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Pancuronium Bromide (Pavulon) Isolation and Identification in Aged Autopsy Tissues and Fluids

ABSTRACT: The isolation and detection of pancuronium bromide was developed for aged autopsy samples to identify and confirm this compound in questioned tissue samples. A novel protocol was optimized for the isolation of the target drug in highly decomposed tissues. Solid-phase extraction (SPE) cartridges containing styrene-divinylbenzene were investigated. This polymer retained quaternary drugs and facilitated sequential elution upon washing with commonly available solvents. The semi-purified SPE samples were prescreened by pyrolysis GC-MS. A candidate specimen was then confirmed by microbore high-performance liquid chromatography/electrospray-ionization/mass spectrometry (μHPLC-ESI-MS/MS) with a triple-quadrupole mass spectrometer. The developed procedures provided a qualitative or semiquantitative (at best) basis for the investigation of difficult cases involving overdoses of polar drugs.

KEYWORDS: forensic science, aged exhumation tissues, Pancuronium bromide (Pavulon), gas chromatography-mass spectrometry (GC/MS), solid-phase extraction (SPE), liquid chromatography-electrospray ionization mass spectrometry (μHPLC-ESI-MS/MS)

Pancuronium Chemistry

Pancuronium bromide is a steroid and diquaternary ammonium salt: 1,1′-(3α,17β-dihydroxy-5α-androstan-2β,16β-ylene)bis [1-methylpiperidinum]-diacetate dibromide (1). The drug (Fig. 1) is stable in powder form; however, the bromine groups are quickly exchanged in an aqueous environment. The compound is water-soluble, yet capable of binding strongly to proteins and complexing with lipid materials. The pharmacokinetic data (2) following a therapeutic dose of the drug indicate that 40% of the parent drug and its metabolites are found in the urine and 11% in the bile. Thirty to 87% of the drug binds to protein. Pancuronium strongly binds to gamma globulin and moderately binds to albumin. Approximately 25% of an injected dose may be recovered as the 3-hydroxy metabolite, while less than 5% is found as the 17-hydroxy metabolite. Both metabolites are significantly less potent than the parent drug, and no published method has provided an isolation protocol or measured the biological activity of the 3,17-dihydroxypancuronium metabolite. The half-life of pancuronium in plasma is in the range of 89–161 min (2). Pancuronium should therefore be effectively eliminated from the blood after 15–27 h (10 half-lives) in patients with normal liver and kidney function. Patients with hepatic difficulties exhibit a doubling of the drug half-life (2).

The typical intravenous dose of pancuronium is 0.04–0.1 mg/kg. Increasing the dose and plasma concentration (3) results in 50% paralysis at 0.09 μg/mL, 80% paralysis at 0.17 μg/mL, and 99% paralysis at 0.22 μg/mL. The intravenous LD_{50} in animals is 0.016–0.15 mg/kg (3), and rabbits appear more sensitive to the drug than rats. Calculated values for humans indicate that a lethal dose of 0.15 mg/kg could be achieved with a single 10.5-mg intravenous injection in a 70-kg individual. Health- compromised patients would be expected to be more sensitive to the drug.

The opposing ammonium groups and lipid nature of pancuronium necessitate unique protocols to isolate the drug from complex biological matrices. Published methods for characterizing pancuronium in plasma and urine samples include colorimetry and fluorometry (4–6), thin-layer chromatography (TLC) (7), high-performance liquid chromatography (HPLC) with an ultraviolet detector (8,9), gas chromatography (GC) with a nitrogen-sensitive detector (10), and pyrolysis probe distillation with chemical ionization mass spectrometry (11). Plasma can be processed utilizing Rose Bengal (5), bromophenol blue (12), sodium perchlorate (6), or with potassium iodide (11) for ion-pair extractions into neutral solvents. These methods are sensitive to 1 ng/mL for fresh plasma and urine samples. Although sensitive, these previously published methods did not address aged autopsy tissue samples, nor characterize the parent drug directly. Therefore, experiments were performed to develop a protocol to optimize the isolation and characterization of pancuronium in more diverse and difficult questioned samples submitted for pancuronium screening, confirmation, and analysis.

Pancuronium Analysis Scheme

An analysis protocol was developed to process and analyze aged autopsy samples. Generally, tissue samples are recorded, weighed, homogenized with buffer, and passed through a solid-phase extraction (SPE) polymer. The polymer is then extracted with solvents, the extracts evaporated to dryness, the residue dissolved in methanol, and the processed sample screened with gas chromatography-mass spectrometry (GC-MS). Positive GC-MS results are then confirmed using a micro-bore chromatography column interfaced to a high-performance liquid chromatograph (μHPLC). The HPLC is coupled to a tandem, triple-quadrupole mass spectrometer with an electrospray ionization source (ESI-MS/MS) tuned to selectively filter the doubly charged molecular ion of pancuronium.

