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## SENSITIVE GAS CHROMATOGRAPHIC ASSAY FOR THE QUANTITATION OF BRETILIUM IN PLASMA, URINE AND MYOCARDIAL TISSUE

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### SUMMARY

A sensitive analytical method has been developed for the quantitation of bretylium in plasma, urine and myocardial tissue. Bretylium and the internal standard, UM-360 (*o*-iodobenzyltrimethylammonium), are extracted and isolated as the iodide salts. Sodium benzenethiolate is added and the mixture heated to 100° for one hour. This results in the formation of 2-bromobenzyl phenyl thioether and 2-iodobenzyl phenyl thioether, which can be separated and quantitated by gas chromatography. Good reliability and reproducibility can be obtained using electron-capture detection with quantities of bretylium as small as 1 ng.

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### INTRODUCTION

Bretylium tosylate (bretylo<sup>l</sup>, *o*-bromobenzylethyldimethylammonium tosylate) is a unique antiarrhythmic agent possessing antifibrillatory actions [1,2]. The pharmacokinetic properties of bretylium are poorly described due to the lack of an analytical method with both specificity and sensitivity for the quaternary. An earlier method used to measure bretylium in urine [3] did not possess the sensitivity needed to measure bretylium levels in plasma. The previous method was based upon the ability of the quaternary to bind methyl orange, and lacked specificity for bretylium. The method used in the present study for quantitation of bretylium is based upon a procedure used for quantitation of acetylcholine [4] as modified for use in quantitating bretylium by Kuntzman et al. [5]. The procedure involves the removal of the *o*-bromobenzyl group from bretylium by sodium benzenethiolate and the formation of an *o*-bromobenzyl phenyl thioether which can be measured with excellent

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sensitivity by electron-capture detection after separation by gas chromatography.

## METHODS

### *General principle*

Bretylium and UM-360 (*o*-iodobenzyltrimethylammonium) are extracted and isolated from samples as the iodide salt. Addition of sodium benzenethiolate results in the formation of *o*-bromobenzyl phenyl thioether and *o*-iodobenzyl phenyl thioether derivatives which can be separated and quantitated by gas chromatography.

### *Apparatus*

A gas chromatograph (Hewlett-Packard Model 7610A) equipped with a  $^{63}\text{Ni}$  electron-capture detector was used. Glass columns (1.83 m  $\times$  4 mm I.D.) with the following packings were employed: 4.3% OV-101 on Gas-Chrom Q (100–120 mesh), 5.0% OV-1 on Gas-Chrom Q (80–100 mesh), 3% OV-17 on Gas-Chrom Q (100–120 mesh), and 4% OV-225 on Gas-Chrom Q (100–120 mesh) (Applied Science Labs., State College, Pa., U.S.A.). Samples (0.1–2  $\mu\text{l}$ ) were injected manually using standard commercial microsyringes (Hamilton, Reno, Nev., U.S.A.). The following temperatures were used: injection port, 250°; column oven, 190°; and electron-capture detector, 250°. The carrier gas, argon–methane (19:1), flow-rate was 50 ml/min.

A Finnigan gas chromatograph–mass spectrometer (electron impact mode) was used for identification of the major chromatographic peaks. Helium was used as the carrier gas. A 3% OV-17 on Gas-Chrom Q (100–120 mesh) (1.7 m  $\times$  2 mm I.D.) column was used. Injection port, column, and detector temperatures were as described above.

### *Extraction*

Bretylium and UM-360 were extracted from biological fluids as the iodide salts using the method of Vidic et al. [6] as modified by Pohlmann and Cohen [7]. UM-360 chloride, 50  $\mu\text{l}$  of a 5.5  $\mu\text{g}/\text{ml}$  solution, and 1 ml distilled water were added to: 1 ml plasma or serum, 1 ml dissolved tissue sample, or 100  $\mu\text{l}$  urine. Chloroform (3 ml) was added and the tubes were vortexed for 10 sec followed by centrifugation at 1500 *g* for 5 min. The chloroform layer was removed and discarded. Potassium triiodide [iodine–potassium iodide–water (1:2:10 w/w/v)], 200  $\mu\text{l}$ , and chloroform, 3 ml, were added. The samples were shaken gently on a reciprocating flat bed shaker (100 rpm) for one hour followed by centrifugation at 1500 *g* for 5 min. The chloroform layer was transferred to a conical glass vial (Reactivial, Pierce, Rockford, Ill., U.S.A.) and evaporated to a dry residue under a stream of dry nitrogen. The presence of elemental iodine in the dry residue does not interfere with subsequent derivatization and quantitation although it can be removed by the addition of 50  $\mu\text{l}$  of 10% ascorbic acid in methanol followed by vortexing and evaporation of the methanol.

