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Short communication

Screening procedure for the analysis of distigmine bromide in serum by high-performance liquid chromatography–electrospray ionization mass spectrometry

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Abstract

A screening procedure was developed for the identification and quantification of distigmine bromide in serum samples by using liquid chromatography (LC)–electrospray ionization (ESI)-mass spectrometry (MS). In this method, distigmine bromide was analyzed in 0.5 mL serum by using pancuronium bromide as the internal standard, and gradient elution was performed using a reversed-phase column and a mixture of 10 mM-ammonium formate and methanol as the mobile phase. A highly sensitive assay could be performed with simple solid phase extraction using a cation exchange cartridge column by carrying out selected ion monitoring analysis in the positive ion detection mode. The procedure was validated in terms of linearity ($0.997 < r^2 < 0.999$ for concentrations ranging from 5 to 250 ng/mL), extraction recovery (83.0% to 89.3%, $n = 5$), and detection limit (S/N ratio, >3 at 2.5 ng/mL). The inter- and intra-day precisions (coefficient of variation; CV%) were <8.5% and <9.7%, respectively. The analytes were evaluated for stability and were found to be stable in serum for 1 week at 4 °C and 4 weeks at −30 °C, and successfully applied to in the analysis of two overdose cases. This method is sensitive and useful for the detection, quantification, and confirmation of distigmine bromide in serum.

Keywords: Distigmine bromide; Serum; Validation; LC–ESI-MS

1. Introduction

{*N*,*N*-Hexa-methylenedi-(1-methyl-3-(methyl-carbamoyl-oxy) pyridinium bromide); Ubretid®; Fig. 1} is a quaternary ammonium compound; it is used as an anticholinesterase drug for the treatment of multiple sclerosis and urinary retention and to reverse paralysis of the small intestine. The side effects of distigmine bromide overdose are nausea, vomiting, sweating, increased salivation, diarrhea, abdominal cramps, and blurred vision [1,2]. Recently, fatal intoxications following a distigmine bromide overdose have been reported [3]; however the related analytical data has not been published.

Although quaternary ammonium compounds are difficult to extract and analyze by conventional analytical methods, some screening methods have been developed for these compounds. Nishikawa et al. [4] described the screening of nine quaternary ammonium compounds in blood samples by using direct inlet electron impact ionization mass spectrometry. However, this method cannot be used to isolate quaternary ammonium compounds.

High-performance liquid chromatography (HPLC) is the most useful technique for the rapid and sensitive analysis of quaternary ammonium compounds. These compounds have been quantitated using either reversed-phase-HPLC [5] or LC–mass spectrometry (MS) [6–9]. In this study, an LC–electrospray ionization (ESI)-MS (LC–ESI-MS) method was developed to analyze distigmine bromide in human serum by using pancuronium bromide as the internal standard (IS). This method was validated and used for the analysis of distigmine bromide in samples from cases of clinical and forensic poisoning.

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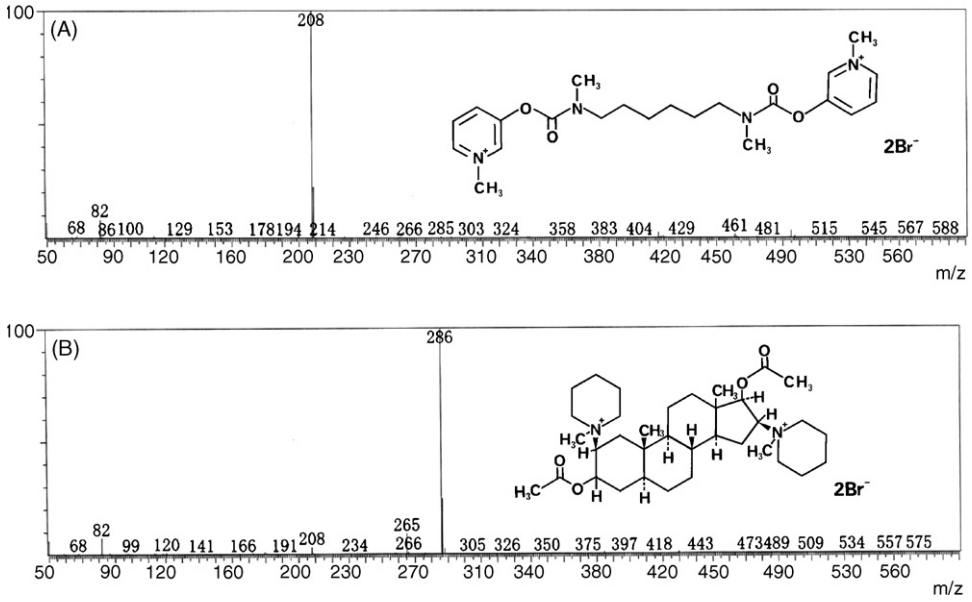

