D. Wittmer,[1] *M.S., S. Atwell,*[2] *M.S., and*
W. G. Haney, Jr.,[3] *Ph.D.*

Quantitative Extraction of Tubocurarine, Gallamine, and Decamethonium from Biological Materials

Forensic analysis of skeletal muscle relaxants such as tubocurarine and gallamine has been hampered by the fact that these quaternary ammonium compounds are not readily extractable from the biological matrix. Several techniques have been presented to effect this extraction, but these appear to result in either erratic, incomplete extraction [*1*] or in nonselective extraction [*2*].

Use of ion exchange resins for purification of these compounds [*3*] appears to yield somewhat more reproducible results, although overall recovery is less than complete and the nature of the elution process leads to contaminants which interfere with chromatographic characteristics of these drugs. It was felt, therefore, that a technique based on classical solvent-solvent extraction would be more suitable for use in routine screening of biological materials for these drugs.

As a result, a procedure giving high and predictable recovery of these compounds from biological materials has been developed. It relies on ion pair formation with the anion of picric acid, extraction of the ion pair, and subsequent examination of the residue using previously developed chromatographic procedures.

Experimental

Materials

d-tubocurarine chloride and gallamine triethiodide were obtained as United States Pharmacopeia Reference Standards, and decamethonium bromide was obtained from Burroughs-Wellcome. Solutions of picric acid ($3.0 \times 10^{-2}M$) were prepared by dissolving an appropriate amount of picric acid, previously recrystallized from ethanol, in phosphate buffer pH 6.5. Sodium 9,10-dimethoxyanthracene-2-sulfonate was used as a solution ($1.0 \times 10^{-2}M$) in phosphate buffer pH 6.5.

Extraction Procedure

Various quantities of test drug, as noted in Table 1, were added to 10.0 ml of urine. The pH was adjusted to about 9 to 10 with dilute sodium hydroxide solution, the mixture was extracted with 30 ml of chloroform, and the chloroform extract was used to

Received for publication 22 April 1974; revised manuscript received 6 June 1974; accepted for publication 8 June 1974.

[1] Research associate, School of Pharmacy, University of Missouri, Kansas City, Mo.
[2] Laboratory supervisor, Toxicology Laboratory, The Childrens Mercy Hospital, Kansas City, Mo.
[3] Assistant professor of pharmaceutical chemistry, School of Pharmacy, University of Missouri, Kansas City, Mo.

TABLE 1—*Efficiency of extraction of selected skeletal muscle relaxants from biological material.*

Drug	Concentration, μg/ml or μg/g	Material	Recovery	Recovery, %	Standard Deviation, %
Tubocurarine	0.01	urine	0.0093	93	9.74
		blood	0.0096	96	8.37
		liver	0.0086	86	9.43
	0.04	urine	0.0423	105	6.30
		blood	0.0384	96	6.13
		liver	0.0355	89	7.17
	0.08	urine	0.0834	104	4.64
		blood	0.0823	102	3.96
		liver	0.0702	87	5.16
Gallamine	0.01	urine	0.0097	97	7.43
		blood	0.0103	103	8.44
		liver	0.0092	92	8.97
	0.04	urine	0.0383	96	6.46
		blood	0.0412	103	7.19
		liver	0.0395	98	6.14
	0.08	urine	0.0784	98	4.30
		blood	0.0826	103	4.91
		liver	0.0807	101	5.46
Decamethonium	0.01	urine	0.0094	94	9.31
		blood	0.0099	99	8.77
		liver	0.0100	100	8.11
	0.04	urine	0.0422	106	6.34
		blood	0.0378	95	6.66
		liver	0.0394	99	7.46
	0.08	urine	0.0784	98	4.57
		blood	0.0814	102	4.16
		liver	0.0776	97	5.02

screen for other drugs or discarded. The pH of the aqueous solution was adjusted to pH 1 to 2 with dilute hydrochloric acid, the solution extracted with 30 ml of chloroform, and the chloroform extract discarded. Picric acid solution (4.0 ml) was added to the chloroform-washed urine, and the mixture was extracted with 30 ml of 96/4 dichloromethanel-pentanol solution. The extract was evaporated to dryness in a stream of dry nitrogen, and the residue was reconstituted in methanol and applied to one of several chromatographic systems.

Blood (20.0 ml) was treated in the same manner after an initial deproteinization using one volume of 20% trichloroacetic acid (TCA), followed by centrifugation and filtration of the supernatant.

