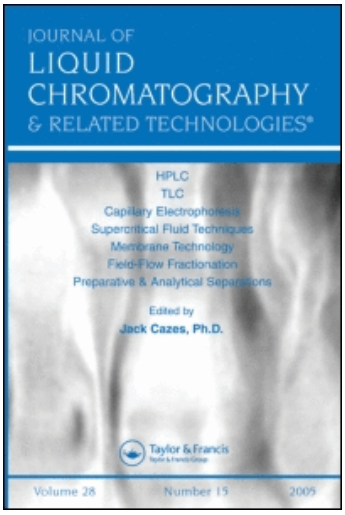

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Determination of Acetylcholine and Choline in Human Plasma Using High-Performance Liquid Chromatography Combined with an Immobilized Enzyme Reactor

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DETERMINATION OF ACETYLCHOLINE AND CHOLINE IN HUMAN PLASMA USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY COMBINED WITH AN IMMOBILIZED ENZYME REACTOR

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ABSTRACT

A method for assaying Acetylcholine (ACh) and Chloine (Ch) simultaneously in human plasma by high-performance liquid chromatography with electrochemical detection (LCEC) combined with an immobilized enzyme reactor is described for the first time in this paper. Human plasma samples were obtained from 12 volunteers free of neurological and psychological disease. The volunteers were from 22 to 41 years old (mean 30.1 years), eight being males. Extraction of ACh and Ch from plasma was performed according to the liquid cation-exchange method. Concentrations of ACh ranged from 57.0 to 179.6 pmol/ml (mean and S.D. of 121.6 and 40.9 pmol/ml respectively). The present method, LCEC combined with an immobilized enzyme reactor is rapid, simple and sensitive compared with the biological methods, GC-MS and RIA for analyzing ACh and Ch in plasma.

INTRODUCTION

Acetylcholine (ACh), which was first documented by Dale and Dudley(1) in 1929, is identified as a neurotransmitter. The presence of ACh has been demonstrated not only in the central nervous system, sympathetic and parasympathetic nervous system, but also in non-neural tissues of a variety of organs, such as lung, liver, kidney, and placenta(2). Previously, the ACh activity of human blood has been investigated by bioassay method, while gas chromatography-mass spectrometry (GC-MS) and radio-immunoassay (RIA) have been assayed for ACh in human plasma. However, the results of these studies are variable. In 1983, Potter et al.(3) first reported a method for assaying ACh and choline (Ch) by high-performance liquid chromatography with electrochemical detection(LCEC) and determined them in brain tissue of rats. This assay method for ACh and Ch is based on the separation of ACh and Ch on a polymer gel column, followed by their enzymatic conversion to hydrogen peroxide through postcolumn immobilized enzyme reaction with acetylcholine esterase and choline oxidase. Until now ACh and Ch have not been determined simultaneously by LCEC in human plasma, because of their very low concentration in plasma. In this paper we describe a method for assaying ACh and Ch extracted from human plasma by a liquid cation-exchange(4) procedure combined with LCEC coupled with an immobilized enzyme reactor.

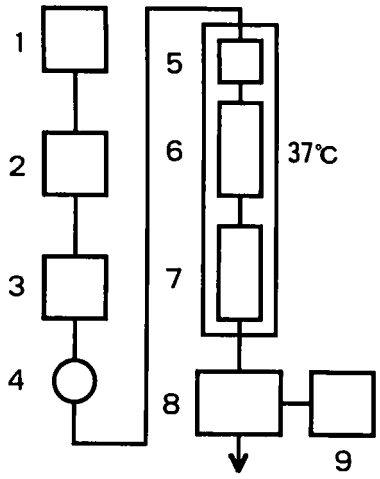
MATERIALS AND METHODS

Chemicals

Physostigmine and tetraphenylboron were obtained from Sigma (St. Louis, MO, U.S.A.). 1-Decanesulphonic acid sodium salt, choline chloride, acetylcholine chloride and 3-heptanone were obtained from Tokyo Kasei (Tokyo, Japan). Tetramethylammonium chloride, disodium hydrogenphosphate, sodium dihydrogenphosphate and other chemicals were obtained commercially. Ethylhomocholine (EHC), which was used as an internal standard, was synthesized with 3-dimethylamino-1-propanol and iodoethane(3).

