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QUANTITATION OF PANCURONIUM, 3-DESACETYLPANCURONIUM, VECURONIUM, 3-DESACETYLVECURONIUM, PIPECURONIUM AND 3-DESACETYLPIPECURONIUM IN BIOLOGICAL FLUIDS BY CAPILLARY GAS CHROMATOGRAPHY USING NITROGEN-SENSITIVE DETECTION

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SUMMARY

A sensitive and specific capillary gas chromatographic (GC) assay was developed for the quantitation of the quaternary ammonium steroidal neuromuscular blocking drugs pancuronium (PANC), vecuronium (VEC) and pipecuronium (PIP), as well as the metabolites 3-desacetylpancuronium (3-desPANC) and 3-desacetylvecuronium (3-desVEC) in plasma, bile and urine; the putative metabolite 3-desacetylpipecuronium (3-desPIP) was extracted and quantitated only in urine. The procedure employed a single dichloromethane extraction of the iodide ion-pairs of the monoquaternary or bisquaternary ammonium compounds (including internal and external standards) from acidified, ether-washed biological fluid followed by the formation of stable O-*tert.*-butyldimethylsilyl derivatives at the 3-hydroxy steroidal position of the metabolites. An automated capillary GC system fitted with a nitrogen-sensitive detector and an integrator was then used to analyze and quantitate both parent compounds and their derivatized metabolites. Optimal extraction, derivatization and GC conditions, as well as short-term stability and recoveries of these drugs and metabolites in plasma, are reported. Electron ionization mass spectrometry combined with GC was used to confirm the identities of compounds eluted from the column. The assay demonstrated a 10^3-fold linear range up to 5000 ng/ml for PANC, VEC, 3-desVEC and PIP, and lower limits of detection with adequate precision of 2 ng/ml for PANC, VEC and PIP, and 4 ng/ml for 3-desVEC; 3-desPANC was linear from 8 to 500 ng/ml while 3-desPIP was linear from 25 to 1000 ng/ml. The precision (coefficient of variation) of the calibration curves for underivatized drugs and their derivatized metabolites over the linear ranges was 2–20% and the reproducibility of the assay over a range of clinical concentrations of these drugs found in human plasma was 5–16% for PANC, 2–4% for VEC and 6–11% for PIP. No interferences were detected in the assay of plasma samples from 106 surgical patients.

INTRODUCTION

The quaternary ammonium steroidal compounds pancuronium (Fig. 1,I), vecuronium (VI) and pipecuronium (XII) are intermediate-acting and long-acting

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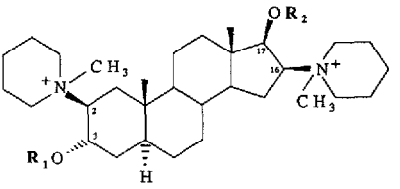
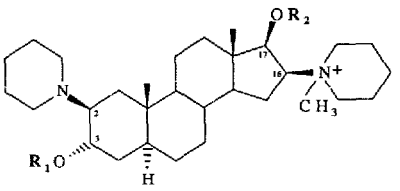
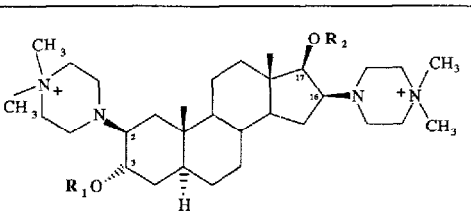
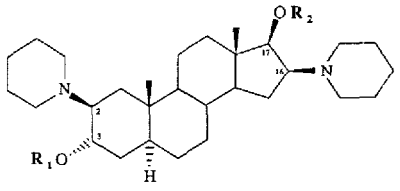
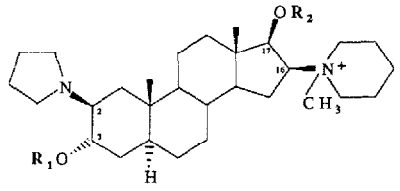
	NAME	R_1	R_2	
I	Pancuronium	Acetyl	Acetyl	
II	3-Desacetyl-pancuronium	H	Acetyl	
III	17-Desacetyl-pancuronium	Acetyl	H	
IV	3,17-*Bis*-desacetyl-pancuronium	H	H	
V		Propionyl	Propionyl	
VI	Vecuronium	Acetyl	Acetyl	
VII	3-Desacetyl-vecuronium	H	Acetyl	
VIII	17-Desacetyl-vecuronium	Acetyl	H	
IX	3,17-*Bis*-desacetyl-vecuronium	H	H	
X		Propionyl	Propionyl	
XI		Butyryl	Acetyl	
XII	Pipecuronium	Acetyl	Acetyl	
XIII	3-Desacetyl-pipecuronium	H	Acetyl	
XIV	17-Desacetyl-pipecuronium	Acetyl	H	
XV	3,17-*Bis*-desacetyl-pipecuronium	H	H	
XVI		Acetyl	Acetyl	
XVII		Acetyl	Acetyl	

