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Simultaneous determination of pancuronium, vecuronium and their related compounds using LC–ESI-MS

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Abstract

A simultaneous determination method of quaternary amino steroidal muscle relaxants, pancuronium (PAN), vecuronium (VEC), and 17-monodesacetyl pancuronium (17-OH-PAN), 3,17-bisdesacetyl pancuronium (3,17-OH-PAN), 3-monodesacetyl vecuronium (3-OH-VEC), 3,17-bisdesacetyl vecuronium (3,17-OH-VEC) in human serum was developed using liquid chromatography–electrospray ionization-mass spectrometry (LC–ESI-MS). The weak cation exchange cartridge was useful for the extraction of these compounds. Under optimized LC–ESI-MS conditions, these compounds were almost fully separated within 6.5 min. Linear responses over the concentration range 0.25–50.0 ng/mL were demonstrated for each compound.

The developed method successfully detected VEC, 3-OH-VEC and 3,17-OH-VEC in serum intravenously administered with VEC. The level of 3-OH-VEC was higher than other compounds. This suggested that 3-OH-VEC was useful as a forensic probe in VEC administration.

Keywords: Pancuronium; Vecuronium; LC–ESI-MS; Toxicology; Forensic analysis; Determination

1. Introduction

Muscle relaxants are widely used in clinical anesthesia to induce skeletal muscle relaxation, including respiratory muscles [1]. It is therefore, very important to manage patients with a mechanical ventilator to prevent malpractice. As muscle relaxants can be lethal without a mechanical ventilator (the intravenous LD_{50} of PAN and VEC in mice are 0.047 and 0.051 mg/kg, respectively), their use is sometimes examined in forensic laboratories in cases of murder [2–6] and suicide [7–12].

Most muscle relaxants currently used in Japan are amino steroidal non-depolarizing neuromuscular blocking agents, vecuronium 1-[(2β, 3α, 5α, 16β, 17β)-3, 17-bis(acetyloxy)-2-(1-piperidinyl) androstan-16-yl]-1-methylpiperidinium bromide) and pancuronium (1,1′-[(2β, 3α, 5α, 16β, 17β-bis(acetyloxy) androstan-2,16-diyl] bis-1,1′-methylpiperidinium dibromide). It is thought that these compounds may be metabolized to 3-desacetyl, 17-desacetyl and 3,17-desacetyl-form in humans, respectively. It is well known that 3-desacetyl-form is an active metabolite and manifested as prolonged paralysis in patients.

From forensic and clinical toxicological viewpoints, muscle relaxants are very important compounds. However, because of the presence of quaternary amine moieties, and the lack of a UV chromophore and thermal stability, these compounds are difficult to extract and analyze by conventional analytical methods.

The quantification of muscle relaxants in biological specimens has been reported using high-performance liquid chromatography (HPLC) with a fluorometer [12,13]

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electrochemical detector [14], LC–MS(/MS) [7–9,15,16] or gas chromatography (GC) with a nitrogen-sensitive detector [17,18] and direct CI-MS [19]. However, reports of the determination methods of PAN and VEC, including their metabolites in sera, are limited. Furthermore, there are no reports of the simultaneous determination of these compounds. We have therefore, developed a simultaneous determination method for PAN, VEC and these metabolites using LC–ESI-MS. This method could provide forensic evidence of PAN and VEC use.

2. Materials and methods

2.1. Subjects

Informed consent was obtained from the patient before operation. The patient had received 604 mg of VEC intravenously for 6 days (4 mg/h) at surgery for partial liver transplantation. After 3, 14, 24 and 31 days, 1 mL of serum sample was collected and stored at −30 °C until analysis.

2.2. Materials

PAN, 17-OH-PAN, 3,17-OH-PAN, VEC, 3-OH-VEC, 3,17-OH-VEC and 3,17-bispropionyl pancuronium (as the internal standard, IS) were supplied by N.V. Organon Co. (The Netherlands) and were stored at 4 °C in the dark. The structures of these compounds are shown in Fig. 1. Acetonitrile was LC–MS grade from Wako Pure Chemical Industries (Osaka, Japan). Ammonium formate was purchased from Fluka (Osaka, Japan). Formic acid was purchased from Kanto Chemical Co. Ltd (Tokyo, Japan). Other chemicals used were of analytical grade.