Materials and Methods

Sample Acquisition

Tissue samples from exhumations were logged into the chain-of-custody system and assigned a unique number for cross-referencing

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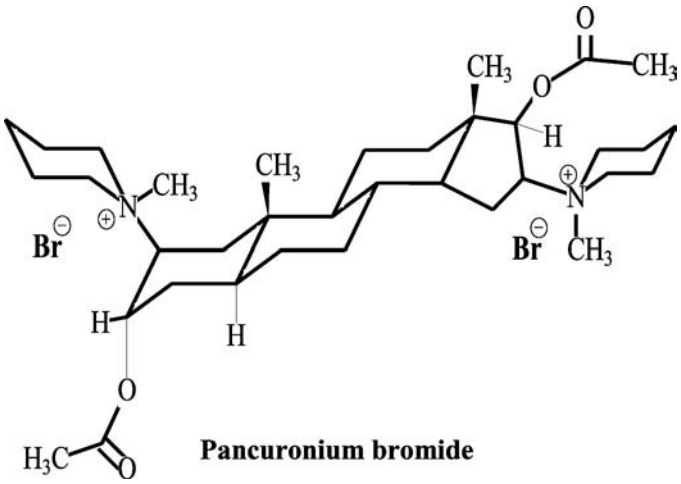

FIG. 1—*Structure of Pancuronium bromide (Pavulon) revealing two quaternary ammonium group separated by a highly lipid steroid structure. The combination of two ammonium groups and the lipid nature of the steroid ring system generate a drug with very unusual chemical and mass spectral properties.*

purposes. Blank solvent samples and control tissue samples (spiked pig liver) were likewise analyzed in a manner identical to the questioned samples. Initial studies were conducted with fresh, store-bought liver, while later experiments used the same material that had been aged via storage for several weeks at room temperature (in a fume hood). Commercial embalming fluids identical to those used prior to burial of the questioned bodies were obtained and subjected to the analysis protocols along with the exhumation tissues.

Reagents and Extraction Supplies

All reagents and solvents were of analytical-grade or better. Sodium dihydrogenphosphate (NaH_2PO_4), HPLC-grade water and pancuronium bromide were obtained from Sigma Chemical Co. High-purity (GC-MS) methanol was obtained from Burdick and Jackson. Solid-phase extraction cartridges [styrene divinylbenzene (SDvB)] were obtained from J.T. Baker, (Holland).

Sample Preparation

In order to efficiently process a large number of complex samples in parallel, a new protocol was developed to avoid liquid-liquid ion-pair solvent extractions. Preliminary investigations explored different solid-phase extraction (SPE) cartridges. Five commercial SPE cartridges (Nexus, Florisil, SDvB, Oasis, and CN) and one silica gel cartridge were prepared. Twenty mL of a 250-ng/mL pancuronium solution in 0.8 M sodium dihydrogenphosphate were initially passed through each of the six SPE cartridges under reduced pressure. When dry, each cartridge was subsequently eluted with a variety of solvent combinations and assessed with TLC to determine the most efficient system for the isolation of pancuronium. From these studies, the styrene-divinyl-benzene (SDvB) SPE cartridge was found to efficiently retain the analyte. The parent drug was optimally recovered from the SDvB cartridge by a series of tandem leaching procedures. These were water (1.5 mL, to remove salts); methanol (3 mL, to remove extraneous organic compounds); and finally acetic acid/water (1:1, 3 mL) to elute and isolate pancuronium. Pancuronium was consistently recovered in yields exceeding 90% by the acetic acid/water extraction. Further studies with pig liver samples, aged from weeks to months under ambient conditions, also revealed that only the SDvB column extraction efficiencies were not degraded when exposed to increased amounts of biological contamination. Most background contamination could be removed by the water and methanol treatments, and the pancuronium extract could then be efficiently collected by the 50% acetic acid/water extraction. Evaporation of this final extract by heating at 100°C under a stream of helium gas produced the final sample. The isolated pancuronium could then be redissolved in methanol for analysis. Two μL of this solution were used for all mass spectral analyses.

Tissue samples from 10 to 40 g of questioned specimens were prepared for analysis. The size of the sample was determined by the total quantity of specimen submitted. The weighed sample was thawed and cut into small, 0.5-cm^3 sections for homogenization in a stainless steel Waring tissue blender along with an equal volume of 0.8 M NaH_2PO_4buffer in HPLC-grade-water (pH 4.5). After blending the tissue and buffer to a consistent paste, the material was vacuum-filtered at room temperature through a sterile, 0.8-μm Nalgene biological filter. The filtration time ranged from one hour to four days, depending upon the level of tissue putrefaction and mucous content. The amber-to-yellow filtrate was collected and refrigerated prior to SDvB-SPE extraction. The tissue residue was removed from the Nalgene filter and frozen for archival storage.

A series of 1.5-mL SDvB cartridges was placed in their vacuum SPE holders with corresponding liquid-sample reservoirs. A separate SPE cartridge was used for each tissue sample. Approximately 10–40 mL of the NaH_2PO_4buffer/tissue extract were slowly passed through the SPE cartridge at a rate of one drop/second. After the sample had completely passed through the column, the SPE resin bed was washed with 1.5 mL of water to remove inorganic salts. The aqueous fractions were removed from the SPE apparatus and archived.

The SPE vacuum chamber was next fitted with clean 4-mL collection vials for the SDvB solvent extractions. The SDvB columns were extracted with 1.0-mL methanol aliquots three times. During each extraction, the cartridges were allowed to leach and equilibrate for 5.0 min prior to elution. The three methanol extractions were then combined, evaporated to dryness, and archived.

Another SDvB-SPE extraction process was then conducted with collection into a clean 4-mL vial. The SPE cartridge was finally eluted with 3 × 1.0-mL aliquots of 50% acetic acid/water. Again, for each 1.0-mL extraction, the cartridge was allowed to leach and equilibrate for 5.0 min prior to elution. The vials containing 3.0 mL of acetic acid/water extracts were transferred to a Reacti-Therm Heating Module. The heating block was set at 100°C, and evaporation of the extraction solvents to dryness was performed under a stream of pure helium gas. Each sample residue obtained from the SDvB-SPE extraction was then reconstituted in 200 μL of methanol for subsequent analysis by GC-MS and μHPLC-ESI-MS/MS.

