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Screening procedure for eight quaternary nitrogen muscle relaxants in blood by high-performance liquid chromatography–electrospray ionization mass spectrometry

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Abstract

A screening procedure was developed for the identification and the quantification of eight quaternary nitrogen muscle relaxants, including d-tubocurarine, alcuronium, pancuronium, vecuronium, atracurium, mivacurium, rocuronium and mebezonium, in blood samples. The procedure involves ion-pair extraction with methylene chloride at pH 5.4, reversed-phase HPLC separation and electrospray ionization mass spectrometry detection. The procedure was validated in terms of linearity ($0.929<r<0.998$ for concentrations ranging from 0.1 to 10 mg/l), repeatability ($6.9<\text{RSD}<17.8\%$ at 1 mg/l, $n=8$), relative extraction recovery (46.0 to 91.1% at 1 mg/l, $n=8$) and limit of detection (S/N ratio >5 for all the target compounds at 0.1 mg/l). The screening test was found satisfactory and applied in two fatal deaths. In the first case, toxicological investigations on biological fluids collected during the autopsy revealed the presence of vecuronium (1.2 and 0.6 mg/l in blood and urine, respectively) and its desacetylated metabolite, 3-hydroxy-vecuronium (4.4 and 0.7 mg vecuronium equivalent/l in blood and urine, respectively). In the second forensic case, blood analysis showed high levels of mebezonium (6.5 mg/l). The developed procedure was found suitable for forensic investigations.

Keywords: Quaternary nitrogen muscle relaxants

1. Introduction

Quaternary nitrogen muscle relaxants can sometimes be involved in forensic laboratories in cases of suicide or homicide. Because of the lack of chromophore, thermal stability and also because of the presence of a permanent positive charge, quaternary nitrogen muscle relaxants are difficult to extract and to analyse by conventional analytical methods. For example, a few procedures were reported for the determination of vecuronium in blood using high-performance liquid chromatography (HPLC) with ultraviolet, fluorescence, or electrochemical detection or gas chromatography (GC) with nitrogen-sensitive detection after laborious extraction techniques and time consuming derivatization step [1–4].

Clinical and/or forensic problems may require univocal proof of the presence of these quaternary nitrogen muscle relaxants. Currently, several fatal intoxications following suxamethonium, rocuronium, pancuronium or atracurium administration have been reported [5–10], but analytical data are generally poor. Postmortem investigations including vec-

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uronium data were previously reported [11], but the common thermolytic decomposition product measured by the developed procedure (GC–mass spectrometry, MS) was not able to distinguish between vecuronium and pancuronium intoxications. In 1983, Bertol et al. [12] published post mortem investigations for mebezonium but concentrations were measured using thin-layer chromatography–ultraviolet spectrophotometry. In 1996, a suicidal attempt by intracardiac injection of T61® was reported by Hantson et al. [13], but only delayed hepatotoxicity was reported in their paper. In early 2002, the detection of seven quaternary nitrogen muscle relaxants in biological fluids using ion-trap LC–electrospray ionization (ESI) MS was proposed by Kerskes et al. [5], but their screening test did not include d-tubocurarine, alcuronium and mebezonium.

In the reported study, a screening procedure for eight quaternary nitrogen muscle relaxants, including d-tubocurarine, alcuronium, pancuronium, vecuronium, atracurium, mivacurium, rocuronium and mebezonium, using high-performance liquid chromatography–electrospray ionization mass spectrometry is presented. The procedure has been validated and applied to two fatal deaths following vecuronium and mebezonium exposure.

2. Experimental

2.1. Chemicals

Methylene chloride, acetonitrile and methanol were HPLC grade (Merck, Germany). Formic acid (HCOOH) was Normatom grade (Prolabo, France) and ammonium formate (NH_4COOH) was analytical grade (Fluka, Germany).

The pH 3.0 buffer (HPLC mobile phase) was prepared using a 2 m*M* NH_4COOH solution adjusted to the desired pH with HCOOH.

