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# SIMPLE METHOD FOR THE DETERMINATION OF CHOLINE AND ACETYLCHOLINE BY PYROLYSIS GAS CHROMATOGRAPHY

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## SUMMARY

An improved purification procedure is described for the simultaneous assay of endogenous choline and acetylcholine by pyrolysis gas chromatography, particularly for providing a simple and effective method for propionylation of choline in the presence of acetylcholine. The reaction was carried out in acetonitrile solution prepared by dissolving the evaporated residue of the supernatant of brain homogenate. Thus samples for propionylation were prepared without the use of ion-exchange chromatography.

## INTRODUCTION

Methods for the simultaneous assay of choline and acetylcholine by gas chromatography (GC) were developed [1—4] after instrumental assay methods for acetylcholine had been established [5—9]. These methods commonly include a demethylation procedure to produce volatile derivatives and acylation of choline to propionylcholine by reaction with propionyl chloride. For simultaneous demethylation of choline and acetylcholine, chemical or pyrolysis methods are generally used for GC and/or gas chromatography—mass spectrometric (GC—MS) analysis. However, chemical demethylation is complex, time-consuming, and requires meticulous anhydrous conditions. Therefore, pyrolysis has been generally applied to the GC separation of non-volatile quaternary compounds. On the other hand, complete esterification of choline with propionyl chloride by allowing the mixture to stand at room temperature for 5—30 min is not always reproducible and successive purification procedures with ion-exchange chromatography are necessary. In this paper, we have

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presented an effective method for direct propionylation of the amine in acetonitrile solution and confirmed its reproducibility. Also, samples for propionylation were prepared without the use of ion-exchange chromatography.

## MATERIALS AND METHODS

### *Apparatus and materials*

A Model NJE 2601 Metabostat (Shin-Nihon Musen, Japan) was used for microwave irradiation. The output of the device is adjustable from 0 to 5 kW at 2.45 GHz. A stabilized power supply maintains a constant power output from the magnetron 2M12 (New Japan Radio Company) even if input voltages vary by 10% from 200 V a.c. (60 Hz, rms). The magnetron is water-cooled and a thermal switch is used to monitor temperatures. The duration of irradiation can be set from 0.1 to 9.9 sec in 0.1-sec units with high reproducibility. GC analyses were carried out on a Shimazu Model 3BF chromatograph equipped with a hydrogen flame ionization detector, and a Model PYR-MS pyrolyzer developed as a result of cooperative effort between Kotaki-Shoji Co., Tokyo, Japan and the authors' group. The filament consists of 80% platinum and 20% radium, and is coil-shaped to allow the sample to be placed at the point of maximum heating. MS analysis was by a pyrolysis gas chromatograph—chemical-ionization quadrupole mass spectrometer (JEOL-Q1OA PGC/CI/QMS) coupled with a basic JMA-980A computer system and a high-speed graphic output system which includes a KSR-733 silent printer, and multiple ion detection capabilities. Both gas chromatographs were equipped with glass columns, 1.2 m × 3 mm I.D., packed with 5% OV-101, 5% dodecyldimethylenetriamine succinamide (Jenden Phase) on 80—100 mesh Gas-Chrom Q (Applied Science Labs., State College, Pa., U.S.A.).

The carrier gas was nitrogen for routine GC analyses and helium for the combined GC—MS studies. The flow-rate for both systems was 25 ml/min. The temperature of the columns was 85°, the injection port was at 130°, and the detector at 200°.

### *Animals*

Sprague—Dawley rats were obtained from Nihon Clea Co. and housed two per cage. The lights were automatically turned on at 8.00 a.m. in 12 h light—dark cycles. The rats were exposed to microwave irradiation at a level of 5 kW for 1.5 sec.

### *Chemicals*

All the common chemicals employed were reagent grade, obtained from either Wako Pure Chemical Industries (Tokyo, Japan) or Sigma (St. Louis, Mo., U.S.A.). Propionyl chloride was obtained from Tokyo Kasei Co. (Tokyo, Japan).

### *Extraction from tissue*

The principle is based on the procedures of Stavinoha et al. [8] and of Maruyama and Hosoya [10, 11] except for the propionylation procedure. An outline of the procedure is given in Fig. 1. Rats were sacrificed by microwave