Fig. 1. Mass spectra and chemical structures of (A) distigmine bromide and (B) pancuronium bromide.

2. Experimental

2.1. Chemicals

Standard distigmine bromide was obtained from Torii Pharmaceutical Co. Ltd. (Tokyo, Japan), and pancuronium bromide was purchased from Sigma–Aldrich Inc. (MO, USA). The chemical structures of these compounds are show in Fig. 1. A stock solution of distigmine bromide (1 mg/mL) and working solutions (10 and 1 μg/mL) were prepared in methanol. All other chemicals were obtained from Merck (Darmstadt, Germany). Prior to use, all the solutions were filtered through a 0.45 μm membrane filter.

Solid phase extraction (SPE) cartridges along with 500 mg of Bond Elut® CBA from Varian (CA, USA) were used for extraction. Purified deionized water was prepared using the Milli-Q Academic Quantum Purification System (Millipore, MA, USA).

2.2. Calibration curves and quality control samples

Calibration curves were prepared by analysis of 0.5 mL blank serum to which known amount of distigmine bromide had been added (5, 10, 50, 100, and 250 ng/mL). The quality control (QC) samples were separately prepared in blank serum at concentrations of 7.5, 90, and 200 ng/mL. The spiked serum samples (standards and quality controls) were extracted following by SPE in the following manner.

2.3. Extraction procedure

Twenty-five microliters of pancuronium bromide (1 μg/mL) was added to 0.5 mL of the serum sample. Subsequently, 4 mL methanol was added to each sample. The protein fraction was separated by precipitation after vortexing and centrifugation. The samples were centrifuged at 3000 rpm for 10 min. The supernatant was transferred to another clean tube, and 4 mL deionised water was added to each tube. Subsequently, the samples were mixed well and then applied to the SPE cartridges.

The SPE cartridges were conditioned with 1 mL each of methanol and water. The samples were applied to the conditioned cartridges and were passed through the cartridges at a flow-rate of 5 mL/min using a vacuum pump. The cartridges were then washed with 1 mL distilled water and 1 mL 50% methanol and dried under vacuum (1 min). The residual compounds were eluted with 1.5 mL 50 mM hydrochloride–methanol. The solution thus obtained was evaporated to dryness under a stream of nitrogen at room temperature. The dry residue was dissolved in 0.1 mL 10 mM formic ammonium solution (pH 6.4) and filtered through a 0.45 μm membrane filter prior to injecting into the LC system.

2.4. LC–ESI-MS conditions

A Shimadzu 8000α mass spectrometer equipped with an ESI source (Shimazdu Corp., Kyoto, Japan) and an LC 10 HPLC system (Shimazdu, Tokyo, Japan) were used for LC–MS analysis. Isolation was performed using an XTerra MS C_{18} column (150 mm × 2.1 mm, 3.5 μm; Waters, Japan), and the column temperature was maintained at 50 °C. The mobile phase consisted of methanol and 10 mM formic ammonium and was delivered at a constant flow rate of 0.3 mL/min. Gradient elution was applied. The binary mobile phase consisted of the following mobile phases: (A) 100% methanol and (B) 10 mM formic ammonium solution (pH 6.4). Initially, the mobile phase comprised 100% B; it was gradually reduced to 80% at 8 min. It was then gradually returned to 100% at 10 min. A 10 μL aliquot of the treated sample was used for analysis.

