Journal of Chromatography, 414 (1987) 167–173
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3417

Note

Determination of acetylcholine and choline in perchlorate extracts of brain tissue using liquid chromatography–electrochemistry with an immobilized-enzyme reactor

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(First received August 15th, 1986; revised manuscript received September 16th, 1986)

Several types of assay for the measurement of acetylcholine (ACh) and choline (Ch) have been reported in recent years based on radioisotopes [1,2] or gas chromatography [3–5]. Recently Potter et al. [6] developed a liquid chromatographic (LC) technique using electrochemical detection (ED). This innovative method was based on the separation of ACh and Ch by reversed phase chromatography and treatment of the separated analytes with a post-column infusion of acetylcholinesterase and choline oxidase. The hydrogen peroxide formed was detected amperometrically at a platinum electrode. A modified version of this method has also been presented [7]. The major disadvantage of both of these LC methods was that the continuous infusion of enzyme solution resulted in a lowered response at the working electrode owing to dilution of the analyte and passivation of the electrode surface by the added proteins. These problems have been overcome to a large degree by using immobilized-enzyme reactors [8–11].

We have developed an immobilized-enzyme column that is reusable for at least two months and 300 tissue samples without any loss in enzyme activity [10]. However, post-column infusion of an alkaline buffer into the analytical stream, in order to attain maximum enzyme activity, was required. The purpose of the present study was to increase the signal-to-noise ratio by omitting the mixing of acidic mobile phase and alkaline infusion buffer before passage through the immobilized-enzyme column. Also, perchloric acid extracts were directly injected

0378-4347/87/$03.50

into the LC system, without further clean-up. This also allowed the extracts to be used for the assay of amines (catecholamines and indoleamines). These metabolites as well as amino acids (including γ-aminobutyric acid) were determined in the same extracts using other LC methods.

EXPERIMENTAL

Materials

Acetylcholine chloride and choline chloride were purchased from Sigma (St. Louis, MO, U.S.A.). Sodium 1-octanesulphonic acid (SOS) was purchased from Aldrich (Milwaukee, WI, U.S.A.). Tetramethylammonium chloride (TMA) and disodium ethylenediaminetetraacetate (EDTA) were purchased from Wako (Osaka, Japan). All other chemicals were analytical-reagent grade. Ethylhomocholine (EHC) was synthesized and purified in our laboratory following the method of Potter et al. [6].

LC–ED system

The system consisted of an LC-304 analyzer system (Bioanalytical Systems, Lafayette, IN, U.S.A.) with an automatic sample injector (KSST-601, Kyowa Seimitsu, Tokyo, Japan), guard and chromatographic columns, an immobilized-enzyme column (BAS-Japan, Tokyo, Japan), a column heater (LC-22/23A, Bioanalytical Systems), electrochemical detector (LC-4B, Bioanalytical Systems), which was installed with a platinum working electrode (TL-10A, Bioanalytical Systems), and a chromatographic data processor (7000B, System Instrument, Tokyo, Japan). The mobile phase consisted of vacuum-degassed 50 m*M* sodium phosphate, pH 8.3, 1 m*M* TMA and 70 μM SOS, at a flow rate of 1 ml/min. The guard column (50 mm $\times$ 4.6 mm I.D.) and the chromatographic column (150 mm $\times$ 4.6 mm I.D.) were filled with styrene polymer, PLRP-S (7-μm particles, Polymer Laboratory, Stow, OH, U.S.A.). The preparation of the immobilized-enzyme column was described in detail in a previous paper [10]. The chromatographic and immobilized-enzyme columns were maintained at 37°C with a column heater. The applied potential at the working electrode was +500 mV versus Ag/AgCl.

Preparation of brain extracts

The heads of Wistar rats were irradiated with a focused microwave apparatus for 1.15 s (9.5 kW microwave, 2450 MHz, New Japan Electronics, Tokyo, Japan). The brains were rapidly removed and homogenized in ice-cold 0.2 *M* perchloric acid containing EDTA (0.01%) and EHC (10 μM) as an internal standard. The homogenate was frozen and thawed before centrifugation at 10 000 *g* for 20 min. The supernatant was filtered through a Millipore Type HA 0.45-μm membrane and then rinsed with diethyl ether. Then 5 μl of the aqueous layer were injected directly into the LC system. For recovery experiments, aged female Wistar rats were used.