The methanol fractions were used for initial broad chemical and drug screening by GC–MS, while the 50% acetic acid/water fractions were assayed for pancuronium by both GC-MS and μHPLC-ESI-MS/MS. Because of the quantity of proteins, amino acids, and organic materials extracted from the SPE column, re-dissolution of some residues also required an ultrasonic bath and mechanical agitation.

Mass spectral analyses required removing all vestiges of precipitate from the SPE extracts. This was accomplished by

transferring exactly 100 μL of the SDvB eluent into microvials for centrifugation at 4,000 rpm. Eighty μL of the resultant particulate-free liquid were then transferred to a 400-μL conical vial for mass spectral analysis.

Thin–layer chromatography (TLC) was developed to optimize the extraction of control samples prior to GC-MS screening. SDvB cartridges were spiked with 10-100 μg of pancuronium standard and processed. Five-μL aliquots of the final 200-μL methanol solutions were spotted on TLC plates and developed to monitor the recovery of pancuronium from the SPE cartridges. Silica-gel TLC plates (Alltech Nano-Sil-20-UV254) were eluted using a published (7) solvent system of pyridine/butanol/acetic acid/water/ammonium chloride. Pancuronium was visualized using Dragendorff's spray reagent (bismuth subnitrate/acetic acid) as an orange spot at an R_f value of 0.27. The limit of detection for pancuronium by the TLC method was approximately 500 ng. TLC was therefore used to optimize the SDvB–SPE method.

Instrumental

All mass spectrometer instrumentation was mass calibrated prior to the analyses of experimental samples. Standard chromatographic protocols were followed to ensure the quality of the data and proper interpretation of the results. All analyzed samples were first screened for indicative compounds using a Hewlett-Packard (HP) 5973 GC-MS, with auto-sampler, under electron-ionization (EI) mode. The auto sampler allowed hundreds of samples to be screened efficiently for target compounds. The HP 5973 GC–MS was mass calibrated with perfluro-t-butylamine (PFTBA) and manufacturer-recommended tuning parameters. A standard pancuronium solution (10 μL of 2.5 ng/μL concentration) was assayed to obtain qualitative calibration data, while methanol solvent was used for blank determinations. A methanol blank was processed at the beginning, in the middle, and at the end of each series of samples.

The GC-MS was scanned continuously from 55 to 500 amu at 1.5 scans/s. Two-microliter aliquots were the experimental volumes. Ultrahigh-purity helium was the carrier gas (1 mL/min), and the instrument was programmed to thermally degrade pancuronium at 300°C in a glass-lined injection port of the gas chromatograph. The oven was held at 170°C for 5 min and ramped at 20°C/min to 300°C. Pyrolysis products were separated on a 15-m capillary column (0.25-mm i.d., 0.25-μm film, DB-5MS) and identified using commercial mass-analysis software. The presence of pancuronium was indicated by first generating a mass-chromatogram plot of m/z 340 from the GC-MS total ionization data, and secondly, through inspection of the complete mass spectral data of the extracted m/z 340 chromatographic peak. Positive preliminary results for pancuronium were taken to be mass chromatogram m/z 340 peak intensities greater than 10 times background at a retention time of 7.9–8.0 min. Other indicative fragment ions were m/z 98, 124, 286, 356, and 415. These ions provided further indication that pancuronium could be present in a sample. Manual examination of the mass spectral data and fragment-ion ratios generated from unknown samples, when compared to standard pancuronium data, was then utilized to further support preliminary pancuronium findings.

Confirmation of pancuronium in samples that had screened positive by pyrolysis GC-MS was made using μHPLC-ESI-MS/MS: a Thermo Finnigan TSQ 700 instrument was equipped with a microbore HPLC column (Zorbex, 1.0 × 115-mm C-18 column packed with 5-micron beads and 100 Åpore size, Michrom BioResources, Inc., Auburn, CA). The instrument utilized ICIS 8.3.0 SP2 for OSF1 (V4.0) software and was mass calibrated by following established Finnigan procedures using a standard mixture of myoglobin and MRFA (L-methionyl-arginyl-phenylalanyl-alinine) in combination with strict elution and mass-fragment, ion-selection criteria for all sample analyses. Prior to the analysis of each sample set, a 25-ng pancuronium standard in methanol was analyzed to assure instrument stability, sensitivity, and accuracy. The electrospray needle was operated at 4.8 kV, the temperature of the heated capillary inlet port was maintained at 200°C, nitrogen sheath gas was set to 35 psi, the offset energy of the collision cell was −23 V, and argon (2 mTorr) was used as the collision gas. Every questioned sample was preceded in an analytic series by a solvent blank, and each series was terminated with a pancuronium standard to re-verify instrument performance. To further insure that no false positive results were generated, aged tissue samples were intermittently extracted and placed into the sample stream for processing. All of these acceptance criteria were satisfied for all runs, and no false-positive results were observed.

A positive confirmation for pancuronium was taken to be an μHPLC-ESI-MS/MS mass chromatogram m/z 430 peak intensity greater than 10 times background. The m/z 430 product ion is a characteristic fragment ion of pancuronium and is exclusively derived from the doubly charged molecular ion (m/2z 572 = m/z 286). The presence of m/z 100 and 207 product ions was also required to confirm pancuronium in questioned samples. Fragment ions were identified and their intensity ratios verified manually. Additionally, peak ratios from mass chromatogram plots of key ions were evaluated by the analyst and used to make compound identification. Mass spectral data were further assessed through independent comparisons to authentic pancuronium MS/MS electrospray ionization data. In practice, the four major fragment ions of pancuronium (m/z = 100, 207, 430, and 472) were assessed, and the various ion intensities of a putative positive sample had to match those of the standard to approximately ± 20%. Positive confirmation thus constituted ion intensities > 10 × background with ion-ratio variations no larger than 20%.