ESI-MS in the selected ion monitoring mode was used for quantitative mass spectrometric detection. The following optimized MS conditions were selected: gas flow, 4.5 L/min; curve

desolvation line (CDL) temperature, 250 °C; block temperature, 200 °C; detector voltage, 1.5 kV, and probe voltage, 4.5 kV. The CDL voltage was fixed in tuning. Mass vacuum was achieved by using a turbo molecular pump. MS was performed in the positive mode to generate *m/z* 208.1 for distigmine bromide and *m/z* 286.3 for IS; the dwell time for both was 0.3 s. Instrument control and data acquisition and analysis were performed using the Class 8000 version 1.2 data system software.

2.5. Method validation

The linearity of the method by which distigmine bromide was analyzed in human serum was determined in the concentration range of 5–250 ng/mL. Calibration curves were prepared by measuring the peak area ratios (peak area analyte/peak area IS) versus the analyte concentrations in serum and were fitted to the equation $y = a + bx$ by weighted least-squares regression (weighting = $1/x$). Standard curves were used to calculate the analyte concentrations in the unknown and QC samples from the measured peak area ratios.

In order to evaluate the linearity, serum calibration curves were prepared and assayed on three different days. The accuracy and precision were also assessed by analyzing the QC samples at three concentrations on three separate validation days. The accuracy was expressed by the formula (mean observed concentration)/(spiked concentration) × 100%, and the precision was expressed using relative standard deviation (RSD).

Recovery from fortified serum was evaluated by analyzing two sets of QC samples, each consisting of five replicates at three QC concentrations. Set A consisted of blank serum fortified with distigmine bromide at three concentrations in the QC solutions prior to extraction and IS. Set B consisted of blank serum with IS added to blank serum; three QC solutions were added to the eluate after SPE. Comparison of mean three QC peak areas, (set A/set B) × 100, gave the overall analyte recoveries expressed as a percentage.

The stability of distigmine bromide in serum was assessed at room temperature, 4 °C, −30 °C and three freeze–thaw cycles. Five replicates of the QC samples were processed at three levels and stored in a 1.5 mL centrifuge tube; they were then assayed to assess the stability of distigmine bromide in the processed samples. The sample was considered to be stable when the mean value was within 15% of the theoretical value at each concentration.

2.6. Application

2.6.1. Case 1

A 69-year-old woman was admitted to the emergency department of our university hospital when she lost consciousness. Biological examination was unremarkable, except for a cholinesterase activity of 0 U/L (normal, 180–430 U/L). Atropine was administered due to the presence of bradycardia. Serum, urine and stomach wash samples were collected for toxicological analysis. Although organophosphate and/or carbamate pesticide poisoning was strongly suspected, screening of the gastric contents and urine samples by using gas chromatography–nitrogen phosphorus detector did not reveal their presence. Subsequently, distigmine bromide poisoning was suspected since Ubretid® had been prescribed for urine retention. Distigmine bromide analysis revealed its presence. The patient recovered after 19 days and was transferred to another hospital. She had attempted to commit suicide by an overdose of Ubretid®.

2.6.2. Case 2

A 34-year-old man who was known to have been using methamphetamine was found dead in his living room at home. In the previous month, he had informed his family about his intentions of committing suicide. Neither signs of violence nor needle marks were found on this body. However, 140 Ubretid® (distigmine bromide, 5 mg tablets) packets were found in the house. Autopsy findings were unremarkable, except for multivisceral congestion. A post-mortem blood sample was collected for toxicological analysis, and it was stored at −30 °C until analysis.

3. Results

3.1. Calibration curves

The calibration curve for the assay was constructed by analyzing a series of blank serum samples spiked with distigmine bromide at concentrations ranging from 5 to 250 ng/mL. The calibration curve of distigmine bromide was linear over concentrations ranging from 5 to 250 ng/mL. This result was obtained by performing a regression linear analysis of the peak-area ratios (y) of distigmine bromide to IS versus the distigmine bromide concentrations. The regression equation was $y = 0.0043x + 0.9172$, and coefficient of determination (r^2) was >0.997. The experimental peak-area ratios were interpolated on the calibration curve, and the concentrations were back-calculated. The mean back-calculated concentrations approached the spiked concentrations by an RSD of <15%.