Liver, muscle, and lung tissues were mixed with solutions of the test drugs, homogenized, and deproteinated, and the resulting filtrate was treated as above.

Chromatographic Analysis [3]

Paper chromatographic analysis was effected on Whatman Number 1 paper with *n*-butanol:acetic acid:water (40:10:40) as solvent. Extracts were also chromatographed on System I—Alumina Woelm, acid (chloroform:methanol, 80:20); System II—Alumina Woelm, basic (methanol:chloroform, 75:25); and System III—Alumina G Merck (methanol:acetic acid:water, 92:3:3).

Analysis of Drugs Eluted from Chromatographic Plates

Qualitative analysis of test drugs on developed chromatograms was effected by examination under ultraviolet light, by spraying with iodoplatinate solution [4], and by comparing R_f values to those of drug standards. Detection limits in System I, as determined by successive application of decreasing amounts of the drugs, development, and visualization were: gallamine, 0.3 μg; tubocararine, 0.5 μg; and decamethonium, 0.7 μg per application.

Quantitation of drugs in plate areas (System I) was accomplished by carefully transferring the area containing the drug to a centrifuge tube containing phosphate buffer pH 6.5 (3.0 ml). The mixture was shaken for two minutes, centrifuged, and an aliquot (2.0 ml) removed and mixed with methylene dichloride (4.0 ml) and sodium 9,10-dimethoxyanthracene-2-sulfonate solution (1.0 ml). The mixture was again shaken for two minutes, centrifuged, and an aliquot (3.0 ml) of the extract was removed. Tetrabutylammonium hydroxide (two drops of a 25% methanol solution) was added, and fluorescence determined (excitation, 368 nm; emission, 448 nm). Quantities were determined by reference to calibration curves.

Determination of Extraction Efficiency

For each concentration of the drugs listed in Table 1, a 20 by 20-cm thin-layer chromatographic (TLC) plate was divided into eight equal sections. Aliquots (10 μl) of drug solution in methanol containing 0.01, 0.02, 0.04, 0.08, and 0.10 μg/μl of the drug were applied to Sections 1-5, and the concentrate (see above) was applied to Section 6. Section 7 was used as a blank, and 3.0 μg of the drug were applied to the last section as a marker. The plate was developed (System I) and dried in a stream of dry nitrogen. Section 8 was visualized with iodoplatinate solution with the remainder of the plate carefully covered. Areas 0.5 cm above and below the area corresponding to the visualized drug were removed and quantified as above. Recovery was calculated from calibration curves so prepared.

Results and Discussion

Suitability of several counterions for use in this extraction was investigated before picric acid was chosen. While hexanitrodiphenylamine [5] and bromothymol blue [6] appeared to give complete extraction of gallamine and decamethonium, extraction of tubocurarine was in each case incomplete. This is apparently due to the fact that these two reagents must be used at a pH higher than 7.7 to avoid extraction of the un-ionized reagent. At pH 7.7 it is likely that phenolic hydroxyls of tubocurarine would have a tendency to ionize, diminishing extraction efficiency. At pH 6.5 phenolic ionization would be suppressed, resulting in more complete recovery.

It is possible to use picric acid in the final determination of these quaternary ammonium compounds as eluted from the TLC media [7]. However, molar absorptivity of picrate ion pairs is such that the limit of reliable determination is 1×10^{-7} equivalents (0.393 μg of tubocurarine for example). Since it was a purpose of this work to examine extraction efficiency at submicrogram levels, a counterion yielding higher sensitivity was used for the final analysis. Sodium 9,10-dimethoxyanthracene-2-sulfonate has been shown [8] to give reliable data for $10^{-8}M$ solutions.

Interference of the counterion with chromatographic characteristics of the drugs was initially a subject of concern [9]. However, R_f values for the drugs (tubocurarine: 0.83, 0.65, 0.72; gallamine: 0.52, 0.74, 0.92; and decamethonium: 0.24, 0.81, 0.81, in TLC

Systems I, II, and III respectively) applied as picrate ion pairs did not differ from those of authentic samples. In addition, picric acid has an R_f value in the range from 0.87 to 1.0 in these systems, so that it does not interfere with chromatographic interpretation.

Seven calibration curves prepared for the drugs had the characteristics shown in Table 2. These calibration curves were prepared from TLC System I because of the greater separation of drugs in this system, and, also, because residual developing solvent does not interfere with pH adjustment.

