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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF DOXACURIUM, A NEW LONG-ACTING NEUROMUSCULAR BLOCKER

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SUMMARY

A rapid and sensitive high-performance liquid chromatographic procedure was developed for the analysis of the new, long-acting neuromuscular blocker doxacurium in the plasma and urine of dog and man and in the bile of dog. Samples were prepared on solid-phase extraction cartridges containing a methyl (C_1) bonded phase and were chromatographed on a 15 cm reversed-phase column (C_1) using a mobile phase of 0.05 M monobasic potassium phosphate–acetonitrile (30 · 70, v/v). The compound was detected at 210 nm with a lower limit of quantitation of 10 ng/ml. An inter-assay accuracy of 90–92% was obtained for the analysis of the drug from biological fluids. The method was applied to studies of doxacurium after intravenous administration to dog and man.

INTRODUCTION

Doxacurium chloride, *trans,trans*-2,2′-[succinylbis(oxytrimethylene)]bis-[1,2,3,4-tetrahydro-6,7,8-trimethoxy-2-methyl-1-(3,4,5-trimethoxybenzyl)-isoquinolinium] dichloride (Fig. 1), is a potent non-depolarizing, long-acting
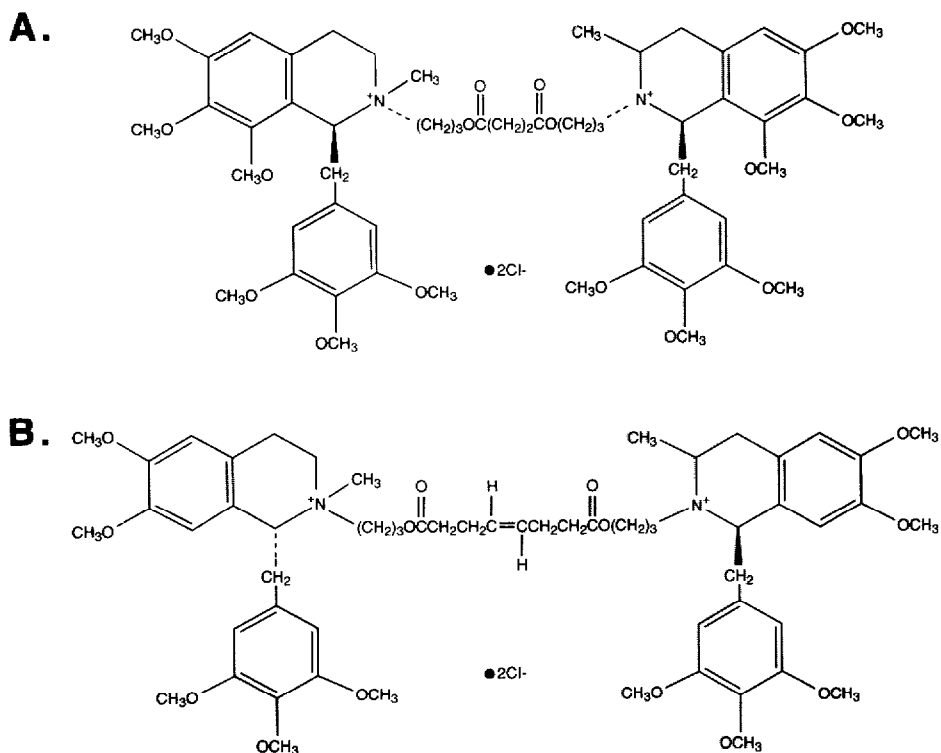
Fig 1 Structures of doxacurium (A) and the internal standard, mivacurium (B).

neuromuscular blocking agent with minimal cardiovascular side-effects at doses equivalent to several multiples of the ED_{95} (the effective dose at which 95% of the animals respond pharmacologically) and exhibits no adverse effects after repeated dosing [1–3]. The analysis of doxacurium, a bis-quarternary compound, by reversed-phase high-performance liquid chromatography (HPLC) is reported here. Several HPLC methods have been developed to assay neuromuscular blockers in biological fluids. These include the use of cation-exchange [4], normal-phase [5], and reversed-phase packings [6–8]. Thin-layer chromatography has also been used [9]. The resolution and sensitivity of these procedures proved inadequate to support pharmacokinetic and metabolic studies of doxacurium; therefore, a reversed-phase chromatographic procedure was developed in conjunction with solid-phase sample preparation. The method was applied to the analysis of doxacurium in biological fluids after intravenous administration of the drug to dog and man.

