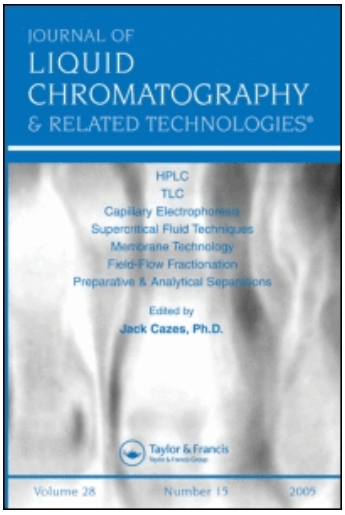


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# ANALYSIS BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY OF THE WATER-SOLUBLE PRECURSORS OF CHOLINE AND ETHANOLAMINE PHOSPHOGLYCERIDES IN THE RAT BRAIN

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## ABSTRACT

The simultaneous quantitation of PC, PE, CDP-choline and CDP-ethanolamine has been performed by high performance liquid chromatography of purified rat brain extracts. The water-soluble intermediates of the phosphoglyceride biosynthesis were extracted from the rat brain by homogenization in 1 M PCA and purified by chromatography on neutral alumina and Dowex 1. This procedure removes almost all of the UV-contaminating substances and inorganic phosphate, which interfere in the analytical chromatographic process. The high performance liquid chromatography was carried out using the styrene-type Aminex A-14 resin. The elution was performed for 5 min with 0.1 M 2-amino-2-methyl-1-propanol, 0.02 M NaCl, pH 10.3 buffer, and then with 0.1 M 2-amino-2-methyl-1-propanol, 0.17 M NaCl, pH 11.0 solution, until the end of the run. Satisfactory separations of PC, PE, CDP-choline and CDP-ethanolamine were achieved within 30 min. 5'-CMP can also be dosed. The cytidine-containing compounds have been detected and quantified by UV

absorption at 280 nm, while PC and PE by phosphorus determination in the chromatographic fractions. The method allows the quantitative determination in subgram amounts of wet tissue.

## INTRODUCTION

Phosphorylcholine (PC), cytidine-5'-diphosphocholine (CDP-choline), phosphorylethanolamine (PE) and cytidine-5'-diphosphoethanolamine (CDP-ethanolamine) are the metabolic intermediates for the synthesis of choline and ethanolamine phosphoglycerides, respectively (1-5). The evaluation of their concentrations in tissues is very often required for a better understanding of their role in the regulation of phospholipid biosynthesis, together with the quantitative determination of cytidine-5'-phosphate (5'-CMP) which, in some cases, can reverse the activity of choline and ethanolamine phosphotransferases (6-9).



Usually, the separation and the quantitation of the above mentioned water-soluble intermediates has been achieved by combining column and paper chromatography (10-12), or by consecutive column chromatographies (4). Thin-layer chromatography has also been used (13,14). Such procedures are time-consuming and large tissue amounts are always required for the analyses.

This paper reports a method for the simultaneous separation and for the quantitative determination of the water-soluble intermediates of the glycerophosphatide synthesis. using high performance liquid chromatography (HPLC) on a conventional ion-exchange polystyrene resin.

## MATERIALS

PC, PE, CDP-choline, CDP-ethanolamine, and 5'-CMP were obtained from Sigma Chem. Co. (St. Louis, Mo., USA).

Phosphoryl-[methyl-$^{14}$C]choline (S.A. of 40-60 mCi/mmole), phosphoryl-[1,2-$^{14}$C]ethanolamine (S.A. of 15-25 mCi/mmole), cytidine-5'-diphospho[methyl-$^{14}$C]choline (S.A. of 40-60 mCi/mmole) were obtained from New England Nucl. Corp. (Frankfurt, West Germany). Cytidine-5'-diphospho[1,2-$^{14}$C]ethanolamine (S.A. of 28 mCi/mmole) was obtained from the Radiochemical Centre, (Amersham, Bucks. England).

Aminex A-14, $Cl^-$ (20 ± 3 μ) and Dowex 1-x 4, $Cl^-$, (100-200 mesh) were purchased from Biorad Lab. (Richmond California, USA).

2-amino-2-methyl-1-propanol (MAP), was obtained from E. Merck (Darmstadt, West Germany). Other chemicals were obtained from commercial sources.

## METHODS

### Extraction and Purification Procedures

Male Sprague-Dawley rats were killed by decapitation. The brain was rapidly removed, weighed and homogenized in ice cold 1 M perchloric acid (1:5, w/v), at 0-4°C, using a glass-teflon homogenizer. The intervals between killing and homogenization were always controlled and kept constant. In some experiments rats were killed by direct immersion in liquid nitrogen.