To further reduce residual matrix contamination from the extracted tissue samples, and to aid in the mass spectral confirmation, all samples were separated into constituent species with a microbore HPLC column interfaced to a high-performance liquid chromatograph (μHPLC). Following injection of the sample onto the μHPLC column, a gradient elution of the system was programmed. A dual-solvent system of A: acetonitrile, isopropanol, water, acetic acid, and heptafluorobutyric acid [1-mL/1-mL/98-mL/100-μL/100-μL], and B: acetonitrile, isopropanol, water, acetic acid, and heptafluorobutyric acid [80-mL/10-mL/10-mL/80-μl/100-μL was used (13). Solvent system A was transitioned to solvent system B over a period of 30 min at a flow rate of 50 μL/min.

To assure lack of carryover from one sample to the next, a solvent blank was analyzed before and after each questioned sample. The retention time of pancuronium under these chromatographic conditions was approximately 16.5–17.5 min, depending upon the complexity of the sample (Fig. 2). Decreasing concentrations of standard solutions were successfully analyzed to an approximate (on-column) limit-of-detection of 1 ng/10 μL injected (based on standard addition into extracts of post-mortem embalmed tissues).

Results

Post-mortem tissue samples can exhibit widely varying degrees of decay and contamination (e.g., putrefaction biochemicals; insect

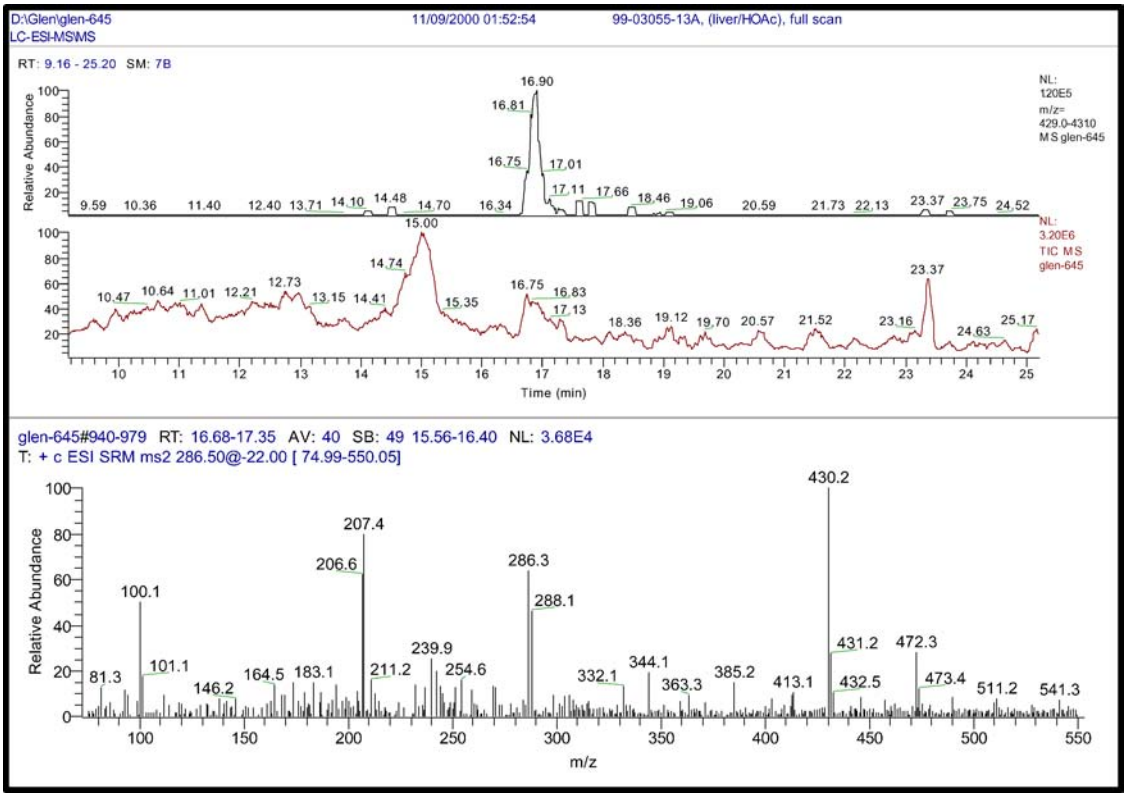

FIG. 2—*μHPLC-ESI-MS/MS conformational analysis of a typical lung tissue extract that had indicated positive for pancuronium by pyrolysis GC-MS prescreening. The mass chromatogram plot of m/z 430 maximized at 16.9 min (upper trace) and generated the confirming mass spectrum (bottom plot) with daughter ions at m/z 100, 207, 430 and 472.*

infestation; saturation with embalming fluids that include formaldehyde, dyes, etc.). In this investigation, all questioned samples contained high background concentrations of extraneous compounds that precluded or seriously reduced the isolation efficiency of previously published methods. To further complicate the measurement of pancuronium, the recovery of the target drug was expected to be very low because of its known high protein binding and the appreciable lengths of time between death and sample collection. Initially, literature review and laboratory experiments evaluated well-established methods that implemented ion-pair formation and liquid-liquid extraction (8–12) to isolate pancuronium. However, these techniques were found to be very problematic for aged autopsy tissues and were abandoned due to the formation of large amounts of persistent emulsions requiring centrifugation and further processing. In addition, samples processed with those protocols also extracted a significant amount of salt (e.g., KI) that clogged the electrospray ionization source of the μHPLC-ESI-MS/MS.

