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Short Communication

Liquid chromatography with electrochemical detection for quantitation of bound choline liberated by phospholipase D hydrolysis from phospholipids containing choline in rat plasma

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ABSTRACT

This report describes the optimal conditions for the determination of bound choline in rat plasma. The method used was based on the liberation of choline by phospholipase D from phospholipids containing choline in plasma, followed by high-performance liquid chromatographic analysis. Normal concentrations of total, free and bound choline in rat plasma were found to be 1278.7 ± 132.5, 11.5 ± 2.2 and 1267.2 ± 125.5 nmol/ml, respectively. The described procedure has the advantages of rapidity, specificity, excellent precision and the need for only a small amount of the sample.

INTRODUCTION

The uptake of choline (Ch) into the brain has been shown to be linearly related to its concentration in plasma [1–3]. The Ch in plasma exists both as free Ch and as bound Ch, such as phosphatidyl Ch. We felt it necessary to determine both types of Ch in order to clarify the relationship between plasma and brain Ch during synthesis of a neurotransmitter, acetylcholine (ACh), in the brain [4].

Recently, liquid chromatography with electrochemical detection (LC–ED) has became widely accepted as an excellent technique for the determination of Ch, particularly because of its simplicity and sensitivity [5–8]. However, phospholipids containing Ch cannot be detected by this method because phosphorylcholine, a binding Ch substance, is not an appropriate substrate for the choline oxidase used in LC–ED [5].

We set out to develop a new rapid and sensitive method for the determination of bound Ch derived from phospholipids containing Ch in plasma by combining the LC–ED technique with the enzymic method using phospholipase D; the phospholipids in plasma are hydrolysed by phospholipase D *in vitro*, and an aliquot of the supernatant containing the liberated Ch is subsequently subjected to LC–ED for quantitation. This re-

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port describes the optimal conditions for the liberation of Ch from plasma phospholipids in enzymic hydrolysis prior to LC–ED determination.

EXPERIMENTAL

Reagents

Phospholipase D (EC 3.1.4.4) isolated from *Streptomyces chromofuscus* and choline chloride were purchased from Sigma (St. Louis, MO, USA). Ethylhomocholine iodide (EHC), N,N-dimethyl-N-ethyl-3-amino-1-propanol, was prepared according to a method reported previously [5,9]. Other reagents were of the highest purity available from commercial sources.

Determination of total, free and bound Ch in plasma

Male Wistar rats (190–200 g, eight weeks) were anesthetized with intraperitoneal injection of 35 mg/kg sodium pentobarbital. Blood was withdrawn from the abdominal aorta using 5 U of heparin per ml blood as anticoagulant. The plasma was obtained after centrifugation at 1000 *g* and 4°C for 15 min.

Total Ch. A 10-μl aliquot of plasma was mixed with 100 μl of 0.1 *M* Tris–HCl buffer (pH 8.0) containing 1.0 mg of $CaCl_2$, 1.0% of Triton X-100, 10 U of phospholipase D and 10 nmol of EHC as internal standard (I.S.). The mixture was incubated at 37°C for 10 min. The enzymic reaction was stopped by the addition of 100 μl of 1.0 *M* $HClO_4$. The mixture was then passed through a 0.45-μm Millipore filter. Aliquots (5 μl of the filtrate) were injected into the LC–ED system for quantitation [8].

Free Ch. A 50-μl aliquot of plasma was mixed with an equal volume of 1.0 *M* $HClO_4$ containing 1.0 nmol of EHC as I.S. of Ch; 10-μl aliquots of filtrate, passed through a 0.45-μm Millipore filter, were then injected into the LC–ED system for quantitation [8].

Bound Ch. Bound Ch levels were obtained by subtracting free Ch levels from total Ch levels.

