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Journal of Chromatography B, 656 (1994) 59–64

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Determination of plasma choline by high-performance liquid chromatography with a postcolumn enzyme reactor and electrochemical detection

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Abstract

A method for the determination of choline in human plasma is described, involving rapid purification of plasma samples and analysis by high-performance liquid chromatography using an on-column enzyme reactor with electrochemical detection. The linearity of the method was tested at choline levels from 3.5 to 28.6 μM in plasma. The recovery was 86% and was independent of the analyte concentration. The inter-assay precision (as coefficient of variation) and accuracy (as the deviation of the concentration found from the theoretical value) were always below 12% in the whole concentration range. The method was applied to the determination of plasma choline levels in eight healthy volunteers after intramuscular administration of L-α-glycerophosphorylcholine (1 g) or a placebo. Mean plasma choline levels in the placebo group ranged from 10.6 to 12.0 μM. After drug administration, the plasma choline level reached 35.1 μM in 30 min, then decreased gradually. Plasma choline levels became comparable in the treated and placebo groups 6–8 h after administration.

1. Introduction

L-α-Glycerophosphorylcholine (Fig. 1) is a precursor of choline-containing phospholipids, mainly phosphatidylcholine and sphingomyelin, and can be considered as a choline and acetylcholine precursor for the central nervous system [1–4]. The compound is completely absorbed after oral administration, and widely metabolized when given either intramuscularly or orally to humans and animals. One of the main plasma metabolites is choline, which is detectable until 6–8 h after oral and intramuscular administration [3,4].

In order to follow the "choline step" of

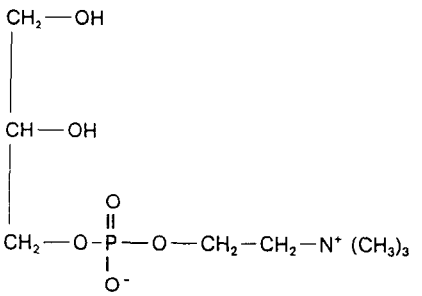

Fig. 1. Structure of L-α-glycerophosphorylcholine.

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0378-4347/94/$07.00
SSDI 0378-4347(94)00070-L

L-α-glycerophosphorylcholine metabolism in humans, we have developed a method for the measurement of plasma choline levels. Although choline can be measured by different methods such as bioassay, gas chromatography with mass spectrometric or nitrogen-sensitive detection radioenzymatic assay, chemiluminescence assay and HPLC with UV detection [5–9], most of these methods are expensive and/or time consuming, because they require long extraction procedures or difficult derivatizations.

The method described here is based on a very simple sample pretreatment, followed by HPLC separation combined with an immobilized enzyme reactor and electrochemical detection [10,11].

2. Experimental

2.1. Chemicals

Choline chloride, purity *ca.* 99% (Sigma, St. Louis, MO, USA), was recrystallized from methanol. Choline oxidase (EC 1.1.3.17) from *Alcaligenes* species and catalase (EC 1.11.1.6) were purchased from Sigma. All other reagents were of ACS or equivalent grade from Merck (Darmstadt, Germany).

Ethylhomocholine (N,N-dimethyl-N-ethyl-3-amino-1-propanol) was chosen as an internal standard (I.S.) and prepared as described by Potter *et al.* [12] by crystallization after incubation of equal volumes of N,N-dimethyl-3-amino-1-propanol and iodoethane.

2.2. HPLC instrumentation

The HPLC apparatus consisted of an L 6000 reciprocating pump (Merck–Hitachi, Darmstadt, Germany), an AS-2000A autosampler (Merck–Hitachi) and a Coulochem II electrochemical detector with a Model 5040 platinum analytical cell (ESA, Bedford, MA, USA). The detector was coupled to a computer terminal (Maxima 820 data system; Waters, Milford, MA, USA) for data acquisition and evaluation. The analytical column was reversed-phase Cyano Spheri-5, 5 μm (100 × 4.6 mm I.D.) (Brownlee, Santa Clara, CA, USA) and the bioreactor column was an Aquapore AX-300 anion-exchange cartridge (Brownlee), 7 μm (30 × 2.1 mm I.D.). The mobile phase was 20 m*M* sodium hydrogenphosphate – 10 m*M* tetramethylammonium chloride adjusted to a final pH of 7.1 with 65% phosphoric acid. The flow-rate was 0.7 ml/min, with continuous degassing with helium. The platinum electrode was set at a potential of +300 mV.