Fig. 1. Structures of compounds I–XVII (pancuronium, vecuronium, pipecuronium, their deacetylated analogues, and suitable GC internal standards).

competitive neuromuscular blocking drugs which may be metabolized in humans to their respective 3-desacetyl, 17-desacetyl and 13,17-bisdesacetyl analogues (see Fig. 1). However, a sensitive and specific assay for the detection and accurate quantitation of these potential metabolites in biological fluids has not been available. Such an assay would not only allow pharmacokinetic analysis of each drug, but help clarify its pharmacodynamic–pharmacokinetic relationship, particu-

larly since 3-desacetylpancuronium (II) and 3-desacetylvecuronium (VII) are reported to have neuromuscular blocking activity [1,2]. The formation of deacetylated metabolites of XII and their neuromuscular blocking activities have not yet been reported for any species.

Although gas chromatography (GC) requiring prior thermal dequaternization has been used to quantitate certain quaternary ammonium compounds [3], it has not been applied to I, VI or XII. Fluorescent dye complex formation with I or VI and their deacetylated analogues has been utilized for the analysis of these drugs, with semi-quantification of the analogues requiring subsequent thin-layer chromatography (TLC) [4–6]. However, such assays using fluorescence detection without TLC suffer from lack of selectivity and reagent instability. Selectivity but not sensitivity has been enhanced using normal-phase liquid chromatography with UV detection [7]. Typically, the sensitivities of these assays are 20–50 ng/ml of plasma for parent compounds and 150 ng/ml for deacetylated analogues, making them inadequate for pharmacokinetic analysis where low drug dosages are administered.

We have previously reported a highly sensitive and specific assay for the quantification of I and VI in serum utilizing double organic ion-pair extractions and chemical ionization selected-ion mass spectrometry (CIMS) [8] in pharmacokinetic studies in normal surgical patients [9–11]. This sophisticated technique is not, however, readily available for routine laboratory use nor easily adaptable for the simultaneous quantification of potential deacetylated metabolites. We now report the development and validation of a routine automated capillary GC procedure allowing high throughput for the separate determinations of I and II, VI and VII or XII and XIII in biological fluids. We include studies on the derivatization conditions and chromatographic characteristics of the deacetylated analogues of each drug.

EXPERIMENTAL

Reagents

Dichloromethane (glass-distilled; Burdick and Jackson Labs., Muskegon, MI, U.S.A.), diethyl ether, acetone (AR; Mallinckrodt, St. Louis, MO, U.S.A.), acetonitrile (HPLC), granular potassium iodide (USP-FCC; J.T. Baker, Jackson, TN, U.S.A.), sodium dihydrogenphosphate (Aldrich, Milwaukee, WI, U.S.A.) and N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) (Pierce, Rockford, IL, U.S.A.) were obtained commercially. Saturated potassium iodide (6 *M*) was prepared just prior to use; 0.8 *M* sodium dihydrogenphosphate was stored at 4 °C.

Standard and reference compounds

Pancuronium, {1,1′-[(2β,3α,5α,16β,17β)-3,17-bis(acetoxy)androstan-2,16-diyl]bis-1,1′-methylpiperidinium} dibromide (I); 3-desacetyl- (II), 17-desacetyl- (III) and 3,17-bisdesacetylpancuronium (IV); vecuronium, {1-[(2β,3α,5α,16β,17β)-3,17-bis(acetoxy)-2-(1-piperidinyl)androstan-16-yl]-1-methylpiperidinium} bromide (VI); 3-desacetyl- (VII), 17-desacetyl- (VIII) and

3,17-bisdesacetylvecuronium (IX); and compounds V, X, XI, XVI and XVII which were used as internal standards, were supplied by Dr. D.S. Savage (Organon Scientific Development Group, Newhouse, U.K.). Pipecuronium, {2β,16β-bis-(4′-dimethyl-1′-piperazino)-3α,17β-diacetoxy-5α-androstane} dibromide (XII), was supplied by Dr. C. Beach (Organon, West Orange, NJ, U.S.A.); 3-desacetyl- (XIII), 17-desacetyl- (XIV) and 3,17-bisdesacetylpipecuronium (XV) were supplied by Gedeon Richter (Budapest, Hungary). Pancuronium purity was certified at 93.8%, vecuronium at 98.4% and pipecuronium at 100%; the purity of all other standard compounds was assumed to be 100% for analytical purposes.