RESULTS

Conditions determined for an optimal enzymic reaction

The factors we examined for optimal enzymic reaction to liberate Ch from bound Ch were the concentrations of the enzyme, the emulsifier and the $CaCl_2$, the plasma volume (substrate), the incubation time, and the pH. Because the substrates, phospholipids containing Ch in this enzymic reaction, were lipids in plasma, Triton X-100 was used as an emulsifier or dispersing agent to increase the affinity between the substrate and the enzyme, phospholipase D. Optimal conditions for the enzymic reaction were examined according to the following procedure: a 50-μl aliquot of plasma was mixed with 100 μl of incubation mixture consisting of 0.1 *M* Tris–HCl buffer (pH 7.8) containing 0.25 mg of $CaCl_2$, 10 U of phospholipase D, 0.2% Triton X-100 and 10 nmol of EHC as I.S., and incubated at 37°C for 20 min. The effect of each factor on the enzymic reaction was examined by changing the concentration and/or range of each factor with the other conditions fixed.

The optimal concentration of the emulsifier was examined in the range 0–1.0%. As the concentration of the emulsifier was increased, the level of Ch liberated by the enzymic reaction also increased. The maximum responses were observed at 0.8–1.0% concentration of the agent.

The effect of amount of the enzyme was examined in the range from 0 to 10.0 U. The maximum reaction yield was observed at an enzyme concentration of 2 U, and above. The effect of pH was examined in the pH range 4–10. Maximum responses were observed in the pH range 6–9. The rate of the enzymic reaction at pH 4 and 5 was only 2.5% of the maximum response. At pH 10, the rate decreased to 28% of the maximum. With regard to the incubation time for enzymic reaction, the reaction was rapidly increased by increasing the incubation time, and reached maximum levels at incubation for over 5 min at 37°C. The maximum levels were maintained for incubation up to 60 min.

Varying the concentration of $CaCl_2$ in the

range 0–6 mg was found to have no effect on the enzymic reaction in normal plasma. In order to counteract the deficiency of calcium ions in some abnormal samples, a sufficient amount (1.0 mg) of the agent was selected for addition to the incubation mixture.

The effect of the phospholipid level in the plasma sample on enzymic hydrolysis was investigated by varying the amount of plasma between 5 and 200 μl. The reaction was linear for 5–60 μl of added plasma, and only became independent of the amount of plasma at levels greater than 70 μl. Accordingly, within this range of added plasma, the concentration of the liberated Ch (nmol/ml) obtained by calculation from absolute levels was found to be constant.

In line with the results reported above, we selected and fixed the optimal conditions for the enzymic reaction leading to liberation of Ch from the phospholipids containing Ch in plasma: 10 μl of plasma were added to 100 μl of incubation mixture, consisting of 0.1 *M* Tris–HCl buffer (pH 8.0) containing 1.0 mg of $CaCl_2$, 10 nmol of EHC as I.S., 10 U of phospholipase D and 1.0% Triton X-100, and incubated at 37°C for 10 min.

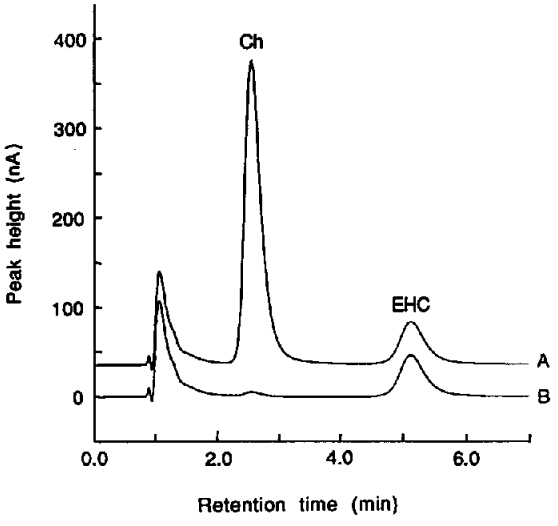

Fig. 1. Typical chromatograms obtained after enzymic (A) and non-enzymic (B) reaction with phospholipase D. A 10-μl aliquot of plasma was mixed with 100 μl of 0.1 *M* Tris–HCl buffer (pH 8.0) containing 1.0 mg of $CaCl_2$, 10 nmol of EHC, 1.0% Triton X-100 and either 10 U of phospholipase D or no enzyme, and incubated 37°C for 10 min.

TABLE I

NORMAL CONCENTRATIONS OF TOTAL, FREE AND BOUND CHOLINE IN RAT PLASMA

Values represent the mean ± S.D. for ten animals. Each type of Ch in plasma was determined according to the procedures described in Experimental.

