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DETERMINATION OF PICOMOLE AMOUNTS OF CHOLINE AND ACETYLCHOLINE IN BLOOD BY GAS CHROMATOGRAPHY–MASS SPECTROMETRY EQUIPPED WITH A NEWLY IMPROVED PYROLYZER

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SUMMARY

Blood levels of choline (Ch) and acetylcholine (ACh) have previously not been confirmed because of the difficulty in measuring these compounds. In this paper a recently developed method for the assay of Ch and ACh, which employs a chemical ionization (isobutane) quadrupole mass spectrometer (JEOL QH 100) equipped with a new type of pyrolyzer is reported. The correlation between the mass fragmentogram peak ratios and the amounts of compounds was good from 0.5 to 4 pmol. The assay limit for quantitation was *ca.* 0.3 pmol for Ch and ACh. The values for Ch from rat whole blood, serum and red blood cells were 10.88 ± 1.46, 6.72 ± 1.02 and 4.56 ± 0.53 nmol/ml, respectively, and for ACh the respective values were 3.56 ± 0.86, 1.69 ± 0.16 and 1.87 ± 0.83 nmol/ml. In human whole blood the levels of Ch and ACh were 22.55 ± 3.97 and 3.23 ± 0.23, respectively.

INTRODUCTION

A major obstacle in clarifying the neurophysiological role of choline (Ch) and acetylcholine (ACh) has been the delay in the development of a simple assay method, even though the role of ACh as a neurotransmitter was demonstrated more than 50 years ago. Among the large number of analytical methods, gas chromatographic (GC) and GC–mass spectrometric (MS) techniques have been essential for use in the accurate estimation of neurotransmitters in biological specimens, as described in a previous report[1]. Specifically, pyrolysis–GC (Py–GC) and Py–GC–MS procedures, principally based on the method of Szilagyi *et al.*[2] and other investigators (3–6) have provided excellent accuracy, sensitivity and reproducibility for these measurements. We have been working on these assay methods for *ca.* 10 years, with the results being an improved method which has been applied to the measurement of picomole amounts of Ch and ACh in blood. The levels of these compounds in blood have previously been determined by several investigators, but the results have not been in good agreement. In this paper, this recently developed method for the assay of Ch and ACh in rat and human blood, incorporating a new type of Py–GC chemical ionization (isobutane) quadrupole MS with hyperbolic rods is reported. In addition, a newly improved type of pyrolyzer for the demethylation reaction has been used to produce the volatile derivatives.

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MATERIALS AND METHODS

Materials

Animals. Male Sprague-Dawley rats were obtained from Nihon Clea and housed two per cage. The lights were automatically turned on at 8:00 a.m. in a 12-h light–dark cycle.

Chemicals. All the common chemicals employed were reagent grade, obtained from either Wako (Tokyo, Japan) or Sigma (St. Louis, MO, U.S.A.). Propionyl chloride was purchased from Tokyo Kasei (Tokyo, Japan).

Apparatus

Pyrolyzer. The pyrolyzer, a Model PYR-MS, was developed as a result of cooperative efforts between Kotaki Shoji (Tokyo, Japan) and the authors' group. The pyrolyzing system consists of three parts: the filament holder, the switching connector and the power source. The pyrolyzing system is designed to be applicable to most types of gas chromatograph, and does not allow flash-back of the carrier gas to occur during the procedure of inserting or removing the filament holder. This technique is successfully carried out by means of a switching connector, as shown in Fig. 1. For this method a few microliters of sample are placed on a filament which is made of 80% platinum and 20% rhodium, as described in the previous report[1]. The filament is either V-shaped or in the form of a coil to allow the sample to be placed at the point of maximum heating. After evaporation of the solvent, using the proper conditions set on the power supply, the filament holder is attached to the switching connector using the screw holder, while keeping the switch closed. Then, the switch is opened and the filament holder is inserted into the end of the holder so that the top of the filament sits immediately in front of the column in the gas chromatograph. The duration of pyrolysis and the amount of current utilized are adjustable on the power source. The temperature of the filament at a particular setting of the power source was determined by employing a 0.3-mm chromel–alumel thermocouple welded to the filament of the pyrolyzer. The resultant electromotive force was recorded as the filament was heated and the values were converted to temperature by use of "Chart of Electromotive Force of Chromel–Alumel Thermocouple".

