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LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION FOR THE DETERMINATION OF CHOLINE AND ACETYLCHOLINE IN PLASMA AND RED BLOOD CELLS

FAILURE TO DETECT ACETYLCHOLINE IN BLOOD OF HUMANS AND MICE

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SUMMARY

An assay is described for the measurement of choline in plasma and red blood cells using liquid chromatography, an enzyme reactor and electrochemical detection after a simple sample pretreatment. The intra-assay coefficient of variation for choline was 6.2 and 3.8% in plasma and in red blood cells, respectively. Using this method we have re-investigated the presence of acetylcholine in blood constituents. We were not able to demonstrate acetylcholine with a limit of detection of 10 pmol per ml of plasma or per ml of red blood cells.

INTRODUCTION

Choline (Ch) is present in all animal tissues and is involved in several important biochemical pathways. Acetylation of Ch results in formation of the neurotransmitter acetylcholine (ACh), and phosphorylation of Ch results in phosphorylcholine, an important precursor for phospholipids.

The measurement of Ch content in blood constituents is of interest in experimental precursor loading therapy with Ch or lecithin of patients with Alzheimer's disease [1]. Furthermore, the very pronounced rise in Ch levels in red blood cells

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(RBCs) of lithium-treated patients with manic-depressive disorder has prompted the investigation of this parameter in a variety of psychiatric and neurological disorders [2]. Also, an animal model for the lithium-induced rise of Ch in RBCs has recently been developed in the mouse, providing the possibility for comparison of this effect of lithium on RBCs with possible effects on brain cells [3].

Until now it has not been possible to evaluate the significance of physiological concentrations of ACh in blood, since there is no agreement about the correct actual value of this concentration. Highly diverging ACh concentrations have been reported in the literature, ranging from non-detectable levels [4] up to 3500 pmol/ml in blood [5–7].

Several methods, including gas chromatography–mass spectrometry (GC–MS) and radioenzymatic assays [8], have been employed to quantify Ch and ACh in biological tissues. These methods require expensive equipment and/or are complicated and time-consuming. Recently Potter et al. [9] reported a liquid chromatographic (LC) method for the analysis of Ch and ACh. Separation of Ch and ACh was achieved by reversed-phase LC. Subsequently, these components were mixed with an enzyme solution in a reaction coil in order to produce the electrochemically detectable hydrogen peroxide. An improvement on the LC method involved the immobilization of enzymes [10–13] to reduce the waste of expensive enzymes and to render the post-column enzyme addition superfluous. Recently, we reported [14] a simplified and optimized measurement of Ch and ACh, with high sensitivity and good stability characteristics.

The present paper describes the measurement of Ch in plasma and RBCs by means of LC, an enzyme reactor and electrochemical detection. By using this sensitive method we have also re-investigated the presence of ACh in the blood of humans and mice.

EXPERIMENTAL

Materials

Acetylcholine chloride, choline chloride, acetylcholine esterase (EC 3.1.1.7, type VI-S from electric eel, 260 I.U./mg) and choline oxidase (EC 1.1.3.17) were obtained from Sigma (St. Louis, MO, U.S.A.). All other chemicals were of analytical grade and were purchased from Merck (Darmstadt, F.R.G.). Water, purified by a Milli-Q system (super-C cartridge, ion-Ex cartridges, organex-Q cartridge, 0.2-μm filter; Millipore, Bedford, MA, U.S.A.) was used for aqueous solutions.

Stock solutions of Ch and ACh were prepared in 0.01% acetic acid; fresh standards were prepared weekly with an appropriate dilution of the stock solution in 0.001% acetic acid. Standard solutions were stored at 4°C.

Analytical system

The biological samples were analysed by injection of the purified samples into the analytical system equipped with a 20- or 100-μl loop; the latter loop was installed when the highest sensitivity for the detection of ACh was required. The analytical system has been described previously [14] and consisted of a liquid chromatograph (LKB 2150; LKB, Bromma, Sweden), a pulse damper (SSI, 12-