0.5-2.0 g of tissue samples were used for the extraction. The homogenate was centrifuged at 15.000 x g for 10 min, and the clear supernatant removed. The pel-

let was again extracted with 5 ml of 1 M perchloric acid (PCA). The combined extracts were diluted with distilled water (1:1, v/v).

This solution was passed through a small alumina column (1 cm i.d. x 3 cm) previously equilibrated with 0.5 M PCA. The column was washed with 5 ml of 0.5 M PCA and then with 20 ml of distilled water. The water-soluble intermediates were finally eluted from the washed alumina with 20 ml of 1 M ammonia solution. The collected ammonia-eluate was diluted (1:1) with distilled water and then adsorbed, at 90-120 ml/h, onto a Dowex 1-x 4 column (1 cm i.d. x 6 cm), formate form, previously equilibrated with water. After washing with 30 ml of distilled water, the elution of the water-soluble intermediates was carried out with 40 ml of 0.1 M formic acid. The formic acid eluate was concentrated to dryness under vacuum, at 40°C, on a rotary evaporator. The residue was dissolved in 2-3 ml of 0.1 M MAP, 0.02 M NaCl, pH 10.3 ± 0.02 buffer (Buffer A). 0.1-0.5 ml of the reconstituted extract was used for the simultaneous determination by HPLC of the water-soluble intermediates of the phosphoglyceride synthesis.

In other experiments the extraction was carried out essentially as described by Sundler (17). The brain was homogenized in 20 volumes of chloroform-methanol (2:1, v/v) and extracted for 1 h at room temperature. The lipid extract was then equilibrated with 0.2 volume of water and the phases separated by centrifugation. The upper-phase was transferred into a flask and the

organic phase washed with 0.1 volume of methanol-HCl (1:1, v/v) and with 0.1 volume of methanol-1% NaCl (1:1, v/v). The washings were pooled with the first upper-phase. The lipid-free insoluble residue was extracted twice with 5 volumes of 1 M PCA. The PCA extract was added to the pooled aqueous-methanolic washings, thus constituting the total extract of the water-soluble intermediates, which was centrifuged at 15,000 x g and processed as described above. This extraction procedure is used if the analysis of lipids in the same tissue sample is also required.

The two extraction procedures reported above give identical results as far as the water-soluble intermediates are concerned, and may be used for the extraction of the hydrosoluble intermediates from other tissues.



High Performance Liquid Chromatographic Analysis

a) Apparatus. The analytical procedure was carried out by using a PYE-UNICAM Model LC 20 liquid chromatograph, equipped with an UV detector operating at 280 nm, and with a flow cell of 1 mm in diameter and a path length of 10 mm. The detector output was connected to a Philips Model PM 8220 recorder, with a chart-speed of 30 cm/h. A thick walled glass column (0.4 cm i.d. x 30 cm), jacketed for temperature control and packed with Aminex A-14 resin, was used for the chromatographic separations.

b) Column Preparation. Aminex A-14, $Cl^-$, was suspended in 3 M HCl and then treated as previously described (15). The final height of the resin bed was 23 cm. The column was washed for 5 min with 0.3 M HCl at 1.2 ml/min,

and then conditioned for 15 min with Buffer A, at the same flow rate.

c) Elution Buffers. Buffer A, used for the column equilibration and for the first chromatographic step, was prepared by adjusting a 0.1 M MAP, 0.02 M NaCl solution to pH 10.3 ± 0.02 with 3 M HCl. The second eluent (Buffer B) was 0.1 M MAP, 0.17 M NaCl solution, pH 11. The buffers were both degassed under vacuum at 50°C and their pH rechecked.

d) Chromatographic Elution. The sample (e.g. purified tissue extract or calibration mixture) was adsorbed onto the column under nitrogen pressure. The elution was performed at 50°C with Buffer A for the first 5 min and then with Buffer B until the end of the run. The flow rate was kept at 1.2 ml/min and 1.2 ml fractions were collected.

Prior to a new analysis, the column was regenerated for 5 min with 0.3 M HCl and then equilibrated for 15 min with Buffer A.

e) Detection, Identification and Quantitation of the Chromatographic Peaks. In routine analyses, cytidine-containing compounds were detected by their UV absorption at 280 nm, while PC and PE were detected by measuring the phosphorus content of the chromatographic fractions.

The eluted compounds were identified by their respective retention times determined by calibration runs. In the preliminary experiments the retention times of PC and PE were obtained by using radioactive PC

and PE, and counting the radioactivity in small amounts of the eluted fractions.

