II.4.6 Muscle relaxants

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Introduction

Muscle relaxants can be classified into the peripheral-and central-acting drug types (*Table 6.1*). The drugs of the peripheral type are also called neuromuscular blocking agents. The peripheral-acting muscle relaxants are being used for muscle relaxation upon endotracheal intubation and/or general anaesthesia for surgical operation. The central-acting muscle relaxants are used for treatments of painful muscle contracture caused by locomotorial disorders, and for relaxation of muscle stiffness caused by psychotic tension or by neurosis. The cases of poisoning by the peripheral-acting muscle relaxants often take place, because of its strong suppressive action on respiration. However, their poisoning is rare at the scene of medical treatments, because the risk is usually avoided by artificial respiration. Most of poisoning incidents due to the peripheral-acting muscle relaxants are intentional and/or homicidal. In this chapter, therefore, analytical methods only for the peripheral-acting muscle relaxants (*Table 6.2*) are dealt with. Since these drugs are quaternary ammonium salts, special care should be taken for their stability and efficient extraction [1].

TLC analysis

Reagents and its preparation

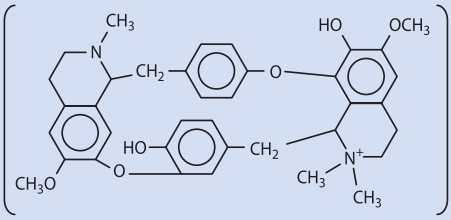
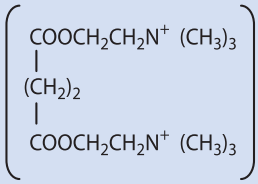
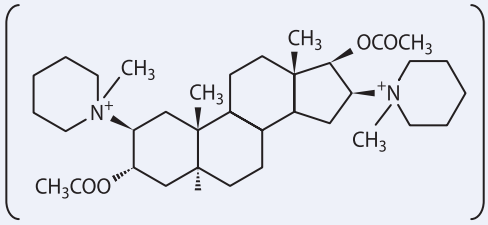
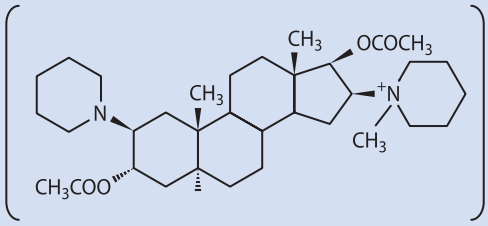
- Tubocurarine chloride, suxamethonium chloride (succinylcholine chloride) and pancuronium bromide can be purchased from Sigma (St. Louis, MO, USA). For vecuronium bromide, pure powder is not commercially available; ampoule solution for medical use

Table 6.1
Classification of muscle relaxants

muscle relaxants	peripheral-acting	non-depolarizing type tubocurarine chloride (alkaloid type), pancuronium bromide and vecuronium bromide (other types) depolarizing type suxamethonium chloride (choline type)
	central-acting	chlorphenesin carbamate, phenprobamate and methocarbamol (carbamate type) chlorzoxazone (chlorzoxazone type) chlormezanone, dantrolene sodium, pridinol mesilate, afloqualone, eperisone hydrochloride, tolperisone hydrochloride, baclofen and tizanidine hydrochloride (other types)

Table 6.2

Peripheral-acting muscle relaxants

a) alkaloid type	
tubocurarine chloride	 $Cl^- \cdot HCl \cdot 5H_2O$
b) choline type	
suxamethonium chloride	 $2Cl^- \cdot 2H_2O$
c) others	
panucuronium bromide	 $2Br^-$
vecuronium bromide	 Br^-

(Musculax) can be obtained from Japan Organon-Sankyo (Tokyo, Japan). Each standard compound is dissolved in methanol just before use[a].

