GLC Determination of Bretylium in Biological Fluids

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Abstract □ Bretylium [(*o*-bromobenzyl)ethyldimethylamine] is a quaternary ammonium compound used as the tosylate salt for treatment of ventricular fibrillation in humans. A sensitive assay was developed for the determination of low bretylium concentrations in plasma and urine. The internal standards were (*p*-bromobenzyl)ethyldimethylammonium *p*-toluenesulfonate and (*o*-methoxybenzyl)ethyldimethylammonium *p*-toluenesulfonate. Samples were deproteinized with acetonitrile and extracted with methylene chloride. After the evaporation of the organic phase, the residue was reacted with sodium 2,4,5,-trichlorothiophenolate in methanol. This procedure yielded volatile compounds with excellent electron-capture capabilities for the GLC analysis. The assay sensitivity is 5 ng/ml. The extraction recovery of bretylium as determined by a direct radioactivity measurement was 90 and 97% for plasma and urine, respectively. The method is highly reproducible with no significant day-to-day variations. Comparisons of 60 standard plasma samples, 25 standard urine samples, and plasma samples from a dog that received [^{14}C]bretylium showed excellent agreement between the GLC method and direct radioactivity measurement of bretylium.

Keyphrases □ Bretylium—determination in biological fluids by GLC and direct radioactivity measurement, humans □ GLC—analysis of bretylium in biological fluids, humans □ Radiochemistry—analysis of bretylium in biological fluids, compared to GLC determination, humans

Bretylium [(*o*-bromobenzyl)ethyldimethylamine] is a quaternary ammonium compound used as the tosylate salt for the treatment of ventricular fibrillation in humans (1, 2). Various methods for its determination have been reported (3–7). The most promising method is a GLC analysis (6) utilizing sodium thiophenolate to react with bretylium to produce *o*-bromobenzyl phenylthio ether. However, it is tedious and lacks sufficient sensitivity.

This paper reports a simplified and sensitive GLC method for the determination of bretylium in small plasma and urine samples. The new extraction procedure minimizes the variability in assay recovery and reproducibility. To validate this method, the results of the GLC method were compared with direct radioactivity measurement of [^{14}C]bretylium in standard samples as well as in samples collected from a dog that received the labeled drug.

EXPERIMENTAL

Materials and Methods—A stock solution of bretylium tosylate[1] (I) (10 mg/ml as a quaternary ammonium compound) was prepared in purified water. This solution was used to prepare standard plasma and urine samples containing 5–250 ng of bretylium/ml. The stock solutions of the internal standards, (*p*-bromobenzyl)ethyldimethylammonium *p*-toluenesulfonate[2] (II) and (*o*-methoxybenzyl)ethyldimethylammonium *p*-toluenesulfonate[2] (III) (each containing 1 μg/ml as the quaternary ammonium compound), were prepared in purified water. The glassware used in solution preparation and in the assay was washed with soap, soaked in 4 *N* HNO_3 for 24 hr, rinsed with purified water, and finally rinsed with methanol.

Sodium 2,4,5-trichlorothiophenolate (IV) was used as a reacting agent. It was synthesized by a method similar to that described previously for the preparation of sodium thiophenolate (6, 8). The reagent was only moderately hygroscopic and, when stored in a drying oven, was stable for at least 6 months.

Table I—Recovery of [^{14}C]Bretylium from Plasma and Urine

[^{14}C]Bretylium Added[a], ng/ml	Percent Recovery from Plasma[a]	Percent Recovery from Urine
4.75	90.8 (3.68)	
9.33	89.2 (1.03)	97.3
24.10	90.2 (1.23)	96.3
49.8	89.3 (1.56)	97.4
73.7	90.3 (1.30)	96.1
98.7	92.7 (1.07)	99.3
Mean	90.4 (1.66)	97.2
CV	1.41 —	1.17

[a] Mean of five determinations. The [^{14}C]bretylium concentration was determined by direct scintillation counting of each sample. The numbers in parentheses are the coefficients of variation in percent.