Stock standard solutions (1 mg of each compound per ml) and, by serial dilution, working calibration and internal standard solutions were prepared in acetonitrile and stored at −20°C. V and XVII were used as internal standards for I, II, XII and XIII analyses, while X or XI and XVII were used for VI and VII analyses.

Apparatus

We used a gas chromatograph equipped with an automatic injector, a split/splitless injection system operated in the splitless mode and a nitrogen-sensitive detector (Models 5890A and 7672A; Hewlett-Packard, Santa Clara, CA, U.S.A.). Detector output was monitored by a recording integrator (Model 3392A, Hewlett-Packard). The chemically bonded fused-silica capillary columns tested were Durabond-5 (DB-5) columns with stationary phases coated at 0.1- and 0.25-μm film thickness (15 m×0.32 mm I.D.; J & W Scientific, Rancho Cordova, CA, U.S.A.) and an Ultra 2 cross-linked 5% phenylmethyl silicone column (12.5 m×0.31 mm I.D. and 0.17-μm film thickness; Hewlett-Packard). An Organomation N-Evap drying apparatus and Eberbach shaker were used for organic extractions.

Drug stability conditions

The stability of each drug was examined in normal human plasma at different temperatures (23 and 37°C) and under various pH conditions (pH 5.0, 6.0 and 7.4) over a period of 40 h. A phosphate–citrate–mannitol buffered solution (pH 4.0) [12] of each standard compound was prepared and added to plasma solutions of different pH to a final concentration of 500 ng/ml. Six 1-ml aliquots were taken at 0, 3, 21 and 40 h after each drug addition, acidified to pH 5.4 with sodium dihydrogenphosphate and frozen at −30°C prior to extraction and analysis.

Recoveries

Absolute and relative recoveries of I, VI, VII and XII were determined in human plasma, while absolute recovery of XIII was determined in plasma, buffered urine (50 μl) and buffered bile (200 μl). Known concentrations of each compound in equal volumes of acetonitrile were added to two sets of plasma samples (6×1 ml) and appropriate internal standards (i.e., monoquaternary or bisquaternary) were added to one set prior to extraction (set A) and to the other after extraction but before evaporation of dichloromethane (set B); both drug and internal standards were added to the dichloromethane extracts of a third set of

blank plasma samples representing the total amounts of drug added (set C). Absolute recoveries were estimated by GC comparison of drug/internal standard peak-height ratios obtained from set B versus those obtained from set C, and relative recoveries by comparison of set A versus set C. In addition, relative recoveries of I, V and XII were compared using either saturated 6 *M* potassium iodide or 6 *M* sodium perchlorate in the extraction procedure.

Injection port and detector temperatures

The GC injection port and detector temperatures were optimized for maximal peak-height responses to all compounds by holding the injection port at 270°C and varying the detector temperature between 280 and 320°C. The detector was then maintained at its optimal temperature while the injection port temperature was varied between 240 and 330°C.

Derivatization reaction conditions

The optimal temperatures and times for the complete formation of O-*tert.*-butyldimethylsilyl (O-*t*-BDMS) derivatives of II, III, IV, VII, VIII, IX and XIII were determined using standard compounds in acetonitrile, with known amounts of V as internal standard and 40 µl MTBSTFA. The sealed reaction tubes were allowed to stand for predetermined time periods (up to 80 h) at room temperature, 60 and 100°C, and the degree of derivatization was determined by comparison of peak-height ratios of each standard compound with internal standard.

Biological samples

For determination of drug stability, extraction recoveries and linearity, normal human blood bank plasma was used. For pharmacokinetic analyses, biological fluids were kept on ice until acidification within 1 h of collection. Heparinized blood was centrifuged and each 1 ml of plasma acidified with 1 ml 0.8 *M* sodium dihydrogenphosphate; bile and urine samples were acidified with 1 *M* phosphoric acid to pH 5.0 ± 0.2. All samples were stored at −30°C until analysis.

