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DETERMINATION OF CHOLINE AND ACETYLCHOLINE LEVELS IN RAT BRAIN REGIONS BY LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

Regional choline (Ch) and acetylcholine (ACh) in rat brain were clearly determined by high-performance liquid chromatography with electrochemical detection. The method is based on that of Potter *et al.*: the hydrogen peroxide that is enzymatically produced from both compounds is measured and a successful improvement of the method, particularly for purification, is described. Recoveries were 96.1 ± 1.4% for Ch and 95.6 ± 2.2% for ACh and amounts as low as 10 pmol could be determined. Prior to measuring the compounds, a newly developed magnetic field microwave instrument (10 kW) was utilized for the rapid inactivation of brain enzymes. The levels of Ch and ACh in brain regions were compared with those reported elsewhere.

INTRODUCTION

Clarification of the neurophysiological roles of choline (Ch) and acetylcholine (ACh) has been difficult owing to the lack of simple assay methods for their determination in biological specimens. This difficulty is related to the fact that both of these species are quaternary ammonium compounds. Among several attempted methods, however, gas chromatography–mass spectrometry (GC–MS) has proved useful for the simultaneous determination of both compounds[1–5]. Many workers have investigated procedures using liquid chromatography as a more economical approach, and a method employing this technique has recently been established by Potter *et al.*[6]. The method consists in measuring hydrogen peroxide derived from the reaction of Ch with choline oxidase. The choline oxidase is contained in a separate solution, which is mixed with the effluent from the separation column. ACh eluted from the column is converted into Ch by adding acetylcholinesterase (AChE) to the solution containing choline oxidase. The hydrogen peroxide is directly detected by an electrochemical detector[7,8]. Based on this approach, we have established an improved method incorporating simplified extraction procedure. The levels of Ch and ACh in rat brain regions following killing by either decapitation or microwave ir-

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radiation (MWR) (10 kW) were then determined by this procedure[9] and compared with those obtained using GC–MS[9].

EXPERIMENTAL

Animals

Male Sprague–Dawley rats (Charles River Japan), weighing 340–370 g and aged 10 weeks, were housed five per cage with standard food and water *ad libitum*. The lighting period was from 07:00 to 19:00 (12 h).

Reagents

All reagents were of the highest available purity, purchased from commercial sources, and were used without further purification. The only exception was diethyl ether, which was used for synthesizing ethyl homocholine (EHC), and which was redistilled. AChE Type III, Ch oxidase Type III, 3-dimethylamino-1-propanol (DAP) and N,N-dimethyl(N-ethyl)-3-amino-1-propanol (DMAP) were purchased from Sigma (St. Louis, MO, U.S.A.).

Microwave instrument

A new microwave device (NJE Model 2603) was used to inactivate rapidly brain enzymes. This unit has a power output of 10 kW at 2450 MHZ[10]. In order to achieve an even distribution of microwave heating with a minimum amount of trauma and with a maximum certainity concerning inactivation of appropriate enzymes, a predominately magnetic field distribution was concentrated on the head of the animal rather than the conventional electric field. An integrated tuning system was used to increase the efficiency and the distribution of microwave energy absorption. Also, a newly designed animal container constructed with a water-jacket was used.

Tissue preparation

The rats were killed either by microwave irradiation (MWR) (9.0 kW, 1.1 sec) or decapitated by guillotine. The brain was rapidly removed and dissected into seven regions following the method of Glowinski and Iversen[11].

LCEC system

The solvent delivery system consisted of Model 110A and Model 112 pumps (Beckman, Fullerton, CA, U.S.A.). The pumping rates were set at 0.8 ml/min for the mobile phase and 0.5 ml/min for the enzyme-containing solution. The mobile phase consisted of 0.01 *M* sodium acetate adjusted to pH 5.0 with 0.02 *M* citric acid, containing 5% acetonitrile, 1.2 m*M* tetramethylammonium (TMA) chloride and 0.1 m*M* sodium octyl sulphate (SOS). The enzyme solution consisted of 0.2 *M* sodium phosphate buffer (pH 8.5) containing 2 units/ml of AChE and 1 unit/ml of choline oxidase. A Chemcosorb 3 μm C-18 reversed-phase column (100 × 6.0 mm I.D.) (Chemco Scientific, Osaka, Japan) was used for the separation of Ch, EHC and ACh. The reaction coil was composed of a 10-m length of poly(vinyl chloride) tubing of 0.5 mm I.D. The effluent from the column flowed to the reaction coil at a 180° angle through a PTFE tee-connector, while the enzyme solution entered the stream at a 90° angle. The LC-3A amperometric detector (Bioanalytical Systems, Indianapolis,