Some pure standards of quaternary nitrogen muscle relaxants were commercially available: d-tubocurarine chloride and pancuronium bromide were purchased from Sigma (St. Louis, MO, USA), alcuronium chloride from Roche (Basle, Switzerland). All others were prepared using pharmaceutical preparations: Norcuron for vecuronium bromide, Tracrium® for atracurium dibesylate, Mivacron® for mivacurium chloride, Esmeron® for rocuronium bromide and T61® for mebezonium iodide.

2.2. Sample extraction

This screening test was carried out with 1 ml of blood sample mixed with 1 ml of saturated KI solution, 1 ml of 0.8 *M* phosphate buffer (NaH_2PO_4), pH 5.4 and 5 ml of methylene chloride in a 30-ml glass tube. After horizontal shaking (15 min at 100 cycles/min) and centrifugation (10 min at 4000 rpm), the organic phase was transferred to 5-ml borosilicate tube and evaporated. The dry extract was reconstituted in 30 μl of HPLC mobile phase.

2.3. LC–MS method

A 2-μl volume of the extract was injected onto the column (5 μm Nucleosil C_{18} LC Packings, 150×1.0 mm I.D.) protected by a 0.5 μm frit. Each 20-min chromatographic run was carried out with a binary mobile phase of methanol–50 m*M* NH_4COOH, pH 3.0 buffer, using a linear gradient (methanol 30 to 90% in 6 min) generated by a 20-ml dual-syringe HPLC pump (Applied Biosystems Model 140B). The flow-rate was 50 μl/min.

Detection was carried out by a Perkin-Elmer Sciex API-100 mass spectrometer. Nitrogen (purity grade 99.95%) was employed as nebulizing gas. The instrument was operated in the positive ionization mode (ionspray +4250 V). Ions generated in the ion source were sampled into the mass analyzer through a 25-μm orifice held at +40 V. MS data were recorded in the full scan mode (m/z 200 to 720).

The quaternary nitrogen muscle relaxants were identified on the basis of their relative retention time and their specific ions (Table 1).

In a first step, the specimens were extracted without internal standard and the concentration estimated using a external calibration curve. As sufficient sample was available in our cases, blood specimens were re-extracted using mivacurium as internal standard at the final concentration of 2 mg/l.

3. Results and discussion

Under the analytical conditions used, no interfering peak extracted from the biological samples was

Table 1
Retention times (t_R) and positive ion spray fragments (m/z values) for the eight quaternary nitrogen muscle relaxants and vecuronium metabolite, hydroxy-vecuronium

	t_R (min)	m/z
d-Tubocurarine	11.7	609, 552, 521
Pancuronium	12.3	430, 472, 617
Alcuronium	12.4	333, 312, 711
Rocuronium	12.4	487, 530
Hydroxy-vecuronium	12.4	515, 258, 356
Vecuronium	12.7	557, 356, 398
Atracurium	13.2	464, 516, 570
Mivacurium	13.4	514, 600, 671
Mebezonium	15.1	294, 276, 208

The underlined ions were used for quantification.

observed with the eight target drugs. A typical chromatographic profile is shown in Fig. 1.

The validation parameters of the developed procedure are summarized in Table 2. Response detector linearity was observed for concentrations of curares ranging from 0.1 to 10 mg/l (r values ranged from 0.929 to 0.998). Within-run precision ($n=8$) and relative extraction recovery at 1 mg/l ($n=8$) were found acceptable (RSDs ranging from 6.9 to 17.8%, relative extraction recoveries ranging from 46.0 to 91.1%). At the lowest calibration point (0.1 mg/l), the signal-to-noise ratio was always higher than 5 for all the target compounds.

4. Applications

4.1. Case 1

A 34-year-old female nurse in a department of surgery was found dead at the hospital. At the autopsy, the body showed no signs of violence but a needle mark was present on the left arm. Blood and urine specimens were collected and constituted the sixth quality control (domain: forensic toxicology) of the French Society of Analytical Toxicology (S.F.T.A.).