- Synthesis of succinylmonocholine iodide [2]: 2.3 g choline iodide and 5 g succinic anhydride are mixed and fused for reaction by heating the mixture at 140 °C for 1–2 h in an oil bath. After cooling to room temperature, the excessive (not reacted) succinic anhydride is washed with 100 mL acetone, succinyl monocholine iodide is crystallized in the mixture of methanol/acetone/diethyl ether.
- A 0.85-g aliquot of bismuth subnitrate is dissolved in a mixture of 40 mL distilled water and 10 mL acetic acid to prepare "A" solution. A 8-g aliquot of potassium iodide is dissolved in 20 mL distilled water to prepare "B" solution. Then, a mixture of A/B/acetic acid/distilled water (1:1:4:20, v/v) is prepared (Dragendorff reagent).

- A 1-mL aliquot of 10 % platinic chloride solution is mixed with 25 mL of 4 % potassium iodide and 24 mL distilled water (iodoplatinate reagent).

TLC conditions

TLC plates: fluorescent compound-containing silica gel plates (Silica Gel 60 F_{254}, Merck, Darmstadt, Germany).

Developing solvents[b]: ① 0.1 M hydrochloric acid solution/acetonitrile (1:1, v/v), ② methanol/tetrahydrofuran/5 % formic acid solution (7:7:6, v/v), ③ methanol/chloroform/acetic acid (5:4:1, v/v).

Detection reagents [3]: ④ Dragendorff reagent, ⑤ iodoplatinate reagent.

Procedure

i. A solution specimen without dilution or a powder specimen after dissolving in methanol is spotted on a TLC plate.
ii. The spot is developed with a developing solvent in a glass tank.
iii. After development, the plate is dried with a blower, and the spot is located under ultraviolet light at 254 nm.
iv. The plate is sprayed with each reagent[c]. The color and R_f value of the spot are compared with those of the authentic compound for tentative identification.

Assessment of the method

In poisoning incidents with the muscle relaxants, the injection solution is occasionally left on the spot; in such a case, TLC is a simple and rapid method for identification.

The R_f values and the detection limits of the spots are shown in *Table 6.3*.

Table 6.3

R_f value and detection limits of the muscle relaxants observed by TLC

Compound	R_f value			Detection limit (μg)		
	Developing solvents			UV and reagents		
	①	②	③	UV	④	⑤
suxamethonium	0.25	0.15	0.02	–	0.1	0.1
succinylmonocholine	0.37			–	1.0	
choline	0.46			–	0.1	
pancuronium	0.47	0.38	0.10*	–	0.1	0.1
vecuronium	0.51	0.47	0.27*	–	0.1	0.1
tubocurarine	0.59	0.52	0.20*	0.3	0.3	0.2

*: tailing; –: no UV absorption; ①: 0.1 M HCl/acetonitrile (1:1, v/v); ②: methanol/tetrahydrofuran/5 % formic acid (7:7:6, v/v); ③: methanol/chloroform/acetic acid (5:4:1, v/v); ④: Dragendorff reagent; ⑤: iodoplatinate reagent

Direct inlet MS analysis [4]

Reagents and their preparation

- A 1-g aliquot of iodine and 2 g potassium iodide are dissolved in distilled water to prepare 20 mL solution (KI_3).
- A 13.6-g aliquot of potassium dihydrogenphosphate is dissolved in distilled water to prepare 100 mL solution. A 14.2-g aliquot of disodium hydrogenphosphate is dissolved in distilled water to prepare 100 mL solution. Appropriate amounts of the above two solutions are mixed to obtain phosphate buffer solution at pH 5.0.

MS conditions

Instrument: an MS QP-5050 mass spectrometer with a direct inlet probe (Shimadzu Corp., Kyoto, Japan); ionization: electron impact ionization (EI) and chemical ionization (CI) modes.

Probe conditions: temperature program at 40 °C/min from 30 to 350 °C.