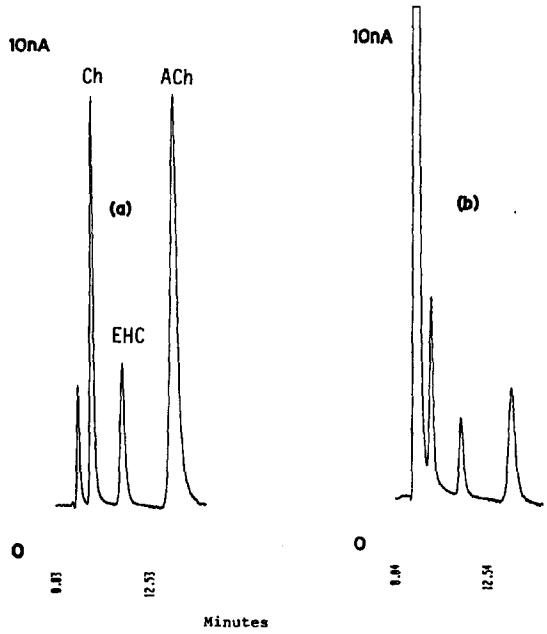

Fig. 1. (a) Chromatogram of a standard containing acetylcholine, ethylhomocholine and choline. Peaks: Ch = choline (4.6 min); EHC = ethylhomocholine (8.6 min); ACh = acetylcholine (15.6 min). Amounts of Ch, EHC and ACh are 50 pmol per 5 μl injected. Detector range was 10 nA full scale. (b) Chromatogram of brain extracts. The extract was prepared from 50 mg of rat whole brain with 1 ml of perchloric acid solution; 5 nmol of EHC were added as an internal standard, and 5 μl of the extract were injected into the LC system

RESULTS AND DISCUSSION

Recently, Potter et al. [6] reported an LC post-column enzyme infusion method for the assay of ACh and Ch; the present assay differs in the use of a reusable immobilized-enzyme column, which was directly connected to the chromatographic column.

Chromatographic conditions

We reported in a previous paper [10] that the optimal pH for the hydrolysis of ACh and Ch was 8–8.5 and that the phosphate buffer and 1.2 m*M* TMA did not interfere with the enzyme activities. We examined the use of a styrene polymer, PLRP-S, as a packing material for LC under weak alkaline conditions (pH 8.3, this study). By using an appropriate flow-rate (1 ml/min) and a phosphate buffer containing 1 m*M* TMA and 70 μ*M* SOS, a good separation was achieved. A representative chromatogram of the external standards is shown in Fig. 1a. The stability of ACh dissolved in the mobile phase at pH 8.3 was checked and the results showed no degradation within 1.5 h (Table I).

Application to tissue samples

An acidic extract, usually of perchloric acid, is directly applicable to the LC system for the determination of catecholamines, indoleamines and amino acids.

TABLE I

STABILITY OF ACETYLCHOLINE IN MOBILE PHASE OF pH 8.3

ACh dissolved in pH 8.3 mobile phase (10 μM) was kept at room temperature, and 100 pmol of ACh were applied to the column at various times. A trace of Ch was found in the solution of commercial ACh chloride (less than 1% of total). Range was 50 nA full scale

Time (h)	Peak height (nA)	
	Ch	ACh
0	0.75	9.57
0.5	0.75	9.39
1.0	0.93	9.39
1.5	1.04	9.36

In the present study, the same acidic extract is also immediately injectable. The straight chromatographic flow-line, without mixing alkaline buffer before the immobilized-enzyme column (see ref. 10), decreased the noise and enhanced the assay sensitivity. The peak height of ACh and Ch was linear from 1 to 500 pmol (Fig. 2). Minimal detectable amounts of ACh and Ch were ca. 1 pmol (signal-to-noise ratio 10:1). A representative chromatogram of the perchloric acid extract of brain is shown in Fig. 1b. Under the conditions described, a good separation was achieved. The tissues were extracted with perchloric acid, and 5 μl of the supernatant were applied to the LC system. The results showed that ACh extracted from 5 mg of rat whole brain was detectable under the conditions described (signal-to-noise ratio 5:1), and good proportionality was obtained between the amounts of Ch and ACh and the tissue weight ($r=0.99$ in both ACh and Ch, Fig. 3).

TABLE II

RECOVERIES OF ACETYLCHOLINE AND CHOLINE ADDED TO RAT WHOLE BRAIN HOMOGENATE

Whole brains of aged female rats were homogenized with 0.2 M perchloric acid and treated as described in Experimental. The tissue concentrations of the homogenates were 50 mg/ml. Ethylhomocholine (EHC) was added to the homogenate as an internal standard at a concentration of 10 nmol/ml. $n=4$

Added compound	Amount added (nmol)	Amount found (nmol)		Recovery (%)
		Ch	ACh	
None	—	1.20 ± 0.01	1.08 ± 0.02	—
Ch	10	12.00 ± 0.25	1.22 ± 0.08	108
ACh	5	1.39 ± 0.04	6.72 ± 0.07	110
None*	—	1.17 ± 0.01	1.09 ± 0.01	—

*Brain homogenate was sonicated for 5 min, and 5 μl of the 10 000 g supernatant of the homogenate were applied to the LC system. The values were calibrated with the internal standard and represent mean ± S.E.