The detection cell was dismantled weekly and the electrode was washed for 10 min with 10 μl of 60% nitric acid, followed by 15 min in 95% ethanol in an ultrasonic bath. The cell was then reassembled and reconnected to the HPLC system.

2.3. Preparation of enzyme reactor

The enzyme reactor was prepared as described by Eva *et al.* [13]. Briefly, choline oxidase was adsorbed on an Aquapore AX-300 anion-exchange cartridge by slow injection of 50 μl of an aqueous solution containing 100 IU/ml of enzyme, through a 1-cm piece of PTFE tubing connected to the cartridge holder, which had been removed from the HPLC system. To prevent fouling of the electrode, the reactor had to be washed with the mobile phase for several minutes after loading, before the electrode was turned on. Under our experimental conditions, the lifetime of the reactor was about 3 days or 100 samples, after which the reactor was regenerated simply by repeating the charging procedure. Each Aquapore AX-300 cartridge was replaced after about 1 month. When the HPLC system had to be stopped for a few days, only water was used to wash the columns, with no organic solvents.

2.4. Subjects and plasma sample collection

Eight healthy male volunteers aged 19–24 years were injected intramuscularly with 1 g of L-α-glycerophosphorylcholine or placebo after an overnight fast. Blood samples were drawn by direct venipuncture at time zero (basal) and then

at 5, 15, 30 and 45 min and 1, 2, 3, 4, 6, 8 and 10 h after dosing, and placed in heparinized tubes. Plasma was separated by centrifugation at 2000 *g* for 15 min and kept frozen until analysis.

2.5. Sample preparation

A 2-ml volume of acetonitrile containing 0.3 μg/ml of ethylhomocholine (I.S.) was added to 0.1 ml of plasma. The mixture was shaken for *ca.* 1 min on a vortex mixer, then centrifuged at 2000 *g* for 15 min. After centrifugation, 1.5 ml of the supernatant were withdrawn and evaporated to dryness *in vacuo*. The residue was dissolved in 0.5 ml of the HPLC eluent and 50 μl were injected into the chromatograph.

Blank plasma samples were prepared by adding 10 μl of 100 IU/ml choline oxidase and 10 μl of 100 IU/ml catalase to 0.1 ml of plasma and incubating at 37°C for 15 min, in order to destroy the endogenous choline.

2.6. Calculation and control samples

As choline is an endogenous plasma component, standard calibration samples were prepared by adding known amounts of choline chloride in the range 3.58–28.65 μ*M* to water. Four concentrations in the working range were measured in duplicate. Calibration graphs were obtained by the least-squares method. Peak-area ratios of choline to the I.S. were used to generate the linear least-squares regression lines. Concentrations of choline in the unknown samples were obtained by interpolation on this calibration graph using peak-area ratios from unknown samples. Blank plasma samples were used to monitor the interferences.

The recovery of choline was checked by comparing the peak-area ratios in water extracts spiked with the compound with the ratios for equal amounts of directly injected choline solution. The I.S. for this assay was added just before injection into the chromatograph.

The assay performance was checked by preparing quality control (QC) samples at the start of the study: known amounts of choline chloride were added to both water and plasma. Choline levels in plasma QC samples were evaluated by subtracting the basal value (endogenous level) for each sample from the found value. Inter-assay (day-to-day) precision [coefficient of variation (C.V.)] and accuracy (calculated as the relative deviation of the mean concentration found from the theoretical concentration) were established by analysing the QC samples simultaneously with the plasma samples on different days.