Procedure

The extraction procedure was similar to that reported for the MS analysis of I and VI [8] which used dichloromethane extraction and back-extraction of drugs from serum as iodide ion pairs. To 1 ml plasma acidified to pH 5.4 with 1 ml 0.8 *M* sodium dihydrogenphosphate, we added 500 ng of the appropriate internal standards in acetonitrile. Acidified urine (50–500 µl) and bile samples (1–100 µl) were diluted to 2 ml with 1 *M* phosphate buffer (pH 5.0) before analysis. All calibration and biological fluid samples received a total of 300 µl acetonitrile prior to extraction with 6 ml of diethyl ether for 10 min. After centrifugation and vacuum removal of both ether and the interface protein precipitate, 1.5 ml of saturated potassium iodide and 5 ml dichloromethane were added to the aqueous layer, and the mixture was shaken for 15 min. Following centrifugation, the upper dichloromethane layer was transferred to a 5-ml conical tube and dried under nitrogen at room temperature. If derivatization was required, the residue was dissolved in 100 µl acetonitrile and 40 µl MTBSTFA, the tube sealed with a PTFE-

lined screw cap and heated at 60°C for 6 h (VII) or for 40 h (II and XIII). The acetonitrile–MTBSTFA was then evaporated before the addition of 50 μl acetone for injection into the gas chromatograph.

GC was carried out on 2 μl of extract using an injection temperature of 300°C with a splitless time of 30 s. Helium was used for septum purge (2 ml/min), carrier gas (velocity 70–100 cm/s) and make-up gas (20 ml/min), and the detector temperature was 320°C. The temperature program routinely used began with an initial temperature of 150°C (0.5 min), was increased by 45°C/min to 286°C, then by 3°C/min to 310°C (2 min). Linear regression analysis was used to construct calibration curves of the standard/internal standard peak-height ratios versus each standard concentration.

Gas chromatography–electron ionization (EI) mass spectrometry

All CG-EI mass spectra were obtained using a VG 70-70H double-focusing mass spectrometer (VG Analytical, Manchester, U.K.) with associated VG 2025 data system. Chromatography was performed on a DB-5 column (10 m × 0.32 mm I.D., 0.25 μm film thickness) interfaced directly to the mass spectrometer. The injection port temperature was 300°C (splitless mode) and helium was the carrier gas (34 kPa head pressure); the interface temperature was maintained at 280°C. The temperature program started at 150°C (0.5 min), was increased by 40°C/min to 280°C, then by 2°C/min to 300°C (10 min). Operational parameters for the mass spectrometer were: source temperature, 220°C; ionization energy, 70 eV; emission current, 200 μA; resolution, 2000; scan speed, 5 s/dec; mass range, 750–200 daltons; and acceleration voltage, 4 kV (3 kV for XII, XIII, XIV and XV).

RESULTS AND DISCUSSION

Drug stability in human plasma

Human plasma samples for pharmacokinetic studies were acidified to pH 5.4 with 0.8 *M* sodium dihydrogenphosphate prior to analysis to ensure stability of I, VI and XII. Fig. 2 shows the stability of I, VI and XII in plasma over 40 h at pH values of 5.0, 6.0 and 7.4 and temperatures of 23 and 37°C (pH 7.4). The order of stability under these conditions was VI > XII > I. All three drugs appeared relatively stable at pH 5.0 while the decrease in VI between 21 and 40 h was not consistent with its relative rates of degradation at higher pH values. At pH 7.4, all drugs were relatively unstable and exhibited enhanced degradation rates at 37°C. Consequently, all biological samples for analysis were stored on ice until acidification within 1 h after collection. I and XII were stable in human plasma for at least four months when stored at pH 5.4 and −30°C.

VI has been reported to be very unstable in human plasma at pH 7.4 (7% remaining after 24 h at 22°C) [13], but we have not observed this degree of instability. MS studies in this laboratory have determined that I and VI were stable for at least 14 h at room temperature in acidified human serum (pH 5–6), but unstable at higher pH (7–8), with I being less stable than VI [8]. While I appeared to be more stable than VI in aqueous solutions [8,12], the reverse appeared true in unacidified normal human plasma.