EXPERIMENTAL

Materials

Monobasic ammonium phosphate, sodium chloride, and sodium pentobarbital were A.R. grade (Mallinckrodt, Paris, KY, U.S.A.), whereas methanol,

water, and acetonitrile were HPLC grade (OmniSolv EM Science, Cherry Hill, NJ, U.S.A.). Phenylmethylsulfonyl fluoride (PMSF) (Sigma, St. Louis, MO, U.S.A.) was used as a non-specific cholinesterase inhibitor and was added to 7-ml EDTA Vacutainer® tubes (No. 6450, Becton Dickinson, Rutherford, NJ, U.S.A.) prior to blood collection. The chromatographic conditions described were used to determine the retention times of other neuromuscular blockers including d-tubocurarine chloride injection USP (Squibb & Sons, Princeton, NJ, U.S.A.), metocurine iodide injection NF (Eli Lily, Indianapolis, IN, U,S.A.), pancuronium bromide injection (Organon, West Orange, NJ, U.S.A.), succinyl chloride, atracurium besylate, and mivacurium chloride [10] (Burroughs Wellcome, Research Triangle Park, NC, U.S.A.). Mivacurium chloride, (E)-$(1R,1'R)$-2,2'-[4-octenedioylbis(oxytrimethylene)]bis-[1,2,3,4-tetrahydro-6,7-dimethoxy-2-methyl-1-(3,4,5-trimethoxybenzyl)isoquinolinium] dichloride, was used as the internal standard, since its structure is very similar to that of doxacurium (Fig. 1).

Apparatus

Bond Elut® solid-phase extraction (SPE) cartridges containing methylsilyl (C_1) reversed-phase material (Analytichem International, Harbor City, CA, U.S.A.) were used for sample preparation in conjunction with a twelve-port SPE vacuum manifold (Supelco, Bellefonte, PA, U.S.A.). A Varian 5060 liquid chromatograph (Varian Instruments, Palo Alto, CA, U.S.A.), equipped with a 150 mm × 4.6 mm Spherisorb column containing 5-μm particles of spherical silica bond with a methylsilyl phase (S5C1) (Keystone Scientific, State College, PA, U.S.A.), was used to separate doxacurium and internal standard from endogenous materials. A precolumn cartridge containing the same bonded phase (Supelco) protected the analytical column. Samples were injected onto the column with a WISP autosampler (Waters Assoc., Milford, MA, U.S.A.), and the analytes were quantitated at 210 nm with a Spectroflow 757 variable-wavelength ultraviolet detector (ABI Analytical, Kratos Division, Ramsey, NJ, U.S.A.). Chromatographic voltage acquisition, peak-area analysis, and data reduction were obtained from a microcomputer interfaced with a PDP-11/70 minicomputer (Digital Equipment, Merrimack, NH, U.S.A.). The purity and identification of the HPLC peaks were confirmed with a 1040M ultraviolet diode-array detector (Hewlett-Packard, Avondale, PA, U.S.A.). The binding of doxacurium to plasma was determined by centrifugal ultrafiltration with the Centrifree micropartition systems (Amicon, Danvers, MA, U.S.A.).

Preparation of standards

Analytical standard solutions (100 μg/ml) were prepared by dissolving doxacurium chloride or the internal standard (mivacurium chloride) in acidified saline–methanol (80:20, v/v) and diluting aliquots of these standards with the same mixture to specific concentrations as needed. The pH of the 0.9%

(w/v) saline was adjusted to 3 with 0.1 M hydrochloric acid prior to use. Standard solutions were stable for several days at room temperature (24–26°C) and for several weeks when refrigerated (3–8°C). Method standards (20–200 ng/ml) were prepared by adding drug and internal standard (200 ng) to 1.5-ml polypropylene tubes (Brinkmann Instruments, Westbury, NY, U.S.A.) containing plasma, urine, or diluted bile (1.0 ml) and were used to establish a regression encompassing the concentrations of the experimental samples. The internal standard was added to all method standards and experimental samples prior to extraction. After chromatoghraphic analysis, the peak-area ratios of doxacurium to internal standard were determined, and a least-squares linear regression was calculated. The lower limit of quantitation of doxacurium or internal standard was 10 ng/ml in plasma or urine and 30 ng/ml in bile at a signal-to-noise ratio of 7.

