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SIMPLIFIED DETECTION OF QUATERNARY AMMONIUM COMPOUNDS BY GAS CHROMATOGRAPHY

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SUMMARY

An analytical method for the measurement of quaternary ammonium compounds in biological fluids has been developed. Samples are prepared by forming the corresponding iodides, which are extracted and isolated. The residue is taken into *n*-hexane or into water and part of the solution obtained is injected onto the gas chromatograph where thermal degradation takes place. The methyl iodide released is measured by a ^{63}Ni electron capture detector. This method is quite sensitive and detects with good reliability and reproducibility as little as 10^{-14} mole quaternary ammonium compound.

INTRODUCTION

Clinical laboratories are in need of a reliable procedure to analyze quaternary ammonium salts because this class of compounds includes the neurotransmitter acetylcholine (ACh) as well as the anticholinesterases edrophonium, neostigmine and pyridostigmine, which are important in the diagnosis and management of myasthenia gravis[1]. Although a number of analytical methods have been used, most of them have serious shortcomings. Bioassay methods are frequently quite sensitive but of questionable specificity[2,3] whereas chemical methods are often very tedious and/or not sufficiently sensitive[4–6]. The analysis by gas chromatography appears most promising, but the methods currently available utilize pyrolysis of the sample and subsequent analysis by mass spectrometry[7]. Owing to complexity and the great expense, these latter methods are unsuitable for most laboratories.

In this paper, we report the development of a simplified gas chromatographic method that avoids the use of pyrolysis and mass spectrometry. We are able to measure acetylcholine, neostigmine and pyridostigmine with acceptable accuracy and requisite sensitivity.

EXPERIMENTAL APPARATUS

A research gas chromatograph (Hewlett-Packard Model 5756 B) equipped with a dual flame detector and a ^{63}Ni detector (splitter ratio 10:1) is used. In the glass columns, 1.8 m × 2 mm I.D., the following packing materials are employed: Porapak Q, 80–100 mesh (Waters Assoc., Milford, Mass., U.S.A.); Chromosorb 101, 80–100 mesh and Chromosorb 105, 80–100 mesh (Johns-Manville, Denver, Colo., U.S.A.); samples of 0.1–1.0 μl are injected manually with a standard microsyringe (Hamilton). The column section within the injection port is tightly packed with copper wool. The following temperatures are used: injection port, 320°; column oven, 145–170° (isothermal operation); flame ionization detector, 320°; electron capture detector, 250°. The gas flow-rates are: carrier gas (helium or nitrogen), 20 ml/min; hydrogen, 20 ml/min; air, 400 ml/min; argon–methane (9:1), 30 ml/min.

The area recorded is measured as the product of height and half the width under the peak or is directly obtained as the output of an electronic integrator (Hewlett-Packard Model 3370A). For positive identification and quantitative calibration the following standards are run under identical conditions: methyl, ethyl and propyl iodides; methyl and ethyl bromides; methyl, ethyl and propyl chlorides; potassium iodide, potassium triiodide in chloroform and iodine in chloroform or *n*-hexane. In addition, extracts of solutions containing known amounts of pyridostigmine, neostigmine or ACh are analyzed.

METHOD OF PROCEDURE AND RESULTS

General principle

The principle of this analytical method is to isolate the quaternary ammonium compound as its iodide from biological fluids. The iodide extract is then thermally degraded to release methyl iodide upon its injection onto the gas chromatographic column.

Extraction

The extraction of the quaternary ammonium compound from biological fluids as its iodide is achieved by a modification of the method of Vidic *et al.*[8] and is performed as follows.

One milliliter of serum or urine is adjusted to pH 6.0 with 1.0 *N* HCl or 1.0 *N* NaOH, extracted twice with 5.0 ml of chloroform, centrifuged at 12,000 *g* at 0° (Sorvall Model 2CB) for 10 min. To the retained aqueous phase are added 100 μl potassium triiodide [iodine–potassium iodide–water (1:2:20; w/w/v)] for the serum extraction and 400 μl of potassium triiodide for the urine extraction. The extract is agitated for 30 sec in a high-speed Vortex Mixer and centrifuged for 10 min at 15,000 *g*. The organic liquid phase is retained, the residue re-extracted in chloroform with the organic liquid phases then being combined. The combined organic extracts are vacuum desiccated over potassium hydroxide pellets for 48 h to remove iodine. The desiccated residue is redissolved in 1.0 ml of *n*-hexane for injection onto the gas chromatography column.

Detection

The methyl iodide released by thermal degradation is separated on the column and measured by the electron capture detector. The parallel trace of the dual flame ionization detector monitors the actual solvent volume injected as well as other organic residue present, thus serving as a quality control.