2.3. Sample preparation and extraction procedure

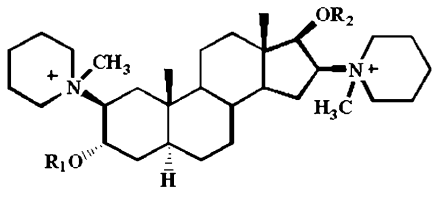
PAN, VEC and their related compounds, as well as suxamethonium, adsorbed to glassware (data not shown) [16] and it is therefore, necessary to use plasticware, such as a polypropylene, for this analysis.

Stock solutions of each standard were prepared in 10 mM ammonium formate pH 3.8 (120 μg/mL) and stored at −80 °C in the dark. To obtain calibration standards for each compound at concentrations of 0.25, 0.5, 1.0, 5.0, 10, 50 ng/mL, the stock solutions were spiked appropriately into drug-free standard human serum. IS was prepared in 10 mM ammonium formate pH 3.8 at a concentration of 100 ng/mL.

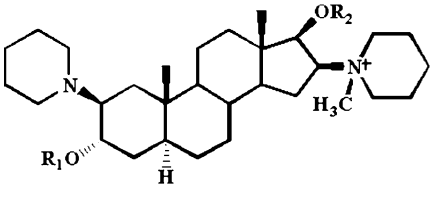
Muscle relaxants and related compounds were extracted from serum on a weak cation exchange cartridge (BondElut CBA, Varian). The cartridge was first conditioned with 1 mL of methanol, followed by 1 mL water. Two hundred microliters of serum sample mixed with 200 μL of methanol for deproteinization and 25 μL of IS solution in a polypropylene microtube were applied to the conditioned cartridge. The cartridge was then washed with 1 mL of water, followed by 1 mL of methanol–water (50:50, v/v). Analytes were eluted with 1 mL of 50 mM HCl–methanol solution. After the total evaporation of methanol under nitrogen stream at room temperature, the residue was reconstituted with 40 μL of mobile phase. An aliquot of 10 μL was injected onto the column.

2.4. LC–ESI-MS condition

For the LC–ESI-MS system, the following instrumentation was used: the HPLC system was a NANOSPACE SI-2 (Shiseido, Japan), and reversed-phase chromatography was performed on a CAPCELL PAK MG II (Shiseido, Japan;



Compounds	R1	R2	M.W.
Pancuronium (PAN)	Acetyl	Acetyl	572.5
3-OH-PAN	H	Acetyl	530.4
17-OH-PAN	Acetyl	H	530.4
3,17-OH-PAN	H	H	488.4
Internal standard (IS)	Propionyl	Propionyl	600.5



Compounds	R1	R2	M.W.
Vecuronium (VEC)	Acetyl	Acetyl	557.4
3-OH-VEC	H	Acetyl	515.4
17-OH-VEC	Acetyl	H	515.4
3,17-OH-VEC	H	H	473.4

Fig. 1. The structures of pancuronium, vecuronium and related compounds. 3,17-Bispropionyl pancuronium was used as the internal standard.

2.0×35 mm, 3 μm) equipped with a guard cartridge CAPCELL PAK MG II (Shiseido, Japan; 2.0×10 mm, 3 μm). Column temperature was set at 40 °C, and the flow rate was 400 μL/min. Each chromatographic run was performed with a linear A/B gradient where solvent A = acetonitrile and solvent B = 50 mM ammonium formate pH 3.8 (solvent A 17 to 34% in 5 min, then back to 17% at 6 min). The injection volume was 10 μL. This HPLC system was coupled without splitting to a ZMD 4000 mass spectrometer (Micromass, UK) with an electrospray ionization probe in the positive ion mode. The capillary and cone voltage were set at 3.00 kV and +20 V (+40 V for in-source CID, which was the optimized value), respectively. The source block and the desolvation temperatures were set at 130 and 400 °C, respectively. The nebulizing gas flow rate was set at 400 L/h. Mass spectral data were collected as either total ion current (TIC) or selected ion monitoring (SIM) at characteristic *m/z* values, which are the base peak ions of each compound (*m/z* 286.2 for PAN, *m/z* 265.2 for 17-OH-PAN, *m/z* 244.1 for 3, 17-OH-PAN, *m/z* 279.2 for VEC, *m/z* 258.1 for 3-OH-VEC, *m/z* 237.1 for 3, 17-OH-VEC, *m/z* 300.2 for IS compound). These *m/z* values mean doubly charged molecular related ions (M^{2+} or $[M+H]^{2+}$).