Assay—*Method I*—To 1 ml of plasma or urine was added 0.1 ml of the internal standard solution containing 100 ng of II and 1 ml of acetonitrile[3]. The sample was vortexed for 10 sec and centrifuged at 4000 rpm for 5 min. The supernate was transferred into a 155 × 13-mm polytef-lined screw-capped culture tube[4], extracted with 5 ml of methylene dichloride[5] by gentle shaking for 30 min, and centrifuged at 4000 rpm for 5 min. Five milliliters of the organic layer was evaporated to dryness in a new culture tube. The residue was dissolved in 3 ml of methanol containing 1.0 μg of IV and allowed to react and evaporate to dryness at 60° with vigorous shaking in a vacuum. The same reaction procedure was repeated twice using 1 ml of the substituted thiophenolate solution. The residue was reconstituted with 200 ml of methanol from which 2 μl was injected into the gas–liquid chromatograph.

Method II—To 0.25 ml of plasma or urine was added 0.1 ml of the internal standard solution containing 100 ng of III, 0.5 ml of 0.1 *N* NaCl solution, and 1 ml of acetonitrile. The sample was vortexed for 10 sec and centrifuged at 4000 rpm for 5 min. The supernate was transferred into a culture tube to which 0.2 ml of 1 *N* NaOH was added. The mixture was extracted with 5 ml of methylene dichloride by gentle shaking for 30 min and was centrifuged at 4000 rpm for 5 min. The samples then were treated as in Method I, except that they were reacted only once with 3 ml of methanolic solution containing 1.0 μg of IV.

GLC Procedure—Analysis was performed on a gas–liquid chromatograph[6] equipped with a 2.08-m × 4-mm i.d. silanized glass column (coiled) packed with 3% OV-225 on 100–120-mesh Supelcoport and a ^{63}Ni-electron-capture detector. The injection port, column, and detector temperatures were maintained isothermally at 270, 250, and 300°, respectively. Argon–methane (95:5 v/v) was used as the carrier gas at a flow rate of 50 ml/min (30 ml/min through the column, 20 ml/min directly to the detector as a scavenger gas).

Data acquisition was performed on a laboratory minicomputer[7]. The peak heights of the derivatized bretylium were divided by the peak heights of the derivatized internal standard, and the ratios were used to construct the standard curve and to determine its linearity.

Animal Study—One male beagle dog[8], 13.1 kg, received [^{14}C]bretylium tosylate[9] ([*benzyl*-7-^{14}C], 0.512 μCi/mg), 10 mg/kg iv. Food was withheld for 4 hr after drug administration. Blood samples (5 ml each) were drawn in heparinized vacutainers through the jugular vein at 4, 6, 8, 12, 16, 20, and 24 hr after dosing. The plasma samples were kept frozen until they

[1] Ganes Chemical Co., New York, N.Y.
[2] Synthesized by the method of Jenden *et al.* (8), Arnar-Stone Laboratories, McGaw Park, Ill.
[3] HPLC grade, E. M. Laboratories, Cincinnati, Ohio.
[4] No. 2511-B3, Wheaton Tubing Products, Millville, N.J.
[5] Nanograde, Burdick & Jackson Laboratories, Muskegon, Mich.
[6] Model 428, Packard Instruments, Downers Grove, Ill.
[7] Model 3352D, Hewlett-Packard, Avondale, Pa.
[8] Laboratory Research Enterprises, Kalamazoo, Mich.
[9] New England Nuclear, Boston, Mass.

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Table II—Reproducibility, Day-to-Day Variations, and Recovery of the GLC Method for Determination of Bretylium in Plasma and Urine