Quantitation of extraction and chromatographic measurement

The yield of the extraction has been determined by comparing the size of the chromatographic peaks obtained following extraction of known amounts of quaternary ammonium salts from biological fluids with those peaks obtained when a known amount of the iodine salt of that compound was injected directly onto the chromatographic column. This method demonstrates that the yield of extraction is consistently 95% or more. In control samples the molar ratio of chromatographically measured alkyl halide to quaternary ammonium salt present in the original samples is unity. Thus, the trace of the electron capture detector is a direct measure of the quaternary ammonium salt concentration, because we have observed that all of the quaternary ammonium iodide injected onto the column is thermally degraded and detected as the appropriate alkyl iodide and by demonstrating that these peaks have the same size as those obtained following direct injection of the appropriate alkyl iodide. With our present gas chromatographic systems we are thus able to measure with good reproducibility as little as 1×10^{-14} mole of quaternary ammonium compound.

Potential problems in the method

(1) Failure to refrigerate blood or serum below 12° results in significant decreases in quaternary ammonium compound concentrations after 5–6 h. This is most likely due to serum cholinesterase activity and refrigeration is particularly critical for neostigmine. (2) Failure to use sufficient potassium triiodide may result in inadequate conversion of all the compound to the iodide, which will result in loss of compound to the aqueous phase in the subsequent extraction. (3) The chromatographic retention time of the appropriate alkyl iodide is sufficiently different from any of the other conceivable thermal degradation products of the quaternary ammonium compounds studied which would capture electrons, but may be sufficiently close to that of iodine to make differentiation extremely difficult. This potential source of error is overcome by the prolonged desiccation step. Control samples of serum and urine (containing no added quaternary ammonium compounds) to which potassium triiodide is added in excess of the amount used for extraction reveal no chromatographic peak for iodine after 24 h of dessication. (4) Choline, which is found in serum, may also be demethylated at the temperature employed. However, choline iodide, which has poor solubility in organic solvents such as chloroform or *n*-hexane, does not appear in the fractions of the extract containing the iodide of neostigmine, pyridostigmine or edrophonium. An extremely small amount of material with an appropriate retention time does rarely appear in blank sera, and although this may be due to methyl iodide released by the degradation of choline iodide, it is present in such small amounts that it is difficult to differentiate it from baseline "noise" and does not alter the values obtained for serum quaternary ammonium compound being measured. (5) None of the solvents being used has column retention times similar to the alkyl iodides being measured.

DISCUSSION

The thermal degradation of quaternary ammonium halides is usually accomplished by pyrolysis of dried sample material[7]. We have achieved this fragmentation by direct injection of organic or aqueous solutions into an overheated injection port. At about 250° the ammonium salts begin cleaving, and with the injection port kept at temperatures of 310° the release of alkyl halide is complete and instantaneous. In order to avoid undercooling through evaporation of water and to facilitate quantitative formation of the volatile alkyl halide we packed the entrance section of the columns with copper wool or fine copper turnings. Practical experience has shown us that this copper wool has to be replaced as frequently as the silicone septum, or after about 30 injections, in order to maintain a stable baseline.

We have tried to achieve separation by using SE-30 or Dexsil 300 GC (Analabs, North Haven, Conn., U.S.A.), as column packing but were unsuccessful because the halide peak appears as a front shoulder of the solvent (*n*-hexane) peak at the temperatures (about 50°) which must be employed with these packings. Aqueous solutions showed a better separation, but the tailing of the water peak rendered this system useless for practical analysis. We have also tested the separation on porous polymers. By selecting a packing material with a suitable polarity, we were able to analyze hexane solutions of pyridostigmine on Porapak Q and on Chromosorb 101, and ACh dissolved in water is readily separated by Chromosorb 105.

It would be desirable to simplify the method to the point where serum could be injected directly into the column, but the treatment of serum or other biological fluids directly with potassium triiodide results in such a wide spectrum of halogenated compounds that to measure specifically for the presence of quaternary ammonium compounds in this manner would be impossible.

Quaternary ammonium anticholinesterases such as pyridostigmine and neostigmine are readily soluble in a number of volatile organic solvents, but ACh is not soluble in any suitable organic solvent and therefore must be dissolved in water. Whereas Chromosorb 101 clearly separates methyl iodide from the organic solvent peak, Chromosorb 105, which is a more polar packing material, is required for aqueous solutions. Chromosorb 105 is not suitable for the separation of pyridostigmine or neostigmine because of the coincident peaks of the organic solvent and methyl iodide with this packing materials.

Further increase of injection port or column temperatures does not result in the appearance of other fragmentation products which are detectable by the electron capture detector, despite the detection of additional organic peaks by the flame ionization detector.

This analytical method has the requisite simplicity and reliability to be utilized by any gas chromatography laboratory interested in studying these compounds.

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