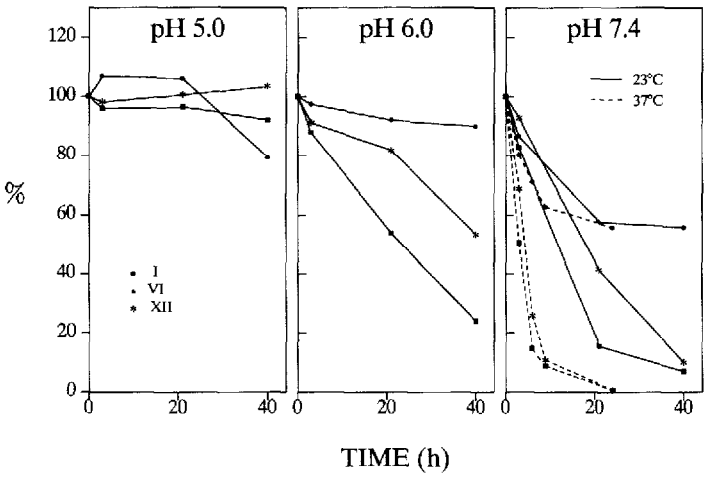


Fig. 2. Stability of I, VI and XII in human plasma at various pH values and temperatures. Values were derived using XI as internal standard and normalized to percentage of initial concentration at each pH value.

Absolute and relative recoveries

Absolute recoveries of I, VI, VII and XII from human plasma ranged from 72 to 81% at both high and low concentrations (see Table I). Relative recoveries ranged from 99 to 107% with good precision for I, VI and VII while those for XII were lower (87–93%), possibly due to its structural dissimilarity with the internal standard (V) used. Absolute recovery of XIII was <15% from plasma and buffered bile, but 76% from buffered urine. Dual internal standards were rou-

TABLE I

ABSOLUTE AND RELATIVE RECOVERIES OF I, VI, VII AND XII EXTRACTED FROM HUMAN PLASMA

Recoveries of I and XII were estimated using 500 ng V (internal standard) while those of VI and VII were estimated with 500 ng X (internal standard) ($n=6$).

Compound	Concentration added (ng/ml)	Absolute recovery (mean ± S.D.) (%)	Relative recovery (mean ± S.D.) (%)
I	562	76.2 ± 4.0	100.6 ± 2.9
	70.3	75.6 ± 3.7	100.8 ± 5.4
VI	659	77.3 ± 3.6	101.1 ± 3.6
	82.4	74.7 ± 5.1	99.2 ± 3.5
VII	541	80.4 ± 4.0	104.4 ± 8.7
	67.6	81.4 ± 5.9	106.8 ± 3.2
XII	589	74.7 ± 4.0	87.2 ± 4.3
	73.6	71.7 ± 3.9	92.5 ± 5.0

tinely used to monitor both recoveries and GC characteristics; however, only V, X and XI were used in quantitation and their choice depended on the quaternary nature of the drug analyzed. Reliable and accurate absolute recoveries could be estimated only when standard drugs and internal standard were added in acetonitrile to dichloromethane extracts of blank plasma and not when they were dried down alone and injected directly into the GC system. In the latter case, absolute GC peak heights of unextracted drugs were markedly decreased indicating either glass absorption or a requirement of extracted iodide for ion-pair formation before reconstitution in acetone. When the relative recoveries of I, V and XII were compared at pH 5.4 using either iodide or perchlorate as the pairing ion, iodide provided greater recoveries for all three compounds.

Injection solvent

The most suitable injection solvent for these drugs and their derivatized analogues should allow maximum solubility, maintain stability and cause minimal deterioration of, or interference with, the GC column and the nitrogen-selective detector. We compared the GC responses of structurally representative compounds (I, II, V, VII, VIII, IX, XII, XIII, XVI and XVII) in acetone, methanol, ethanol, isopropanol, 2-butanone, 3-butanone, ethyl acetate and benzene, and determined that acetone provided optimal responses for quaternized compounds under the chromatographic conditions of the assay. The decreased GC responses of most compounds observed with the other solvents probably reflected decreased solubilities, as suggested by concomitantly increased GC responses to non-quaternized XVI. 2,4-Pentandione caused considerable column damage on two occasions and was subsequently excluded.