0125), a cation exchanger (100 mm × 3.0 mm I.D., reversed-phase Chromspher 5 C_{18}, modified for cation exchange with lauryl sulphate; Chrompack, Middelburg, The Netherlands), an injector (Rheodyne 7152; Rheodyne, Cotati, CA, U.S.A.), a guard column (reversed-phase, 10 mm × 1.9 mm I.D.; Chrompack), an enzyme reactor (10 mm × 1.9 mm I.D., Hypersil APS-2), activated with glutaraldehyde and loaded with enzymes according to Damsma et al. [14] and an electrochemical detector (Amor; Spark Holland, Emmen, The Netherlands). The guard column, the analytical column and the enzyme reactor are all fitted in one cartridge holder (Chrompack). The mobile phase consisted of 0.2 *M* potassium phosphate buffer (pH 8.0) and 0.005 *M* potassium chloride, filtered at 0.45 μm. The detector was equipped with a platinum working electrode, an Ag/AgCl reference electrode and an auxiliary electrode. Hydrogen peroxide was detected at +500 mV.

Blood sample preparation

Plasma and RBCs were separated by centrifugation (800 *g*, 15 min). The interface was discarded and the RBCs were washed with three volumes of saline, containing neostigmine (5 μ*M*), and again centrifuged (800 *g*, 15 min). The supernatant was discarded. Ethylhomocholine [9] was added to both blood fractions, serving as internal standards.

Plasma (150 μl) and RBCs (150 μl) were deproteinized with 1 ml of 0.4 *M* perchloric acid (30 min). After centrifugation (5500 *g*, 1 min) a 750-μl aliquot of supernatant was taken for further treatment. The excess of perchloric acid was removed by addition of potassium acetate (36 μl, 10 *M*, 5 min), followed by centrifugation (5500 *g*, 1 min). The supernatants were used for immediate analysis by LC or were stored at 4 °C.

During the whole blood preparation procedure the samples were kept on ice or kept at 4 °C.

Mice blood experiments

Trunk blood (1–2 ml) samples were collected from decapitated mice. Each sample was taken from two mice and collected in vials containing 35 U.S.P. units sodium heparin and neostigmine bromide (final concentration 10 μ*M*). It was shown that even after incubation for 5 min this concentration of neostigmine was sufficient to prevent hydrolysis of ACh added to the total blood. After separation each plasma and RBC sample was divided in two portions: one served as a control and ACh was added to the other. In a second series of experiments, mouse blood was collected without the addition of neostigmine; this blood was divided into two portions; again, one served as a control and ACh (10 nmol/ml) was added to the other. Subsequently, these samples were incubated for 7 min at 37 °C, after which plasma and RBCs were processed as described above.

Human blood experiments

Venous blood from volunteers was collected by venipuncture in heparinized vacuum tubes (Venoject, Leuven, Belgium), to which physostigmine salicylate (1 m*M* final concentration) was added. After separation of the blood constituents, Ch was determined by two different procedures. One set of samples was

analysed by LC, another was analysed by a radioenzymatic procedure as described in detail previously [3,15]. The samples analysed by LC were also investigated for the presence of ACh.

RESULTS

Chromatography

Chromatograms obtained from plasma and RBCs of mice and a standard mixture (Fig. 1) show that the use of a cation exchanger connected to an enzyme reactor results in good separation of Ch, ACh and ethylhomocholine. The identity of these compounds is confirmed by their chromatographic properties. In addition, samples were occasionally assayed without the enzyme reactor; the complete disappearance of the quaternary components strongly supports the specificity of the method. To lower the detection limit for ACh we increased the loop volume to 100 μl; the limit of detection for ACh was then 100 fmol per 100-μl sample.

The reproducibility of the sample treatment was determined by analysis of seven separately prepared blood samples derived from one blood sample of a lithium-treated patient. The mean values ($\pm$S.D.) for plasma Ch and RBC Ch were: 10.8 ± 0.8 μM (coefficient of variation = 6.2%) and 167 ± 6.3 μM (coefficient of variation = 3.8%).

