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High-performance liquid chromatographic assay of L-α-glycerophosphorylcholine using a two-step enzymic conversion

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ABSTRACT

A high-performance liquid chromatographic method using an enzymic reactor for determination of L-α-glycerophosphorylcholine in pharmaceutical forms is described. The procedure includes incubation of L-α-glycerophosphorylcholine with glycerophosphorylcholine phosphodiesterase (EC 3.1.4.2), giving choline and glycerophosphate, and subsequent chromatography of choline with a post-column enzymic reactor and electrochemical detection. The results obtained show a close linearity of the whole assay from 2 to 150 nmol/ml L-α-glycerophosphorylcholine, the sensitivity being 2 pmol per 20 μl of injected sample. The precision of the method in the analysis of L-α-glycerophosphorylcholine in pharmaceutical forms, ampoules and capsules, was 1.34 and 1.21%, respectively.

INTRODUCTION

L-α-Glycerophosphorylcholine is an intermediate in the catabolic pathway of phosphatidylcholine, which leads to the generation of choline [1,2], and in this respect it can be regarded as a choline precursor. L-α-Glycerophosphorylcholine can also be formed in the synthesis of phosphatidylcholine, which takes place by transacylation of two molecules of lysophosphatidylcholine [1].

Recent studies carried out by our group with L-α-glycerophosphoryl-[1,2-^{14}C]choline in the rat showed that this water-soluble compound was well absorbed after oral administration, and that its major metabolite in the blood was choline; in the brain, [^{14}C]choline and phosphatidyl[^{14}C]-choline have been found [3].

These pharmacokinetic findings were paralleled by experimental and clinical

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evidence that this compound, when administered either orally or parenterally, is effective in improving the behavioural and cognitive symptoms related to impaired cholinergic functions [4–6]. The interest in the use of this compound in the therapy of disorders of memory formation and retention prompted us to develop an analytical method for the determination of L-α-glycerophosphorylcholine in biological fluids and in pharmaceutical forms.

This paper describes a method for the quantitative determination of L-α-glycerophosphorylcholine in pharmaceutical forms. The assay is based on a two-step procedure.

(1) Incubation of L-α-glycerophosphorylcholine in the presence of glycerophosphorylcholine phosphodiesterase giving choline and glycerophosphate.

(2) High-performance liquid chromatographic (HPLC) analysis of choline, using a post-column reactor with immobilized choline oxidase and an electrochemical detector measuring the enzymically produced hydrogen peroxide [7,8].

EXPERIMENTAL

Chemicals

Choline chloride, *ca.* 99% purity (Sigma, St. Louis, MO, U.S.A.), was recrystallized from methanol. L-α-glycerophosphorylcholine, *ca.* 99% purity, was from Chemi (Frosinone, Italy). Choline oxidase from *Alcaligenes* species and glycerophosphorylcholine phosphodiesterase from mould were purchased from Sigma. All other reagents of ACS or equal grade were purchased from Merck (Darmstadt, Germany).

Enzymic hydrolysis of L-α-glycerophosphorylcholine and sample preparation

A 100-μl sample containing an unknown amount of L-α-glycerophosphorylcholine in 0.1 *M* phosphate buffer at pH 8.0 was hydrolysed to choline and glycerophosphate by incubation at 37°C for 20 min with 10 μl of glycerophosphorylcholine phosphodiesterase (EC 3.1.4.2) (10 I.U./ml). A 2-ml volume of acetonitrile containing ethylhomocholine (2.5 μg/ml) was added as internal standard, and centrifuged at 2000 *g* for 15 min. After centrifugation, 1.5 ml of the supernatant were withdrawn and evaporated to dryness *in vacuo*. The extract was then reconstituted with 1.5 ml of the HPLC eluent, and 20 μl were injected into the chromatograph.

The analyses of L-α-glycerophosphorylcholine pharmaceutical forms (ampoules and capsules) were carried out with fixed amounts of each preparation dissolved in 0.1 *M* phosphate buffer (pH 8.0) and diluted to a theoretical concentration of 100 nmol/ml; 100 μl of these solutions were then incubated as described above.

HPLC system

The HPLC apparatus consisted of a reciprocating pump (L 6000 Merck Hi-

tachi), a six-port rotary valve (Negretti 220, Southampton, U.K.) with a 20-μl loop, a reversed-phase column (Cyano Spheri-10, 10 μm, 30 mm × 4.6 mm I.D., Brownlee, Santa Clara, CA, U.S.A.), an anion-exchange cartridge (Aquapore AX 300, 7 μm, 30 mm × 2.1 mm I.D., Brownlee) and an electrochemical detector with a platinum working electrode (M 20 Gynkotek, Germering, Munich, Germany). The oxidation potential was +0.5 V *versus* an Ag/AgCl electrode. The mobile phase was 15 m*M* sodium hydrogenphosphate and 0.5 m*M* tetramethylammonium chloride at a final pH of 7.