Optimal GC injection conditions

Quaternary ammonium compounds appear to require thermal dequaternization in order to achieve good resolution, peak shape and sensitivity by GC [3,14]. This has been achieved chemically [15] or thermally using a pyrolysis chamber prior to GC [16]. However, these techniques are imprecise and time-consuming, and chemical dequaternization requires extremely anhydrous conditions. Although EIMS of the eluting GC peaks of authentic quaternized compounds indicated they had undergone N-demethylation (see *GC and GC–EIMS of I, VI, XII and their derivatized analogues*), this detection technique cannot distinguish between N-demethylation occurring prior to GC or after elution of the compounds into the mass spectrometer source. Our observation that I, VI and XVI co-elute under the same GC temperature conditions (see Table II) suggested that these compounds undergo thermal dequaternization in the injection port. However, when I, VI and XVI were injected on-column both simultaneously and separately at an oven temperature of 50°C followed by the routine temperature program used, we found that all three compounds co-eluted, indicating that in this mode they may undergo thermal dequaternization at a particular column temperature before chromatography.

The splitless rather than split injection mode was used to enhance the sensitivity of GC detection of all compounds. Although on-column injection produced a four-fold increase in sensitivity of detection over splitless injection, this mode

could not be automated at the time of assay development. Injection port temperatures ranging from 240 to 340 °C were tested for maximal detector responses using integrated peak heights. Maximal responses occurred at 290 °C for I, V, VI, VII, XVI and XVII, and at 300 °C for II, VIII and IX; XII and XIII demonstrated maximal sensitivity at 310 °C. We selected an injection port temperature of 300 °C and detector temperature of 320 °C for analyses of all drugs and analogues.

Derivatization conditions for deacetylated analogues

Investigation of potentially suitable derivatives of the deacetylated analogues of I, VI and XII which would demonstrate enhanced volatility and acceptable GC characteristics focused on O-trimethylsilylation (O-TMS) and O-*t*-BDMS. It was necessary that these derivatives be stable under the storage and chromatographic conditions to be used. O-TMS derivatives which were readily formed under mild heating conditions were unstable in acetone whereas O-*t*-BDMS derivatives were stable for at least one month at room temperature. Each O-*t*-BDMS-derivatized analogue also gave rise to a single, symmetrical peak well separated from its parent drug and other derivatized analogues (see Fig. 3).

Derivatization was studied at 22, 60 and 100 °C for up to 80 h and showed that analogues of I and XII were much more difficult to derivatize than those of VI. Derivatization at high temperatures for long periods may cause unnecessary decomposition (e.g., deacetylation) of either drug or internal standard, resulting in artificially lowered levels of drug and artificially elevated levels of derivatized analogues. Optimal derivatization conditions for the 3-desacetyl analogues, therefore, were determined as the time and temperature necessary to achieve

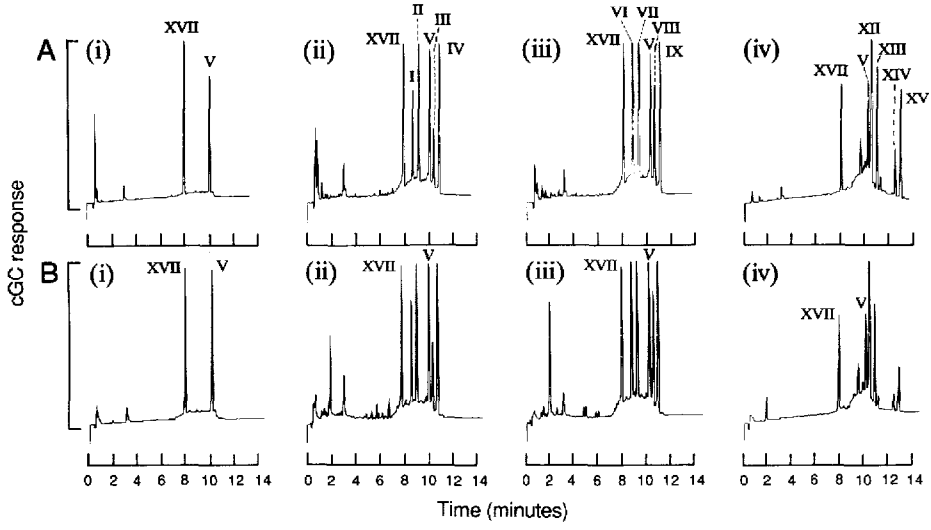


Fig. 3. Representative capillary gas chromatograms of (i) internal standards (V and XVII), (ii) I and its O-*t*-BDMS-derivatized analogues, (iii) VI and its O-*t*-BDMS-derivatized analogues and (iv) XII and its O-*t*-BDMS-derivatized analogues on (A) DB-5 (15 m) and (B) Ultra 2 (12.5 m) columns. Peak identification is shown in Fig. 1; sample peaks in (A) correspond with those in (B) and GC conditions are described in text.