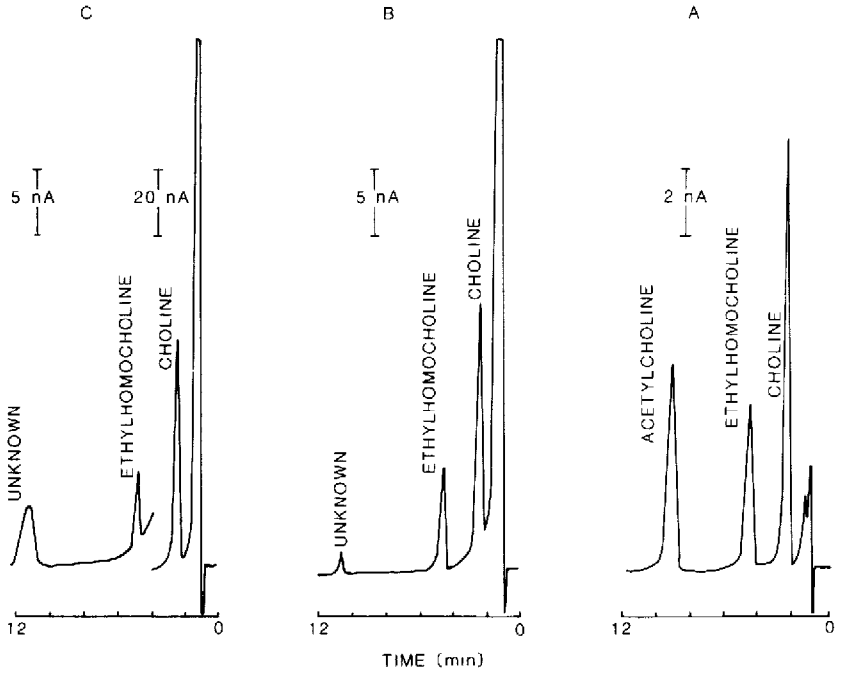

Fig. 1. Chromatograms of (A) a standard mixture of choline, ethylhomocholine and acetylcholine (20 pmol/20 μl), (B) plasma and (C) RBCs.

Comparison of LC and radioenzymatic analysis of Ch in plasma and RBCs

Plasma and RBCs from five human volunteers were analysed for their Ch content by two different analytical methods. The results are presented in Table I. The group means indicate that no systematic differences exist between the two analytical methods. Also, there is a clear reasonable agreement between individual samples. The plasma and RBC levels of Ch are comparable with those reported by Hanin et al. [5].

Presence of ACh in blood constituents

Human blood was analysed for the presence of Ch and ACh. To protect the sampled blood from the action of acetylcholinesterase, we collected the blood in vials containing physostigmine. We were not able to detect any ACh, either in

TABLE I

COMPARISON OF LC AND RADIOENZYMATIC (RE) ASSAYS FOR THE MEASUREMENT OF CHOLINE IN HUMAN BLOOD CONSTITUENTS OF FIVE CONTROLS

Values are nmol/ml of plasma or RBCs.

Control	Plasma		RBCs	
	LC	RE	LC	RE
1	9.9	7.8	10.9	12.8
2	9.9	8.6	99.0	87.6
3	11.7	11.9	16.6	20.0
4	6.7	5 6	8.8	10.4
5	9.1	9.9	10 4	10.8
Mean ± S.D.	9.5 ± 1.8	8 8 ± 2.3	29.9 ± 39	28.3 ± 33

TABLE II

AMOUNT OF CHOLINE AND ACETYLCHOLINE IN PLASMA AND RBCs OF MICE, AND THE EFFECTS THEREON OF NEOSTIGMINE, ACETYLCHOLINE ADDITION AND INCUBATION

Incubation at 37°C for 7 min. All values are the average ± S.D. of five determinations. N.D. = not detectable: detection limit 10 pmol/ml. Ch values are expressed as nmol/ml and ACh values as pmol/ml.

	Plasma		RBCs	
	Ch	ACh	Ch	ACh
Control + neostigmine	7.8 ± 1.3	N.D.	46.4 ± 5.9	N.D.
Control + neostigmine + ACh (200 pmol/ml of plasma; 120 pmol/ml of RBCs)	8.0 ± 1.6	180 ± 7	47.4 ± 7.1	110 ± 8
Incubation	13.5 ± 1.3	N.D.	42.1 ± 4.3	N.D.
Incubation + ACh (10 nmol/ml of blood)	25.0 ± 2.6	N.D.	46.3 ± 6.7	N.D.

human plasma or in human RBCs. The overall detection limit of ACh in blood constituents is ca. 10 pmol per ml of plasma or RBCs (taking into account that the samples are diluted 8.05 times during sample preparation).

In view of the failure to detect ACh in human blood the role of acetylcholinesterase was further investigated in mouse blood. To prevent hydrolysis of ACh a reversible acetylcholinesterase inhibitor, neostigmine, was added to the blood collection vials. The levels of Ch in plasma and RBCs (Table II) were in the same range as reported previously [3], but again we were not able to detect any ACh. To investigate whether ACh was preserved during the sample preparation, we added ACh to plasma (200 pmol/ml) and RBCs (120 pmol/ml). The results show that the added ACh was recovered and could be quantified (Table II). The mean recovery ($\pm$S.D.) of ACh added to plasma was $90 \pm 2.4\%$ ($n=5$) and of ACh added to RBCs $90 \pm 5.8\%$ ($n=5$).