Gas Chromatography-Mass Spectrometry (GC-MS) Screening for Pancuronium

Salts are not amenable to gas-chromatographic analysis. However, a methanol solution of Pavulon injected into a 300°C inlet port of a Hewlett-Packard 5973 GC-MS generated a number of indicative chemical species. A pure standard showed that the major pyrolysis product was a species consisting of the pancuronium parent less two quaternary methyl groups (Fig. 3; retention time of 17.5 min). Indicative mass spectral data for this compound yielded the molecular ion at m/z 542 and characteristic ions at m/z 467 and 124.

In addition to the major pyrolysis product, a group of more volatile components was also produced in the hot GC injector port (Fig. 3; retention time 6.0–9.5 min). One isomeric set of compounds yielded a molecular ion at m/z 415 and unique fragment ions at m/z 340 and 356. Although other thermal degradation compounds were also generated, at low pancuronium concentrations the appearance of m/z 340 was more intense under these GC-MS conditions and was very indicative of the presence of pancuronium in questioned samples. Figure 4 depicts the possible ion-formation pathways corresponding to the observed mass spectral fragmentation pattern generated from a pancuronium pyrolysis product of molecular weight 415.

When a mass chromatogram plot of m/z 340 was generated from GC-MS data from a pancuronium-containing biological sample (Fig. 5), a characteristic "fingerprint" peak provided an indication at ~9.0 min that pancuronium was present in the sample. This method of data display allowed the assay of complex mixtures for low concentrations of pancuronium that would otherwise be effectively masked by the GC-MS total ionization baseline. To demonstrate the effectiveness of this GC-MS screening procedure, an acetic acid/water extraction sample from decomposed human lung tissue was spiked with pancuronium at a concentration of 5 ng/μL and re-analyzed a second time as a spiked control sample. Although previously negative for pancuronium, the specimen now gave a positive indication of pancuronium by the thermal degradation GC-MS techniques. A ballpark sensitivity for this technique can be estimated from the fact that the experiments were conducted

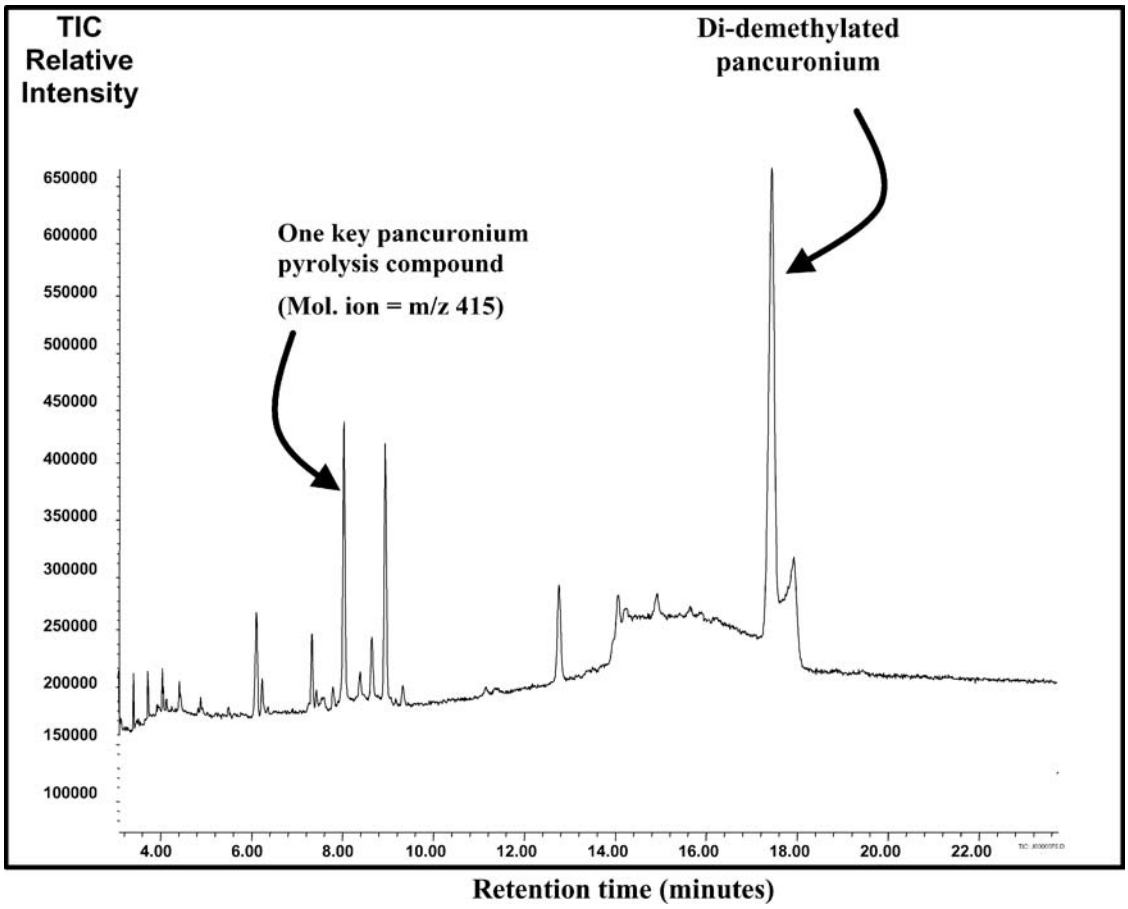

FIG. 3—*GC-MS total ionization plot of pancuronium thermal decomposition products. The di-demethylated pancuronium was observed at 17.5 min. Additional thermal decomposition compounds were observed between 6.0–9.5 min. A key indicator compound of pancuronium, with molecular weight m/z 415, was dominant in positive samples.*

with 20 g of homogenized lung tissue, spiked with 20 ng/g of pancuronium, to give a GC-MS total-ion chromatogram S/N > 10:1.