Apparatus and chromatographic conditions

This assay method is based on the separation of ACh and Ch on an AC-GEL, followed by their enzymatic conversion to hydrogen peroxide through a post-column immobilized enzyme reaction with acetylcholine esterase and choline oxidase(5). The procedure is outlined in Fig.1. LCEC system consisted of an 880-50 degasser (Jusco, Tokyo, Japan), an EP-10 pump, a CB-100 amperometric detector equipped with a WE-PT platinum electrode (Eicom, Kyoto, Japan), a Rheodyne 7125 injector with a 200 μl sample loop (Berkeley, CA, U.S.A.), a prepak guard column (5 x 4 mm I.D., Eicom), and an Eicom-pak AC-GEL (polymer gel, 150 x 6 mm I.D., Eicom), an AC-Enzymepak immobilized enzyme column (5 x 4mm I.D., Eicom)(5). The mobile phase was 0.1 M sodium phosphate buffer (pH



1. Solvent Reservoir
2. Degasser
3. Pump
4. Sample Injector
5. Precolumn
6. Column
7. Immobilized Enzyme Column
8. Electro Chemical Detector
9. Recoder

FIGURE 1. Diagram of LCEC system

8.3) containing 1.2 mM tetramethylammonium chloride (TMA) and 300 mg/l 1-decanesulphonic acid sodium salt, which was filtered through a 0.22 μm membrane filter (Millipore, Bedford, MA, U.S.A.). The LCEC separation and enzymatic reaction were performed at 37 ℃. The flow rate was 1.0 ml/min. The electrode potential was set at +450 mV against a Ag/AgCl reference electrode for the detection of hydrogen peroxide. Under these conditions the retention times were: Ch, 6.8 min; EHC, 8.3 min; ACh, 12.8 min.

Sample and extraction

Samples of human plasma were obtained from 12 volunteers free of neurological and psychological disease. The volunteers

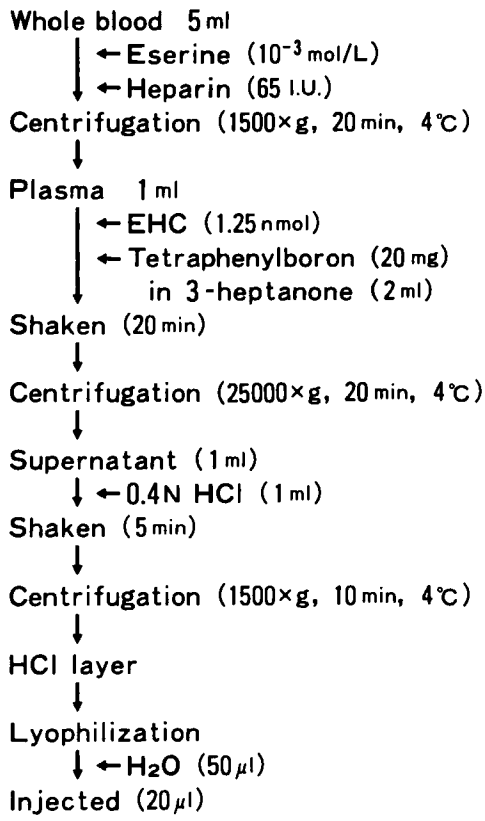


FIGURE 2. Extraction of ACh and Ch from human plasma

were from 22 to 41 years old (mean 30.1 ± 5.8 years), eight being males. We collected 5 ml of human whole blood in a glass tube (Terumo, Tokyo, Japan) containing 65 I.U. of heparin and 1 mmol of physostigmine. Extraction of ACh and Ch from plasma was performed immediately after collection according to the liquid cation-exchange method(4) as shown in Fig.2. Briefly, the tubes

were centrifuged at 1500 g for 20 min at 4 ℃ to separate the plasma. After addition of 1.25 nmol of EHC and 2 ml of 3-heptanone containing 20 mg of tetraphenylboron, the plasma mixture was shaken for 20 min at room temperature and centrifuged at 25000 g for 20 min at 4 ℃. Then 1000 μl of the supernatant were taken and added to 1 ml of 0.4 M hydrochloric acid. The mixture was shaken for 5 min and centrifuged at 1500 g for 10 min at 4 ℃. Finally the organic layer was discarded by aspiration and the acid layer collected, lyophilized and stored at 4 ℃. Samples were dissolved in 50 μl of distilled water and 20 μl injected into the LCEC system.