Sample preparation

Experimental samples and method standards were prepared for chromatography with solid-phase extraction cartridges in conjunction with a vacuum manifold. The cartridges were preconditioned by passing methanol (3 ml) and distilled water (3 ml) through the reversed-phase packing sequentially. Care was taken to ensure that the packing remained wet between solvent applications. The biological fluid (1 ml) (plasma, bile diluted 1:3 with distilled water, or urine) was then drawn through the cartridge slowly over 30 s under partial vacuum. The endogenous material was cleared from the bonded phase by sequential washes (3 ml) of water, acetonitrile, methanol, and water. The retained doxacurium and the internal standard were eluted from the bonded phase into 1.5-ml polypropylene tubes with 1 ml of a solution of methanol–acidified (pH 3) 0.05 M monobasic potassium phosphate (80:20, v/v). The eluate was mixed thoroughly and a 300-μl aliquot transferred to a polypropylene limited-volume (400 μl) insert for injection (20–100 μl) by the autosampler.

Chromatography

The HPLC mobile phase was 0.05 M monobasic potassium phosphate (pH 3)–acetonitrile containing 0.5% (v/v) methanol (30:70, v/v). The solvent was pumped through the C_1 reversed-phase column at 1 ml/min eluting the internal standard and doxacurium with retention times of 340 and 400 s, respectively. All compounds were detected by ultraviolet absorbance at 210 nm with a limit of detection of 1 ng of drug injected into the column (signal-to-noise ratio of 5).

Pharmacokinetic analysis

Mean plasma concentrations of doxacurium versus time were fitted to a two-compartment intravenous bolus model using a non-linear least-squares regression analysis computer program, NONLIN [11]. The beta half-life, total body

clearance, and apparent steady-state volume of distribution were calculated by the computer using the equations described by Gibaldi and Perrier [12].

Dogs

Male Mongrel dogs weighing 8–15 kg were anesthetized with sodium pentobarbital, 30 mg/kg intravenously, and administered doxacurium chloride at 9 μg/kg intravenously. The trachea was cannulated and the animals were ventilated with room air using a Harvard Apparatus respiration pump. Arterial blood pressure was measured from a cannula in the femoral artery connected to a Statham P23 transducer, and heart rate was determined from the ECG (lead II) using a Grass tachograph. The animals were fasted for 12 h prior to the experiment. Urine and bile were collected from cannulas placed in the bladder via the urethra and common bile duct, respectively.

Humans

Three male patients at Massachusetts General Hospital (Boston, MA, U.S.A.) gave written informed consent to be administered doxacurium chloride (80 μg/kg) during routine surgery. The weight of the subjects ranged from 75 to 80 kg and their age from 21 to 40 years. Arterial and venous cannulae were implanted after patients received local anesthesia. General anesthesia was induced with fentanyl (6–8 μg/kg) and thiopental (6–8 mg/kg), and the trachea was intubated under topical anesthesia. Arterial pressure, heart rate, and twitch were simultaneously recorded. After achieving a stable baseline for at least 15 min, doxacurium was injected as a rapid bolus (5–10 s).

Blood samples

Blood samples of approximately 5 ml (dog) were taken prior to drug administration and at 2, 5, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 120 min post-dose from an indwelling venous cannula. For humans, samples (5 ml) were drawn at 1, 2, 5, 10, 20, 30, 60, 120, 240, and 360 min post-dose. Blood was drawn by syringe and transferred into Vacutainer tubes containing EDTA (liquid) and PMSF (50 μl). The latter was prepared by dissolving 70 mg PMSF into 1 ml of dimethylformamide (Pierce, Rockford, IL, U.S.A.). The blood was centrifuged at 3000 g for 10 min to harvest plasma, which was transferred to separate tubes and shipped on dry ice to Burroughs Wellcome.

RESULTS

Accuracy and precision

The mean inter-assay accuracy and precision for the determination of doxacurium in plasma were 96.9 ± 1.8 and 92.9 ± 3.1% (mean ± S.D.), respectively (Table I). Replicates of doxacurium at various concentrations ranging from 10 to 2000 ng/ml were prepared by adding drug and internal standard to drug-

TABLE I

INTER-ASSAY ACCURACY AND PRECISION FOR THE ANALYSIS OF DOXACURIUM IN HUMAN PLASMA

Added (ng/ml)	Found (mean ± S D.) (ng/ml)	Accuracy (%)	Precision (%)	n
10	9 9 ± 1.4	99.2	85.4	12
20	19.2 ± 1.8	95.8	90.6	12
40	41 8 ± 3.0	95.6	92.8	12
60	61.9 ± 2.7	96.8	95.7	12
80	83.2 ± 3.8	96.0	95.4	12
100	104.0 ± 3.8	96.0	96.4	12
200	199.1 ± 12.2	99.5	93.9	11
500	475.1 ± 26.8	95.0	94.4	11
1000	1008.3 ± 59.5	99.2	94.1	6
1500	1525.2 ± 135.3	98.3	91.1	6
2000	2098.7 ± 161.5	95.1	92.3	6