IN, U.S.A.) was equipped with a platinum working electrode with the potential being set at +0.6 V *vs.* an Ag–AgCl reference electrode. The output of the detector was set at 10–100 nA full-scale, depending on the concentrations of ACh and Ch.

Extraction from tissue

The basic extraction was based on the procedure of Maruyama *et al.*[4], which is much less complex than that described by Potter *et al.*[6]. Dissected tissue was homogenized with 1 ml of 15% formic acid in acetone containing 10 nmole of ethyl homocholine (EHC) as the internal standard. For homogenization an ultrasonic cell disruptor (Sonifier, Model 200; Branson, U.S.A.) at 13% power output (20 W) for 30 sec was used. After standing in ice for 30 min, the homogenate was centrifuged at 20,000 *g* at 4°C for 15 min. The supernatant solution was washed once with 1 ml of diethyl ether. After discarding the ether, the aqueous portion was dried with a stream of nitrogen. The residue was dissolved in 200 μl of distilled water and centrifuged at 10,000 *g* using a Beckman Microfuge for a few minutes to separate the insoluble residue. An aliquot of the supernatant was them used for direct injection into the chromatograph (normal extraction).

If minimization of the initial solvent peak was required, further purification was accomplished as follows. The supernatant was transferred into a small tube and 20 μl of potassium triiodide solution (2 g of KI and 1.8 g of I_2 in 10 ml of distilled water) were added and mixed well on a vortex mixer. After centrifugation using a Beckman Microfuge at 10000 *g* for 3 min, the supernatant was decanted and the triiodide precipitate was dissolved in 200 μl of distilled water. To remove excess of triiodide, approximately 30 mg of macroporous AG 1-X8 (Cl^-) were added to the solution and the mixture was shaken for 5 sec on the vortex mixer. After centrifugation with the above equipment at 10000 *g* for 3 min, 5–10 μl of supernatant were used for injection into the chromatograph (extended extraction).

Effect of temperature on the stability of Ch and ACh

Amounts of 20 nmole each of Ch and ACh were dissolved in 1 ml of distilled water (pH 5.6) or 0.2 *M* sodium phosphate buffer (pH 7.4) and heated at 95°C for various periods of time in a water-bath. After the solution had cooled to room temperature, 50 nmole of EHC (in 50 μl) were added as an internal standard. An aliquot of the solution was then injected into the chromatograph. The peak-height ratios of Ch and ACh to the internal standard were compared for the heated and unheated solutions and the effect of heating was evaluated.

RESULTS

Chromatographic conditions providing effective hydrogen peroxide production

A successful assay of Ch and ACh by this system requires the selection of appropriate conditions for adequate production of hydrogen peroxide from the reaction with choline oxidase. A simple test for this purpose can be carried out by eliminating the separation column. This will allow the establishment of the ratio of mobile phase to enzyme solution and the appropriate length of tubing. Likewise, the percentage conversion of ACh to Ch and that of Ch to hydrogen peroxide can be also determined. The results of such experiments are shown in Table I.

TABLE I

DETERMINATION OF OPTIMAL CONDITIONS FOR HYDROGEN PEROXIDE FORMATION

Flow-rate (ml/min)			*pH of combined fluid*	*Peak height (cm)**			*Conversion of ACh to Ch (%)*	*Conversion of Ch to H_2O_2 (%)*
Mobile phase	*Enzyme solution*	*Combined fluid*		*H_2O_2*	*Ch*	*ACh*		
0.8	0.1	0.9	7.42	6.8 (76)	3.5 (39)	3.0 (33)	86	51
0.8	0.2	1.0	7.70	6.9 (77)	6.4 (72)	5.6 (61)	88	93
0.8	0.3	1.1	7.85	7.2 (80)	7.2 (81)	6.7 (73)	93	100
0.8	0.4	1.2	7.92	8.9 (99)	9.0 (101)	9.3 (101)	103	101
0.8	0.5	1.3	8.00	9.0 (100)	8.9 (100)	9.2 (100)	103	99

* Values in parentheses, %.