### *Derivatization*

Sodium benzenethiolate (3 mg/ml in ethyl acetate), 200  $\mu\text{l}$ , was added and

the vial capped securely. The samples were refluxed in a sand bath heated to 100–120° for one hour and allowed to cool at room temperature. The ethyl acetate was removed under a stream of dry nitrogen and the residue dissolved in 100  $\mu$ l hexane or cyclohexane. Injection of the ethyl acetate after derivatization and cooling to room temperature reveals the presence of late peaks which interfere with subsequent injections. These peaks were removed by evaporation of the ethyl acetate and dissolution of the residue in cyclohexane or hexane.

#### *Preparation of samples for analysis*

Male mongrel dogs weighing 14.0–16.3 kg were anesthetized with intravenous sodium pentobarbital, 30 mg/kg. A left external jugular vein cannula was inserted for drug administration and a second cannula was inserted into the inferior vena cava through the right femoral vein for withdrawal of blood samples for bretylium analysis. Blood was withdrawn into commercially prepared heparinized containers (Vacutainer, Becton-Dickinson, Toronto, Canada). A bolus of bretylium tosylate (6 mg/kg) was administered and plasma samples obtained at appropriate intervals. The animals ( $n = 3$ ) were sacrificed 12 h after the administration of bretylium and 300–350-mg sections of atrial, left ventricular, and right ventricular myocardium were removed for analysis of bretylium concentration. A 12-h urine sample was removed from the bladder. The myocardial samples were dissolved in 500  $\mu$ l of 12 *N* sodium hydroxide with 800  $\mu$ l of 7 *N* hydrochloric acid added after tissue dissolution. The above experiments were designed to demonstrate the ability of the assay to detect bretylium in biological fluids and tissue.

## RESULTS

#### *Quantitation of bretylium and extraction efficiency*

Electron-capture detection was used for the measurement of the halogenated thioethers. Typical chromatograms obtained from injected standards and plasma are shown in Fig. 1. Peak height ratios (*o*-bromobenzyl phenyl thioether:*o*-iodobenzyl phenyl thioether) were used for quantitation. Standard curves were prepared for plasma, urine and tissue by the addition of known quantities of bretylium to the samples. A linear correlation exists for samples representing bretylium plasma concentrations of 25–8000 ng/ml;  $r = 0.998$ . Linearity also exists for standards representing as little as 1 ng/ml bretylium or as much as 20  $\mu$ g/ml bretylium; 1–20 ng/ml ( $r = 0.992$ ) and 2–20  $\mu$ g/ml ( $r = 0.995$ ). The quantity of UM-360 added to the last two standards was modified to facilitate measurement of peak heights. The injected sample volume was varied so the detector response ranged from 0.1–2.0  $\cdot 10^{-10}$  A and retained a linear response ratio. Standard curves for urine, 10–200  $\mu$ g/ml ( $r = 0.999$ ) and myocardial tissue, 1–20  $\mu$ g/ml ( $r = 0.990$ ) also were linear.

Bretylium, 25–8000 ng, and UM-360, 250 ng, dissolved in methanol, were added to conical glass vials and the methanol evaporated under a stream of dry nitrogen. These samples were run in parallel with plasma bretylium standards representing 25–8000 ng/ml bretylium. UM-360, 250 ng, dissolved in methanol, was added to the dry chloroform extract of plasma and the methanol evaporated under dry nitrogen. Recovery of bretylium from plasma using

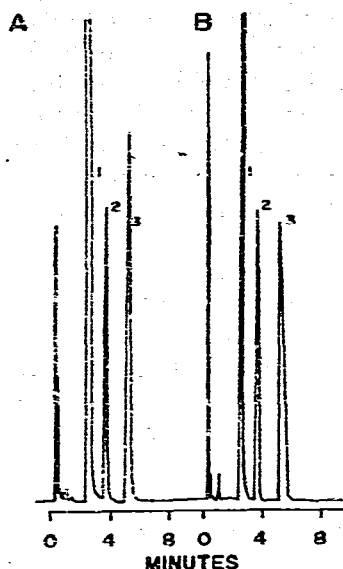


Fig. 1. Gas chromatograms from standards and plasma. Sample A was prepared from stock solutions of bretylium and UM-360. Peaks: 1 (retention time 2.9 min) = benzenethiol disulfide, a contaminant formed from sodium benzenethiolate derivatization; 2 (retention time 3.6 min) = *o*-bromobenzyl phenyl thioether, a derivative of bretylium; 3 (retention time 5.3 min) = *o*-iodobenzyl phenyl thioether, a derivative formed from UM-360.

potassium triiodide extraction as described previously was  $95.9 \pm 3.0\%$ .