RESULTS

ACh, Ch and EHC in human plasma could be measured with high sensitivity using this LCEC system. The peak areas increased linearly as shown in Fig.3 with increasing volume injected for ACh from 0.3 pmol to 5 nmol and for Ch from 1 pmol to 5 nmol. These ranges cover the ACh and Ch concentrations in all samples examined. The absolute recoveries(n=3) of added ACh (125 pmol per ml), Ch (1.25 nmol per ml) and EHC (1.25 nmol per ml) were ACh 65.0 %, Ch 64.1 % and EHC 69.0 %. Fig.4 shows typical chromatograms of human plasma sample (containing 1.25 nmol of EHC as internal standard) and of an authentic standard sample (156

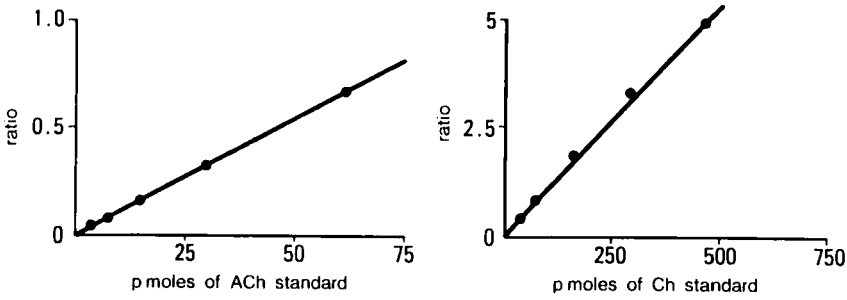


FIGURE 3. Standard curves for ACh and Ch. Samples (n=3) are solved in 5 μl distilled water.

$$\text{ratio} = \frac{\text{area of ACh or Ch}}{\text{area of 153 pmol EHC}}$$

pmol of Ch , 156 pmol of EHC and 15.6 pmol of ACh). The peaks of Ch, EHC and ACh from the plasma sample were clearly separated and were identified by comparison with the authentic samples. Concentrations of ACh in plasma from 12 normal subjects ranged from 57.0 to 179.6 pmol/ml. The mean and standard deviation were 121.6 ± 40.9 pmol/ml. The plasma concentration of Ch in 12 human subjects ranged from 12.2 to 42.7 nmol/ml (mean 20.0 ± 8.2 nmol/ml). The results of all subjects are shown in Table 1.

DISCUSSION

Our assay system was based on the HPLC procedure of Potter et al.(3). They first developed an assay for ACh and Ch by LCEC

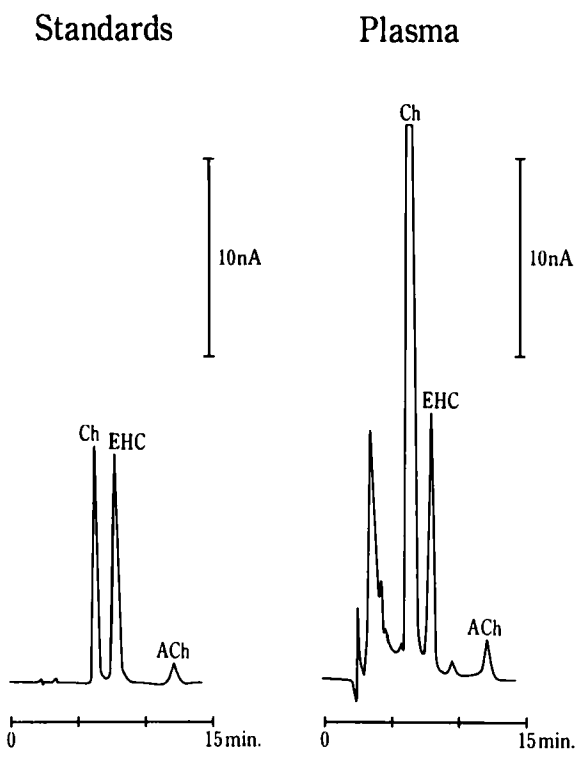


FIGURE 4. Chromatograms of standards and a human plasma. Standards contained 15.6 pmol of ACh and 156 pmol each of Ch and EHC. The human plasma extraction contained 1.25 nmol of EHC as an internal standard.