irradiation and the brains were removed from the skull. Extraction of choline and acetylcholine was carried out with 3 ml of 15% 1 *N* formic acid in acetone, using 100 $\mu$l of 0.3 m*M* butyrylcholine as the internal standard, in a cold Polytron homogenizer at 6800 rpm for 20 sec. After standing in ice for 30 min, the homogenates were centrifuged at 16,000 *g* at 0° for 15 min. The supernatant solutions were washed twice with 1 ml of diethyl ether. After discarding the ether the aqueous portion was dried. The residue was dissolved in 100 $\mu$l of acetonitrile and 300 $\mu$l of propionyl chloride were added. This solution was allowed to stand at 60° for 40 min for complete acylation. The solvent was then evaporated with a nitrogen stream and the residue was dissolved in 200 $\mu$l of distilled water. Twenty microlitres of potassium peroxide solution (2 g of KI and 1.8 g $I_2$ in 10 ml water) were added to the solution and mixed well on the flushing mixer. After centrifugation by Beckman Microfuge for 3 min at 10,000 *g* the supernatants were removed and the precipitates were dissolved in 50 $\mu$l of acetonitrile. To remove excess iodide approximately 5 mg of macroporous AGI-X8-$Cl^-$ was added to the solution and the mixture was shaken for 5 sec. For assaying endogenous acetylcholine and choline, 2 $\mu$l of the mixture were put on the platinum ribbon of the pyrolyzer and pyrolyzed at 2 A for 7.5 sec.

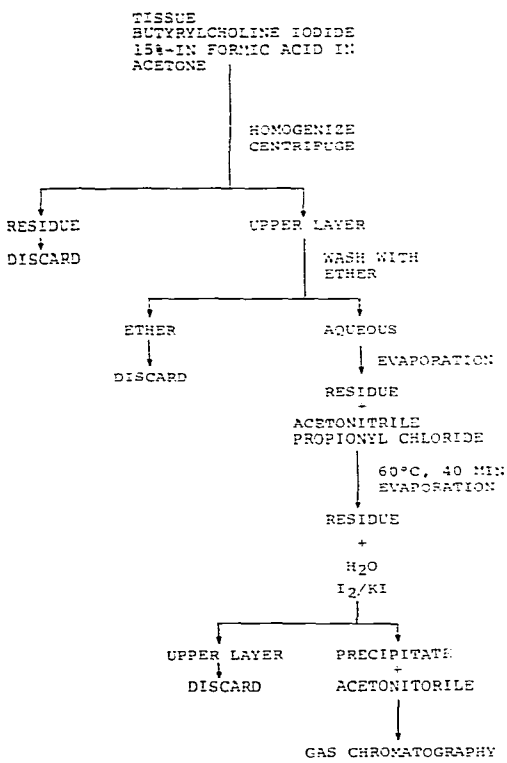


**Fig. 1. Extraction procedure for choline and acetylcholine from brain tissue.**

## RESULTS AND DISCUSSION

### *Conditions for propionylation of choline*

In order to determine the critical conditions for the unsuccessful propionylation of choline, standard samples in each tube were prepared as follows: 300 $\mu$l of propionyl chloride were poured into small tubes in which 100 $\mu$l of 0.3 m*M* acetylcholine iodide, 100 $\mu$l of 0.3 m*M* choline and 100 $\mu$l of 0.3 m*M* butyrylcholine iodide, all in acetonitrile, had been placed. The tubes were then allowed to stand at room temperature for 10, 20, 30, 40 and 60 min, respectively, and the percentage yield of the product in each sample was measured by GC after treating the samples with potassium periodide solution to precipitate the quaternary ammonium compounds. The results are shown in Fig. 2. The reaction gradually increased with time and reached a maximum, 92.0%, after 40 min. Next, the samples prepared as above were used to test the effect of temperature on maximum yield as shown in Table I. From this experiment, the reaction was complete after 40 min at 60°. The stability and reproducibility were confirmed by repeating the analysis on samples refrigerated at 4° for two weeks.

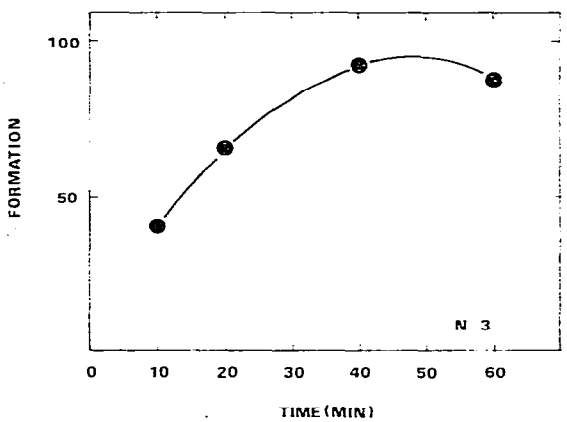


Fig. 2. Effect of reaction time on propionylation at room temperature (23°).

