Journal of Chromatography, 206 (1981) 327–332
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,429

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION OF PHOSPHOLIPID PRECURSORS AND THEIR DIRECT MEASUREMENT BY AUTOMATIC PHOSPHORUS ANALYSIS

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(Received October 15th, 1980)

SUMMARY

In this report we have described an improved method for the analysis of phosphorylated metabolic intermediates of the phospholipid synthesis or breakdown in biological samples. The application of high-performance liquid chromatography enables the separation of the most common metabolites within less than 1.5 h, and the direct connection to the automatic phosphorus analyzer makes a simultaneous quantitation possible. The method is currently being applied to studies of metabolic differences in animal and human tissues.

INTRODUCTION

Anion-exchange chromatography as applied to the analysis of the total glycerophosphatides in animal tissues was introduced by Hubscher and Hawthorne[1], who studied the water-soluble phosphate esters released from phospholipids by mild alkaline hydrolysis. An ammonium formate gradient was used to elute the phosphate esters from a Dowex 1-X2 column and quantitation was obtained by phosphorus determination[2]. Subsequently, related methods were developed including that of Lairon *et al.*[3], who applied a high-performance liquid chromatographic (HPLC) method to separate the phosphorus-containing compounds which can be derived from lecithin by hydrolysis, *i.e.* glyceryl phosphorylcholine (GPC), glyceryl phosphate (GP), phosphorylcholine (PC) and orthophosphate (P_i).

Some of the hydrolysis products that have been identified are involved in phospholipid biosynthesis in living tissues and thus the basic method can be applied to studies of lipid metabolism. Separation and direct quantitation of two groups of phospholipid precursors from rabbit and rat heart tissue was described by Geiger and

0021-9673/81/0000–0000/$02.50

Roberts[4], and their method was applied by Warden *et al.*[5] to studies with quiescent and serum-stimulated mouse fibroblasts.

Determination of the phospholipid precursors may be used to reveal or study some diseases characterized by metabolic errors in the phospholipid synthesis as found, for instance, in connection with certain kidney diseases[6]. Moreover, these precursors may have some importance as a measure of the level of natural lipid hydrolysis and turnover, and therefore reflect changes which occur in food and food products during storage.

In this report we describe the analysis of individual phospholipid precursors in biological samples. The method includes an HPLC separation of the precursors and direct monitoring of the eluate by the use of the Bessman phosphorus analyzer[7].

EXPERIMENTAL

All reagents were of A.C.S. or equal grade. In addition, the authentic standard compounds GPC, PC, glyceryl phosphoryl ethanolamine (GPE), phosphoryl ethanolamine (PE) and cytidine diphosphate choline (CDP-choline) were purchased from Sigma (St. Louis, MO, U.S.A.), and the anion-exchange resin AG MP-1 (200–400 mesh) from Bio-Rad Labs. (Richmond, CA, U.S.A.).

Depending on the elution system to be used, the resin was slurried in 1 *N* hydrochloric acid or 1 *N* acetic acid and washed with glass-distilled water, then washed with 1 *N* sodium hydroxide and again with water. The procedure was repeated and the final washing was made with 1 *N* acid (HCl or CH_3COOH) in which the resin was stored as a 1:1 (v/v) slurry after degassing.

Columns (15 × 0.3 cm I.D.; Altex, Berkeley, CA, U.S.A.) were partially filled with glass-distilled water, then packed by successive additions of small amounts of prewashed resin slurry. Between additions water was pumped through the column at a maximum pressure of 200 p.s.i. A similar column, partially filled with resin (*ca.* 10 cm), was used as a precolumn for sample loading. After packing the column was washed by pumping 10 m*M* ammonium tetraborate adjusted to pH 10.0 with ammonium hydroxide or 50 m*M* acetic acid adjusted to pH 10.0 with ammonium hydroxide, depending on the further elution system.

Individual standard compounds or standard mixtures were prepared in 0.5 m*M* ammonium tetraborate. The biological samples of chicken embryo retina and neonatal rat lung tissue were prepared by the method of Khym[8].

Samples (50–200 μl) were introduced on the top of the resin by means of a precision syringe (Hamilton, Reno, NV, U.S.A.) and then the sample was drawn into the resin by vacuum. A small volume of elution solvent was then used to rinse the sample residues from the column walls into the resin. When the resin was used in Cl^- form a linear gradient elution was applied (elution system I). The gradient was produced by connecting two similar chambers with 20 ml of solution in each chamber. The solvent was pumped at a flow-rate of 0.3 ml/min from the mixing chamber containing a 10 m*M* ammonium tetraborate solution adjusted to pH 10.0 with ammonium hydroxide into which a 50 m*M* ammonium tetraborate–100 m*M* ammonium chloride solution at pH 9.0 (adjusted with ammonium hydroxide) flowed. If the resin was used in the CH_3COO^- form, the elution system II was applied. In this case the elution was performed with 50 m*M* acetic acid adjusted to pH 10.0 with ammonium

hydroxide until the GPC peak was recorded on the chromatogram (elution time *ca.* 22 min), then the elution was continued with 50 m*M* acetic acid at pH 5.0 (adjusted with ammonium hydroxide) until all the other precursors were eluted from the column.