The water-soluble intermediates present in the biological extracts were identified by comparing their retention times with those of standards, or by chromatographic co-elution experiments with radioactive compounds.

The quantitation of cytidine-containing compounds was carried out by using the chromatographic procedure of peak area measurements, while the quantitation of PC and PE peaks by phosphorus determination in the chromatographic fractions. The concentration of the water-soluble intermediates in the sample analysis was determined from their relative calibration factors, obtained by chromatographying known amounts of synthetic compounds, processed as the biological extracts.

f) Chromatographic Calibration Mixture. Separate standard solutions of PC, PE, CDP-choline, CDP-ethanolamine and 5'-CMP, containing 2,5 mg/ml of each compound in 0.01 M HCl, were prepared. The concentrations of the cytidine derivatives were determined by their absorbance at 280 nm (16), while those of PC and PE by phosphorus determination (4). Aliquots of these solutions, containing 40-80 nmoles of CDP-choline, CDP-ethanolamine and 5'-CMP, and 400-800 nmoles of PC and PE, were mixed and, after their dilution to 10 ml with 0.5 M PCA, were processed as mentioned above for the biological extracts. Aliquots of the solution, obtained after the treatment of the mixture on alumina and Dowex 1, were used for the chromatographic calibration.

## RESULTS AND DISCUSSION

### Chromatographic Resolution

A separation of the synthetic water-soluble intermediates of the phosphoglyceride biosynthesis is shown in Figure 1. The peaks of PC and PE, as obtained from

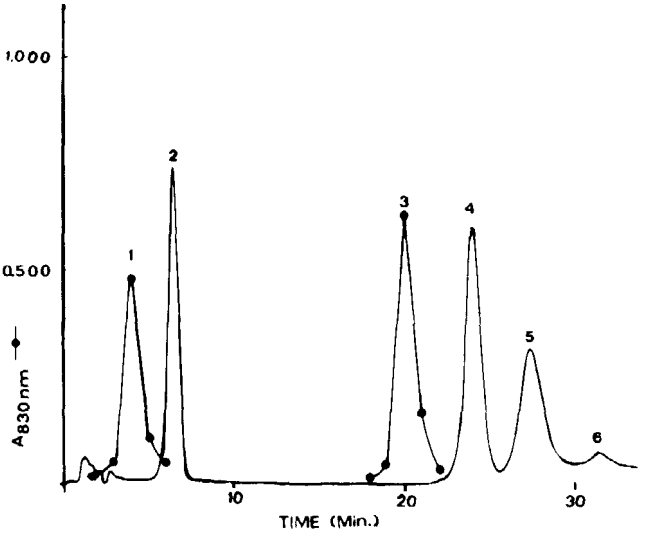


Figure 1. HPLC of the synthetic water-soluble intermediates of the phosphoglyceride biosynthesis. The synthetic calibration mixture was processed as described for the brain extract. The elution was carried out for 5 min with Buffer A and then with Buffer B until the end. Flow rate: 1.2 ml/min; temperature: 50°; column: Aminex A-14; detection: absorbance at 280 nm (—) and at 830 nm (-●-). The overall chromatographic profile was obtained by drawing the phosphorus peaks of PC and PE on the UV chromatogram of the cytidine-containing compounds, which was recorded at 0.160 aufs.
1. PC (100.4 nmoles); 2. CDP-choline (17.5 nmoles); 3. PE (122.5 nmoles); 4. 5'-CMP (25.3 nmoles); 5. CDP-ethanolamine (22.5 nmoles); 6. Unknown.

the phosphorus content of the chromatographic fractions, were drawn on the chromatogram of the recorded UV-absorbance of CDP-choline, CDP-ethanolamine and 5'-CMP. The separation of these compounds is complete, and the chromatographic process may be used for an accurate quantitation of the eluted substances.

Overall recoveries of CDP-choline, CDP-ethanolamine, PC and PE are 78 ± 4.1; 79 ± 3.0; 82 ± 4.2; and 80 ± 3.1, respectively, when compared to the unprocessed standards or to the brain samples spiked with radioactive water-soluble precursors.

Determination of the Water-Soluble Precursor Pool

The quantitative determination by HPLC of the water-soluble intermediates of choline and ethanolamine phosphoglyceride synthesis is complicated by the presence in the tissue extract of several interfering compounds. The direct chromatography of the unpurified extract after the removal of PCA, in the form of potassium salt, is not suitable for the precursor quantitation. Previous experiments have shown that, under our chromatographic conditions, the compounds which co-elute, or interfere with the intermediates that are to be quantitatively determined, are the purine and pyrimidine bases and the nucleosides. This circumstance has induced us to look for a procedure that could give a tissue extract free from such components.