3. Results and discussion

3.1. Mass spectra of muscle relaxants and related compounds

The mass spectra of muscle relaxants and related compounds are shown in Fig. 2. Each mass spectrum revealed a base peak ion at *m/z* 286.2 for PAN, *m/z* 265.2 for 17-OH-PAN, *m/z* 244.1 for 3, 17-OH-PAN, *m/z* 279.2 for VEC, *m/z* 258.1 for 3-OH-VEC, *m/z* 237.1 for 3, 17-OH-VEC, *m/z* 300.2 for IS, respectively, corresponding to doubly charged molecules (M^{2+} or $[M+H]^{2+}$). Singly charged molecules (M^{+}) of VEC (*m/z* 557.5), 3-OH-VEC (*m/z* 515.4) and 3, 17-OH-VEC (*m/z* 473.4) were also found in the spectra. In Fig. 2, each mass spectrum also contains a peak at *m/z* 99.9. This suggests the fragmentation of methylpiperazine moieties.

Furthermore, we applied the in-source CID technique to strongly identify the muscle relaxants and related compounds at single stage LC–MS. The observed characteristic fragment ions are shown in Table 1. These fragment ions were assigned by their structures and would be very useful for the identification of muscle relaxants in a sample.

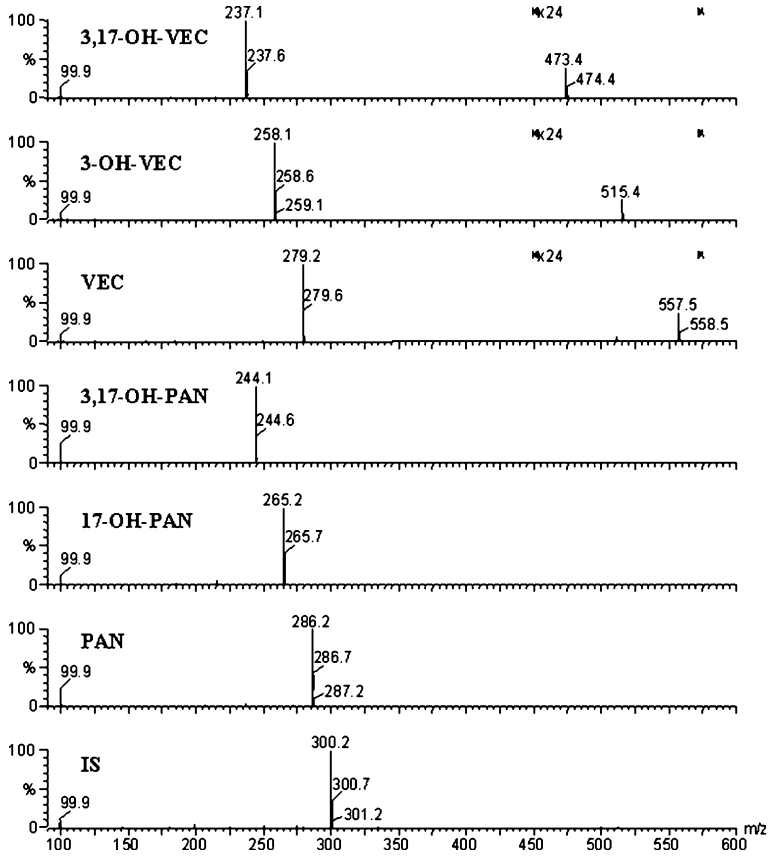


Fig. 2. Mass spectra of pancuronium, vecuronium and related compounds. These are the result of mixture of standard solutions. The concentration of each compound was of 0.5 μg/mL.