The detection cell was daily disassembled, and the platinum electrode was washed for 10 min with 10 μl of 35% nitric acid. The cell was then reassembled and reconnected to the HPLC system.

Ethylhomocholine [N,N-dimethyl-(N-ethyl)-3-amino-1-propanol] was chosen as an internal standard and prepared as described by Potter *et al.* [7] by crystallization after incubation of equal volumes of dimethyl-3-amino-1-propanol and iodoethane.

Preparation of the enzyme reactor

Adsorption of choline oxidase (EC 1.1.3.17) on the anion-exchange cartridge [8] was achieved by slow injection of 50 μl of a water solution containing 100 I.U./ml enzyme onto the cartridge via a 1-cm piece of PTFE tubing connected to the cartridge holder after this was removed from the HPLC system. To prevent fouling of the electrode, the reactor should be washed with the mobile phase for several minutes after loading before the electrode is turned on.

Under our experimental conditions the lifetime of the reactor was three days. Regeneration of the reactor was achieved simply by repeating the charging procedure described above. In order to prolong the life to one week the bioreactor should be refrigerated at 4°C in 15 m*M* phosphate buffer (pH 7.0) containing 0.5% sodium azide.

RESULTS AND DISCUSSION

Under the described conditions, the linearity of the L-α-glycerophosphorylcholine assay has been verified in the range 2–150 nmol/ml. The regression curve was constructed by linear regression fitting, and its mathematical expression was $y = 2.38x - 0.04$ ($r = 0.9989$) where y is the concentration of the samples and x is the choline/internal standard peak-height ratio. Comparison with an equivalent series of choline samples showed that the L-α-glycerophosphorylcholine and choline assays display identical dose–response curves and a close correlation (Pearson's correlation coefficient $r = 0.9997$, $p < 10^{-6}$). These results clearly indicate that the hydrolysis of L-α-glycerophosphorylcholine took place and that it can be quantitatively determined as choline.

Typical chromatograms of a non-hydrolysed sample containing 100 nmol/ml L-α-glycerophosphorylcholine (blank), a choline standard (100 nmol/ml) and a

real sample of L-α-glycerophosphorylcholine prepared as described are shown in Fig. 1.

Under our experimental conditions, the assay sensitivity was 2 pmol per 20 μl of injected hydrolysed L-α-glycerophosphorylcholine.

The repeatability and the reliability of both the reactor and the instrumental system were tested by repeated injections ($n = 6$) of choline samples (100 nmol/ml) into the chromatograph. The coefficient of variation (C.V.) obtained using this procedure was 1.15%.