adequate and reproducible derivatization without causing significant degradation (i.e., >5%) of drug and/or internal standard. Some thermal decomposition of both drug and internal standard was observed at 100°C, precluding accurate quantitation at this derivatization temperature. Such decomposition was significantly reduced during derivatization at 60°C, allowing accurate and precise determination of II (from 8 to 500 ng/ml), VII (from 4 to 5000 ng/ml) and XIII (from 25 to 1000 ng/ml in buffered urine). Because of the relatively mild derivatization conditions required for VII, we performed routine analyses of VI and VII with derivatization at 60°C for 6 h (1.5% degradation of VI to VII). Routine analyses of I and XII were carried out without prior derivatization; however, if II or XIII were to be determined, derivatization was carried out at 60°C for 40 h.

GC and GC–EIMS of I, VI, XII and their derivatized analogues

Several fused-silica capillary columns (10–30 m) with different chemically bonded stationary phases and film thicknesses were assessed for chromatographic suitability. Because these high-molecular-mass quaternary ammonium steroidal compounds eluted at relatively high temperatures (>280°C), short (10–15 m) columns were necessary to minimize the required chromatographic analysis time. A comparison of Ultra 2 and DB-5 columns of similar length, internal diameter, stationary phase and film coating revealed equivalent chromatographic characteristics (see Fig. 3). Both the Ultra 2 (12.5 m) and DB-5 (15 m) columns selected provided optimal sensitivity and stability and 15-min analysis time per sample, although more precise results were obtained with the Ultra 2 column. Table II lists the relative GC retention times of suitable internal standards, I, VI, XII and their O-*t*-BDMS-derivatized analogues on both columns.

Relative GC retention times and EI mass spectra of peaks were used to confirm

TABLE II

RELATIVE GC RETENTION TIMES OF DRUGS AND THEIR O-*t*-BDMS-DERIVATIZED ANALOGUES ON ULTRA 2 (12.5 m) AND DB-5 (15 m) COLUMNS USING SPLITLESS INJECTION

Compound	Relative retention time	
	Ultra 2	DB-5
XVII	0.796	0.765
I/VI/XVI	0.874	0.856
II/VI	0.920	0.911
XI	0.989	0.987
V	1.000	0.999
X	1.000	1.000
XII	1.039	1.038
III/VIII	1.044	1.049
IV/IX	1.086	1.095
XIII	1.088	1.094
XIV	1.246	1.252
XV	1.288	1.295

the identities of each drug and its O-*t*-BDMS-derivatized analogues. These quaternary ammonium compounds will not give rise to true molecular ions since dequaternization occurs either in the GC injection port or in the mass spectrometer source, resulting in $(M-CH_3Br)^+$ or $(M-2CH_3Br)^+$ ions; only XVI will yield an $(M)^+$ ion. All drugs and their respective derivatized analogues yielded appropriate $(M-xCH_3Br)^+$ ions and fragmentation patterns consistent with their molecular structure, except 17-O-*t*-BDMS-XIV which gave rise to a major fragment ion indicating loss of HO-*t*-BDMS, acetic acid and a methyl radical, but no $(M-2CH_3Br)^+$ ion.

GC–EIMS analysis of an extracted and O-*t*-BDMS-derivatized plasma sample from a patient given VI (100 μg/kg, intravenously) verified that the compounds eluting with the same retention times as those of VI and derivatized VII were identical to authentic compounds.