TABLE III

INTER-ASSAY VARIABILITY OVER LONG-TERM USAGE OF IMMOBILIZED-ENZYME REACTOR

ACh, Ch and EHC (100 pmol each) were injected into the LC system. Using the same immobilized-enzyme column, determinations were carried out on five experimental groups during the first month and on four during the following month. The immobilized-enzyme column was refrigerated when not in use. The mean number of samples in each test run was 24. The standard solution containing ACh, Ch and EHC was injected as the first sample in each group and calibration was carried out using EHC as an internal standard. The same standard was also injected as the last sample as an indicator of change during the test run. The value shown in table are the mean ±S.E. of the value obtained from the standard injected as the last application to the LC system in each experimental group.

Term (month)	*n*	Amount (pmol)	
		Ch	ACh
First	5	102.3±3.7	98.5±1.8
Second	4	100.2±1.0	96.1±1.7

Recovery experiments

Known amounts of ACh and Ch were added to the rat brain homogenate and extracted as described in Experimental. The recovery of internal standard (EHC) added to all the homogenates was ca. 96%. The results of the recovery experiments are shown in Table II. The content of ACh in 50 mg of rat whole brain was 1.20 nmol, and the standard error of mean was only 1–2%. Sonication had no apparent influence. The recoveries of Ch and ACh were 108 and 110%, respec-

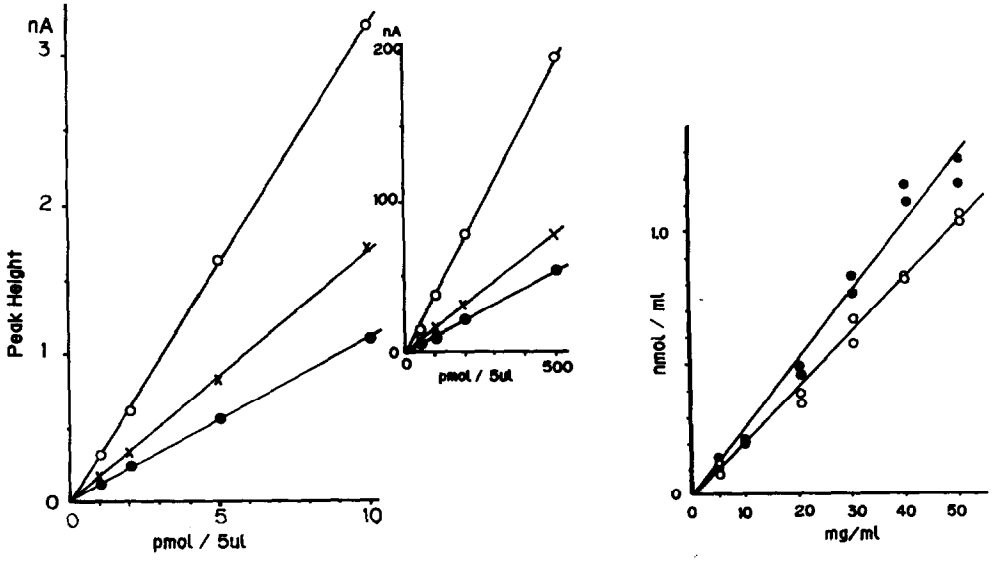

Fig. 2. Standard curves for acetylcholine (●), ethylhomocholine (×) and choline (○).

Fig. 3. Relationship between concentration of acetylcholine (○) and choline (●) and the weight of sample tissue homogenized.

tively. It has been observed that only 3–5% of total ACh and Ch was extracted, with a second extraction, from the residual of first extraction. The values of ACh and Ch were calculated using a calibration from the ratio of the peak height of Ch or ACh to that of EHC. The previously reported continuous enzyme infusion methods lead to a decrease in sensitivity apparently caused by the passivation of the working electrode. In the present procedure, some decrease in the original sensitivity was also observed, but this was readily overcome by cleaning the working electrode with a dichromate–sulphuric acid solution, following the method of Anton [12].

Inter-assay variability over long-term usage

Determinations in nine experiments (ca. 220 samples) were carried out over two months with the same immobilized-enzyme column. The variances of the values of ACh and Ch in a standard solution were compared between groups tested in the first and second months. No significant difference was observed, indicating no significant change in the enzyme response ratios of ACh and Ch to EHC added as an internal standard during the two months. In a separate evaluation, results of ten sequential determinations on the standard showed a very small intra-assay variance (Ch 105.9 ± 1.8 pmol and ACh 102.1 ± 0.8 pmol). These results indicated good reproducibility over at least two months.

CONCLUSION

A method of determining ACh and Ch by coupling a styrene polymer chromatographic column, immobilized-enzyme column and electrochemical detector has been presented. Perchlorate extracts were injected directly into the chromatograph in a weakly alkaline mobile phase. The minimal detectable amounts of ACh and Ch were ca. 1 pmol. The sensitivity of the assay permits ca. 5 mg of brain tissue to be used. This procedure will provide a simple, rapid and sensitive means for studying the central cholinergic nervous system.

ACKNOWLEDGEMENTS

The authors thank Dr. Y. Omori, Director of Biological Safety Research Center, National Institute of Hygienic Sciences, Dr. P. Kissinger, Professor of Chemistry, Padue University, and Dr. C. Duda, Bioanalytical Systems, for their helpful advice.

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