TABLE I

EFFECT OF TEMPERATURE ON PROPIONYLCHOLINE PRODUCTION

Values represent the percentage formation of propionyl ester, mean ± S.E. ($n$ = 3).

| Heating time (min) | Temperature (° C) | | |
|---|---|---|---|
| | 23 | 40 | 60 |
| 10 | 35 ± 5.3 | 45 ± 0.4 | 76 ± 1.9 |
| 40 | 56 ± 0.6 | 82 ± 1.1 | 102 ± 2.2 |

*Sensitivity and standard curves*

Using this method, propionyl choline and acetylcholine were separated, and their sharp peaks were observed within 15 min on the gas chromatogram as the pyrolyzed products (Fig. 3). Peaks derived from biological samples were compared with those from the standards by pyrolysis GC—MS analysis [12].

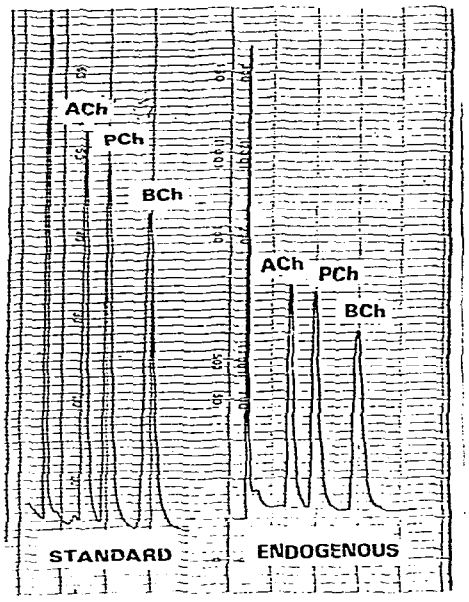


Fig. 3. Typical gas chromatogram of propionylcholine (PCh) and acetylcholine (ACh) as pyrolized esters of dimethylaminoethanol. Peaks of standard compounds of PCh and ACh are seen on the left and those derived from endogenous compounds are shown on the right. Butyryl choline iodide (BCh) is used as internal standard.

The correlation between the ratio and amounts of the compounds was excellent. The measurement for acetylcholine was more sensitive than propionylcholine; however, propionylcholine could be determined down to the 30—240 picomole range.

*Recovery from tissue*

The efficiency of the assays described was tested by studying the recovery of choline added to brain homogenate. The results of the experiment are shown in Table II. The total recovery for choline represents the 38.0 ± 0.96 nmoles of added standard plus the endogenous amount of the compound extracted from 1 ml of brain homogenate. With the procedure shown in Fig. 1, a normal level of acetylcholine was also assayed without interference. When the added standard was subtracted, endogenous choline was estimated at 8.1 ± 0.98 nmoles/ml of homogenate. The total recovery of added choline by this method was therefore 99.6 ± 4.2%. In order to confirm reproducibility, further experiments assaying the endogenous compounds from rat brain after decapitation or by microwave irradiation were carried out (Table III). Assayed levels after microwave irradiation were 25.5 ± 3.76 for choline and 26.9 ± 1.30 nmoles/g

TABLE II

RECOVERY OF CHOLINE ADDED TO BRAIN HOMOGENATE

Choline and butyrylcholine (30 nmoles) were added to 1 ml of brain homogenate and extracted in the manner shown in Fig. 1. The values (nmoles/ml homogenate) represent the mean ± S.E. from three determinations.

| Amines | Normal endogenous | Added | Theoretical total | Assayed total | Recovery (%) |
|---|---|---|---|---|---|
| Choline | 8.1 ± 0.98 | 30 | 38.2 ± 0.96 | 38.0 ± 0.96 | 99.6 ± 4.2 |
| Acetylcholine | 8.3 ± 0.00 | — | — | 8.3 ± 0.29 | — |

TABLE III

ACETYLCHOLINE AND CHOLINE LEVELS (nmoles/g WET WEIGHT) IN RAT WHOLE BRAIN

The values represent the mean ± S.E. of five determinations. Choline as its propionyl ester and acetylcholine were assayed by pyrolysis GC using butyrylcholine as an internal standard.

| | Acetylcholine | Choline |
|---|---|---|
| Microwave (5 kW for 1.6 sec) | 26.9 ± 1.3 | 25.5 ± 3.8 |
| Decapitation | 14.4 ± 1.3 | 172.3 ± 30.7 |

tissue for acetylcholine, and those by decapitation were 172.3 ± 30.7 and 14.4 ± 1.29 nmoles/g tissue, respectively. The levels of the amines after microwave irradiation or decapitation were clearly in accord with those reported by Stavinoha [2, 13]; that is, levels of the amines were found in approximately equimolar amounts in rat whole brain, and the variations previously reported [14—16] were not seen.

In summary, we have provided an improved method for the esterification of choline in the presence of acetylcholine and demonstrated the usefulness of pyrolysis gas chromatography for assaying both amines simultaneously in brain tissue.

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