Electrospray-MS/MS Analysis for Pancuronium Confirmation

Electrospray mass spectral analysis for pancuronium was developed to confirm the presence of the pancuronium parent drug in post-mortem samples. Using a Thermo Finnigan TSQ 700 tandem mass spectrometer, a control solution (200 ng/μL) of a pancuronium standard in water/methanol/acetic acid (50 mL/50 mL/5 mL) was infused into the ESI source. The full-scan mass spectrum revealed minor fragments and an abundant m/z 286 ion (Fig. 6). This ion is undoubtedly the doubly charged 572 amu pancuronium molecular ion recorded at one-half the molecular weight. Other fragment ions at m/z 472 (M^+-100) and m/z 430 (M^+-100-42) indicated losses of a methylpiperidine ring and ketene group, respectively (Fig. 6, insert). Electrospray ions at m/z 651 and 653 indicated the addition of bromine ($M^+ + 79/81$) to form singly charged pancuronium, while the ion at m/z 631 ($M^+ + 60$-1) likely resulted from the addition of an acetate anion to the doubly charged parent ion.

The doubly charged molecular ion, m/z 286, formed during electrospray ionization is stable and abundant. It was therefore chosen for selective reaction monitoring by MS/MS. Following ESI of pancuronium, only the m/z 286 was filtered through the first quadrupole of the TSQ 700. In the second quadrupole, this ion was allowed to fragment further through collisionally induced dissociation in argon gas (2 mTorr) to generate characteristic secondary product ions. These ions were then filtered and measured by the third quadrupole to yield selective MS/MS data for pancuronium (Fig. 7). Ions at m/z 472, 430, 207, and 100 were used to further confirm the presence pancuronium in questioned samples. Mass chromatogram plots were also extensively used to manually highlight and confirm that pancuronium was present through ion-ratio assignments.

Discussion

Previous investigators focused on the identification of pancuronium to assist in an inquiry following attempted murder (14), to confirm the drug in a suicide investigation (6), and in other cases with access to postmortem blood, serum, and urine analyses (15). In those reports, tissue samples were not significantly aged, decayed, or exposed to environmental, fungal, or bacterial influences. In the present study, a significantly larger number of samples were successfully assayed for pancuronium after exposure to various external factors that resulted in widely variable states of decomposition and biological and chemical exposures. The use of SPE was integral to routine sample preparation and concentration of

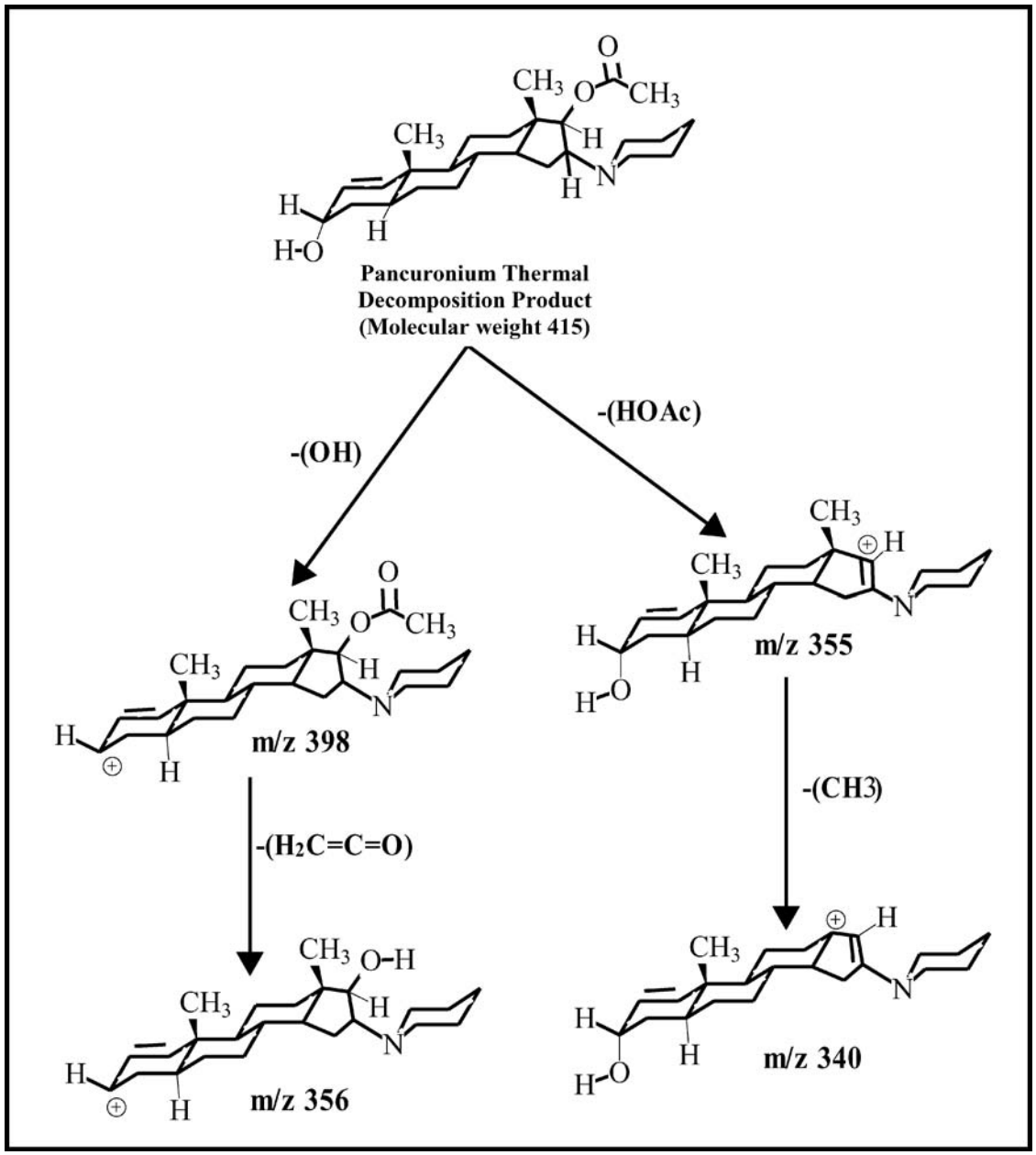

FIG. 4—*Proposed fragmentation pathways of characteristic mass spectral ions from a unique thermal decomposition product of pancuronium (molecular weight 415).*