Procedure

i. A 1-mL volume of urine, 1 mL of the phosphate buffer solution (pH 5.0), 100 μL KI_3 solution and 1 mL dichloromethane are placed in a glass centrifuge tube with a ground-in stopper, which had been treated with silane, and shaken vigorously for 3 min for extraction.
ii. After the tube is centrifuged, the organic phase (lower layer) is transferred to a small glass vial with a silicone cap. The organic extract is evaporated to dryness under a stream of nitrogen at room temperature.
iii. The residue is dissolved in 50 μL dichloromethane and a 3-μL aliquot of it is placed in a sample tube of the direct inlet probe followed by the evaporation of the solvent.
iv. MS analysis is performed in the EI mode and in the CI mode with isobutane as reagent gas.

Assessment of the method

Fragment ions for the muscle relaxants observed in both EI and CI modes are shown in *Table 6.4*.

KI_3 was used as an ion-pairing reagent for extraction of the ionized drugs; other organic ion-pairing reagents can be used [5], but KI_3 is suitable for the mass spectral measurements, because the inorganic KI_3 does not almost interfere with the measurements.

Table 6.4

Principal fragment ions of the muscle relaxants detected by direct inlet MS

Compound	*m/z* (relative intensity, %)	
	EI	CI
suxamethonium	58 (100), 71 (30)	191 (100), 261 (45)
pancuronium	467 (100), 340 (40)	416 (100), 543 (70), 483 (30)
vecuronium	425 (100), 467 (50)	374 (100), 501 (75), 543 (20)
tubocurarine	298 (100), 594 (25)	264 (100), 306 (40), 320 (35)

LC/MS/MS analysis

Reagents and their preparation

- A 0.63-g aliquot of ammonium formate is dissolved in distilled water to prepare 1,000 mL solution. The pH of the solution is adjusted to 6 by adding formic acid or ammonia water (10 mM, pH 6.0).
- A 0.83-mL volume of concentrated hydrochloric acid is diluted with distilled water to prepare 100 mL solution, followed by addition of 100 mL methanol (0.1 M hydrochloric acid solution/methanol, 1:1, v/v).

LC/MS/MS conditions

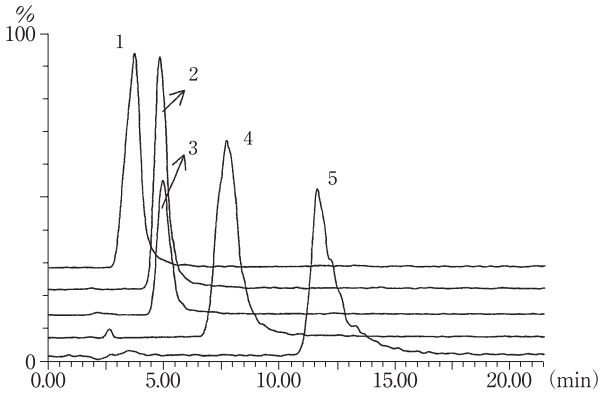
Instrumental conditions; instrument: a Quattro LC/MS instrument (Micromass, Manchester, UK); interface: electrospray ionization (ESI); ionization: positive mode; capillary voltage: 3.7 kV; cone voltage: 25 V; ion source temperature: 100 °C; collision gas: argon (2.3 e^{-3} mbarr); collision energy: 23 V.

HPLC column[d]: TSK gel VMpak-25 (75 × 2.0 mm i. d., Tosoh, Tokyo, Japan); mobile phase: 15 mM ammonium formate solution/acetonitrile (30:70, v/v); its flow rate: 0.08 mL/min.

Procedure

i. A 5-mL volume of methanol, 5 mL distilled water and 10 mL of 10 mM formate buffer solution (pH 6.0) are passed through a Bond Elut CBA cartridge (Varian, Harbor City, CA, USA)[e] to activate it.
ii. Urine or tissue supernatant[f] is directly poured into the cartridge; serum is diluted 2-fold with distilled water, and a 1–3 mL volume of the solution is mixed with an equal volume of 10 mM formate buffer solution (pH 6.0), followed by application to the cartridge.
iii. The cartridge is washed with 2 mL distilled water.
iv. A target compound is eluted from the cartridge with 2 mL of 0.1 M hydrochloric acid solution/methanol (1:1, v/v).
v. An fixed volume of the eluate[g] is injected into LC/MS/MS.

