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Note

Improvements in the gas chromatographic analysis of acetylcholine and choline

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Since the mid-sixties, there have been several reports of chemical methods for the determination of choline and acetylcholine. The chemical methods have the advantages of specificity, selectivity, reliability and in some cases sensitivity over bioassay methods. An excellent review of the chemical methods in current use has been compiled by Hanin [1].

Several reports in the literature of spurious rat brain choline and acetylcholine values were apparently due not only to the method of determination but to the post-mortem activity of several enzymes in the brain [2]. The use of high-power (5 kW) microwave irradiation has stabilized the values obtained for physiological levels of choline and acetylcholine [3].

In general, chemical methods can be divided into three groups: isotope derivative, fluorescence, and chromatographic methods. Gas chromatographic methods for cholinergic compounds differ primarily in their means of detection — mass spectrometry [4], nitrogen [5] or flame ionization [6] — and by their means of volatilizing acetylcholine and choline — demethylation [7] or pyrolysis [8].

This note describes advances in the gas chromatographic analysis of acetylcholine and choline and the incorporation of these techniques into previously reported methods [9].

EXPERIMENTAL

Materials

2,2',4,4',6,6'-Hexanitrodiphenylamine (dipicrylamine), butyryl chloride and 4-dimethylamino-3-methyl-2-butanone were obtained from Aldrich, Milwaukee, Wisc., U.S.A. Triton X-100 and 10% OV-17 on Gas-Chrom Q were obtained from Applied Science Labs., Richmond, Calif., U.S.A. Tris(hydroxymethyl methylaminopropane sulphonic acid) (TAPS) was obtained from Calbiochem, San Diego, Calif., U.S.A.

Synthesis of butyrylcholine iodide

Butyryl chloride (0.15 moles, 16.1 g) was added to 100 ml of acetone in a 500-ml round-bottomed flask in an ice-bath. 2-(N,N-Dimethylamino)ethanol (0.14 moles, 12.5 g) was dissolved in an equal volume of acetone and added slowly with stirring. The solution was stirred overnight under anhydrous conditions, cooled in an ice—acetone mixture and filtered in a büchner funnel. The white precipitate was washed two times with cold acetone and dried under vacuum.

The yield of the N,N-dimethylamino ethylbutyrate formed was 65%. Anhydrous diethyl ether (100 ml) was then added to the compound in a separatory funnel. Sodium hydroxide (0.91 moles, 3.6 g) was dissolved in 50 ml of water and then added to the solution. The water layer was then washed three times with 25 ml of ether. The ether extracts were combined and washed two times with 50 ml salt-saturated water. The ether layer was then filtered through a funnel containing anhydrous sodium sulfate. Methyl iodide (0.10 moles) was then added to the ether solution. Yellow crystals were precipitated from the solution after storing it in the freezer for 3½ h. The crystals were isolated, washed with acetone and tested for contamination by choline, which proved to be less than 1%.

Preparation of the column

OV-17 (10% w/w) on Gas-Chrom Q was pre-sifted through an 80-mesh sieve. Three grams of the 10% OV-17 were added to 10 ml acetone in a 100-ml round-bottomed flask. Triton X-100 (300 mg) was dissolved in 10 ml methanol and added to the flask. The suspension was blown dry with a nitrogen stream while rotating the flask by hand. The column packing was then dried in an oven for 12 h at 100°. The material was then put in a silanized glass column (1.2 m × 2 mm I.D.) and conditioned for 24 h at 150°.

A Beckman GC-65 dual-column gas chromatograph equipped with flame ionization detector was used. The gas flow-rates were: helium 40 ml/min, air 250 ml/min, and hydrogen 50 ml/min. The conditions were: column temperature 115°, injection port 200°, detector 225°.

Analysis of acetylcholine and choline

A gas chromatographic—mass spectrometric procedure for the micro-estimation of acetylcholine and choline [4, 9] was modified to allow for the gas chromatographic assay of acetylcholine and choline using butyrylcholine as an internal standard. Utilization of dipicrylamine to extract acetylcholine and choline into dichloromethane was retained. A new column material utilizing readily available materials (10% Triton X-100 and 10% OV-17 on Gas-Chrom Q) gave good results.

Following dissection of the brain from the skull, the brain was weighed and 50 nmoles of butyrylcholine were added to 4 ml acetone—1 *N* formic acid (85:15) for homogenization and analysis of whole brain. For half a brain or less, 2 ml acetone—1 *N* formic acid (85:15) were used. The homogenate was centrifuged at 26,000 *g* for 20 min. Two milliliters or the entire supernatant were transferred to a 10-ml screw-capped centrifuge tube. Two volumes (i.e. 4 ml) of diethyl ether were added to the tube, vortexed and centrifuged for