Mass spectrometer. Mass spectral studies were accomplished using a JMS-QH100 gas chromatograph– chemical ionization (CI) quadrupole mass spectrometer which was recently developed as a result of cooperative efforts between Japan Elec-

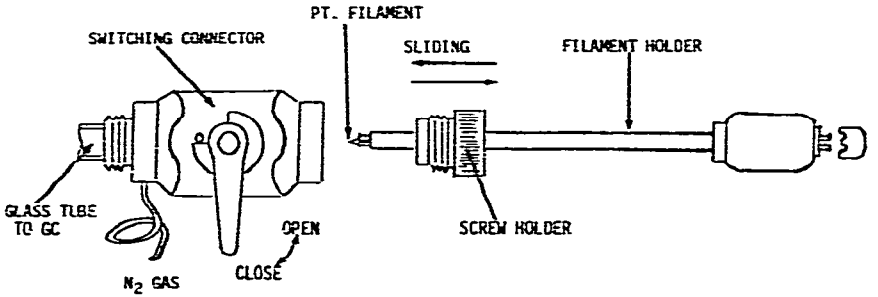

Fig. 1. Diagram of pyrolyzer showing parts of switching connector and filament holder. Explanation is given in the Materials and methods section.

tron Optics Laboratory and the authors' group. This instrument is primarily designed to provide accurate and rapid quantitation of organic compounds, particularly those derived from biological specimens. The resolution of this mass spectrometer, 1600 ($M/\Delta M_{1/2}$) at m/z 800, is accomplished by utilizing large diameter hyperbolic rods for the quadrupole mass analyzer. A single ion source for CI is used. For CI operation one of three kinds of reagent gases can be alternatively selected within 20 sec. For control of the system, three 16-bit microcomputers function in individual modes. The first one is designed to control the opening and the closing of the solvent valve and for adjusting the temperatures of the GC, the GC–MS interface and the ion source. The second computer is mainly used for monitoring ions for mass fragmentographic analyses. The last computer is designed for processing of PFK calibration, normalization of mass signals, background subtraction and quantitative calculations using peak height or peak area values from the mass fragmentograms. The analysis time is divided into three blocks for each injection, so that the four channels which are employed in each block for mass fragmentography permit the selection of twelve different ions over the entire scan. These operations are controlled by real-time dialogic communication between the computers and an operator via a display screen and keyboard. For the measurements described in this paper the ion energy was 7.5 V with an ionizing voltage of 250 eV and ionizing current of 300 μA. Isobutane was used as the reagent gas for CI and was maintained at a pressure of 0.9–1.1 Torr.

Gas chromatograph. The gas chromatograph was attached to the mass spectrometer was equipped with a glass column (1.0 m × 3 mm I.D.) packed with 5% OV-101 and 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 helium for the GC–MS studies, with a flow-rate of 30 ml/min. The temperature of the column was 169°C, the injection port and the interface were 180°C and the ion source was 170°C.

Methods

Extraction procedure. Rat blood was collected from the tail vein by means of a glass pipetter. Human blood was drawn from the cephalic vein. The blood sample (200 μl) was transferred to a glass tube (100 × 15 mm) which contained 5 μl of 3 m*M* [$^2H_9$]ACh as an internal standard and 0.3 ml of 2 m*M* physostigmine. After addition of 1 ml of 0.4 *N* perchloric acid the mixture was stirred vigorously to effect protein denaturation. After centrifugation at 1500 *g* at room temperature for 10 min the supernatant was adjusted to pH 4.0 with 7.4 *N* potassium acetate. The sample was centrifuged again at 1500 *g* at room temperature for 5 min and the supernatant was washed with ether. The remaining aqueous phase was lyophilyzed. The residue was treated with propionyl chloride to form the propionyl ester of choline and further purified by the procedure which is outlined in Fig. 2. This method has previously been reported as an improved propionylation procedure for the simultaneous assay of endogenous Ch and ACh[1].

Assay for ACh-esterase activity in blood. AChE activity was measured by the spectrophotometric method of Ellman *et al.*[7].