3. Results and discussion

Examples of chromatograms from a blank plasma sample (A), a standard sample containing choline 7.2 μ*M* (B) and a plasma extract from volunteers treated with L-α-glycerophosphorylcholine (C) are shown in Fig. 2. The extract of blank plasma sample does not show any peak that could interfere with analysis. The retention times of choline and the I.S. were *ca.* 5 and 8 min, respectively.

Acetonitrile was used as an extracting–denaturing solvent starting from 0.1 ml of plasma; under these conditions, the mean recovery, summarized in Table 1, was 86% (C.V. = 6.7%) for choline over the whole concentration range and 92% (C.V. = 3%) for the I.S. ethylhomocholine at the working concentration. The choline recovery was independent of concentration over the range investigated, so the regression analysis showed high linearity in plasma ($r > 0.99$). The limit of quantification (LOQ) was assumed to be the lowest validated point for QC samples in water, *i.e.* 3.58 μ*M*, using 0.1 ml of sample.

Water and human plasma QC samples, spiked with different amounts of choline, were assayed with each of the HPLC runs in support of this study. The inter-assay precision (C.V.) was 8.3% at the LOQ in the water and 10.8% at the endogenous level in plasma. Accuracy, $[(F - A)/A] \cdot 100$, calculated from the deviation of the mean concentration found (F) from the nominal value (A), indicated an inter-assay variation of 11.9% at the LOQ in water and below 3.5% in plasma QC samples. The results, summarized in Table 2, indicated that the method has accept-

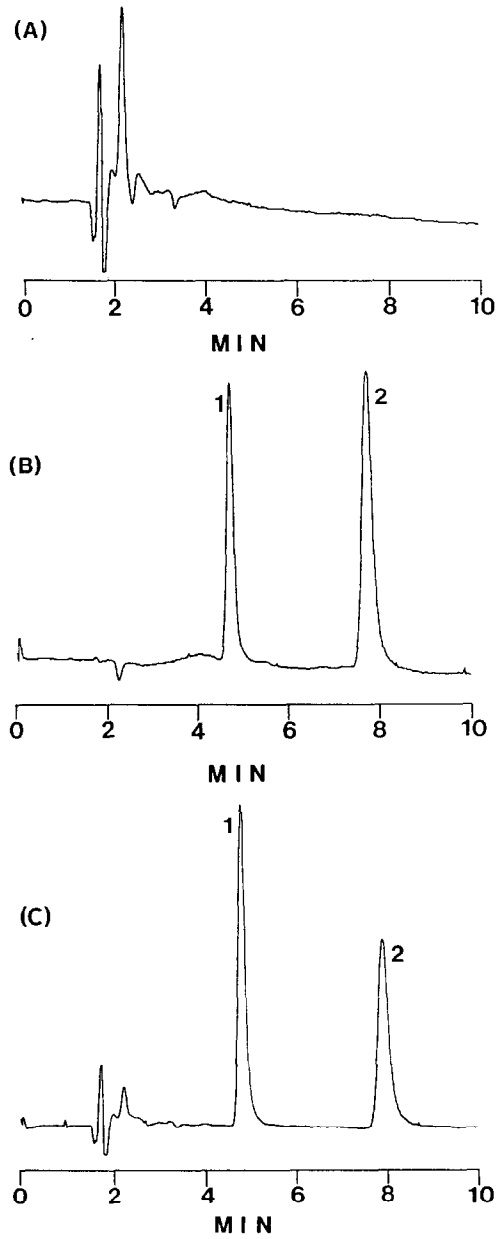

Fig. 2. Typical chromatograms of (A) a blank plasma sample (prepared as described in the text), (B) a choline standard sample prepared in water (7.16 μM) and (C) a plasma sample from healthy volunteers. Peaks: 1 = choline; 2 = ethylhomocholine (internal standard). Detailed chromatographic conditions are described in the text.

able reliability, as the precision and accuracy were <15% at all concentrations tested [14].