Figure 6.1



Mass chromatogram for some muscle relaxants obtained by LC/MS/MS. 1: succinylmonocholine (*m/z* 145); 2: vecuronium (*m/z* 356); 3: pancuronium (*m/z* 430); 4: tubocurarine (*m/z* 521); 5: suxamethonium (*m/z* 130).

Assessment of the method

By this method, the muscle relaxants in urine, serum and tissue homogenate can be extracted, but especially suxamethonium (succinylcholine) is rapidly hydrolyzed in blood by the action of cholinesterase; since it is very difficult to detect the drug from blood, its detection should be made with a urine specimen. In animal experiments for suxamethonium administration to rats, it was possible to detect the drug from the liver, kidney and heart [6].

Figure 6.1 shows mass chromatograms for some muscle relaxants obtained with product ions formed by LC/MS/MS. *Table 6.5* shows principal ions of product ion mass spectra for the drugs.

The peripheral-acting muscle relaxants generally have ionized structures of quaternary ammonium salts. Therefore, LC/MS with an ESI interface is most suitable for obtaining molecular ions. It is also possible to analyze them by thermospray ionization [5], atmospheric pressure chemical ionization and frit fast atom bombardment ionization [6] methods; however, in these methods, fragment ions are generally more intense than the molecular ones. As a characteristic of the ESI method, polyvalent ions can appear; in this method, bivalent molecular ions appear for the 4 muscle relaxants (*Table 6.5*). However, it should be noted that spectrum patterns are different according to the types of instruments and conditions, even if

Table 6.5

Principal product ions for the muscle relaxants observed by MS/MS analysis

Compound	Precursor ion	Product ion *m/z* (relative intensity, %)
suxamethonium	M^{2+}	130 (100), 158 (35), 204 (15)
succinylmonocholine	M^{+}	145 (100)
pancuronium	M^{2+}	430 (100), 206 (50), 332 (30)
vecuronium	$[M+H]^{2+}$	356 (100), 398 (40), 249 (25)
tubocurarine	M^{2+}	521 (100), 254 (80), 552 (70)

their interfaces are equally ESI; the patterns should be checked on every occasion of instrumental analysis.

The detection limits of the drugs in the selected reaction monitoring mode were 2–20 ng/mL.

Poisoning cases, and toxic and fatal concentrations

The depolarizing-type muscle relaxant suxamethonium is bound with acetylcholine receptors of the neuromuscler junctions to produce continuous depolarization, resulting in temporary muscle contraction followed by muscle flaccidness. The non-depolarizing type muscle relaxants, such as tubocurarine, pancuronium and vecuronium, are also bound with acetylcholine receptors of the neuromuscler junctions competitively with acetylcholine to inhibit depolarization, resulting in muscle flaccidness. All of the above muscle relaxants act on the diaphragmatic muscle to suppress respiration; only in their therapeutic doses, the spontaneous respiration stops resulting in danger of life without any artificial respiration. Suxamethonium is usually administered in the dose of 0.8–1.0 mg/kg to gain muscle flaccidness in about 1 min; the muscle activities recovers after several minutes. Pancuronium is administered in the dose of 0.08–0.1 mg/kg to produce muscle flaccidness in 1–2 min, which continues for 40–60 min.

Acute toxic affects of suxamethonium, pancuronium and vecuronium expressed as their LD_{50} values (mg/kg) were: 0.53 (rabbits, intravenous) [7], 0.036–0.047 (mice, intravenous) [8, 9] and 0.051 (mice, intravenous) [10], respectively. Usually the LD_{50} values are lowest by their intravenous administration; by the subcutaneous and intraperitoneal injections, the LD_{50} values are several to ten times higher. By oral administration, the values are several hundred to several thousand times higher; it is said that the absorption of the muscle relaxants from the digestive tract is very low.