#### *Mass spectroscopy and identification of major peaks*

A total ion chromatogram and mass spectra of the three major peaks are given in Fig. 2. Peak 1 of the total ion chromatogram, with a base peak ( $m/e = 109$ ) corresponding to cleavage of the disulfide bond and a second peak ( $m/e = 218$ ) representing the molecular species was shown to be benzenethiol disulfide. Peak 2, with a base peak ( $m/e = 171$ ) corresponding to fragmentation of the thioether with formation of the resonance stabilized bromotropylium ion and a second peak representing the molecular species ( $m/e = 280$ ) was shown to be *o*-bromobenzyl phenyl thioether. Peak 3, with a base peak ( $m/e = 217$ ) corresponding to formation of an iodotropylium ion by fragmentation of the thioether, was shown to be *o*-iodobenzyl phenyl thioether.

#### *Canine plasma, urine and myocardial tissue samples*

Plasma levels of bretylium taken at appropriate intervals over a period of 12 h after a bolus intravenous injection of bretylium tosylate, 6 mg/kg, are given in Fig. 3. Plasma bretylium levels varied from a maximum of nearly 20  $\mu\text{g/ml}$  just after bretylium administration to a minimum of just over 2  $\mu\text{g/ml}$  at 12 h.

Myocardial tissue levels of bretylium were measured at the time of sacrifice, 12 h after the administration of bretylium (Fig. 4). Atrial tissue levels of bretylium were lower than those seen in right and left ventricular myocardium. A seven-fold ratio of ventricular myocardial tissue to plasma, bretylium concentration ( $\mu\text{g/g}$  myocardial tissue: $\mu\text{g/ml}$  plasma) was seen.

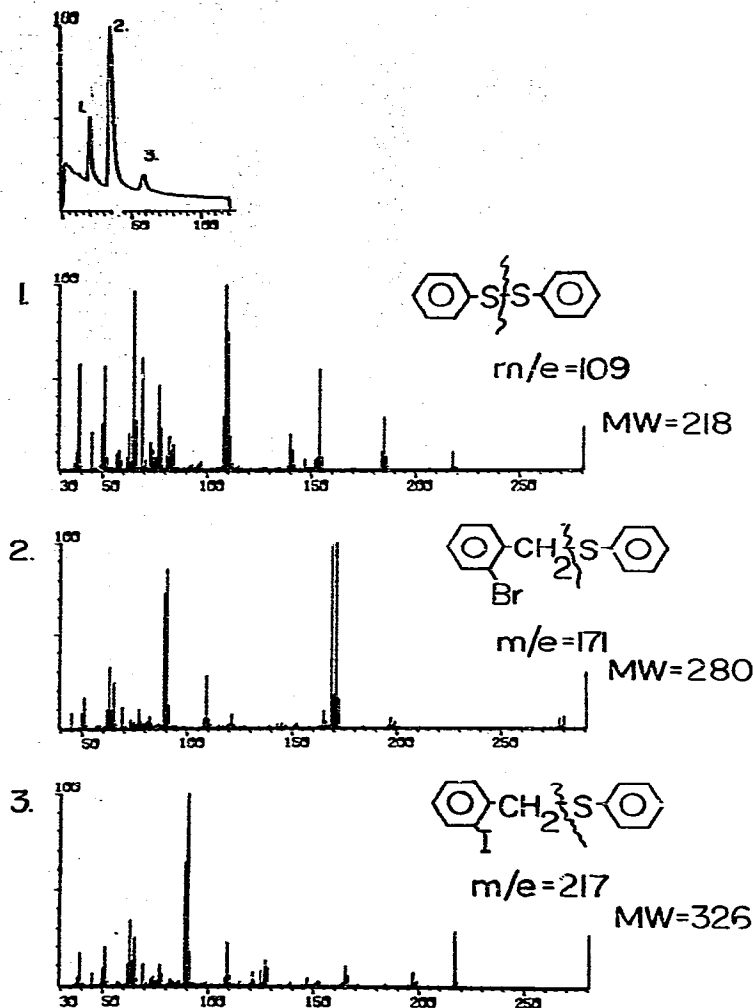


Fig. 2. Mass spectroscopic analysis. The three major peaks measured by electron impact mass spectroscopy corresponded to the three peaks measured by electron capture in Fig. 1. 1, ( $m/e=109$ ,  $m/e=218$ ), benzenethiol disulfide; 2, ( $m/e=171$ ,  $m/e=109$ ,  $m/e=280$ ), *o*-bromobenzyl phenyl thioether; 3, ( $m/e=217$ ,  $m/e=109$ ) *o*-iodobenzyl phenyl thio ether.