Linearity and sensitivity

The assay showed good linearity in plasma, urine and bile over 2–5000 ng/ml for I, VI and XII, 8–500 ng/ml for II and 4–5000 ng/ml for VII. The lower limit of linearity (signal-to-noise ratio > 3) for VI was unaffected by the mild derivatization reaction conditions used to measure VII simultaneously, whereas the sensitivities for I and XII were affected by the prolonged heating at 60 °C necessary to measure II and XIII in the presence of their respective parent drugs. In addition, the absolute sensitivity of the assay for I was adversely affected by the presence of interfering compounds present in some batches of internal standard V which coeluted with I by GC. Consequently, quantitation of both I and XII in biological fluids was performed routinely without derivatization. Using derivatization conditions of 60 °C for 40 h, good linearity and precision could be demonstrated for II from 8–500 ng/ml of plasma; the variability observed above this range was due to either variable recovery or incomplete derivatization. Because of the low and variable recoveries of XIII from plasma and buffered bile (< 15%), linearity was demonstrated only in buffered urine (50 μl) from 25 to 1000 ng/ml. Accurate determination of II and XIII concentrations in biological fluids required the inclusion of separate calibration samples of I with II, and XII with XIII. Finally, linearity of both VIII and IX was demonstrated from 30–2000 ng/ml, while that of the comparable analogues of both I and XII has not yet been determined.

Reproducibility

The precision of the assay for I, VI, VII and XII at various concentrations in plasma samples from human subjects participating in pharmacokinetic studies is summarized in Table III. No precision data on II are presented since levels above 15 ng/ml were not detected in these plasma samples. The relative contributions of the reproducibility of GC injection of the three drugs to the overall precision of the assay also are shown.

Reference method comparison

Using plasma samples obtained from a patient given I by infusion (25 μg/kg over 7 min), we compared drug concentrations estimated by CIMS as a reference method [8] with those by GC (see Fig. 4). Linear regression was used to evaluate

TABLE III

ASSAY AND GC REPRODUCIBILITY IN PATIENT PLASMA SAMPLES

Plasma concentrations were estimated from calibration curves and the GC injection precision was measured as a component of assay precision.

Compound	Assay precision			Precision of GC injection
	n	Concentration (mean ± S.D.) (ng/ml)	C.V. (%)	C.V. ($n=10$) (%)
I	10	85.8 ± 6.2	7.2	2.4
	10	65.1 ± 3.0	4.6	–
	10	18.0 ± 2.8	15.6	5.2
VI	4	809 ± 19	2.3	2.1
	7	85 ± 3.0	3.6	2.1
VII	4	44.3 ± 0.8	1.9	–
	7	13.9 ± 0.4	2.7	–
XII	5	329 ± 18.7	5.7	2.1
	5	83.3 ± 5.8	6.9	3.1
	13	26.6 ± 3.0	11.2	3.8

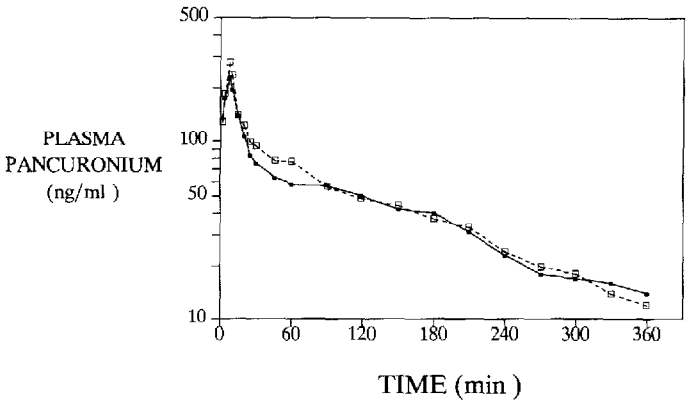


Fig. 4. Comparison of human plasma pancuronium (I) concentrations in timed samples analyzed by CIMS □ and GC ■ after infusion of I (25 μg/kg over 7 min).

results at the same time points and yielded a line $y=0.86x+4.3$ ($r^2=0.981$) indicating a strong linear relationship between the data points generated by the two assays.

CONCLUSION

We have developed an automated, highly selective and sensitive capillary GC assay useful for the separate determination of I and II, VI and VII, and XII and XIII in biological fluids. The assay allows high throughput of samples but cannot be used to quantitate simultaneously I and VI and their respective deacetylated analogues. However, although only one muscle relaxant is usually administered

to surgical patients at a time, the assay would allow simultaneous quantitation of XII in the presence of either I or VI. The assay has demonstrated good precision and sensitivity to 2 ng/ml for each drug and a dynamic (10^3-fold) linear range for I, VI, VII and XII, and 10^2-fold for II and XIII. It has been applied to the pharmacokinetic analysis of these drugs in normal surgical patients and in patients with impaired renal or hepatic function and allowed analysis of new quaternary ammonium steroidal neuromuscular blocking drugs of similar structure. The ability to link the GC output to a mass spectrometer has also allowed confirmation of VII as a metabolite of VI in humans.

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