3.2. Limits of detection and quantification

The limit of detection (LOD) for this assay was 2.5 ng/mL distigmine bromide in the serum; this was measured on the basis of signal/noise (S/N) ≥3. The limit of quantification (LOQ) for the method was defined as the lowest concentration of distigmine bromide measured in five replicates with acceptable precision (±20% RSD) and accuracy (±20%). LOQ assessment was set at 2.5 ng/mL of distigmine bromide in the serum, and at this level, the RSD was 13.2%. The chromatogram used to determine LOQ is shown in Fig. 2.

3.3. Precision

Drug-free serum spiked with distigmine bromide with at three QC concentrations in the QC samples was used for the precision studies. Table 1 summarizes the precision of the assay and its accuracy in serum. The intra- and inter-day assay variabilities were determined by analyzing five parallel samples and

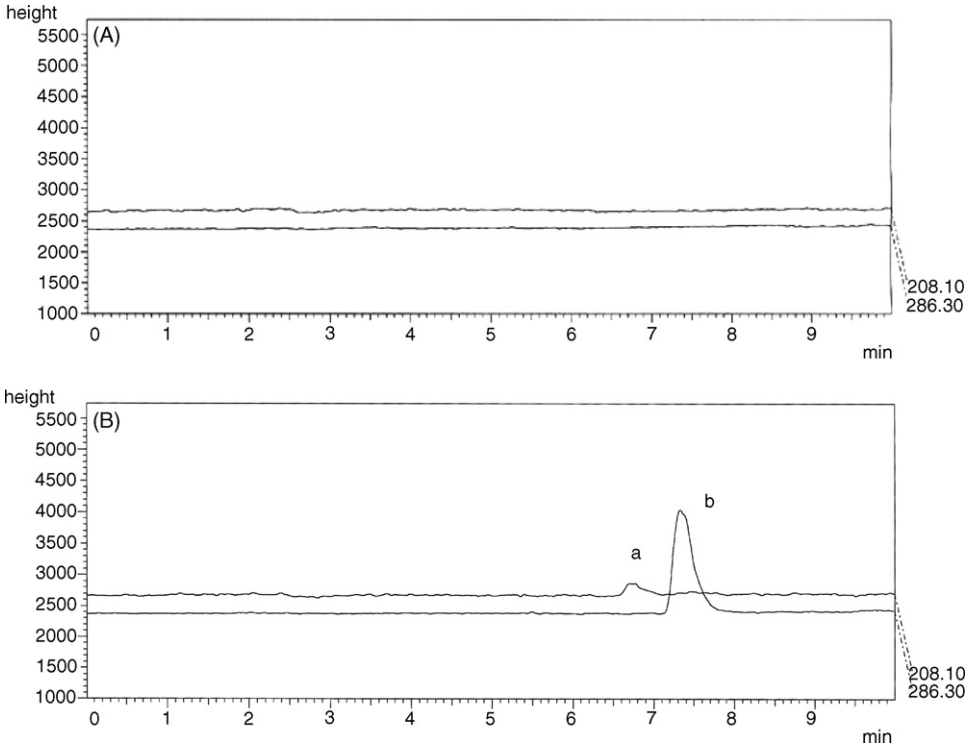

Fig. 2. LC–ESI-MS chromatograms after extraction using the CBA SPE column. Chromatograms of the blank serum (A) serum samples spiked with distigmine bromide for determination of LOQ and IS (25 ng) (B). Peaks of (a) distigmine bromide and (b) IS.

samples on five consecutive days, respectively. Precision was evaluated by using the intra and inter-day RSD. The RSDs for intra- and inter-day assays were <9.7% and <8.5%, respectively ($n = 5$).