RESULTS

Pyrolysis conditions for ACh, propionylcholine (PCh) and butyrylcholine (BCh)

In order to determine the optimum pyrolysis conditions for ACh, PCh (to be

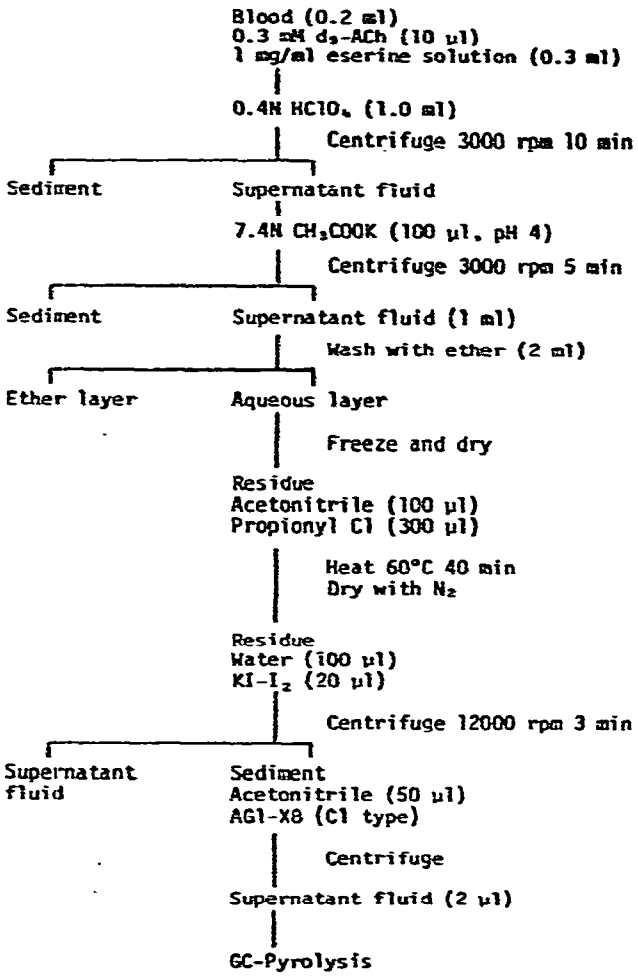

Fig. 2. Flowsheet for simultaneous extraction procedure of choline and acetylcholine from rat and human blood.

derived from Ch) and BCh (to be used as an internal standard for GC assays), the relationship between the peak heights of the pyrolyzed compounds and the filament temperature was ascertained, as shown in Fig. 3. Rapid and effective demethylation of the three compounds was accomplished by supplying 2.5 A for 7.5 sec. The temperature of the filament which produced the maximum peak height was 216°C.

Mass fragmentograms and standard curves for ACh and PCh

The ions at *m/z* 132, 138 and 146, corresponding to M + 1 for demethylated ACh, deuterated ACh ($^2H_9$, the internal standard for MS analyses) and PCh were used. As shown in Fig. 4, these substances eluted within one minute for standards and blood samples. The peak height ratios of standard ACh and PCh to [$^2H_9$] ACh were automatically calculated following the scan of the selected ions, providing the standard curves for ACh and PCh. An excellent correlation between the peak height ratio