The concentrations of pancuronium in suicidal cases by its intravenous injection were 0.3 and 0.9 μg/mL in blood and urine, respectively [11]; and 0.26 and 2.0 μg/mL in blood and urine, respectively [12].

The analysis of suxamethonium in a patient receiving intravenous injection of the drug at 2 mg/kg showed its concentrations in blood plasma of about 40, 2.4 and 0.5 μg/mL at 0.5, 4.5 and 15 min after the injection [13]. By intravenous injection of 1 mg/kg suxamethonium, its concentrations of 44.4 μg/mL and 80 ng/mL in blood plasma were obtained 47.5 s and 7 min after the injection [14]. After intravenous administration of 0.5 mg/kg tubocurarine to 7 subjects, 4.49–61.4 μg/mL of the drug was found in their urine within 24 h after the administration [15]. After intravenous injection of 0.1 mg/kg vecuronium in a patient, blood plasma concentrations of the drug at about 4,000 ng/mL immediately after injection and at only 5 ng/mL 5 h after were detected [16]. After intravenous injection of 4 mg pancuronium, serum concentrations of the drug were 0.6 and 0.07 μg/mL 5 min and 4 h after the administration, respectively [17].

Notes

a) Since these compounds are easily hydrolyzed under alkaline and neutral conditions, the standard solutions should be prepared just before use. Suxamethonium is easily hydrolyzed in alkaline solution; at above pH 7.5, it is rapidly decomposed by incubation at 37 °C for 10 min [18]. However, there is a report describing that it was stable at 4 °C for 6–8 weeks at pH 5 [19]; it is stable under weakly acidic conditions. The authors have also confirmed that decomposition of suxamethonium is suppressed at pH 4. Succinylmonocholine, a metabolic or decomposition product of suxamethonium, is relatively stable in neutral aqueous solution.

b) On a silica gel TLC plate, the quaternary amino groups tend to adsorb to the silanol group; it is, therefore, essential to use acidic developing solvents for TLC separation of the muscle relaxants.

c) Suxamethonium, pancuronium and vecuronium show no UV absorption; it is difficult to detect their spots under ultraviolet light. The colors of the spots are orange for all drugs (reddish orange for choline) with the Dragendorff reagent and dark brown with the iodoplatinate reagent.

d) For the HPLC column, aqueous type GPC packing material is used. It is preferable to use semimicrocolumns with 2.0 mm internal diameter. Except TSK gel VMpak-25 (Tosoh), Asahi-Pak GS-320 and GF-310 (Shodex, Tokyo, Japan), and Develosil Diol-5 (Nomura Kagaku, Aichi, Japan) can be used with their semimicro-sizes. For each of the above columns, a mobile phase of ammonium formate/acetonitrile or ammonium acetate/acetonitrile can be used. With ODS-type columns, trifluoroacetic acid can be added to a mobile phase as an ion-pairing reagent for analysis of the quaternary amino muscle relaxants [6].

e) For solid-phase extraction of the drugs, weak cation-exchanger cartridges are used. In this case, the packing material is of carboxylic acid-type. When strong cation-exchanger cartridges of sulfonic acid-type packing material are used, it is difficult to elute the drugs because of firm ionic binding. The extraction of the muscle relaxants with Bond Elut C_1 cartridges was also reported [14, 16].

f) The organ tissue is minced; a 3-g aliquot of the minced tissue is homogenized with 4 mL distilled water, deproteinized with 1 mL of 1.2 M perchloric acid solution and centrifuged. The pH of the supernatant fraction is adjusted to 6 with ammonia water before application to the cartridge.

g) When a glassware is used for condensation of the eluate, there is a possibility of loss of the analyte due to its adsorption to the glassware. It is preferable to use a plastic container for condensation under a stream of nitrogen with mild heating (to avoid decomposition of the analyte).

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