2 min. The ether phase was aspirated and residual ether and acetone distilled under a stream of nitrogen in a water-bath at 80° for 5 min. 2 *M* TAPS buffer (0.5 ml) (pH 9.2) was added to 2 ml of 2 m*M* dipicrylamine in dichloromethane and vortexed for 2 min followed by 2 min centrifugation. The upper aqueous layer was discarded and the organic phase transferred to another tube. The dichloromethane was evaporated in a stream of dry nitrogen. The sample was vacuum desiccated for 5 min. To the dried sample was added 0.5 ml 5 m*M* silver *p*-toluenesulphonate in acetonitrile and 50 μl double-distilled propionyl chloride. After mixing, the sample was left at room temperature for 5 min, and the solution evaporated with a stream of dry nitrogen; 0.5 ml of 50 m*M* sodium benzenethiolate and 25 m*M* benzenethiol in butanone was then added to the residue. The air in the tube was displaced by dry nitrogen, and the sealed tube was incubated at 80° for 30 min. The cooled sample was treated with 100 μl 0.5 *M* citric acid and washed three times with 1 ml pentane. Traces of pentane were evaporated with a stream of dry nitrogen. The tube was put in an ice-bath and 25 μl chloroform were added. Then 2 *M* ammonium citrate—7.5 *M* ammonium hydroxide buffer (0.1 ml) was added and the sample mixed for 2 min on a vortex mixer, and centrifuged for 2 min on a clinical centrifuge. A 2-μl aliquot of the chloroform extract was injected into the gas chromatograph for analysis. Reference data for calibration of the assay were obtained as a part of each experiment using 50 nmoles butyrylcholine as internal standard. Peak height ratios were obtained by dividing the acetylcholine and choline peak heights by the butyrylcholine peak height. The recovery of acetylcholine, choline and butyrylcholine was approximately 80% through the method when compared to the direct injection of 50 nmoles of 4-dimethylamino-3-methyl-2-butanone.

RESULTS AND DISCUSSION

Fig. 1 demonstrates the linearity of the method. The insert demonstrates that the method is linear below 10 nmoles. The sensitivity of the method is acetylcholine 0.5 ± 0.01 nmoles (S.E.), choline 0.5 ± 0.03 nmoles (S.E.). Butyrylcholine (50 nmoles) is used as internal standard in all of the tubes. A 50-nmole equimolar mixture of acetylcholine, choline and butyrylcholine gives a peak height ratio of approximately one. The linearity of choline is lost at concentrations above 400 nmoles. This is due to a loss of extraction efficiency above 400 nmoles and is correctable by a double extraction with 2 m*M* dipicrylamine. When the extraction volumes are combined, taken to dryness and the assay continued, the curve then remains linear above 400 nmoles.

Fig. 2A illustrates a tracing of an equimolar mixture (50 nmoles of acetylcholine, choline and butyrylcholine) after injecting 2 μl of chloroform into the gas chromatograph. The equimolar mixture was started at the beginning of the assay in a formic acid—acetone mixture. The peaks are symmetrical and narrow enough to allow for measurement of peak heights as well as area determination if an integrator is available. The efficiency of the column, expressed in theoretical plates per foot, is 113 for acetylcholine, 204 for choline, and 238 for butyrylcholine. The column has been in use six months and has not shown signs of deterioration.

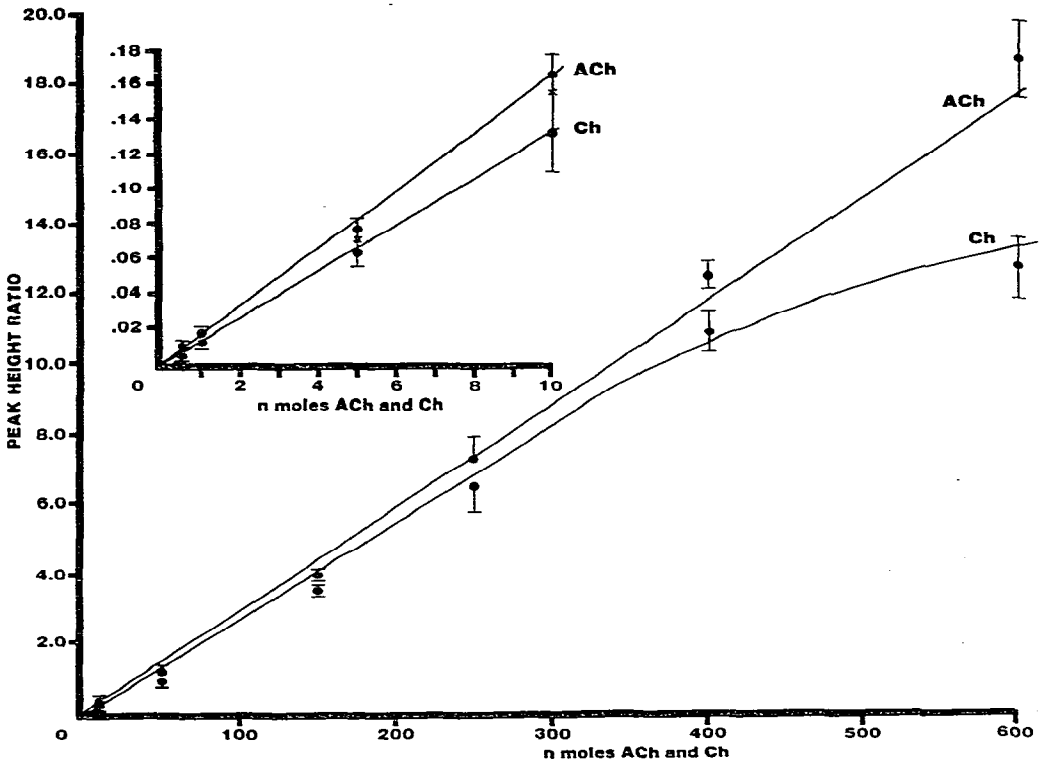

Fig. 1. Peak height ratios of choline (Ch) and acetylcholine (ACh) utilizing 50 nmoles of butyrylcholine (BCh) as internal standard. Insert shows the lower limit of sensitivity of the method (n = 6).