the target quaternary ammonium drug from complex biological matrices.

The present protocols developed for the isolation, screening, and qualitative confirmation of pancuronium in aged exhumation specimens can be applied to small- or large-scale tissue analysis requirements. Injection-port pyrolysis and GC-MS analysis of questioned samples facilitated rapid pre-screening of quaternary ammonium-type drugs. The doubly charged molecular ion of pancuronium and unique analysis capabilities of μHPLC-ESI-TSQ-MS/MS resulted in unambiguous measurements of pancuronium in complex samples. In distinct contradiction to the conclusions of a previous study (16), m/z 286 is the doubly charged molecular ion of pancuronium, and it forms definitive singly charged products ions larger than the m/z 286 ion. These unique data assure confirmation of pancuronium in questioned samples, and the method is particularly important for unambiguous identification in aged autopsy samples.

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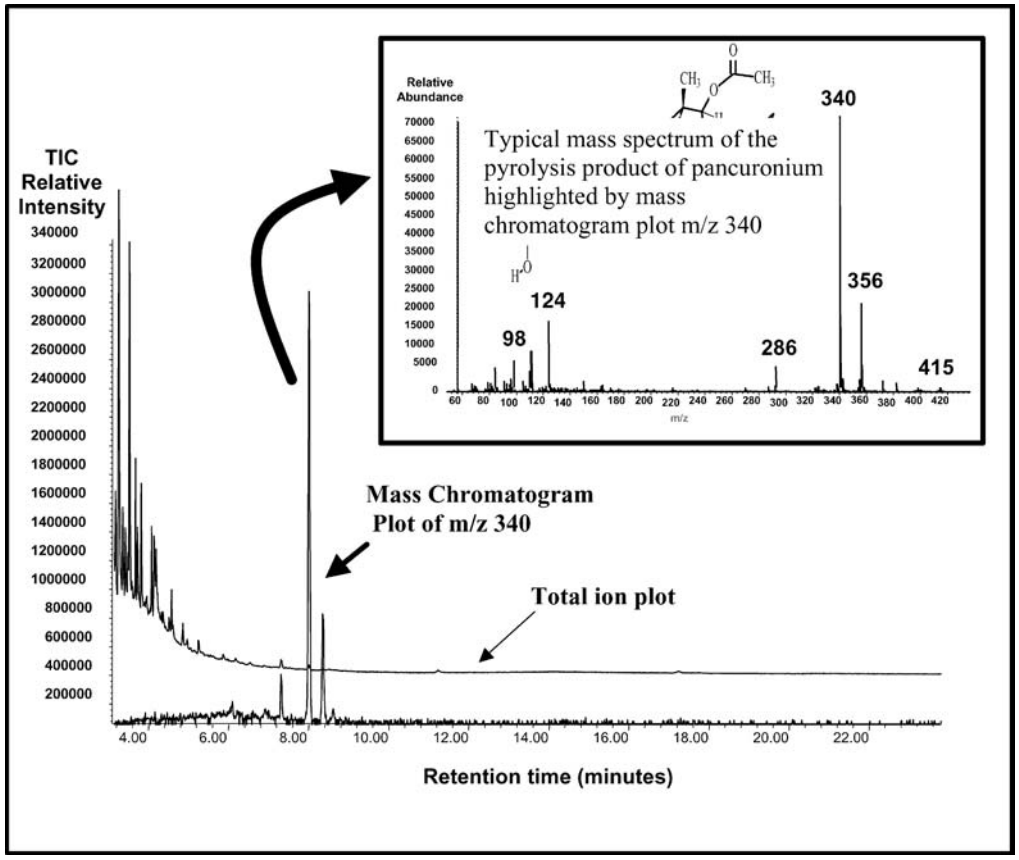

FIG. 5—*Total ionization chromatogram of a tissue extract that was positive for pancuronium (top trace), with an overlay mass chromatogram plot of m/z 340 (bottom trace). Insert is the mass spectrum of the compound that eluted at 8.0 min.*