Screening of blood and urine samples by immunoassay and HPLC–diode array detection (DAD) did not reveal any drugs. Toxicological investigations for fentanyl derivatives by GC–MS [14], benzodiazepines by GC–MS-negative ion chemical ionization (NCI) [15] and volatile substances including propofol by headspace (HS) GC–MS [15] remained also negative.

A self-injection was highly suspected due to the presence of a recent needle mark on the left arm of the female nurse who died in the workplace (hospital). The screening for the eight quaternary nitrogen muscle relaxants revealed the presence of vecuronium and its active metabolite, 3-hydroxy-vecuronium, in both blood and urine specimens. The full scan chromatogram of the post-mortem blood sample is presented in Fig. 2. Vecuronium concentrations were 1.2 and 0.6 mg/l in blood and urine, respectively. As standard solution of 3-hydroxy-vecuronium was not available, the metabolite was identified on the basis of its relative retention time and specific mass spectra previously published [16]. For quantitative determination, the same response factor was used for the metabolite and the parent drug. Metabolite concentrations were 4.4 and 0.7 mg vecuronium equivalent/l in blood and urine, respectively.

Our concentrations are higher than those presented by Giroud et al. [11]. These authors reported post-mortem blood concentrations ranging from 62 to 709 ng vecuronium or pancuronium equivalent/ml (mean 221 ng equivalent/ml in 16 cases) and urine concentrations ranging from 65 to 300 ng vecuronium or pancuronium equivalent/ml (mean 86 ng equivalent/ml in three cases). In this study, no metabolites were identified and the thermolytic decomposition product measured by GC–MS was not able to distinguish between a vecuronium or a pancuronium intoxication.

In our case, vecuronium concentration in blood was slightly higher than the therapeutic ranges (0.4 to 1.0 mg/l). The absence of respiratory assistance and medical control might have contributed to death.

4.2. Case 2

A 58-year-old male veterinarian was found dead outside in a garden. Near the corpse, a syringe and an empty bottle of T61® (veterinary drug used for euthanasia of dogs, cats and other animals containing mebezonium, embutramide, tetracaine and demethylformamide) were found. The deceased was known to be depressive and an accompanying letter explained his suicidal intentions. During external body examination, no sign of violence was noted.