A 12-h urine sample was collected and the excreted bretylium quantitated. The total 12-h excretion of bretylium was  $23.4 \pm 3.9$  mg ( $50.6 \pm 8.3\%$  of the administered dose).

All samples were performed in duplicate with a mean difference of  $3.8 \pm 1.8\%$  between samples.

## DISCUSSION

The method of Kuntzman et al. [5] has a number of problems which prevent its general use for pharmacokinetic studies. (1) A large number of sample transfers and extractions are necessary for the isolation of bretylium and the elimination of substances from plasma and urine with similar retention

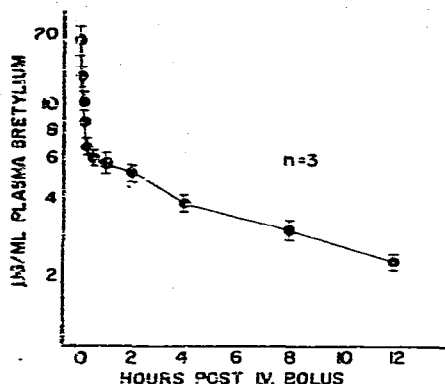


Fig. 3. Plasma bretylium concentrations. Plasma bretylium concentrations in an anesthetized dog after an intravenous (i.v.) bolus injection (6 mg/kg) of bretylium tosylate (mean  $\pm$  standard error of the mean).

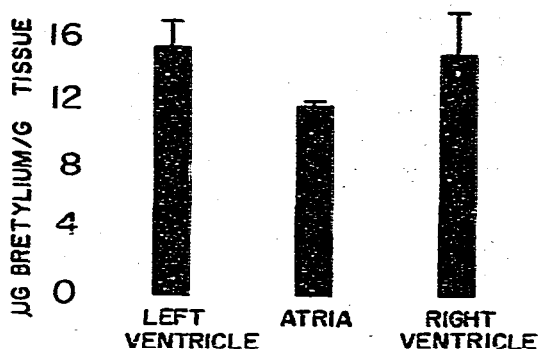


Fig. 4. Myocardial tissue concentrations of bretylium. Bretylium concentrations in canine myocardial tissue, 12 h after a single intravenous bolus injection of 6 mg/kg bretylium tosylate (mean  $\pm$  standard error).

times to those of the derivatized product and external standard. (2) Quantitation of the *o*-bromobenzyl phenyl thioether is possible only by the addition of an external standard, occurring after the extraction and isolation of bretylium. (3) The smallest detectable quantity of bretylium, 70 ng, is greater than the plasma levels necessary for observation of linear first-order pharmacokinetics in man. (4) The poor chromatographic separation of *o*-bromobenzyl phenyl thioether from benzenethiol required the use of conditions which resulted in long retention times for the *o*-bromobenzyl phenyl thioether (14 min) and the external standard (18 min).

The assay procedure for bretylium as given in this paper overcomes many of the problems occurring with the previous method. A single sample wash, followed by chloroform extraction of bretylium as the triiodide salt, is the only procedure necessary for the isolation of bretylium in a relatively pure form. Derivatization followed by evaporation and dissolution of the halogenated thioethers yields a clean chromatogram with no interfering peaks. This is in contrast to the multi-step procedures previously used to isolate bretylium in a relatively pure form [3] and to obtain a derivatized product with sufficient purity to achieve a clean chromatogram [5].

The use of an internal standard undergoing identical isolation and derivatization procedures as the measured product, bretylium, eliminates the problems which can occur due to inaccurate volume transfers occurring prior to the addition of an external standard. Also, the addition of an internal standard serves as a control for both the extraction procedure and derivatization procedure. The failure to detect bretylium in a sample, therefore, can be attributed to the absence of bretylium in a specimen, thus eliminating the possibilities of faulty extraction or derivatization.

The assay is both specific and sensitive for bretylium. Quantities of bretylium in excess of 1 ng are measured easily. The bretylium plasma levels in the

anesthetized dog were considerably higher than are seen in humans at similar dosages of bretylium [5]. However, we still have shown the ability to measure bretylium in quantities as small as one nanogram from plasma standards.

Benzenethiol and *o*-bromobenzyl phenyl thioether were easily separated by a number of column packings (see Apparatus) with retention times for the *o*-bromobenzyl phenyl thioether of 3.2–5.0 min and for *o*-iodobenzyl phenyl thioether of 4.2–6.2 min. No peaks with higher retention times were seen. This allows relatively rapid quantitation of injected samples.

The above assay possesses the required specificity, sensitivity and ease of sample preparation needed for pharmacokinetic studies of bretylium in man and animals.

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