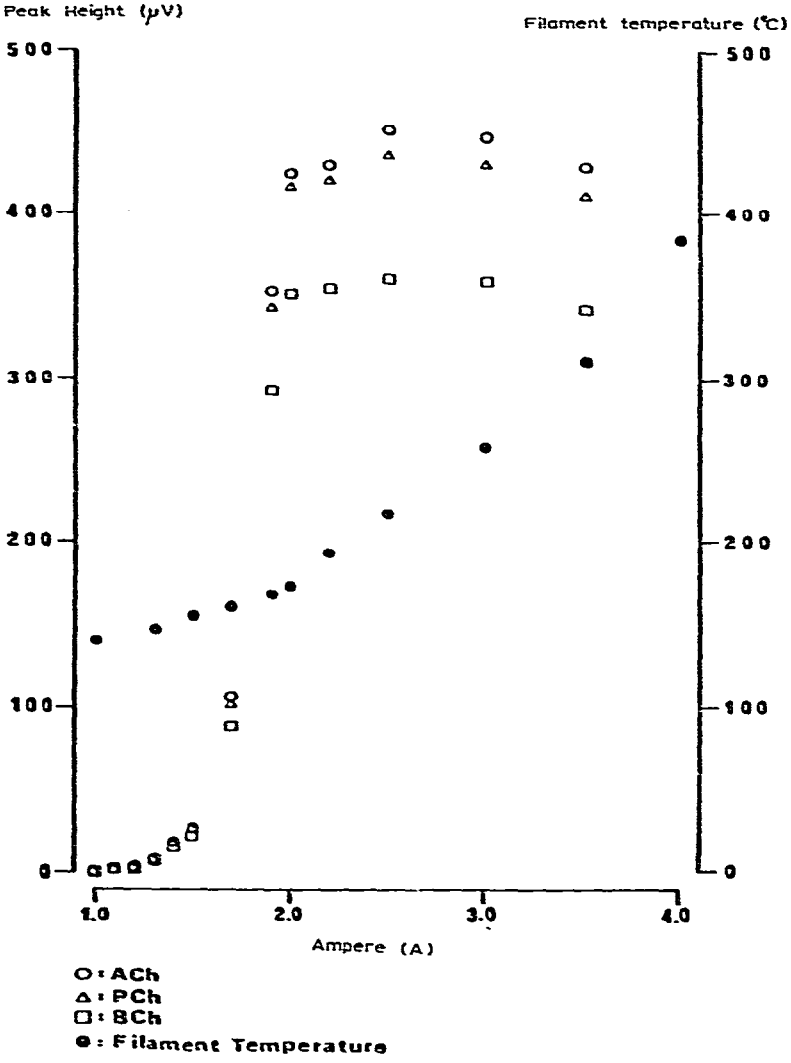

Fig. 3. Effect of temperature and current on demethylation of esters of choline.

and compound amount was obtained, with correlation coefficients of 0.9998 for ACh and 0.9991 for PCh found, as shown in Fig. 5. Linearity was seen in the range 0.5–4.0 pmol, with no significant changes in sensitivity observed for either compound. The assay limit for quantitation was *ca.* 0.3 pmol for ACh and PCh. To test the applicability of this method for assaying endogenous compounds, blood samples were spiked with various amounts of each standard and assayed by pyrolysis GC–MS. The results were parallel to the standard curves. Clear linearity was again obtained, with correlation coefficients of 0.9997 for ACh and 0.9946 for PCh being calculated.

Total recovery from rat blood

The efficiency of the assay being described was examined by studying the re-

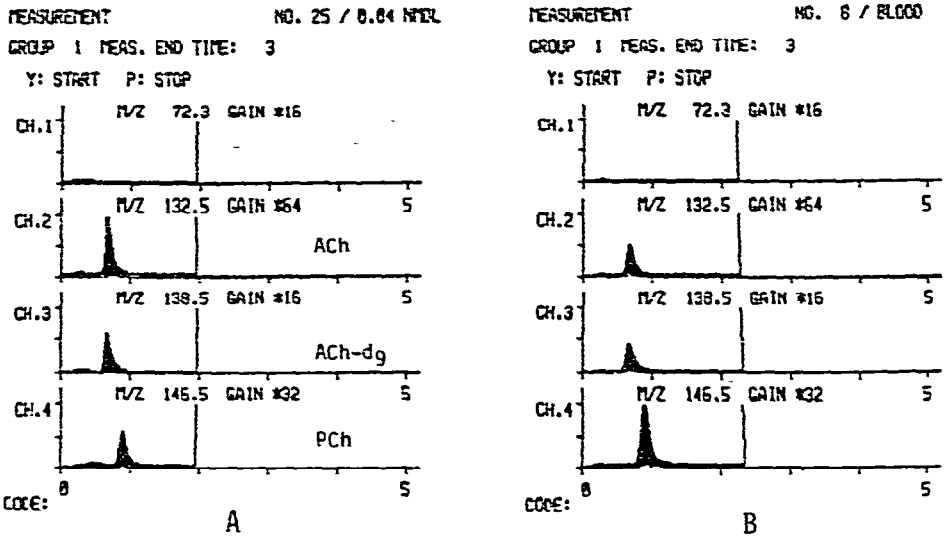

Fig. 4. Mass fragmentogram (A) of standard acetylcholine (ACh), [$^2H_9$]acetylcholine ([$^2H_9$]ACh), and propionylcholine (PCh) compared to the mass fragmentogram (B) of blood with added [$^2H_9$]ACh. The choline in the sample was propionylated.

covery of Ch and ACh added to blood. The results of the experiment are shown in Table I. The total recovery of Ch and ACh through this procedure ranged from 98.3 to 114.3%.

Levels of Ch and ACh in rat and human blood

The levels of ACh and Ch measured by this method in rat whole blood, serum and red blood cells, and in human whole blood are shown in Table II. The value of Ch and ACh in rat red blood cells was obtained by subtracting the levels found in serum from those found in whole blood.

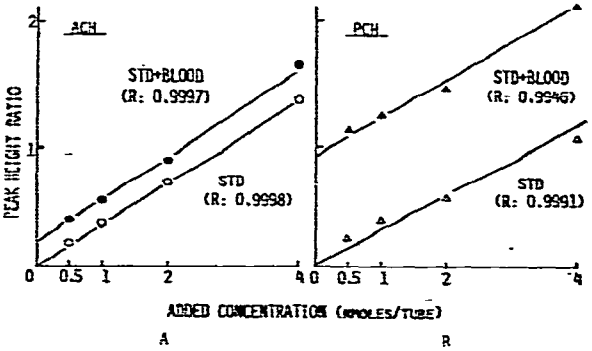

Fig. 5. (A) Calibration curves for standard ACh and standard ACh plus blood. (B) Calibration curves for standard PCh and standard PCh plus blood.