We also investigated whether ACh added to mouse blood, without acetylcholinesterase inhibition, could be recovered from the blood constituents. Blood, to which 10 nmol/ml ACh was added, was incubated for 7 min at 37°C. The results (Table II) demonstrate clearly that no ACh was detectable after incubation of the sample. Thus, the 10 nmol ACh added per ml of blood were completely hydrolysed (more than 99.9%) during the 7-min incubation. As a result, the Ch levels in plasma are increased. It should be noted that the Ch levels of incubated plasma and RBCs are increased compared with control levels. Apparently Ch is produced in these samples, possibly from breakdown of Ch-containing molecules, such as phosphatidylcholine.

DISCUSSION

This study validates the use of LC, with a post-column enzyme reactor and an electrochemical detector, as an analytical alternative for the measurement of Ch in plasma and blood. The method is simple, sensitive and specific. The linearity of the detector response versus the peak height has been reported in a previous study that used the same assay system [14]. We also described a simple sample pretreatment procedure with a good recovery (ca. 90%) and reproducibility. The comparison of the presented LC method with the established radioenzymatic procedure was satisfactory. Recently Webb and Johnson [16] reported the use of a similar analytical procedure for the analysis of Ch in plasma and RBCs. However, they did not immobilize the enzymes in a reactor, and thus wasted the expensive enzymes. In addition, the sensitivity of their method is ca. 100-fold less than the sensitivity of the method reported here.

An interesting finding of the present study is the failure to detect any endogenous ACh (detection limit 10 pmol/ml) in blood from humans and mice. It might be expected that the high activity of acetylcholinesterase in blood is responsible for the absence of ACh. Indeed, when we added large amounts of ACh to blood, we were unable to recover or detect any added ACh after a short incubation at 37°C. However, if we added an acetylcholinesterase inhibitor to the blood fractions, ACh was protected from hydrolysis as shown by the recovery of 90% of the added ACh (Table II). Thus, from the present data we conclude that blood con-

stituents do not contain detectable amounts of ACh, and that this is probably due to the activity of the abundantly available acetylcholinesterase.

The data reported here confirm those of Okonek and Kilbinger [4], who were not able to detect ACh in blood plasma of healthy humans. In plasma of patients with severe nitrostigmine intoxication, however, they measured ACh levels up to 200 pmol/ml in the early phase of complete cholinesterase inhibition. These ACh levels are in the lower range of the values reported by other authors [5–7] for human blood without in vivo cholinesterase inhibition. These investigators [4] also reported that ACh levels were no longer detectable when cholinesterase inhibition was still over 95%. Our results not only confirm the absence of ACh in human blood plasma, as shown by Okonek and Kilbinger [4], but extend these findings to human RBCs and mouse plasma and RBCs.

Our data are clearly at variance with several studies reporting a large range of concentrations of ACh in blood or blood constituents, e.g. Hanin et al. [5] reported values of 28 pmol ACh per ml of plasma and 94 pmol per ml of blood, Hasegawa et al. [6] reported 3560 pmol per ml of blood and Watanabe et al. [7] reported 490 pmol per ml of blood. In these studies GC–MS methods were employed to determine ACh. The collection of the blood was very similar to the method reported here, and we used the same concentration of physostigmine as reported by Hasegawa et al. [6] and Watanabe et al. [7]. The sample preparation procedure is comparable in most studies and not essentially different from our method. The most conspicuous difference between the LC and GC–MS methods is the need for additional isolation and derivatization steps prior to chromatographic separation required for the latter method. Thus, these extra steps appear to us the most likely explanation for the divergent findings concerning the amount of ACh present in blood.

In conclusion, this paper describes a method for the determination of Ch in plasma and blood. The sample preparation is simple and the subsequent assay is based on chromatographic separation by cation-exchange LC, followed by a post-column enzymatic derivatization and subsequent electrochemical detection. The procedure is selective, sensitive, reproducible and uses standard analytical laboratory equipment. Employing the LC method, we failed to detect ACh in blood constituents of humans and mice.

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