Table 1
Observed m/z values of molecular-related ions and fragment ions

Compounds	Molecular weight	Molecular-related ions (m/z)	Frangment ions (m/z)								
Pancuronium (PAN)	572.5	286.2	430			236.5			206.6		99.9
17-OH-PAN	530.4	265.2			388.4			215.4		185.5	99.9
3,17-OH-PAN	488.4	244.1			388.4					185.5	99.9
Vecuronium (VEC)	557.4	279.2, 557.5	430.4	398.4		356.4	249.2		206.6		99.9
3-OH-VEC	515.4	258.1, 515.4		398.4	374.4	356.3	249.2				99.9
3,17-OH-VEC	473.4	237.2, 473.4			374.4	356.4					99.9
IS	600.5	300.2		444.4			250.6	213.6			99.9

3.2. Selected ion monitoring

SIM was performed by monitoring the base peak ions for each compound. We initially optimized important factors for the LC condition, such as column length, temperature, flow rate and gradient duration time as described in materials and methods. Fig. 3 shows optimized chromatograms obtained with standard solution and drug-free serum spiked with each standard. Each compound was almost fully separated within 6.5 min.

3.3. Calibration curves

The calibration curves of six standards for each compound in serum were obtained by applying linear regression to logarithmically transformed data of the peak-area ratio analyte/internal standard, and the amount of analyte. Linear response over a concentration range of 0.25–50.0 ng/mL (0.25, 0.5, 1.0, 5.0, 10, 50 ng/mL) was demonstrated ($r = 0.998$–0.999). Recovery at the lowest concentration point, the limit of detection (LOD) and the limit of quantitation (LOQ) of each compound are listed in Table 2. A remarkable loss of 17-OH-PAN was observed at relatively low concentrations (data not shown), preventing the achievement of the parameters listed in Table 2. This is considered one reason why 17-OH-PAN converts to 3,17-OH-PAN during the extraction procedure.

3.4. Application to human serum

We applied this method to a female patient who had received VEC intravenously for 6 days at surgery. Serum samples were intermittently collected for 3 to 31 days after injection. A 200 μL aliquot of these samples was analyzed by LC–ESI-MS and VEC, 3-OH-VEC and 3,17-OH-VEC were successfully detected and determined. Representative chromatogram was shown in Fig. 4. This is the first report of finding 3,17-OH-VEC in human serum.

Concentration–day curves for this patient are shown in Fig. 5. The level of 3-OH-VEC was higher than other compounds, and the levels of compounds decreased each day.

Some clinical reports have described the per day decrease of the VEC level after administration but did not focus 3-OH-VEC [14,20]. Cirimele et al. [9] detected VEC and 3-OH-VEC in the blood of a suicide. VEC and PAN in a serum and whole blood are converted to their 3-OH-forms at room temperature. 3-OH-forms consider to be useful makers for detect the amount of administrated VEC and PAN. Therefore, we also think that the measurement of 3-OH-PAN is very useful as well as 3-OH-VEC. In this study, we could not determine 3-OH-PAN because 3-OH-PAN standard was not available at this time. However, we detected the peaks of molecular related ion and fragment ions of 3-OH-PAN from decomposed samples of standard PAN. We consider that this method will be able to apply the determination of 3-OH-PAN simultaneously.

In conclusion, we have established a simple and sensitive LC–MS system for the simultaneous determination of PAN, VEC and related compounds, and found that 3-OH-VEC is useful for the univocal confirmation of VEC contribution because of its higher content than VEC in human serum.