As described under Experimental, we found the optimum electrode potential to be +0.6 V. The amounts of the test samples were 100 pmol for hydrogen peroxide and 50 pmol for Ch and ACh. These experiments showed that the highest peak of hydrogen peroxide was obtained by setting the pump flow-rate at 0.8 ml/min for the mobile phase and 0.5 ml/min for the enzyme solution. The pH of the combined solution was 8.0. The percentage conversions for ACh to Ch and Ch to hydrogen peroxide under these conditions were 103% and 99%, respectively. The effect of temperature on the peaks was also tested by heating a mixture of Ch (100 nmol/ml) and choline oxidase (2 units/ml) at 15, 22 and 37°C for 1.5, 5, 10, 20 and 30 min. A 10-μl volume of the heated sample was injected into the chromatograph and the peaks were evaluated. No significant variation in the peak height was observed for the range of conditions employed.

Sensitivity and calibration graph

The positive correlation between the observed peak-height ratios for standard amounts of each compound was excellent (Fig. 1). The assay was slightly more sensitive to Ch than ACh. However, amounts as low as 10 pmol could be determined for both amines. Chromatograms of samples containing Ch, EHC and ACh gave clear and sharp single peaks for each component with a total elution time of less than 22 min (Fig. 2).

Ch and ACh concentrations in rat brain regions following killing by two different methods

The levels of Ch and ACh in rat regions, determined following killing by decapitation or microwave radiation (9.0 kW, 1.1 sec) are shown in Table II. ACh levels in the same regions, as reported previously utilizing pyrolysis GC–MS[10], are also

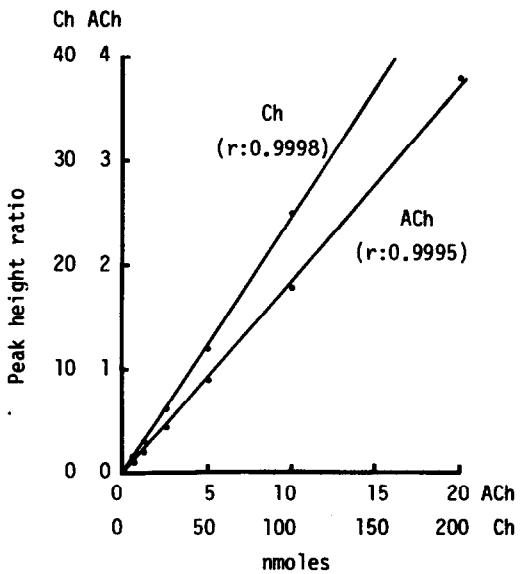

Fig. 1. Calibration graphs for the determination of Ch and ACh by the proposed procedure. r = correlation coefficient.

TABLE II

REGIONAL Ch AND ACh CONCENTRATIONS IN RAT BRAIN (nmoles/g ± S.D.) FOLLOWING KILLING BY TWO DIFFERENT METHODS

Region	*Ch (LC–ED)*		*ACh (LC–ED)*		*ACh (GC–MS)*	
	Decapitation	*MWR*	*Decapitation*	*MWR*	*Decapitation*	*MWR*
Cerebellum	158.6 ± 54.5	19.7 ± 7.2**	4.5 ± 0.8	7.5 ± 2.1*	6.4 ± 1.0	7.3 ± 1.8
Medulla pons	256.4 ± 73.7	15.9 ± 5.1**	15.4 ± 0.8	24.8 ± 3.6**	20.7 ± 3.6	29.3 ± 6.0**
Hypothalamus	272.2 ± 94.9	21.2 ± 5.0**	21.8 ± 1.8	30.1 ± 5.2*	23.6 ± 3.3	38.6 ± 5.1**
Striatum	423.7 ± 101.9	25.1 ± 2.1**	33.3 ± 5.0	76.8 ± 5.3**	34.0 ± 5.9	80.0 ± 9.4**
Midbrain–thalamus	336.1 ± 75.1	19.3 ± 2.9**	21.1 ± 1.5	35.1 ± 3.4**	26.7 ± 4.1	39.5 ± 6.7**
Hippocampus	219.0 ± 28.6	23.2 ± 6.9**	18.0 ± 1.8	28.9 ± 2.1**	15.1 ± 2.3	29.5 ± 3.2**
Cortex	238.0 ± 44.7	14.0 ± 1.5**	8.5 ± 0.9	17.9 ± 1.1**	9.1 ± 2.3	24.1 ± 3.1**