Fig. 2B represents an example of 50 mg of brain tissue taken through the method starting with the homogenization step. The sensitivity of the assay for acetylcholine in brain tissue is approximately 0.5 ± 0.07 nmoles (S.E.). Concentrations in whole brain tissue are 21.90 ± 1.36 nmoles/g (S.E.) for acetylcholine, and 62.15 ± 2.47 nmoles/g (S.E.) for choline (n = 6). This compares favorably with published reports in the literature in which whole brain levels were obtained without the use of microwave irradiation [6, 10].

Improvements in the assay of acetylcholine and choline have lagged slightly behind improvements in the detection devices. The sensitivity, selectivity and quantitative advantages obtained with a gas chromatograph—mass spectrometer tended to compensate for assay deficiencies. It was the purpose of this research to improve the assay to the point where it could reliably be used with a gas chromatograph equipped with a flame ionization detector. The method as used has an approximately 500% cost advantage over the initial purchase price of a gas chromatograph—mass spectrometer. In addition, a gas chromatograph costs much less to operate and requires less expertise. The column selected utilizes commonly available materials. The use of chloroform as the final injection solvent decreases variability over the more volatile dichloromethane required by the mass spectrometer. Consistency has also been enhanced by distillation of the acetone. For the first time, recovery through the method

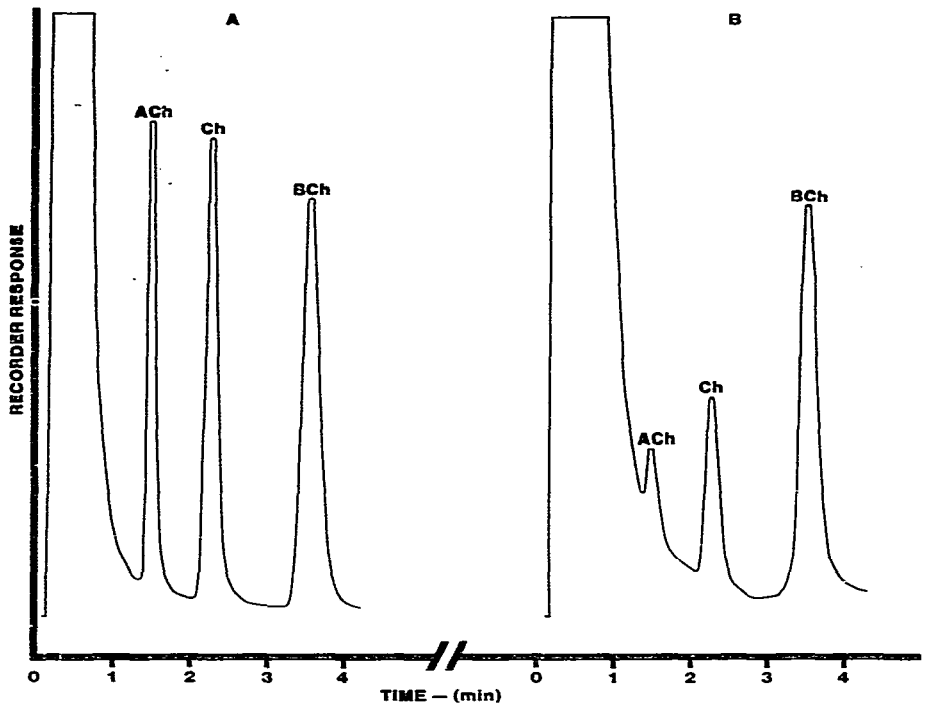

Fig. 2. Chromatogram of a 50-nmole equimolar mixture of acetylcholine (ACh), choline (Ch) and butyrylcholine (BCh) (A), and of a 50-mg rat brain extract (B). Two microliters of the chloroform layer were injected in (A) and (B). Attenuator settings: (A) 100 × 8; (B), 100 × 1. The levels of acetylcholine and choline in (B) were 1.09 ± 0.07 nmoles and 3.94 ± 0.16 nmoles (± S.E.), respectively (n = 7).

(80%) has been quantitated by the use of 4-dimethylamino-3-methyl-2-butanone. This assay and column should be readily adaptable to a nitrogen detector [5] or a stream splitter fitted to the gas chromatograph. These modifications will increase the sensitivity of the assay as well as allowing for the analysis of radioactive choline and acetylcholine.

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