TABLE II

INTER-ASSAY ACCURACY AND PRECISION FOR THE ANALYSIS OF DOXACURIUM IN DOG URINE AND BILE

Added (ng/ml)	Found (mean ± S.D) (ng/ml)	Accuracy (%)	Precision (%)	n
Urine				
10	9.6 ± 0.8	96 2	91.2	11
25	20.2 ± 3.1	80.9	84.9	6
50	41.7 ± 0.7	83.5	98.4	5
100	93.2 ± 6.1	93.2	93.6	19
500	449.5 ± 40.2	89.9	91 0	18
600	573.4 ± 31.7	95.6	94.5	15
1000	922.0 ± 22.1	92.2	97.6	10
Bile				
30	31.8 ± 2.8	93 9	91.2	6
50	44.6 ± 2.1	89.2	95.4	5
100	91.2 ± 3.1	91 2	96.6	6
150	132.4 ± 8.6	88 3	93.5	5
250	228.8 ± 9 0	91.5	96.1	6
500	450.5 ± 16.3	90.1	96.4	6
1000	901.3 ± 65.6	90.1	92.7	5

free human plasma followed by sample preparation and chromatography as described. The concentrations and subsequent recovery of parent compound from these samples were calculated from a linear regression of analytical standards (ng) injected directly into the column versus peak area. The accuracy of the analytical procedure, including solid-phase extraction and chromatography, ranged from 95.0 to 99.5%. The precision ranged from 85.4 to 96.4% and reflects inter-assay variation, since the data were generated over several weeks. The within-assay precision, derived from analysis of eight replicates at each concentration, was 93.9% at 20 ng/ml and 97.3% at 500 ng/ml. In addition, a recovery of $90.7 \pm 4.4\%$ (mean ± S.D.) was achieved for the internal standard from human plasma at a concentration of 200 ng/ml ($n = 20$) and was calculated against direct injections of mivacurium.

Solid-phase extraction and chromatography of doxacurium from dog urine and bile resulted in recoveries similar to those found for plasma (Table II). Specifically, the accuracy ranged from 80.9 to 96.2% over a urinary concentration range of 10–1000 ng/ml. For bile, the accuracy ranged from 88.3 to 93.9% over a concentration range of 30–1000 ng/ml. The high ionic strength of bile necessitated a 1:3 dilution with water prior to solid-phase extraction. The substitution of plasma and urine from the cat and man did not alter the recovery of the drug.

Extraction efficiency and protein binding

The efficiency of eluting doxacurium from the SPE cartridges was investigated independently of chromatography using an aqueous solution of [^{14}C]-doxacurium. The addition of 200 ng of radiolabeled drug (98% purity) to plasma ($n = 4$) followed by solid-phase extraction resulted in a mean recovery of 89.9% of the added radioactivity.

The binding of doxacurium in human and dog plasma was evaluated by ultrafiltration. Five plasma replicates from man and dog were spiked with both labelled and unlabelled doxacurium to obtain concentrations ranging from 50 to 1000 ng/ml, and the samples were then incubated at 37°C for 30 min. After centrifugation and scintillation spectroscopy, the binding was calculated and ranged from 28.0 to 33.7% in human plasma and from 17.3 to 21.1% in dog plasma.

Data from the protein binding study, plasma recovery studies, and the ^{14}C extraction efficiency study indicate that the 10% loss of this compound during sample preparation appears to be due to irreversible partitioning into the solid phase of the extraction cartridges and not to protein binding.