* $P < 0.05$. MWR results compared with decapitation results.

** $P < 0.01$, MWR results compared with decapitation results.

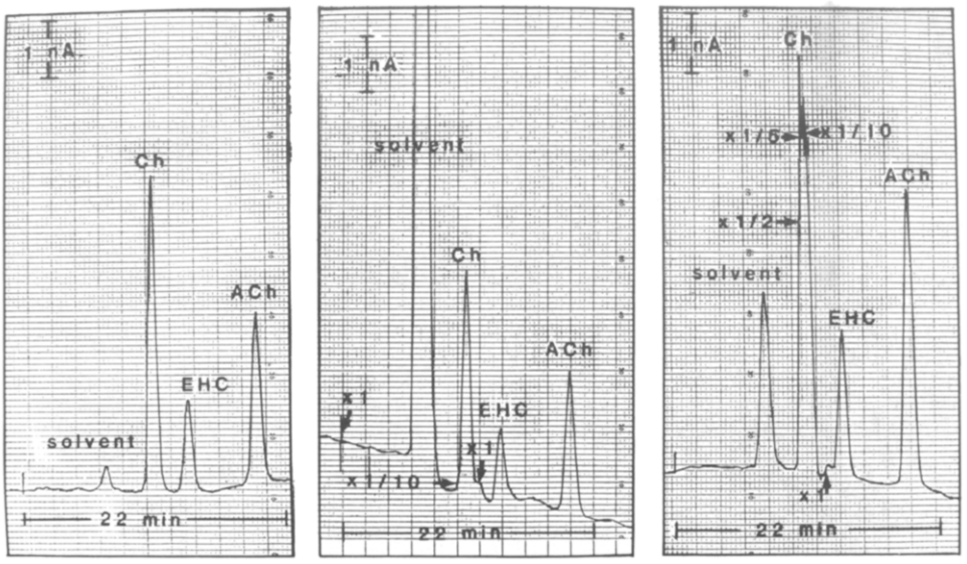

Fig. 2. Typical liquid chromatograms obtained for Ch, ACh and EHC (internal standard) using rat brain homogenates. Peaks of authentic Ch and ACh are showed on the left (A) and those of endogenous amines in the middle (B) and on the right (C). The difference in sample preparation between B (normal) and C (extended purification) is described under Experimental. The arrows show the points at which the sensitivity was switched (manually).

given in Table II for comparison. There are no significant differences in the levels of ACh obtained by the two different assay methods following killing by either of the two methods. The data on Ch obtained by GC–MS are inadequate for comparison. The highest concentration of ACh was found in the corpus striatum, while smaller amounts were found in the midbrain–thalamus, the hypothalamus, the hippocampus, the medulla pons, the cortex and the cerebellum. The currently reported regional levels of ACh following MWR are in complete agreement with those found previously[12–16]. There were no significant differences in the ordering of regions according to the ACh levels for the MWR and decapitation methods of killing. Significantly higher ACh concentrations, however, were noted after MWR in all areas. The highest concentration of Ch was in the corpus striatum, where the largest ACh content was found. There were smaller differences in the observed Ch concentrations than for ACh concentrations among the different regions. However, 8–17.4 times higher Ch levels were found in the decapitation groups than the MWR groups. Hence the levels of Ch were significantly decreased in all areas after MWR. Regional levels of Ch after MWR were in reasonable agreement with those reported by other workers[17–20].