3.4. Recovery

A series of blank serum samples spiked with the three QC levels of distigmine bromide were processed as described in the extraction and assay procedures. Method recovery was determined by comparing the peak-area ratios of distigmine bromide to IS of the extracted samples that were calculated from the calibration curve of distigmine bromide. The extraction recovery of this assay was 76.5–82.4% (Table 1, $n = 5$).

Table 1
Validation characteristics of distigmine bromide in human serum

	Nominal concentration (ng/ml)		
	7.5 ($n = 5$)	90 ($n = 5$)	200 ($n = 5$)
Accuracy (%)	90.45	90.92	101.78
Precision RSD (%)	10.99	4.75	1.17
Intra-day RSD (%)[a]	9.7	5.6	3.6
Inter-day RSD (%)[b]	8.5	3.0	2.3
Extraction recovery (%)[c]	76.5 ± 3.6	82.4 ± 2.6	79.2 ± 1.9

[a] Intra-day accuracy and precision results were obtained from five duplicate samples ($n = 5$) for each concentration of the analyte analyzed on a single day.
[b] Inter-day accuracy and precision results were obtained by analyzing five duplicate samples for each concentration of the analyte on three separate days.
[c] Data are expressed as mean ± SD

3.5. Stability

The stability of distigmine bromide in serum was evaluated by comparing fresh blank human serum spiked with three QC of distigmine bromide at an indicated time. The samples were stored at room temperature (approximately 20–25 °C) for 48 h, 4 °C for 1 week, and − 30 °C for 4 weeks and after three freeze–thaw cycles; they were then processed and analyzed as described in the extraction and assay procedures. At concentrations calculated from the calibration curve, distigmine bromide showed no degradation without in the absence of low-level QC samples (Table 2, $n = 3$). At room temperature, the levels of distigmine bromide in medium- and high-level QC samples were found to be unchanged in the serum for 48 h. At 4 °C and −30 °C, distigmine bromide was stable in serum for 1 and 4 weeks, respectively. Distigmine bromide tended to degrade in low-level QC samples after the three freeze–thaw cycles. These results indicate that samples containing distigmine bromide should be kept at −30 °C if they are to be analyzed more than 1 week after sampling.

4. Discussion

Both distigmine bromide and pancuronium bromide are quaternary ammonium compounds. Their high polarities prevent their extraction with organic solvents. Two sample preparation methods have been reported for the extraction of these compounds from human biological samples [7–9]. One is ion pair extraction [4,7] and the other is SPE [8,9]. Ion pair extraction is as convenient extraction method as SPE. However, blood

Table 2
Stability of distigmine bromide in human serum

Nominal concentration (ng/ml)		7.5	90	200
Room temperature (48 h)	Mean ± SD	ND	73.12 ± 12	197.12 ± 4
	(RSD%)		16.41	2.03
4 °C 1 week	Mean ± SD	4.45 ± 1.15	86.45 ± 6.11	202.45 ± 2.3
	(RSD%)	25.93	7.07	1.14
−30 °C 4 weeks	Mean ± SD	7.2 ± 1.15	73.12 ± 4	202.44 ± 2.32
	(RSD%)	16.04	5.47	1.15
Freeze–thaw (3 cycles)	Mean ± SD	ND	70.45 ± 2.31	201.12 ± 12
	(RSD%)		3.28	5.97

$n = 3$ each.

extracts contained some chromatographic background peaks that made quaternary ammonium compounds analysis using ion pair extract [7]. More importantly, the use of the SPE provided a method for the simultaneous measurement of both the distigmine bromide and the pancuronium bromide in serum and whole blood without background peaks. We selected SPE for sample preparation since it is easier to concentrate the drug from matrices by using SPE than ion pair extraction. We first tested the C_{18} SPE column for two matrices without any significant pretreatment. We noticed that both the compounds almost entirely adhered to the C_{18} column. Therefore, we examined the CBA column that showed good adhesion and recovery. Use of a CBA column effectively removed interfering peaks from the chromatograms of the serum and whole blood extracts. With regard to the results of validation, similar results were obtained for serum and whole blood. The cation exchange cartridge column is suited to the extraction of quaternary ammonium compounds.