The utility of the method was established by determining endogenous choline in healthy volunteers and the levels after administration of L-α-glycerophosphorylcholine (Fig. 3). The mean endogenous plasma choline ranged from 10.6 to 12.0 μM during the observation period, and the coefficient of variation was always moderate at each sampling time, *i.e.* below 24%. These values confirm previous reports of plasma choline levels measured by different methods [15–18].

Table 1
Choline recovery and coefficient of variation for replicate assays ($n = 3$)

Concentration added (μM)	Recovery (%)	C.V. (%)
3.58	92	4.5
7.16	80	1.4
14.33	86	6.5
28.65	84	5.2
Mean	86	6.7

After intramuscular injection of L-α-glycerophosphorylcholine (1 g), the choline levels reached 35.1 μM in 30 min, indicating rapid metabolism to the active metabolite. The plasma concentration then decreased to close to the endogenous levels 6–8 h after dosing. The simple method here described follows the well established HPLC approach using on-line enzymic transformation of choline and electrochemical detection of the hydrogen peroxide. The main difficulty is to fix the choline oxidase to an HPLC-compatible support, to obtain the on-line immobilized enzyme reactor (IMER). Many supports have been described, such as silica, alkylamino-bonded silica and CNBr-activated Sepharose 4B [19–21]. These all establish a covalent bond with the enzyme, allowing the bioreactor to work for 1–3 months. However, we chose a weak anion-exchange cartridge as support, as suggested by Eva *et al.* [13], in which the enzyme is simply adsorbed on the resin. This technique offers some advantages such as low cost, simple preparation and a maximum enzymatic activity during every analytical batch. The eluent must be of weak ionic strength, with no organic solvent, in order to prevent rapid

Table 2
Inter-assay precision and accuracy of the method

Concentration added (μM)	n	Concentration found (μM)	Precision (C.V., %)	Accuracy (%)
Water quality control samples				
3.58	13	4.01	8.28	11.88
10.74	12	10.13	7.12	−5.72
21.49	13	20.95	5.95	−2.51
Plasma quality control samples				
Basal value	13	7.82	10.77	–
Basal value + 7.16	11	7.35[a]	3.12	2.62
Basal value + 14.33	13	13.84[a]	5.86	−3.44

[a] Calculated by subtracting the basal value from the found value for each sample.

degradation of the bioreactor. Under these conditions, about 100 samples can be analysed with one reactor before repeating the enzyme loading procedure.

The cyanopropyl analytical column, with weak negative charges, enabled us to avoid the use of the counter ion in the mobile phase. Some workers use the counter ion with more hydrophobic columns, but in our experience it causes rapid contamination of the platinum working electrode.

Although the extraction procedure is very simple, the addition of ethylhomocholine as an I.S. to the plasma samples not only represents a real control for sample extraction but especially it corrects the loss of bioreactor choline oxidase activity and the decrease in efficiency of the platinum working electrode.

Finally, the sensitivity, repeatability and selectivity render this method suitable, with minor modifications, for the determination of choline in other biological fluids and in tissues.

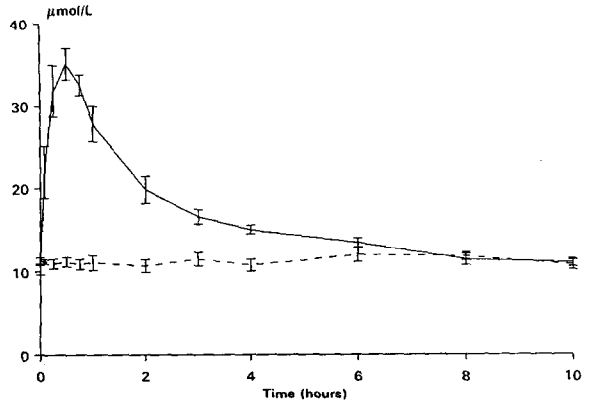

Fig. 3. Mean (±standard error) plasma concentration–time curves for choline after intramuscular injection of L-α-glycerophosphorylcholine (1 g) (solid line) and placebo (broken line) into eight healthy male volunteers.

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