Heat stability of Ch and ACh in distilled water (pH 5.6) or phosphate buffer (pH 7.4)

Testing the heat stability of Ch and ACh is necessary as the brain is substantially heated by MWR. Stability of ACh to heating *in vitro* has been reported previously[21]; a negligible fraction of ACh was decomposed by heating in the range

TABLE III

HEAT STABILITY OF Ch AND ACh IN DISTILLED WATER (pH 5.6) AND PHOSPHATE BUFFER (pH 7.4)

Heating time (min)	*Decomposition rate (%)*			
	Ch		*ACh*	
	*D.W.**	*Buffer***	*D.W.**	*Buffer***
0	0	0	0	0
1	0	0	0	0
2	0	0.5	0	5.4
5	0.4	0.1	10.3	28.0
10	0.3	1.1	17.8	45.6
20	0.6	1.5	33.3	71.0
30	0.9	1.4	41.9	81.0

* D.W.: distilled water, pH 5.6.
** Buffer: 0.2 *M* phosphate buffer, pH 7.4.

37–90°C for 5 min in pH 7.4 phosphate buffer. Similarly, both Ch and ACh were tested for heat stability in this study as described under Experimental. As shown in Table III, only a very small amount of ACh was destroyed at 95°C in 2 min, and Ch was completely stable under these conditions for periods up to 30 min. Stability was calculated by comparing the decrease in ACh and the increase in Ch due to heating.

DISCUSSION

Regarding the chromatographic conditions, the combination of acetonitrile and sodium octyl sulphate shortened the retention times of Ch, ACh and EHC and did not interfere in the enzyme reaction. No prominent effect of tetramethylammonium chloride was observed using the Chemco Scientific reversed-phase column. The maximum peak for each of the compounds was observed when the electrode potential was set at +0.6–0.7 V. The conversion of Ch and ACh to hydrogen peroxide was almost 100%; this maximum value was obtained by reaction of a standard solution of Ch with Ch oxidase for 1.5 min or longer. Therefore, the optimal length of the reaction coil can be estimated as 10 m with a dead volume of approximately 2 ml. Temperature changes between 15 and 37°C did not affect the enzyme reaction. The pH 5.0 mobile phase was pumped at 0.8 ml/min and mixed with the pH 8.5 enzyme solution (0.5 ml/min) in this system. However, the pH of the combined solution must be maintained at 8.0, because this is critical to the proper operation of the enzyme reaction. The simplified extraction procedure exhibited excellent results, as shown in Fig. 2, where no unknown peaks appeared. Recoveries in the extraction procedure were 96.1 ± 1.4% for Ch and 95.6 ± 2.2% for ACh.

Regarding microwave heating of the rat brain, the effect of temperature on the thermal tolerance of Ch and ACh was examined (Table III). As shown here, the compounds were found to be sufficiently heat stable to prevent significant decomposition by microwave heating. The duration of the MWR time (1.1 sec) is negligible compared with the times *in vitro*. Irradiation for 800 msec increased the temperature

of the rat brain to a maximum of 98.3°C with a maximum difference of 6.9°C between areas[10]. It has been reported that endogenous cholineacetyltransferase and AChE, which are involved in the synthesis and degradation of ACh, respectively, are completely decomposed when the brain temperature is increased to 75°C[10,21]. The ACh concentration in rat brain regions was significantly higher following MWR for 1.1 sec compared with that obtained following killing by decapitation. It is well known that decapitation drastically alters the state of ACh in the brain as the ACh is rapidly exposed to post-mortem destruction by cholinesterase. In accordance with this, a considerable amount of Ch was determined in decapitated brains. This result has previously been reported by Stavinoha *et al.*[13] and Weintraub *et al.*[15].

In conclusion, we have developed a very specific, sensitive and accurate procedure for the determination of Ch and ACh in tissue samples. By employing this simpler technique and extraction procedure, the levels in Ch and ACh in rat brain regions were effectively determined. The usefulness of this method was confirmed by a comparison of the regional ACh levels obtained using the proposed method with those obtained using pyrolysis GC–MS. Finally, the 10 kW MWR technique was shown to be effective for the rapid inactivation of rat brain enzymes and, therefore, in preventing post-mortem changes in Ch and ACh. Such inactivation is essential for the proper determination of these two important neurochemicals.

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