TABLE I

TOTAL RECOVERY OF CHOLINE AND ACETYLCHOLINE FROM BLOOD

Choline and acetylcholine were added to blood and extracted in the manner shown in Fig. 2. The values (nmol/ml) represent the mean ± S.E. from three determinations.

	Blood	*Std. (1 nmole)*	*Blood ± std (theorectical)*	*Blood ± std (measured)*	*Recovery (%)**
ACh/[$^2H_9$]ACh	0.119 ± 0.003	0.355 ± 0.030	0.474 ± 0.029	0.545 ± 0.041	114.3 ± 12.02
PCh/[$^2H_9$]ACh	0.973 ± 0.077	0.299 ± 0.028	1.273 ± 0.094	1.237 ± 0.017	98.3 ± 7.96

$$\text{* Recovery} = \frac{\text{blood + std. (measured)}}{\text{blood + std. (theoretical)}} \times 100. \quad n = 3.$$

TABLE II

CHOLINE AND ACETYLCHOLINE LEVELS IN RAT AND HUMAN BLOOD

Values expressed as mean ± S.E. and figure in parentheses shows number of animals. Results of human blood were obtained from three determinations for each sample.

		Ch (nmol/ml)	*ACh (nmol/ml)*	*Ch/Ach (nmol/ml)*
Rat (n = 3)	Whole blood	10.88 ± 1.46	3.56 ± 0.86	3.68 ± 1.29
	Serum	6.72 ± 1.02	1.69 ± 0.16	3.74 ± 0.86
	Red blood cells	4.56 ± 0.53	1.87 ± 0.83	4.56 ± 2.72
Human whole blood	1	45.95 ± 0.25	4.58 ± 0.11	10.04 ± 0.29
	2	17.97 ± 0.35	2.96 ± 0.05	6.03 ± 0.36
	3	11.11 ± 0.11	2.81 ± 0.05	3.96 ± 0.15
	4	15.16 ± 0.23	2.57 ± 0.07	5.85 ± 0.12

DISCUSSION

A newly developed pyrolyzer which prevents flash-back of carrier gas during the application of the sample on the filament has been connected to a GC–MS designed for rapid and effective measurement of relatively low molecular weight (m/z 800 maximum) organic compounds. In this study, [$^2H_9$]ACh was used as an internal standard for analysis of Ch and ACh. No transacylation was observed under the present conditions. With this equipment at least 30 samples can be processed per day by well-trained laboratory technicians.

Systematic studies have not been reported regarding changes in blood levels of Ch and ACh which occur during the isolation and purification of the samples. In our experiments there were significant changes in the blood levels of Ch and ACh when human blood was drawn from the cephalic vein and left at room temperature for 30 sec. Stavinoha *et al.*[8] have reported that an increase in ACh of up to 6.05 ± 1.36 nmol/ml was observed 1 min after intravenous infusion of the compound. The level returned to the original control value by 15 min. Unlike our assay method, Stavinoha *et al.* do not add AChE inhibitors to the blood. To examine the necessity for rapid inactivation of AChE prior to measurement of Ch and ACh in blood, the activity of

this enzyme in rat and human blood was determined and found to be 2.78 μmole/ml·min for human blood and 0.485 μmole/ml/min for rat blood. The data suggested that ACh in human blood can be hydrolyzed six times faster than in rat blood. Theoretically 465 nmole of ACh could be hydrolyzed in human blood per second at room temperature as the maximum figure from the *in vitro* study. This indicates the importance of rapid inactivation of the enzyme before assaying for Ch and ACh in blood.

The levels of Ch and ACh assayed in human blood by our method were 10–200 times higher than those given in Hanin *et al.*'s review[9]. In addition, the mean of the ratio between Ch and ACh in human blood was 57 times higher in our study than that of plasma reported in his chapter. Our measurements of Ch and ACh levels were relatively close to those in Stavinoha's recent report[10].

In summary, the presence of Ch and ACh in rat and human whole blood was clearly confirmed by a newly developed type of Py–GC–MS analysis. The levels of Ch and ACh in the blood were significantly higher than those reported by most other scientists.

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