Compound stability

Doxacurium and the internal standard, mivacurium, were stable in a solution of acidified saline–methanol (80:20, v/v, pH 3). An analytical standard solution containing both compounds was chromatographed daily for five con-

secutive days and the chromatographic peaks showed no degradation in areas or shape. Furthermore, doxacurium appeared to be stable in plasma and urine. For instance, plasma obtained from a dog administered doxacurium intravenously gave identical results when assayed immediately or after two weeks of storage (− 15 °C). In another experiment, plasma and urine from human volunteers were re-analyzed after 60 days of storage (− 15 °C) with no apparent loss of drug. In contrast to doxacurium, mivacurium was very unstable when added to plasma. For example, in vitro experiments using human plasma indicated that the concentration of mivacurium decreased 80% in only 5 min when not protected by the cholinesterase inhibitor PMSF. Non-specific plasma esterases are capable of cleaving the alkyl chain connecting the quaternary nitrogen centers of this compound. Therefore, the use of mivacurium as the
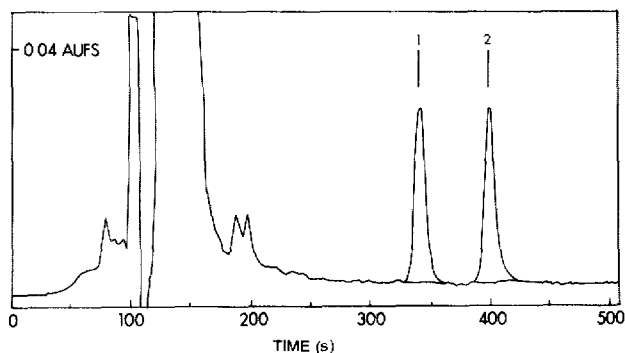

Fig 2 Computer-generated chromatographic scan of a plasma extract taken from a human subject dosed intravenously with doxacurium at 0.08 mg/kg Peaks 1 and 2 represent doxacurium (110 ng/ml) and the internal standard, mivacurium (100 ng/ml), respectively.
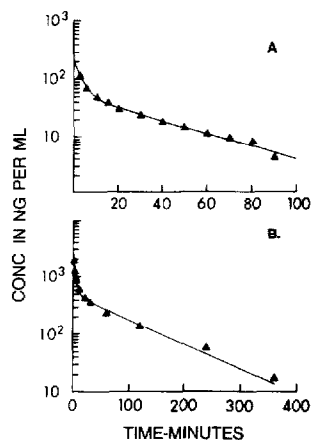

Fig. 3 Model-fitted profiles (solid lines) of mean doxacurium plasma concentrations versus time (▲) after intravenous doses. (A) Dog, 9 0 μg/kg (n = 3), (B) man, 80 0 μg/kg (n = 3).

TABLE III

BILIARY AND URINARY EXCRETION OF DOXACURIUM IN DOGS

The animals were fasted, anesthetized, and received doxacurium intravenously at 9 µg/kg. Bile and urine were collected over 6 h from cannulas placed in the bladder via the urethra and common bile duct, respectively.

Dog No.	Percent of dose excreted		
	Bile	Urine	Total
1	23 8	75.7	99.5
2	25.9	75.5	101.4
3	19 0	73 2	92.2
Mean ± S.D.	22 9 ± 3.5	74 8 ± 1.4	97.7 ± 4.6

internal standard required the addition of an enzyme inhibitor to all plasma samples.

Animal data

Fig. 2 is a computer-generated chromatographic scan of an extract of plasma from a patient treated with doxacurium and shows doxacurium (110 ng/ml) and the internal standard (100 ng/ml) at retention times of 340 and 400 s, respectively. Fig. 3 shows NONLIN-generated profiles that resulted from the fitting of mean plasma concentration–time data from dogs ($n = 3$) and humans ($n = 3$), respectively. Virtually all of the administered doxacurium was eliminated as unchanged drug in dogs. The doses excreted in the urine and bile of dogs were 74.8 ± 1.4 and $22.9 \pm 3.5\%$ (mean ± S.D.), respectively (Table III).

DISCUSSION

Analytical

The procedure presented for the analysis of doxacurium is rapid, sensitive, and selective, and can be used for the quantitation of drug in plasma, urine, and bile. Rapid analysis was achieved using standard, non-silanized glassware, SPE cartridges for sample preparation, and liquid chromatographic conditions requiring only 8 min between injections. A sensitivity of 10 ng/ml was obtained without an eluate concentration step and was reproducible with the aid of an internal standard. Ultraviolet detection at 210 nm was chosen as the quantitation technique of choice over fluorescence. Both techniques provided a lower limit of detection of 1 ng injected into the column at a signal-to-noise ratio of 4–5. However, ultraviolet detection was selected, since drug-free biological fluids were clear of endogenous peaks chromatographically at the retention times of interest, and the availability of fluorescence detectors was limited. Interest-

ingly, Neill and Jones [4] and Simmonds [8] used fluorescence detection to quantitate compounds of similar structure. Although the method was developed for doxacurium, other neuromuscular blockers can be chromatographed using this HPLC system. For instance, the retention times of some blockers used clinically are 155 s for pancuronium, 360 s for atracurium, 220 s for d-tubocurarine, 150 s for succinyl choline, and 355 s for metocurine. Finally, the selectivity of the analytical procedure was verified with a diode-array detector by comparing the ultraviolet profiles of the analytical standards to the chromatographic peaks corresponding to doxacurium and the internal standard in biological samples. In addition, drug-free plasma and urine from dogs and man, along with bile from dogs, were analyzed as described. In all cases, there were no endogenous peaks at the retention times of the compounds of interest.