The phosphorus content of the eluate was monitored continuously by the Bessman phosphorus analyzer (Alsab Scientific, Los Angeles, CA, U.S.A.) as described by Geiger *et al.*[9].

Identification of the peaks on the chromatogram was made according to the retention times of the authentic standards and co-chromatography with the biological samples.

RESULTS AND DISCUSSION

In preliminary studies[10] the AG MP-1 resin was found to be comparable with the mixed resin which was earlier used in our laboratory for the separation of acid-soluble phosphorylated metabolic intermediates[11]. When the mixed resin was used, the glycerol-containing phospholipid precursors, GPC and GPE, were eluted as one group almost within the void volume of the column and the other two identified precursors, PC and PE, were eluted as another group preceding creatine phosphate (CP)[4]. The elution order is somewhat different when using AG MP-1 and the elution systems I or II. The ammonium tetraborate–chloride gradient (elution system I) elutes GPC from the column within the void volume, followed by PC, whereas GPE is eluted later and closer to PE (Fig. 1). In this system CDP-choline is eluted between PE and CP. Although GPC and PC as well as CDP-choline and CP are eluted as well-defined symmetric peaks, both GPE and PE appear as somewhat broad and tailing peaks.

One major advantage of the elution system I is that the final composition of the gradient is the same used to initiate the routine analysis of phosphorylated intermediates[9]. Thus it is possible to continue the elution in order to determine the nucleotide phosphates, ATP, ADP, AMP and CTP, CDP and CMP, which all are central intermediates in phospholipid metabolism. This has already been demonstrated by the preliminary experiments. Some difficulties were, however, encountered when determining sugar phosphates from the same run. Because of the poor stability of sugar phosphates in alkaline solutions used to initiate the elution, increased amounts of P_i and decreased amounts of sugar phosphates were found. This problem is not related to our interest in lipid metabolism, and remains to be studied in detail later.

The tailing effect and the partial overlapping of the GPE and PE peaks interfere with accurate quantitation and therefore another chromatographic system based on the use of AG MP-1 in acetate form was developed (elution system II). A formic acid–ammonium formate system has been widely used in ion-exchange chromatography, apparently because of the high volatility of formic acid which makes it easier to concentrate the collected fractions. A formic acid system was applied also by Hubscher and Hawthorne[1] for the first separation of the hydrolysis products from phospholipids by ion-exchange chromatography. Wells and Dittmer[12] used an ammonium formate gradient at pH 9.5 to obtain good separation of glycerol-containing hydrolysis products of phospholipids, but did not include PC, PE or CDP-choline in their study. By using Dowex 1-X4 (Cl^-) and an ammonium formate gradient at pH 8.5, Lairon *et al.*[3] separated GPC, PC and GP from each other, and the continuation

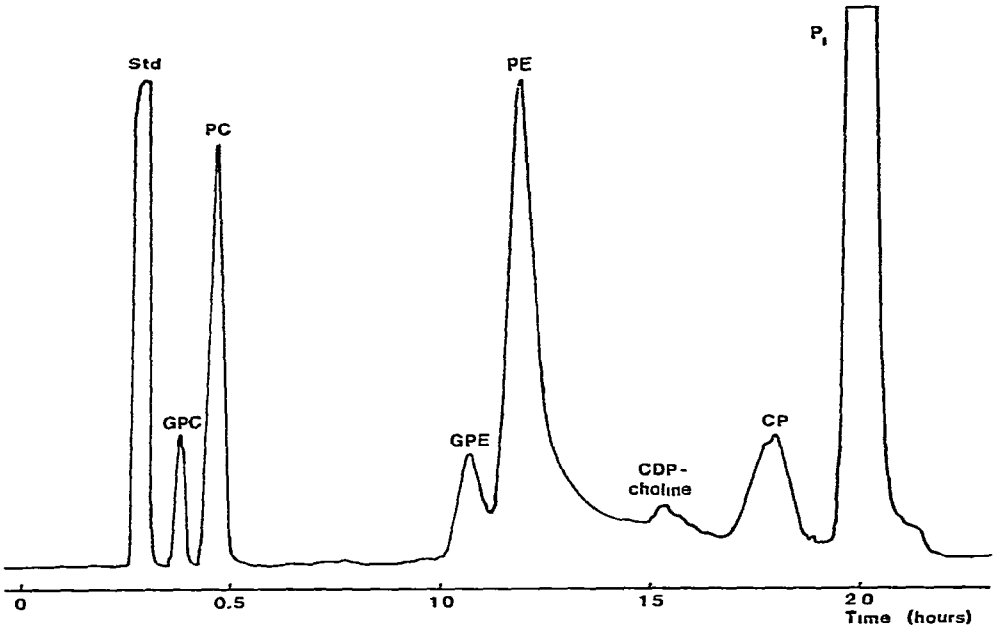

Fig. 1. Separation of phospholipid precursors in a rat lung tissue sample using anion-exchange chromatography in combination with an automatic phosphorus analyzer. An external standard (Std), 20 nmol P_i, at the beginning of the run is used for quantitations. Chromatographic conditions are described in the text.