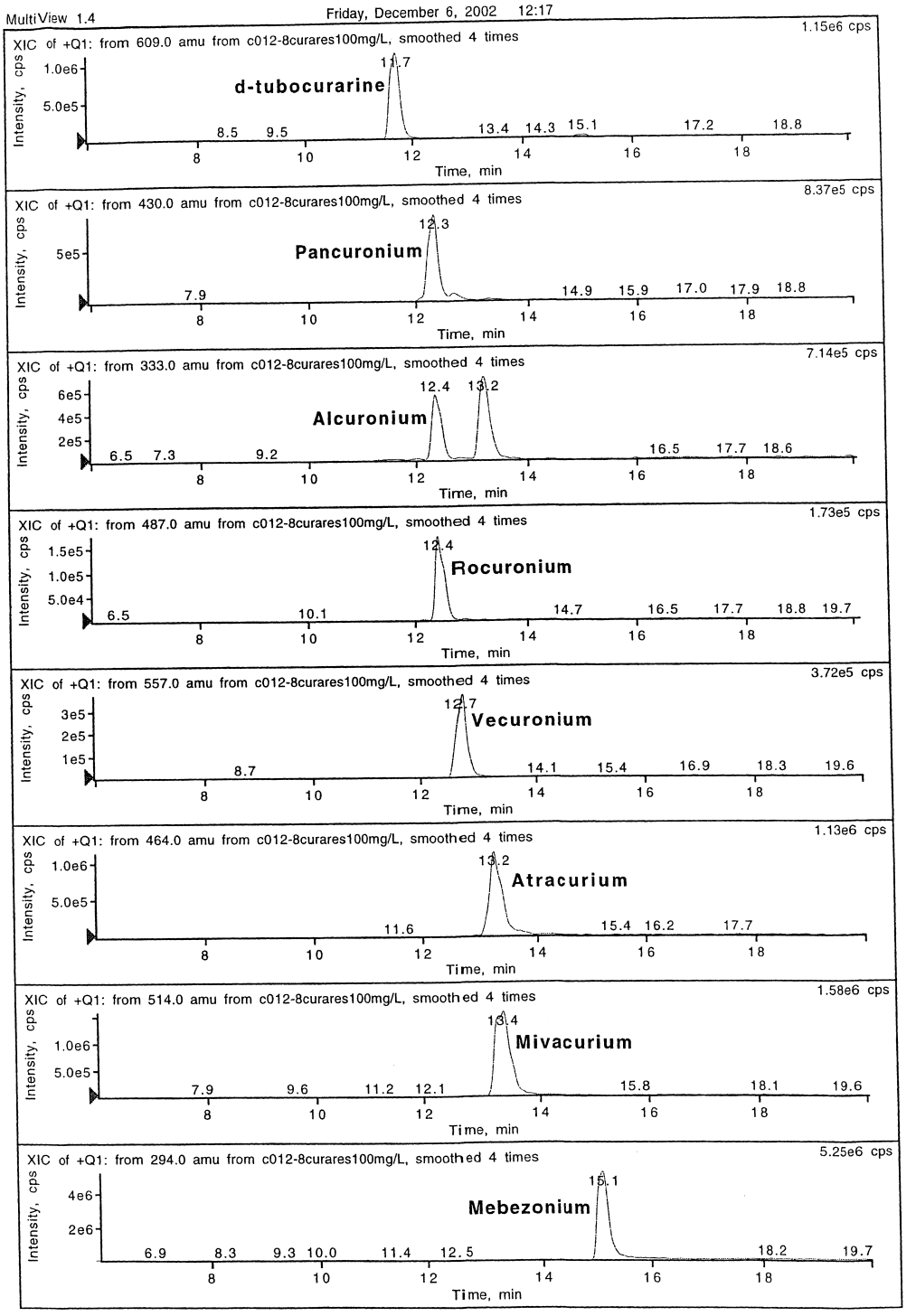

Fig. 1. Chromatographic profile obtained for the eight quaternary nitrogen muscle relaxants.

Table 2
Validation parameters (linearity, within run precision and extraction recovery) for the eight quaternary nitrogen muscle relaxants

	Linearity (r)	Precision (%)	Recovery (%)
d-Tubocurarine	0.975	11.8	47.6
Pancuronium	0.950	6.9	89.8
Alcuronium	0.929	7.7	91.1
Rocuronium	0.998	8.8	85.3
Vecuronium	0.989	17.8	60.7
Atracurium	0.943	15.7	46.0
Mivacurium	0.932	6.9	46.8
Mebezonium	0.981	9.7	75.3

Femoral blood was collected for toxicological investigations. No autopsy was requested.

The screening of post mortem blood by our procedure revealed the presence mebezonium at the concentration of 6.5 mg/l (Fig. 3). This concentration is in the range of those measured by Bertol et al. [12] in three fatalities (4.5, 6.0 and 7.5 mg/l).

The developed procedure appears sensitive enough as a screening test for forensic investigations ($S/N>$ 5 at 0.1 mg/l in full scan mode), but in cases requiring more sensitivity, detector performances can be improved using the single ion monitoring mode for data acquisition.