TABLE 2—*Characteristics of calibration curves prepared for selected skeletal muscle relaxants.*

Drug	Slope, Fluorescence Units/μg	Correlation Coefficient	*y* Intercept
Tubocurarine	863 ± 34	0.982	−0.17
Gallamine	1098 ± 39	0.990	−0.54
Decamethonium	1573 ± 55	0.987	−0.984

Results of extraction studies (Table 1) show that recovery of these drugs from biological material is in most cases complete. Since the standard deviation of results appears in each case to decrease as the amount of drug present increases, a major contributor to the standard deviation must be uncertainty in the analytical method, rather than erratic recovery. Only in the case of tubocurarine extracted from tissue is the recovery less than 90%. This decreased extraction efficiency is evidently due to loss of drug upon deproteination, since tubocurarine added to the supernatant after deproteination was completely recovered.

It should be noted that it is not possible to separate solutions of ion pairs from water using phase-separating filter paper, since filtration gives rise to significant losses. An average of 21% of tubocurarine picrate was lost per filtration, 13% for gallamine picrate, and 8% for decamethonium picrate.

A major advantage of this procedure is that it rids the extract of inorganic ions which may interfere with chromatographic characteristics of the drug. In addition, the procedure is readily appended to current solvent extraction procedures [*10*] used for forensic screening purposes.

Summary

A procedure is described for quantitative extraction of tubocurarine, gallamine, and decamethonium from blood, urine, and tissue. Ion pairs of the drugs are formed with picric acid and are extracted into a dichloromethane/1-pentanol solvent. Qualitative analysis of the extract is effected using thin-layer chromatography. Quantitative analysis is accomplished by elution of TLC areas containing the drug, formation of the ion pair with sodium 9,10-dimethoxyanthracene-2-sulfonate, and analysis of the ion pair by spectrophotofluorometry.

References

[*1*] Stevens, H. M. and Fox, R. H. "A Method for Detecting Tubocurarine in Tissue," *Journal of the Forensic Science Society,* Vol. 2, 1971, pp. 177-182.

[*2*] Mahfouz, M., "The Fate of Tubocurarine in the Body," *British Journal of Pharmacology,* Vol. 4, 1949, pp. 295-303.

[3] Fiori, A. and Marigo, M. "A Method for the Detection of *d*-Tubocurarine, Gallamine, Decamethonium and Succinylcholine in Biological Materials—Modification and Development," *Journal of Chromatography,* Vol. 31, 1967, pp. 171-176.

[4] Sunshine, I., Ed., *Handbook of Analytical Toxicology,* Chemical Rubber Co., Cleveland, Ohio, 1969, p. 794.

[5] Eksborg, S. and Persson, B. A., "Photometric Determination of Acetylcholine in Rat Brain after Selective Isolation by Ion-Pair Extraction and Micro-Column Separation," *Acta Pharmaceutica Suecica,* Vol. 8, 1971, pp. 205-216.

[6] Garrett, E. R. and Tsau, J. "Application of Ion-Pair Methods to Drug Extraction from Biological Fluids I: Quantitative Determination of Biguanides in Urine," *Journal of Pharmaceutical Sciences,* Vol. 61, 1972, pp. 1404-1410.

[7] Gustavii, K. and Schill, G., "Determination of Amines and Quaternary Ammonium Ions as Complexes with Picrate," *Acta Pharmaceutica Suecica,* Vol. 3, 1966, pp. 241-258.

[8] Westerlund, D. and Borg, K. O., "Fluorimetric Determinations by Ion-Pair Extraction, Part V. Studies on Ion-Pair Extraction with the Fluorescent Anion 9, 10-Demethoxyanthracene-2-Sulfonate," *Analytica Chimica Acta,* Vol. 67, 1973, pp. 89-98.

[9] Perdih, A. "Liberation of Acid Dyes from Their Quaternary Ammonium Salts on Chromatograms," *Journal of Chromatography,* Vol. 88, 1974, pp. 370-373.

[10] Mulé, S. J., "Routine Identification of Drugs of Abuse in Human Urine I. Application of Fluorometry, Thin-Layer and Gas-Liquid Chromatography," *Journal of Chromatography,* Vol. 55, 1971, pp. 255-266.

Department of Pharmaceutical Chemistry
School of Pharmacy
University of Missouri
Kansas City, Mo. 64110