Since both distigmine bromide and pancuronium bromide are hydrophilic, the mobile phase must contain a high proportion of aqueous solvent to retain the drugs on the C_{18} column. We first used isocratic elution for the analysis of distigmine bromide and pancuronium bromide with the standard solution; the results were inconsistent. Subsequently, we switched to gradient elution, and it resulted in good separation. Then, we have evaluated column oven temperature and analytical time used when the C_{18} column was used. Distigmine bromide and pancuronium bromide were rapidly eluted from the column by higher temperature. After evaluating several condition, we chose oven temperature at 50 °C for its rapidly and efficient elimination time for both compounds.

Due to its high sensitivity and selectivity, LC–MS is widely used to determine drug concentrations in biological matrices. However, the matrix occasionally affects the assay results, particularly in the case of ESI [10]. In reversed-phase chromatography, most interference peaks in biological matrices were eluted with the solvent front. This problem was solved by de-proteination using methanol and SPE. The separation mechanism in the reversed-phase chromatography system is primarily based on the relative polarity of the analytes. Therefore, it is obvious that hydrophobicity plays an important role in the separation of compounds. However, since distigmine bromide and pancuronium bromide are extremely polar compounds, a highly aqueous mobile phase must be used; this results in un-separated peaks. This problem can be avoided by using gradient elution.

A more sensitive detection is possible by using a HILIC silica column (Waters) than an XTerra MS column for the routine analysis of quaternary ammonium compounds. When a short column is used, the analytical time reduces. In our study, we used an XTerra MS column because this column is also used in the screening of other compounds such as benzodiazepines, barbiturates, tricyclic antidepressants, and organophosphates. The use of an identical analytical column has simplified the screening technique.

The clinical symptoms of organophosphate, carbamate, and distigmine bromide poisoning may be confusing and similar. Therefore, it is very important to distinguish these poisonings. Analytical methods have been described for identifying organophosphate and carbamate identification by using LC–MS [11], gas chromatography [12,13], and gas chromatography–MS [14]. For effective toxicological screening, sufficiently rapid sample screening is as important as sufficient method accuracy. The described method reports valid results within an appropriate time period with both sufficient accuracy and sufficient speed. Thus, in the case of poisoning, the patient's sample can be processed on admission to the emergency room on the same day, and the results can be obtained within few hours. The LC–MS method described here is therefore useful for both toxicological screening of distigmine bromide and subsequent follow-ups.

5. Application of the method and conclusions

The present method has been used successfully for the determination of the distigmine bromide concentrations in cases 1 and 2 following Ubretid® over-dose. The typical serum and whole blood concentrations of distigmine bromide have been provided in Fig. 3. No other drugs or alcohol were identified in these patients. The concentrations of distigmine bromide in cases 1 and 2 were 284.0 and 211.5 ng/mL, respectively. However, these concentrations could not be compared with those observed in a previous report because the literature lacks the data regarding distigmine bromide.

In conclusion, this paper describes a simple, accurate, and specific LC–MS method using CBA column extraction for the analysis of distigmine bromide in human serum. This method can be used for the toxicological screening of distigmine bromide. In addition, it will be useful for the continued development of quaternary ammonium compounds including pancuronium