5. Conclusion

A screening procedure for eight quaternary nitrogen muscle relaxants in biological fluids has been developed. The target compounds detected by HPLC–ESI-MS were d-tubocurarine, alcuronium, pancuronium, vecuronium and its metabolite, hydroxy-vecuronium, atracurium, mivacurium,

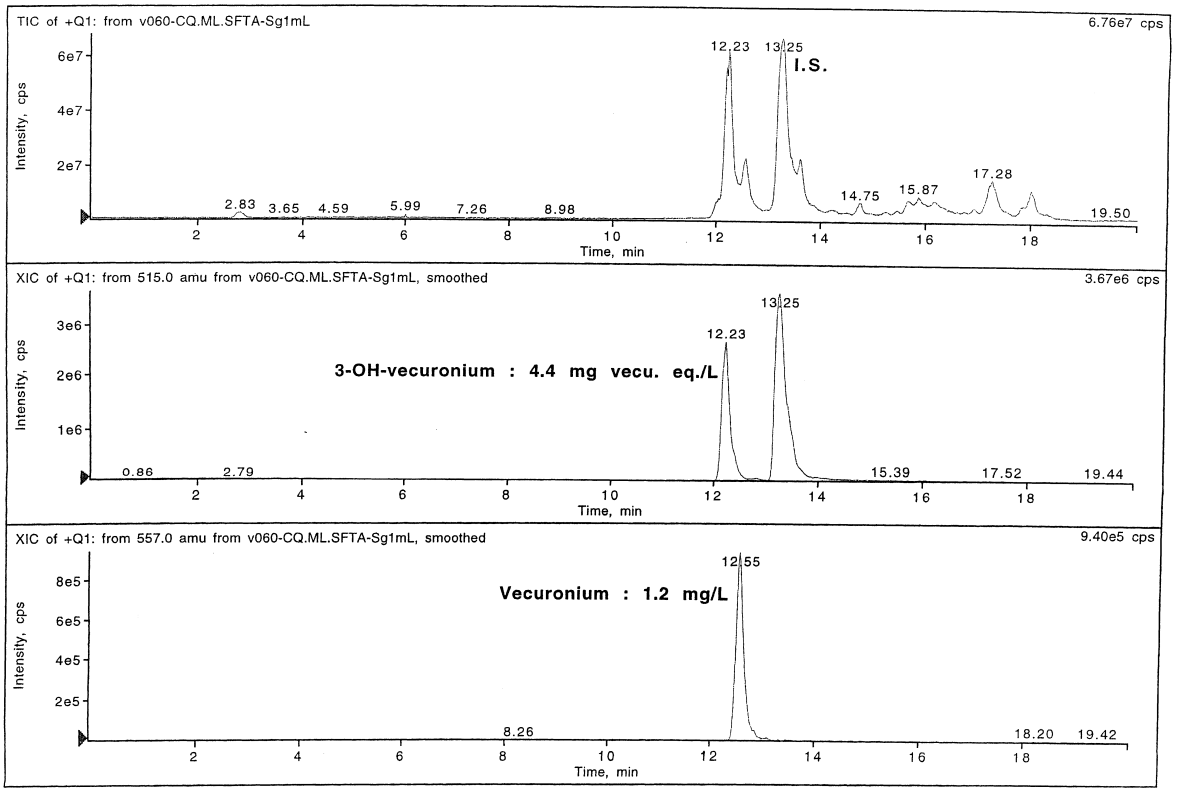

Fig. 2. Full scan chromatogram of the post mortem blood obtained after ion-pair extraction followed by HPLC–ESI-MS detection. At their respective retention times 12.5 and 12.2 min, the specific mass spectra of vecuronium and its metabolite were recorded (I.S.=internal standard=mivacurium).