Since it has been reported that alumina adsorption chromatography has been successfully used for the separation of phosphorylated compounds from bases and nucleosides (17), we have resorted to an aluminum oxide

column for a preliminary clean-up of the brain extract. The reliability of this resolution was checked by running on an alumina column a 0.5 M PCA solution of nucleosides, purine and pyrimidine bases, and labelled water-soluble precursors of the phosphoglyceride biosynthesis. The elution pattern, referred to this experiment, is shown in Figure 2. More than 90% of the

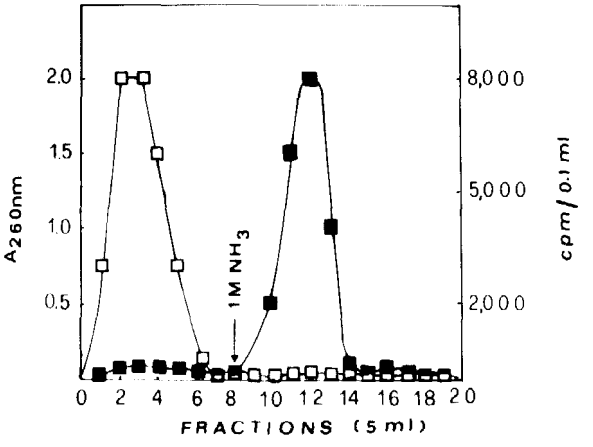


Figure 2. Elution of bases, nucleosides and labelled water-soluble intermediates of the phosphoglyceride biosynthesis from neutral aluminum oxide. A mixture containing 1 µmole of adenine, guanine, uracil, cytosine and their respective nucleosides, and 75,800 cpm [$^{14}$C]-phosphorylcholine, 36,300 cpm [$^{14}$C]-phosphorylethanolamine, 81,250 cpm [$^{14}$C]-CDP-choline, 35,700 cpm [$^{14}$C]-CDP-ethanolamine, in 10 ml of 0.5 M PCA, was applied to a 1 x 3 cm alumina column, equilibrated with 0.5 M PCA. After washing the column with 0.5 M PCA and water, the elution was carried out with 1 M ammonia solution. UV absorbance at 260 nm and radioactivity measurements were carried out on each fraction (5 ml).

initial O.D. and only traces of radioactivity are detectable in the peak obtained by washing the column with 0.5 M PCA and water. On the other hand, almost all of the radioactivity (usually 90%) was recovered within the ammonia eluate. The results obtained show that the phosphorylated compounds, PC, PE, CDP-choline and CDP-ethanolamine, are well separated from free bases and nucleosides. For this reason, the alumina adsorption chromatography can be used to obtain the desired phosphorylated water-soluble precursors free from most of the undesired metabolites. However, the inorganic phosphate is still present in this fraction. In order to avoid its interference with the HPLC analysis ($P_i$ interferes with the PE quantitation), a further purification step is essential. For this purpose, a Dowex 1 chromatography proved to be useful. It must also be pointed out that the anion exchange chromatography step yields the water-soluble precursors in a diluted formic acid solution, which can be evaporated to dryness without any chemical alteration of the solutes.



After these two preliminary chromatographic steps, the sample is suitable for the HPLC analysis.

Figure 3 shows a typical chromatogram of an aliquot of a purified rat brain extract, corresponding to 0.2 g of fresh tissue. Under our experimental conditions, wet tissue amounts of this size are sufficient for accurate determinations. Thus, the sensitivity of this procedure allows the use of tissue amounts smal-

ler than those required by the assay methods previously reported (10-14).

As can be observed from Figure 3, the peaks of the cytidine-containing intermediates are well separated among themselves and from the other interfering components. The contaminating compounds (e.g. nucleotides), present in the extract at concentrations higher than those of the water-soluble intermediates, are eluted after the CDP-ethanolamine peak. At this point, the chromatographic run was stopped and the compounds, still retained on the column, were eluted with 0.3 M HCl. The inspection of the chromatogram