	Concentration	
	nmol/ml	%
Total Ch	1278.7 ± 132.5	100.0
Free Ch	11.5 ± 2.2	0.9
Bound Ch	1267.2 ± 125.6	99.1

Fig. 1 shows typical chromatograms obtained after enzymic and non-enzymic reactions under these optimal conditions. The Ch peak obtained after enzymic reaction represents total Ch, and the peak in the non-enzymic reaction represents free Ch. Because the free Ch peak was negligible compared with that of total Ch, the accurate determination of free Ch levels may be performed by the procedure described in Experimental.

Normal levels of free, bound and total Ch in rat plasma

Normal concentrations of free, bound, and total Ch in rat plasma are shown in Table I. The total Ch level was 1278.7 ± 132.5 nmol/ml (mean ± S.D.), the free Ch level was 11.5 ± 2.2 nmol/ml, and the bound Ch level was 1267.2 ± 125.6 nmol/ml (free Ch subtracted from total Ch). Free Ch and bound Ch levels represented *ca.* 1 and 99% of the total Ch level, respectively.

DISCUSSION

In this study, an LC–ED system was used for the determination of Ch liberated by phospholipase D from phospholipids containing Ch. This system involves two special features for the detection of Ch: one is enzymic detection of Ch using choline oxidase in an immobilized column [5–9], and the other is an adsorption procedure using a glassy carbon pre-column [8]. The use of a pre-column increased the selectivity by trapping electrochemically the active substances in plasma such as catecholamines.

Takayama *et al.* [10] have reported a method for the determination of total Ch in plasma by a colorimetric assay using simultaneous actions of three different enzymes: phospholipase D, choline oxidase and peroxidase. However, each enzyme requires a given set of individual optimal conditions, so that in their method the enzymic conditions for the simultaneous action of the three enzymes can only be the least common multiple for all three enzymes. Because only phospholipase D is necessary for the hydrolysis of the phospholipids in the present method, the examination of enzymic conditions can be focused on phospholipase D alone.

The optimal conditions for the incubation mixture to completely hydrolyse to Ch from the phospholipids, in the plasma volume used for the assay, were selected as 100 μl of 0.1 *M* Tris–HCl buffer (pH 8.0) containing 1.0 mg of $CaCl_2$, 10 nmol of EHC, 10 U of phospholipase D and 1.0% Triton X-100. The optimal plasma volume for the assay was determined to be between 5 and 60 μl. However, this volume range was obtained at 0.2% Triton X, so we selected a 10-μl volume for the actual assay. The incubation mixture for the enzymic reaction included sufficient concentrations for the selected sample volume. When the volume was fixed at 10 μl, the determination of total Ch was observed to be linear between 10 and 7000 nmol/ml. However, because of this lower limit for detecting free Ch, the determination of the free Ch level needs to be performed according to the procedure described in Experimental, if experimental error is to be avoided.

We confirmed the usefulness of the present method by measuring levels in normal rat plasma. The concentration of free Ch determined in rat plasma agreed with results obtained by gas chromatography–mass spectrometry [2,11], colorimetry [12] and LC–ED [3,4]. However, there are few reports indicating the concentrations of total or bound Ch in rat plasma, so it was possible to compare our results with only a single report [12] indicating total Ch in rat plasma by a colorimetric method; here, too, agreement was confirmed. In our results, normal concentrations of total, free and bound Ch in rat plasma were estimated as 1278.7 ± 132.5, 11.5 ± 2.2 and 1267.2 ± 125.6 nmol/ml, respectively. The free Ch level represented only 1% of the total Ch, and bound Ch level represented 99%.

In previous work [4], we were successful in inducing conditions of plasma with no free Ch in rats by intravenous injection of choline oxidase. This treatment caused significantly decreased concentrations of Ch in the regional brain areas examined. However, this treatment did not affect the concentrations of brain ACh at all in the areas examined. Hence, it is suggested that the brain may possess some mechanism for sustaining brain ACh levels to stop the supply of free Ch from circulating to the brain during synthesis of ACh in the brain.

In conclusion, the method described here has many advantages, including rapidity, specificity, excellent precision and the need for only a small sample volume. It may be useful for studies of the neurophysiological, biochemical and pharmacological significance of plasma phospholipids containing Ch.

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