in brain tissue of rats. This method is extensively applied for measuring the concentration in various tissues and body fluids. However, it is difficult to apply this system for measuring ACh concentration in plasma because of its very low level. The liquid cation-exchange method is a known procedure for extracting choline compounds(4). This liquid cation-exchange method is

TABLE 1

Profile of plasma level of acetylcholine and choline of 12 human subjects

Subject	age	sex	ACh pmol/ml	Ch nmol/ml
1	22	M	57.0	12.3
2	23	M	87.3	14.1
3	23	F	125.8	17.5
4	26	F	89.1	19.0
5	26	M	133.5	13.7
6	32	F	73.6	12.2
7	32	F	143.9	24.3
8	33	M	152.2	15.3
9	33	M	173.5	21.2
10	33	M	164.5	42.7
11	37	M	179.6	26.6
12	41	M	78.6	21.0

already avariable for extraction of ACh and Ch from cerebrospinal fluid(6). By using this procedure coupled with the LCEC system, we were able to measure ACh and Ch contents simultaneously in human plasma.

A method for assaying ACh and Ch in plasma by LCEC combined with an immobilized enzyme reactor is reported in this paper for the first time. Until recently, ACh and Ch of blood were measured

by the biological methods GC-MS and RIA. Regarding the biological methods, the activity of ACh in human blood was reported by Diethelm et al.(7) Ratinoff et al.(8) also reported the ACh concentration in plasma using thin-layer chromatography and stated the concentration to be 1.56 ± 0.73 nmol/ml in 1968. Recently, using GC-MS method, Hanin et al.(9) showed that in 10 normal humans the concentration of ACh ranged from 0.007 to 0.140 nmol/ml. Hasegawa et al.(10) also determined the plasma concentration of ACh using GC-MS and reported the concentration to be 3.23 ± 0.23 nmol/ml, these results being 10-200 times higher than those given in Hanin's report. On the other hand, using RIA method, Kawashima et al.(11) determined plasma concentration of ACh in young women and reported a mean ACh value of 2.98 ± 0.62 pmol/ml. Our results using LCEC, 121.6 ± 40.9 pmol/ml, do not differ from Hanin's reports.

The physiological as well as pathophysiological significance of ACh in plasma is still unknown. Recently, Chen et al.(12) suggested a role of ACh related to vasoconstriction and vasodilatation. In short, ACh release two substances from endothelial cells, one is endothelium-hyperpolarizing factor through M1 receptor and the other is endothelium-derived relaxing factor through M2 receptor.

Ch is a precursor of ACh and also a metabolite of ACh. Up to this point, the concentration of Ch, in human plasma was assayed by GC-MS(9) and LCEC(13). Hanin et al. reported the value of Ch

in plasma as ranging from 4.4 to 10.1 nmol/ml by GC-MS method. Webb et al. determined plasma Ch concentrations by LCEC system, based on the HPLC procedure of Potter et al., to be 9.0 ± 2.3 nmol/ml. Our results, 20.0 ± 8.2 nmol/ml, do not differ from either Hanin's or Webb's reports. After ingestion of lecithin, the concentration of Ch in plasma increased significantly(14). These results may suggest that the increase of plasma Ch level cause a parallel change in brain Ch levels, and influences cholinergic activity.

In our experiment, the concentrations of plasma ACh and Ch in the same subjects showed no correlation. The advantage of our method is being able to assay Ch and ACh simultaneously.

The present method, LCEC combined with an immobilized enzyme reactor coupled with cation-exchange method, is rapid, simple and sensitive compared with the biological methods, GC-MS and radioimmunoassay for analyzing ACh and Ch in human plasma. The method described here may become a useful technique in studying the physiological and pathophysiological significance of ACh in plasma.

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