Actual Concentration[a], ng/ml	Plasma Assay[b]			Urine Assay[b]	
	Mean Concentration in Plasma, ng/ml		Mean Overall Percent of Added Concentration[e]	Mean Concentration in Urine[f], ng/ml	Mean Overall Percent of Added Concentration[e]
	Day 1[c]	Day 5[d]			
4.75	4.50 (4.47)	4.81 (4.14)	98.0 (5.37)	—	—
9.33	9.10 (11.6)	9.61 (2.85)	99.7 (9.09)	10.8 (3.87)	115.8 (4.84)
24.1	24.6 (11.5)	25.8 (3.01)	104.0 (9.01)	23.8 (3.81)	98.9 (3.82)
49.8	53.7 (4.66)	51.1 (1.38)	105.7 (4.47)	46.4 (2.97)	93.2 (2.97)
73.7	75.1 (6.06)	72.8 (3.16)	100.7 (5.28)	74.5 (4.50)	101.1 (4.49)
98.7	98.3 (6.46)	101.0 (4.98)	100.7 (5.94)	102.3 (2.22)	103.6 (2.25)
Mean	— (7.45)	— (3.25)	101.5 (6.52)	— (3.47)	102.5 (3.67)
CV			2.8		8.15

[a] [^{14}C]Bretylium added to plasma and urine samples. [b] The numbers in parentheses are the coefficients of variation in percent. [c] $n = 6$. [d] $n = 4$. [e] $n = 10$. [f] $n = 5$.

were assayed. Plasma samples were analyzed by direct counting of the radioactivity *via* a scintillation counter[10] and by the GLC method described under *Method II*.

RESULTS AND DISCUSSION

Bretylium, a quaternary ammonium compound, can be extracted from biological samples by an ion-pair extraction procedure, a complicated and tedious method described previously (6). In the present study, the use of acetonitrile as a deproteinization agent served to remove the proteins, to purify the biological sample, and to enhance the partitioning of bretylium into methylene dichloride, which was used as the extraction solvent. Although acetonitrile is fully miscible with the aqueous solution, it was almost completely partitioned in the organic layer. The recovery of [^{14}C]bretylium from plasma and urine was 90.4 and 97.2%, respectively (Table I). The coefficient of variation for five samples at each concentration (range 4.8–98.7 ng/ml) ranged from 1.0 to 3.7%. There was no correlation between the extraction recovery and plasma concentrations. This approach simplified the previous procedures for bretylium extraction from plasma and may be useful for extracting other quaternary amines from biological fluids.

To determine bretylium by GLC, Kuntzman *et al.* (6) used sodium thiophenolate, which reacts with the quaternary ammonium compound and yields a volatile derivative, *o*-bromobenzylphenylthio ether. The use of this reagent resulted in a sensitivity of ~70 ng/ml when a large volume (3 ml) of the sample was used. To improve this sensitivity, sodium

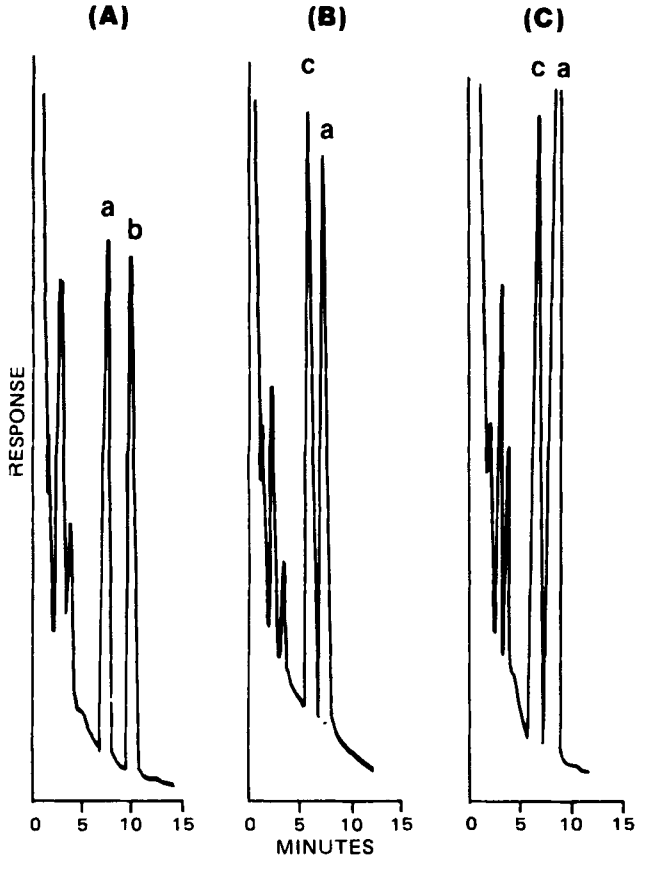


Figure 1—*Typical chromatograms for a blank standard sample spiked with the internal standard for Method I (A) and Method II (B), and a sample from a patient who received bretylium (C), where a, b, and c are the thiophenylether derivatives of bretylium, (p-bromobenzyl)ethyldimethylamine, and (o-methoxybenzyl)ethyldimethylamine, respectively.*