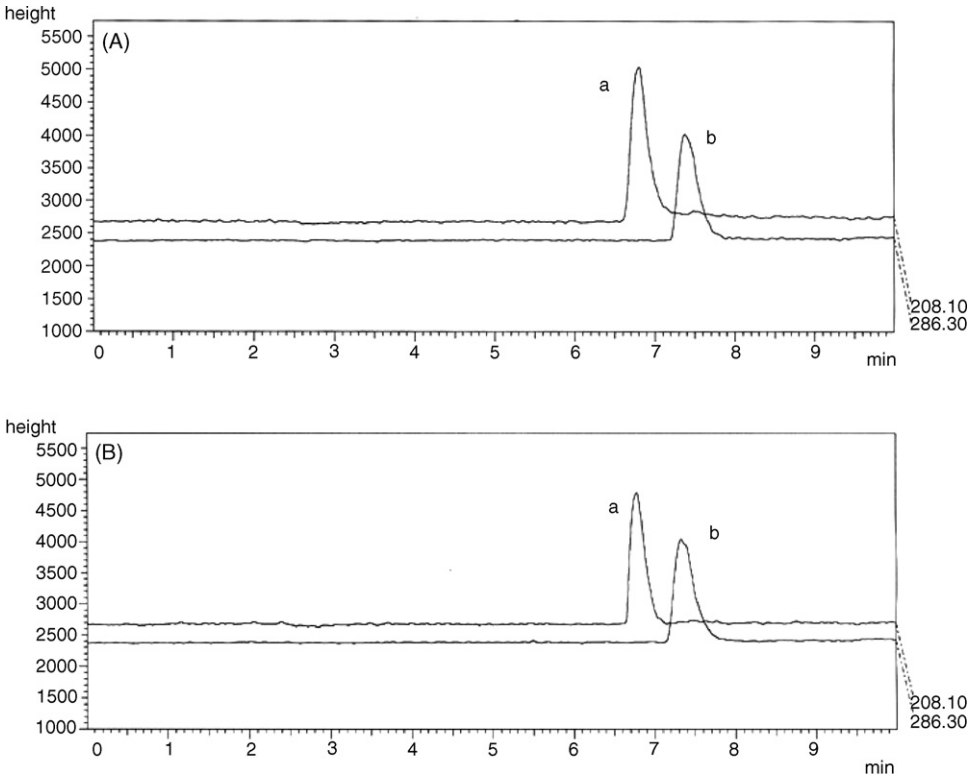

Fig. 3. Chromatograms of case 1 (A) and case 2 (B) along with their mass spectra. Peaks of (a) distigmine bromide and (b) IS.

bromide and/or for forensic toxicological studies involving biological analysis.

References

[1] Y. Tsutsumi, J. Tanaka, T. Miura, H. Yamamoto, H. Kanamori, T. Kawamura, S. Obara, M. Asaka, M. Iwamura, N. Masauzi, Intern. Med. 42 (2003) 1156.
[2] A. Hameed, T.J. Charles, Br. J. Clin. Pract. 48 (1994) 103.
[3] S. Yamanaka, I. Fujita, T. Murota, M. Kawakita, T. Matsuda, Acta. Urologica Japonica 48 (2002) 21.
[4] M. Nishikawa, M. Tatsuno, S. Suzuki, H. Tsuchihashi, Forensic Sci. Int. 51 (1991) 131.
[5] M. Zecevic, L. Zivanovic, A. Stojkovic, J. Chromatogr. A 949 (2002) 61.
[6] O. Quintela, P. Marquet, J.L. Dupuy, J.M. Gaulier, G. Lachatre, J. Anal. Toxicol. 28 (2004) 105.
[7] V. Cirimele, M. Villan, G. Pepin, B. Ludes, P. Kintz, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 789 (2003) 107.
[8] C.H. Kerskes, K.L. Lusthof, P.G. Zweipfenning, J.P. Franke, J. Anal. Toxicol. 26 (2002) 29.
[9] B.D. Andresen, A. Alcaraz, P.M. Grant, J. Forensic Sci. 50 (2005) 196.
[10] S. Souverain, S. Rudaz, J.-L. Veuthey, J. Pharm. Biomed. Anal. 35 (2004) 913.
[11] S. Kawasaki, F. Nagumo, J. Chromatogr. Biomed. Appl. 620 (1993) 61.
[12] H. Tsoukali, G. Theodoridis, N. Raikos, I. Grigoratou, J. Chromatogr. B: Anal. Technol. Biomed. Life Sci. 822 (2005) 194.
[13] S.A. Akgür, P. Öztörk, A. Yemişcigil, J. Toxicol. Environ. Health Part A 66 (2003) 2187.
[14] F. Musshoff, H. Junker, B. Madea, J. Chromatogr. Sci. 40 (2002) 29.