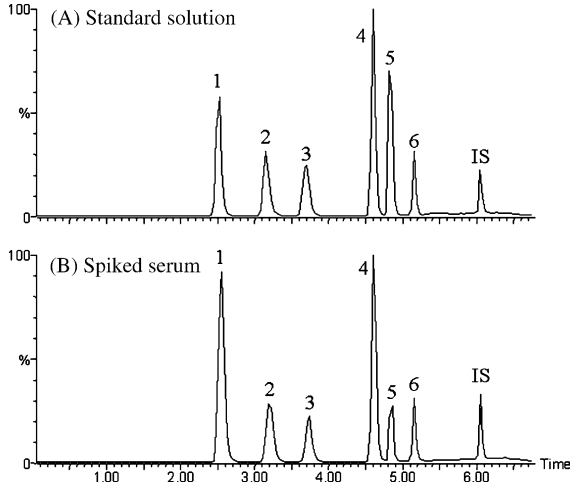


Fig. 3. Representative chromatograms of (A) standard solution and (B) drug-free serum spiked with each standard (0.5 μg/mL). Peaks: 1: 3,17-OH-PAN, 2: 3,17-OH-VEC, 3: 3-OH-VEC, 4: PAN, 5: 17-OH-PAN, 6: VEC.

Table 2
Validation parameters for the determination method of muscle relaxants

	3,17-OH-PAN	3-OH-VEC	3,17-VEC	PAN	VEC
0.25 ng/ml					
C.V. (%)	2.2	7.5	5.7	7.9	6.3
Relative recovery (%)	107	111	107	105	97
2 ng/ml					
C.V. (%)	4.6	10.6	10.1	12.1	9.8
Relative recovery (%)	90	84	90	92	103
50 ng/ml					
C.V. (%)	2.3	4.3	3.8	4.7	3.9
Relative recovery (%)	104	107	104	104	99
LOD (pg/ml)	19.0	68.5	50.3	68.5	50.3
LOQ (pg/ml)	57.7	208.2	152.8	208.2	152.8
Calibration range	0.25–50	0.25–50	0.25–50	0.25–50	0.25–50
r values	0.9993	0.9980	0.9990	0.9991	0.9996

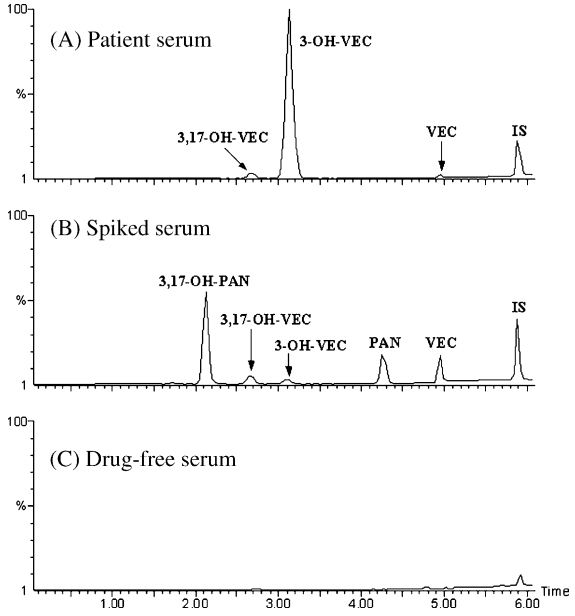


Fig. 4. SIM chromatograms of (A) patient serum, (B) spiked serum (10 ng/mL) and (C) drug-free serum.

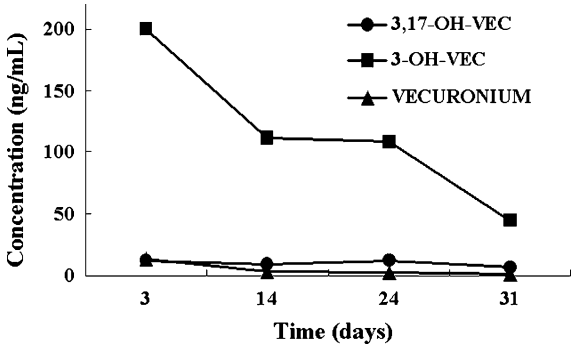


Fig. 5. Variation of serum VEC, 3-OH-VEC and 3,17-OH-VEC each day in a patient after the intravenous infusion of VEC for 6 days (4 mg/h, total amount of VEC 604 mg) at operation.

This method could be helpful in the forensic and clinical fields.

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