[10] Model 2425, Packard Instruments, Downers Grove, Ill.

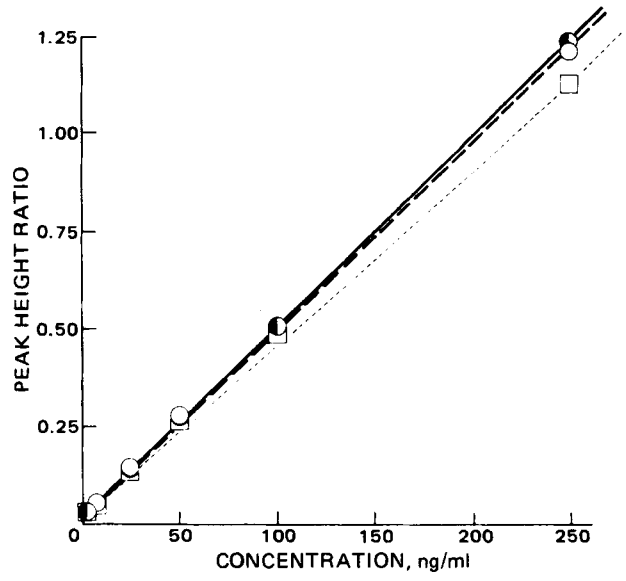


Figure 2—*Reproducibility of the assay of three sets of standard plasma samples determined on Days 1 (●), 3 (○), and 8 (□) by Method II.*

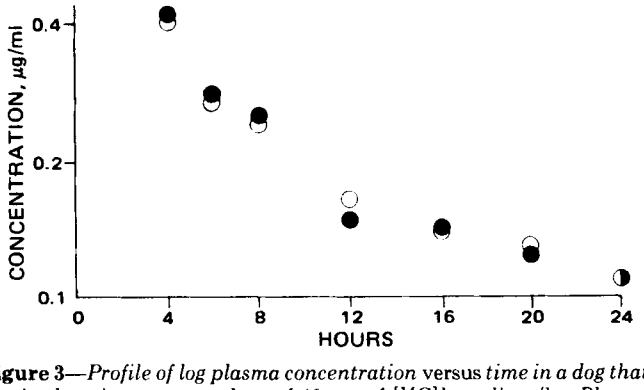
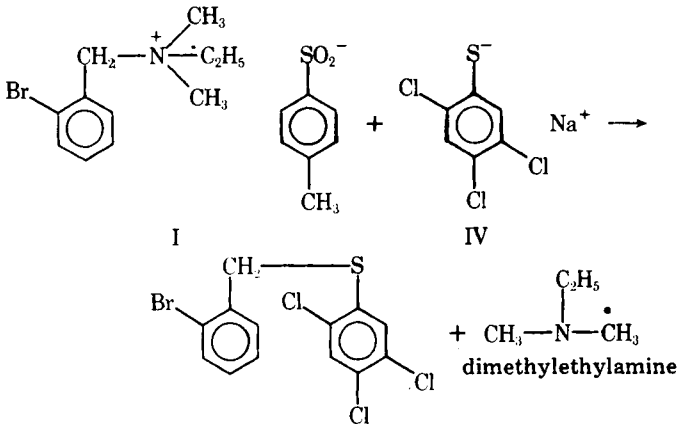


Figure 3—*Profile of log plasma concentration* versus *time in a dog that received an intravenous dose of 10 mg of [^{14}C]bretylium/kg. Plasma samples were assayed by direct radioactivity measurement (○) and by the GLC method (Method II, ●).*