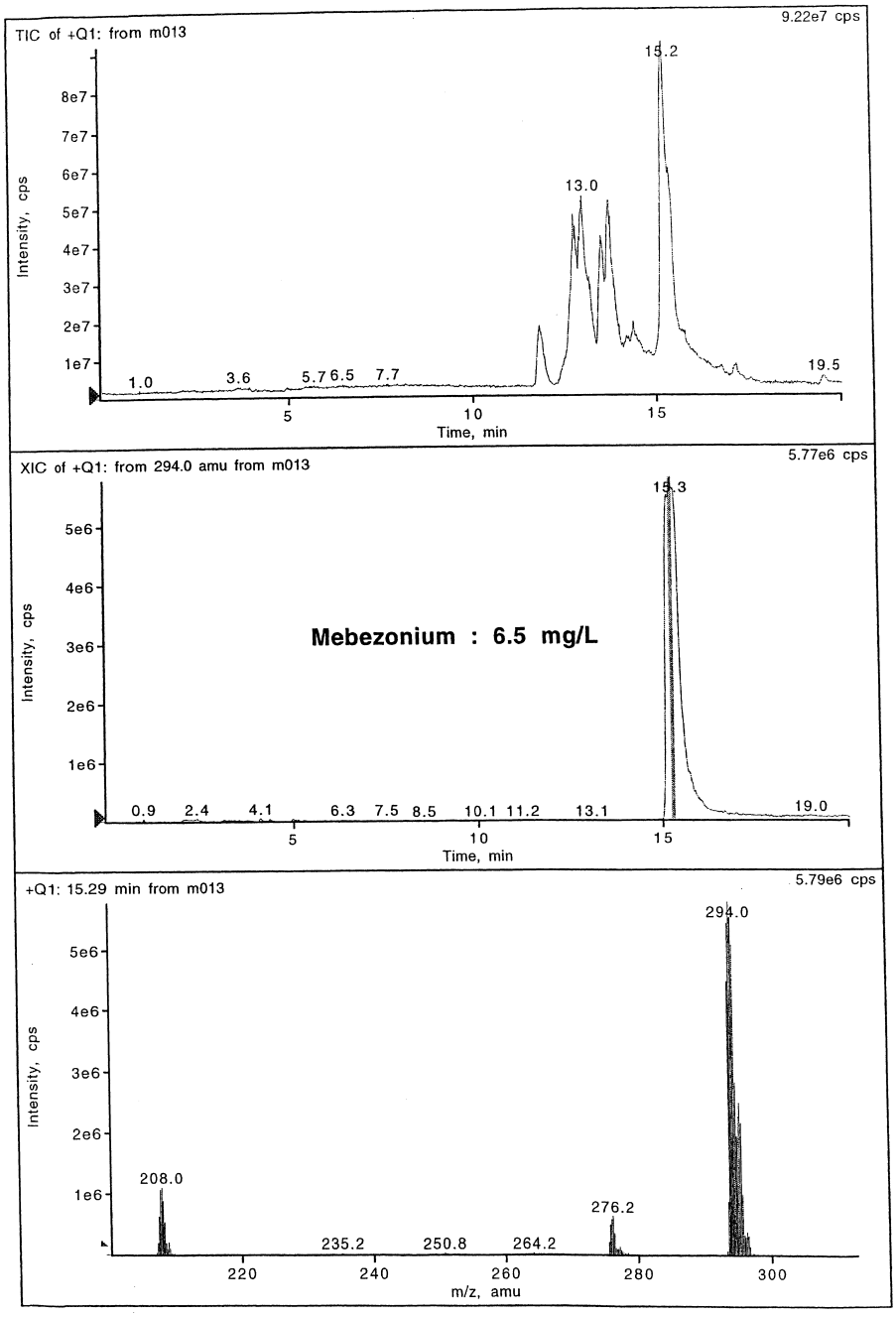

Fig. 3. Full scan chromatogram of the post mortem blood positive for mebezonium. The specific mass spectra of mebezonium was recorded at 15.3 min.

rocuronium and mebezonium. The procedure is robust, sensitive and specific and does not involve laborious extraction techniques and time consuming derivatization step.

The procedure has been validated and applied to two fatal deaths following vecuronium and mebezonium misuse. ESI-MS technology represents the univocal proof of curare contribution in forensic situations.

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