of the elution with a high concentration of HCl released P_i from the resin. They did not study the migration of ethanolamine-containing phosphate esters in this system.

The use of formic acid in the elution mixture is undesirable because of its irritant effect. The high volatility may also interfere with the chromatographic analysis. Furthermore, relatively high concentrations are required for elution. For these reasons we studied the use of acetate instead of formate for the separation of the phospholipid precursors. Various acetate concentrations at different pH values as well as different pH gradients were studied to elute the precursors from the AG MP-1 resin used in the CH_3COO^- form. A simple two-step method applying 50 m*M* acetate at two different pH values, 10.0 and 5.0, was found the most suitable elution system (Fig. 2).

Although Lairon *et al.*[3] used an HPLC method, the separation of the phospholipid components in one sample took more than 4.5 h and the subsequent quantitation of the collected fractions (the total volume of 275 ml) required additional time. With the methods developed in our laboratory the same separation including the quantitation can be accomplished within 1.5 h and the total volume of the eluate is *ca.* 30 ml.

When comparing our two methods we found that the peaks eluted with acetate (Fig. 2) are sharper and therefore easier to quantitate. Moreover, GPE is eluted in this system closer to the origin and well before the PE peak. A disadvantage is the overlapping of GPE and CDP-choline, which were well separated in elution system I.

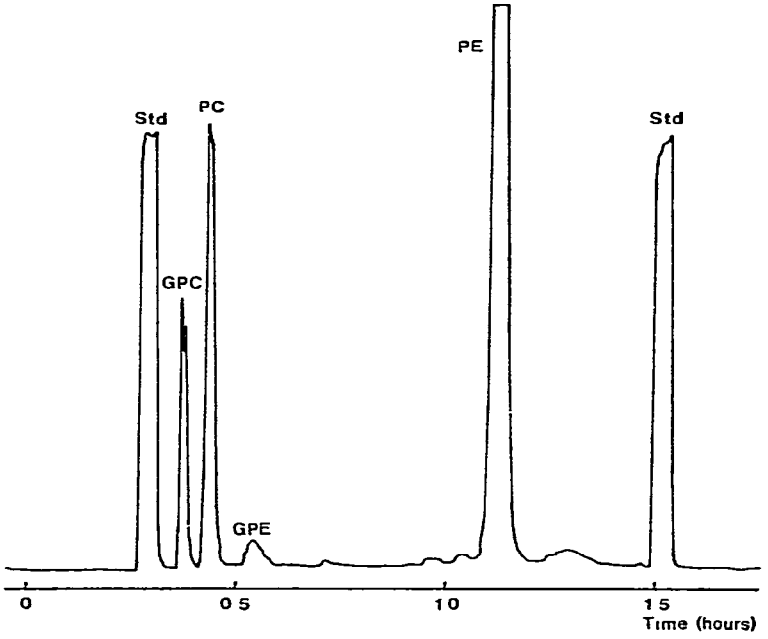

Fig. 2 Separation of phospholipid precursors in a chicken embryo retina sample using anion-exchange chromatography in combination with an automatic phosphorus analyzer. External standards (Std), 20 nmol P_i, are used for quantitations. Chromatographic conditions are described in the text.

The presence of CDP-choline is easy to study by the system I, and thus the quantitative determination of all the components of interest can be achieved.

A chicken embryo retina sample was applied in order to study the reproducibility of the acetate method with a biological sample. The four phospholipid precursors, GPC, PC, GPE and PE, were quantitated from five successive analyses of the sample. The relative standard deviations were 13.5, 4.6, 7.3 and 2.7%, respectively. The high standard deviation for GPC can be explained, at least in part, by its very low affinity for the resin. If the sample contains any acid-soluble phosphorus-containing impurities with no affinity for AG MP-1, they will interfere with the GPC determination. The standard deviations for PC, GPE and PE indicate the good reproducibility of the method.

ACKNOWLEDGEMENTS

The generosity of Dr. Carmel M. Roberts, USC School of Medicine, Los Angeles, and Dr. Sarah Ben-Or, Hadassah Medical School, Jerusalem, for donating the rat lung and chicken retina samples, respectively, is gratefully acknowledged.

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