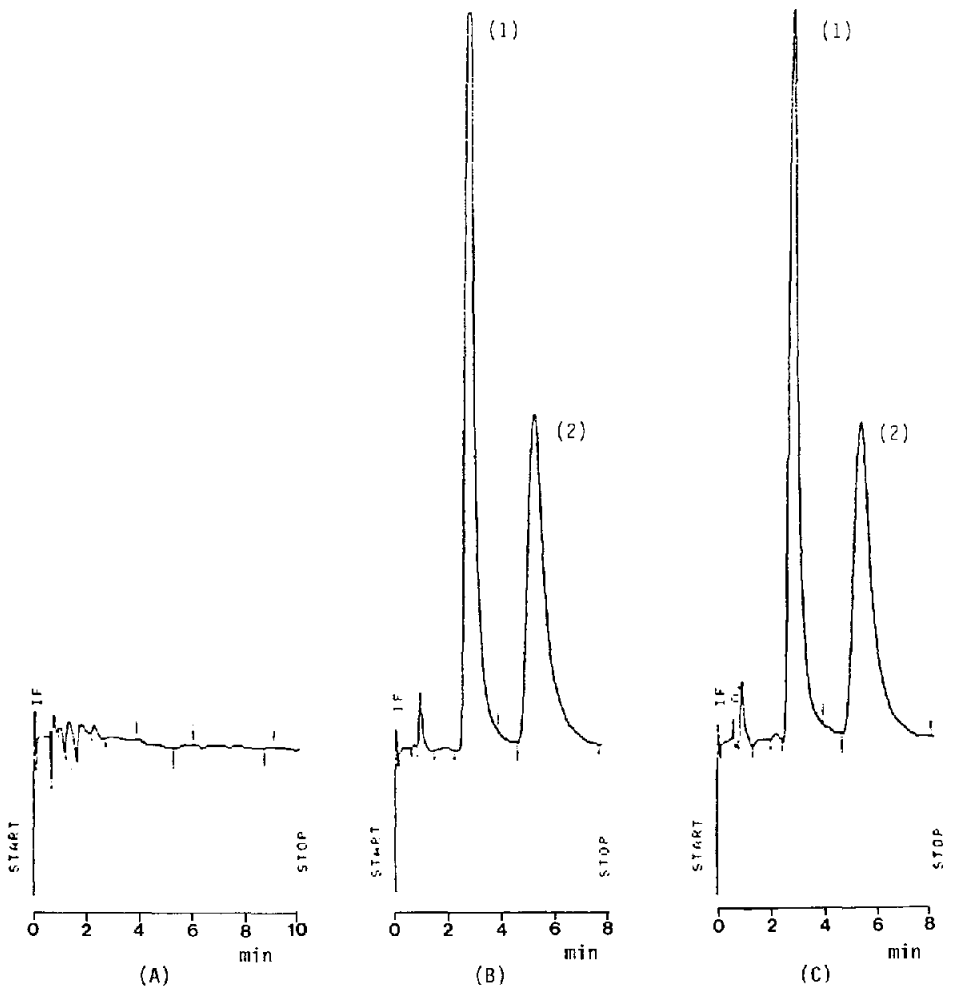

Fig. 1. Typical chromatograms of (A) nonhydrolysed L-α-glycerophosphorylcholine sample (100 nmol/ml), (B) a choline standard sample (100 nmol/ml) and (C) a real sample of L-α-glycerophosphorylcholine preparation (100 nmol/ml). Peaks: 1 = choline; 2 = ethylhomocholine (internal standard). Detailed chromatographic conditions are described in the text.

TABLE I

ASSAY QUALITY CRITERIA (n = 3)

Concentration added (nmol/ml)	Concentration found (nmol/ml)	Recovery (%)	C.V. (%)
2	1.46	73	9.4
4	3.52	88	6.8
8	7.49	94	4.9
20	19.63	98	6.2
40	39.94	100	5.7
80	78.57	98	5.3
100	98.72	99	4.5
150	149.89	100	2.9

The recovery and precision of the assay are given in Table I. The intra-assay and inter-assay C.V., tested at 100 nmol/ml, are detailed in Table II. The precision of the assay applied to the various pharmaceutical forms is reported in Table III. Similarly, satisfactory results were obtained in routine analyses of several batches of L-α-glycerophosphorylcholine ampoules and capsules (Table IV). These results show that this method is relatively simple, rapid, reproducible and suitable for the determination of L-α-glycerophosphorylcholine in pharmaceutical preparations.

The determination of L-α-glycerophosphorylcholine by commonly used analytical procedures, such as colorimetry, spectrophotometry or chromatography, is difficult because this molecule lacks chromophoric groups and is highly polar and thermolabile.

The method described here is based on the enzymic hydrolysis of L-α-glycerophosphorylcholine to choline. This allows the deterination of choline by HPLC using a post-column reactor and electrochemical detection. It thus combines the

TABLE II

INTRA- AND INTER-ASSAY VARIATION (n = 10)

	Concentration added (nmol/ml)	Concentration found (mean ± S.D.) (nmol/ml)	C.V. (%)
Intra-assay	100	100.08 ± 2.097	2.10
Inter-assay	100	99.46 ± 2.239	2.25

TABLE III

DETERMINATION OF L-α-GLYCEROPHOSPHORYLCHOLINE IN DIFFERENT AMPOULES OR CAPSULES IN THE SAME BATCH

Ampoules		Capsules	
Batch	Assay[a]	Batch	Assay[a]
1	98.3	1	100.6
2	98.8	2	102.0
3	99.2	3	101.5
4	97.7	4	100.8
5	101.2	5	98.8
Mean	99.04	Mean	100.74
C.V. (%)	1.34	C.V. (%)	1.21

[a] Mean of two replicates.

selectivity of the enzymic reaction with the high sensitivity of electrochemical detection.

This method is proposed here for the determination of L-α-glycerophosphorylcholine in pharmaceutical preparations. It could also be used (with minor modifications) for the determination of L-α-glycerophosphorylcholine in biological fluids or preparations such as tissue homogenate. Preliminary results obtained in healthy volunteers clearly show that the method allows the determination of L-α-glycerophosphorylcholine in human plasma.

Moreover, the analytical procedure described here could easily be applied to

TABLE IV

ASSAY OF L-α-GLYCEROPHOSPHORYLCHOLINE ON DIFFERENT BATCHES OF PHARMACEUTICAL FORMS

Ampoules		Capsules	
Batch	Percentage of label potency	Batch	Percentage of label potency
1	103.0	1	99.1
2	103.3	2	99.7
3	101.6	3	98.6
4	102.4	4	97.8
5	101.6	5	100.2
Mean	102.38	Mean	99.08
C.V. (%)	0.76	C.V. (%)	0.95

those biological molecules, particularly choline-containing phospholipids, the chromatographic determination of which is easier after selective enzymic transformation.

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