the mobile phase was injected into a reversed-phase column (TSK gel ODS-120A). When chloroform, 1,2-dichloroethane or dichloromethane–1,2-dichloroethane (1:1, v/v) was used as the extraction solvent, a longer evaporation time was required. We found that acetonitrile–water acidified with phosphoric acid was suitable as the mobile phase. The t_R for atropine could be varied by changing either the acetonitrile–water ratio in the mobile phase or the pH of the mobile phase. The precision was excellent and the recoveries were quantitative. Atropine does not have a characteristic UV absorption maximum. We injected 200-μl samples into the chromatograph and measured the absorption at 215 nm, because of the increase of

sensitivity to atropine. This method was very selective for atropine. Ninety-two compounds that we tested did not interfere with the atropine assay. Free tropic acid had a t_R value close to that of atropine, but it was not extracted with dichloromethane from alkalinized samples.

CONCLUSIONS

The method described is sensitive and selective for the determination of atropine in biological specimens. The method consists of two steps: (1) the extraction with dichloromethane of atropine from biological specimens and (2) HPLC analysis of atropine. The t_R for atropine could be varied by changing either the acetonitrile–water ratio in the mobile phase or the pH of the mobile phase. The TSK gel ODS-120A column gave satisfactory results with the mobile phase of acetonitrile–water (2:8, v/v) containing 6 m*M* phosphoric acid.

This method can be applied to the monitoring of atropine in biological samples.

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