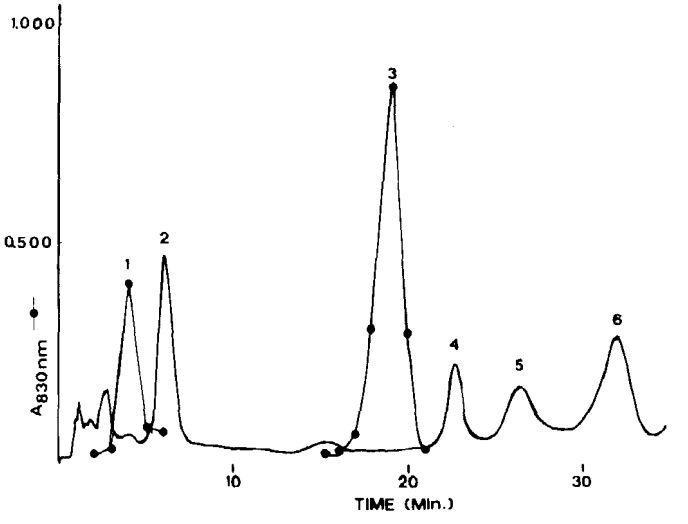


Figure 3. Chromatogram of a purified PCA extract of the rat brain. The amount passed through the column corresponds to 0.2 g of fresh tissue. For the experimental conditions see Figure 1.
1. PC; 2. CDP-choline; 3. PE; 4. 5'-CMP; 5. CDP-ethanolamine; 6. Unknown.

shows that CDP-choline, CDP-ethanolamine and 5'-CMP are eluted as sharp and symmetrical peaks, and no important secondary peaks are present close to them.

Data on the rat brain pool size of the water-soluble intermediates of the phosphoglyceride biosynthesis are reported in Table 1.

The amounts of phosphoryl- and CDP- compounds of choline and ethanolamine in the brain of the rats killed by decapitation (column A of Table 1) agree well with those previously reported (18,19), but they are remarkably higher than those observed by killing the rats by immersion in liquid nitrogen. This difference



TABLE 1

Pool Size of the Water-Soluble Intermediates in the Rat Brain (nmoles/g wet weight)

| Compound | A Decapitation | B Immersion in Liquid Nitrogen |
|---|---|---|
| PC | 280.2 ± 16.8 | 175.7 ± 14.3 |
| CDP-choline | 42.3 ± 5.8 | 11.3 ± 2.4 |
| PE | 880.1 ± 89.3 | 630.5 ± 85.3 |
| CDP-ethanolamine | 31.0 ± 6.2 | 26.8 ± 4.1 |

Results are expressed as mean ± S.D. from nine (A) and six (B) rats.

is more evident in the case of the choline-containing precursors. The obvious explanation for these quantitative differences is that, in the time interval between the decapitation and the homogenization (3 min), the amounts of the water-soluble precursors increase, due to the activity of the enzymes of the phospolipid catabolism. On the contrary, immersing the rat in liquid nitrogen produces inactivation of the enzymes at the time of the sacrifice, and the determined amounts of the metabolites are of the same magnitude as the physiological ones.



## CONCLUSIONS

The aim of this work was to look for a procedure for a rapid and precise determination of the water-soluble precursors of the choline and ethanolamine phosphoglycerides synthesis.

Previous determinations of the water-soluble precursors required very long manipulations of the samples, prior to the quantitation step. In our case, only two main steps proved to be necessary in order to obtain a purified sample suitable for a precise quantitation by HPLC. These steps, namely aluminum oxide adsorption and ion exchange chromatography on Dowex 1, are easy and rapid to carry out and do not require any sophisticated procedure. The alumina column separates the components of the acid soluble pool in two fractions; the first contains free bases, nucleoside and other neutral or cationic compounds, as ethanolamine and choline, while the phosphorylated metabolites are recovered in

the fraction eluted with ammonia. Inorganic phosphate and many nucleotides are removed, from this fraction, by Dowex 1 chromatography. Subsequently HPLC operates the simultaneous separation and the partially automated quantitation of the metabolites investigated. The chromatographic resolution is achieved under conditions where the analytes are anionic enough to be retained by the exchanger and then they are separated by an ion-exchange mechanism, under a step-wise elution system. The complete separation of the water-soluble precursors could not be obtained by isocratic elution, since the chromatographic behaviour of the two couples PC/CDP-choline and PE/CDP-ethanolamine is quite different, owing to the quaternary nitrogen of the choline-derivatives. Therefore, the elution of PC and CDP-choline can be achieved at low ionic strength, while a buffer of higher pH and ionic strength must be used in order to obtain a rapid separation between the ethanolamine-containing intermediates and 5'-CMP. However good separation of CDP-choline, PE, CDP-ethanolamine and 5'-CMP can be obtained isocratically by elution with 0.1 M MAP, 0.05 M NaCl, pH 9.5 buffer, but an overlap between PC and CDP-choline is observed (unpublished observations).



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