Although endogenous material in biological fluids did not interfere with the chromatographic analysis, other problems associated with the analysis of doxacurium required attention. First, the adsorption of quaternary ammonium compounds on the surface of glass is well documented and was a phenomenon displayed by doxacurium also. However, this problem was minimized by using an acidified aqueous salt solution containing methanol, which appeared to reduce substantially the interaction of the quaternary nitrogens with the active silanol sites. For example, concentrations as low as 1 ng/ml doxacurium, prepared in the acidified saline–methanol solution and stored in a glass volumetric flask, showed no drug loss after five days at room temperature. Additional stability experiments indicated that volumetric flasks and limited-volume inserts made of polypropylene could be substituted for those made of glass, if desired. Second, an analytical column containing a C_1 bonded phase was chosen for chromatographic separation. Since these compounds exist as geometric isomers, bonded phases containing longer alkyl chains (C_8 or C_{18}) interact with these isomers causing increased retention times, separation of the isomers, and a marked decrease in assay sensitivity. Although the methylsilyl phase (C_1) was preferred for the analysis of doxacurium during pharmacokinetic studies, a column containing a longer alkyl chain, such as octadecyldimethylsilyl (C_{18}), will be utilized to study the elimination profile of these isomers in the urine of man. Finally, chromatographic interferences from metabolites were not a problem, since doxacurium was eliminated unchanged in the urine and bile.

Factors affecting compound resolution were also investigated. As discussed by Reynolds and co-workers [13,14] the nature and concentration of electrolytes and mobile phase pH influence the selectivity and the capacity (k') of organic cationic solutes markedly in reversed-phase HPLC. These effects have been attributed to the reduction of solute interactions with residual silanols on the stationary phase. In our system, solute concentrations in excess of 0.04 M were required to obtain reasonable and consistent k' values for the bis-quaternary blockers. In addition, the pH of the solute was adjusted to 3.0 and meth-

anol was added as a modifier to reduce peak tailing. Although methanol (0.5–1%) increased resolution and decreased peak tailing of doxacurium in C_1 bonded phases, it had little effect on selectivity in columns containing longer alkyl chains.

Compound stability

Some quaternary ammonium blockers, such as atracurium, are subject to degradation by non-specific plasma and liver esterases [6,15]. Since a similar degradation was observed for mivacurium, the internal standard, an esterase inhibitor (PMSF) was added to all plasma samples. This problem could have been circumvented by choosing an alternative internal standard; however, mivacurium was retained because it has shown marked pharmacological activity as a short-term neuromuscular blocker [16]. As such, we are proceeding with metabolic and disposition studies with mivacurium and will use the current assay for those studies.

Vacutainer tubes containing the inhibitor PMSF should be stored frozen or used immediately, since evaporation of the organic solvent will cause the PMSF to precipitate and reduce its effectiveness. Later studies have indicated that the esterase inhibitor echothiophate iodide (phospholine iodide) may be substituted, since it is more potent and water-soluble than PMSF.

Preliminary pharmacokinetics

The disposition of doxacurium was studied in dogs to provide supporting data for pharmacological studies and was investigated in man to prepare for Phase I clinical studies. After a rapid intravenous bolus, the plasma levels of doxacurium declined biexponentially as noted for other neuromuscular blockers [6,9,17]. The beta half-life of doxacurium in dog and man was 25.7 and 70 min, respectively. The volume of distribution was constant in both species at 0.12 l/kg, while the clearance values ranged from 1.5 ml/min/kg in humans to 3.6 ml/min/kg in dogs. Although the pharmacokinetic parameters obtained for man are preliminary and not statistically significant due to the limited sample size ($n = 3$), we are encouraged, since they are similar to data obtained for other blockers as summarized by Shanks [18]. The current method is being applied to more extensive pharmacological and clinical studies of doxacurium.

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