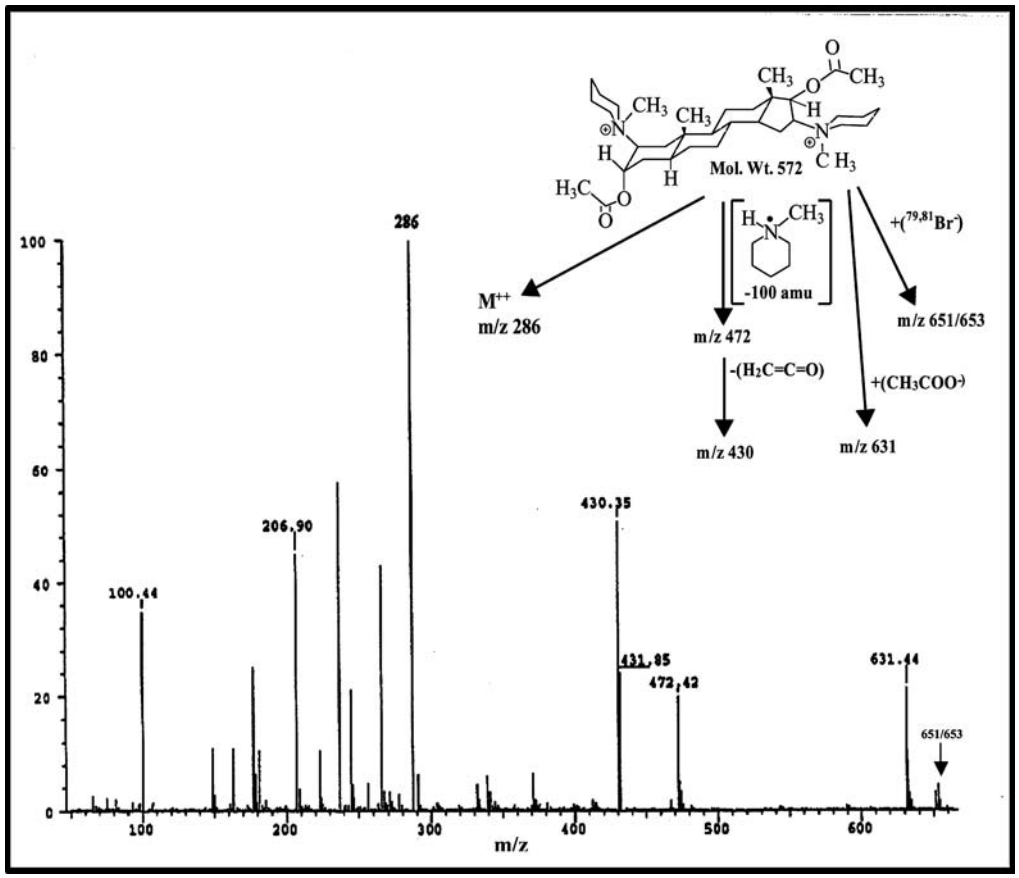

FIG. 6—*Electrospray mass spectral analysis of pancuronium. Characteristic ESI fragment ions are observed. The abundant m/z 286 is the doubly charged pancuronium molecular ion. A proposed MS ion formation scheme is presented for other fragment ions.*

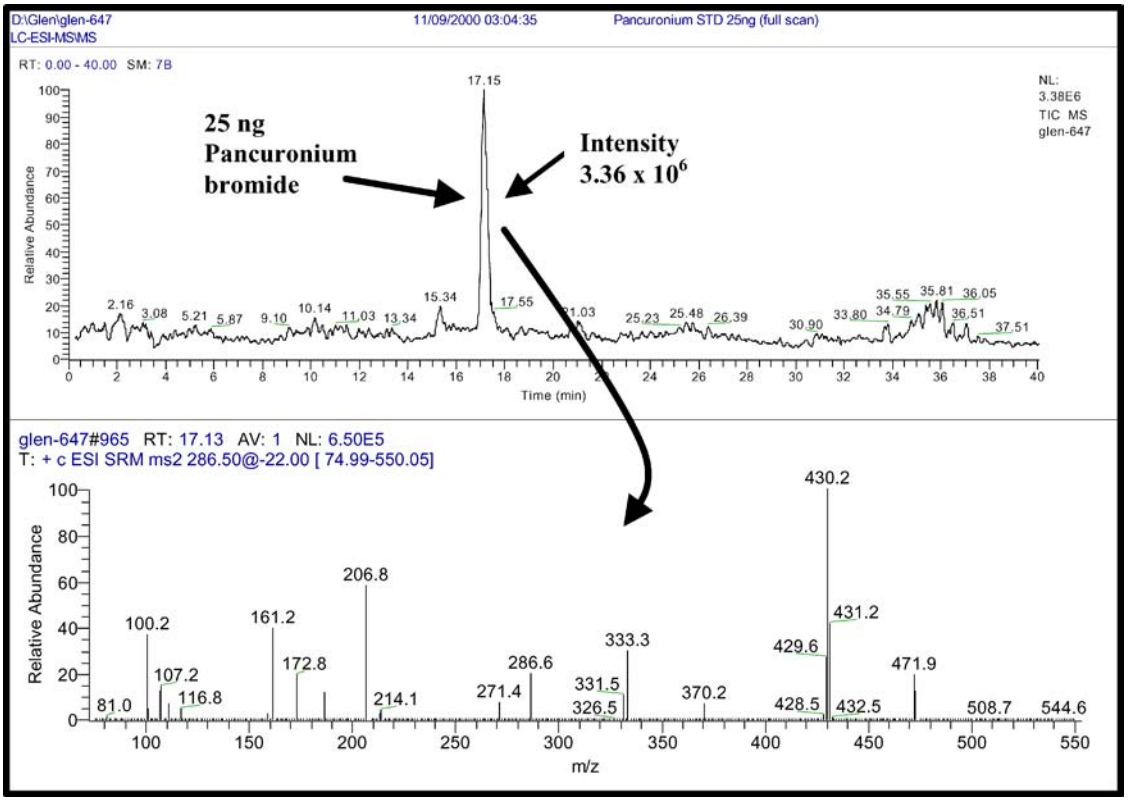

FIG. 7—*μHPLC-ESI-MS/MS mass spectrum of experimental buffer containing 25 ng/uL of pancuronium standard (upper trace). Selective filtering of m/z 286 results in the formation of characteristic pancuronium MS/MS ions to confirm the presence of the drug in questioned samples.*

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