Table III—Precision of the GLC Method for Determination of Bretylium in Plasma

Actual Concentration[a], ng/ml	Concentration Found[b], ng/ml					
	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
4.75	4.49	4.73	4.24	4.52		
9.33	9.38	9.38	7.00	10.00	9.41	9.41
24.1	22.6	27.1	26.1	19.8	26.7	25.3
49.8	51.4	53.6	56.4	57.1	52.2	51.5
73.7	74.9	70.7	68.6	80.1	70.0	78.0
98.7	95.5	103.2	97.9	92.3	108.4	92.7

[a] [^{14}C]Bretylium was used. [b] See mean and coefficient of variation values in Table II.



o-bromobenzyl 2,4,5-trichlorothiophenyl ether

Scheme I

thiophenolate was replaced by IV, which also readily reacts with bretylium and its congeners to produce derivatives extremely sensitive to electron-capture detection. Scheme I shows the derivative. Although the thiophenolate anion can attack at any of the four amine substituents by an S_N2 process, attack at the *o*-bromobenzyl group, as depicted, is preferred due to resonance stabilization of the reaction transition state (9).

In this study, two internal standards, II (Method I) and III (Method II), were used. Compound III reacts more readily with sodium thiophenolate and makes the assay less time consuming. Figure 1 shows typical chromatograms from the assay. The retention times of the phenylthio ether derivatives were 6.1, 7.4, and 9.4 min. Bretylium usually is given with other drugs that may interfere with the assay. To minimize such interference, one of the two internal standards may be selected, depending on the desired retention time.

Under the assay conditions, there were observable impurity peaks at retention times of 2.7 and 3.4 min that did not interfere with the drug or internal standard peak. As expected, qualitative and quantitative GLC showed that the corresponding primary, secondary, and tertiary amines of bretylium do not react with the thiophenolate and have very poor intrinsic electron-capturing ability. The GLC chromatograms showed symmetrical peaks for bretylium and the internal standards, and there were no indications of interference from those amines.

Figure 2 and Table II show the linearity, reproducibility, day-to-day variation, and recovery data of the assay. Table III lists the precision data of the GLC method for the determination of bretylium in plasma. The slopes of the plots of the peak height ratio of bretylium to that of the internal standard *versus* bretylium concentrations in the range of 5–250 ng/ml were 0.00409, 0.00476, and 0.00449 as determined on Days 1, 3, and 8, respectively (10). There was no statistically significant difference in the slopes, intercepts, and individual concentrations determined on two or three occasions during an 8-day period.

In this study, plasma and urine concentrations of 5 ng/ml were determined easily using a 0.25-ml sample. However, the sensitivity can be improved by simple modifications. The GLC assay was compared with a direct radioactivity measurement in 60 standard plasma samples and 25 standard urine samples (Table II). The correlation coefficients between the results of the two methods were 0.999 for plasma and 0.998 for urine samples. Similarly, plasma samples from a dog that received an intravenous dose of 10 mg of [^{14}C]bretylium tosylate/kg were assayed by both the GLC and the direct radioactivity measurement methods. Figure 3 shows the plasma bretylium concentration *versus* time profile. There was excellent correlation between the results of the two procedures, and there was no significant difference between the data (paired *t* test).

A series of standard plasma samples with concentrations ranging from 5 to 250 ng/ml was kept at ambient temperature for 4 days. The GLC determinations showed results of 102.2 ± 10.5% of the corresponding concentrations of controls that were kept frozen during the same period. These data indicate that bretylium is stable in plasma. This result also is consistent with data reported previously on bretylium stability (7).

The proposed procedure is sensitive and requires a single extraction step without any back-extraction or cleaning procedure. This method offers the distinct advantage of requiring very small biological samples. The choice of the internal standard depends on the individual case and drugs involved. Although both internal standards give essentially the same results, the use of the *o*-methoxy congener is less time consuming. This GLC method may be used in single-dose pharmacokinetic studies in humans and animals. It also may be applicable to quality control tests and drug compatibility studies. Other quaternary amines may be extracted and derivatized in a similar manner.

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