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Note

High-performance liquid chromatography in the analysis of the products of phospholipase A hydrolysis of phosphatidylcholine

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In the structural analysis of phosphatidylcholines by means of phospholipase A hydrolysis, it is necessary to isolate free (unesterified) fatty acids released from position *sn*-2 and lysophosphatidylcholine containing the fatty acids of position *sn*-1, free from any residual unhydrolysed phosphatidylcholine, for gas chromatographic analysis of their fatty acid constituents. Thin-layer chromatographic (TLC) methods have generally been used for the purpose (reviewed by Christie[1]). However, high-performance liquid chromatography (HPLC) is increasingly being applied to the analysis of phospholipids[1,2], and can offer advantages in terms of speed, resolution, economy of solvents and cleanliness. An HPLC method is here described for the isolation of phosphatidylcholine hydrolysis products.

EXPERIMENTAL

All solvents were Analar or HPLC grade and were supplied by Fisons (Loughborough, U.K.). *Ophiophagus hannah* snake venom, egg lecithin and synthetic L-α-dipalmitoylphosphatidylcholine were supplied by Sigma (Poole, U.K.).

Pure phosphatidylcholine was prepared from commercial egg lecithin by preparative TLC on silica gel G layers (0.5 mm thick); chloroform–methanol–water (25:10:0.75, by vol.) was the developing solvent. Bands were visualized under UV light after spraying with 0.1% (w/v) 2′,7′-dichlorofluorescein in 95% methanol; the required compound was eluted from the adsorbent with chloroform–methanol–water (5:5:1, by vol.). Phospholipase A hydrolysis was carried out with *O. hannah* venom by the method of Robertson and Lands[3]. The products were separated by TLC with the solvent system described above, or by HPLC.

A model 8770 isocratic HPLC pump (Spectra-Physics, St. Albans, U.K.) equipped with a Knauer differential refractometer (Dr. H. Knauer, Oberursel/Taunus, F.R.G.) was used. The column consisted of a 5 cm × 5 mm guard column and a 25 cm × 5 mm main column packed with Hypersil 5 μm (HPLC Associates, Macclesfield, U.K.). The optimum solvent was found to be acetonitrile–methanol–water (50:45:6.5, by vol.) at 1 ml/min; helium degassing was used. The hydrolysis products from 0.2 to 2 mg phosphatidylcholine could be separated for collection in a single run.

Bands on TLC adsorbents were methylated without prior elution with 3%

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sulphuric acid in methanol[1]. Fractions emerging from the HPLC detector were collected, and after evaporation of solvent were methylated with 1% sulphuric acid in methanol. Methyl esters were subjected to gas chromatographic analysis on a 25-m fused-silica capillary column coated with Silar 5CP (Chrompack U.K., London, U.K.) in a Carlo Erba Model 4130 gas chromatograph (Carlo Erba U.K., Swindon, U.K.) and were quantified by electronic integration.

RESULTS AND DISCUSSION

The procedures developed for the HPLC separation of phospholipids on silica columns have of necessity utilised solvent systems that were compatible with the detection system available, rather than being the optimum for the desired separation. For example, hexane–isopropanol–water mixtures have been widely used as they are suitable for UV detection in the 200–210 nm range[4,5]. In our hands, excellent separations of phosphatidylcholine and lysophosphatidylcholine were obtained with such systems, but it was necessary to add dilute acid or a buffer of pH 6.0 to elute the free fatty acid as a sharp peak. Others have found it necessary to add salts or acids to improve the resolution of the phospholipids with these solvents[6–9].

With refractive-index detection, there is little limitation on the range of solvents that can be used, provided that the required separation can be achieved with an

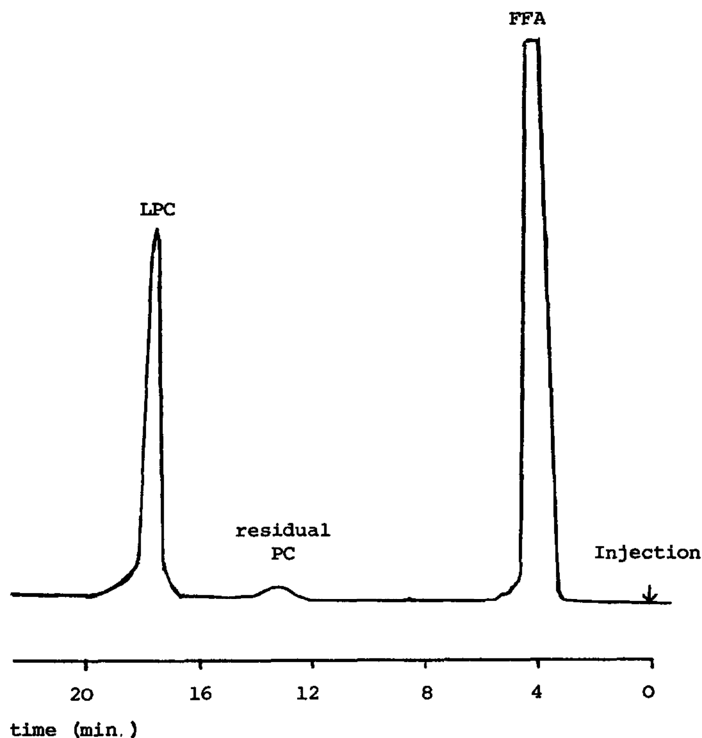

Fig. 1. Separation of the products of phospholipase A hydrolysis of L-α-dipalmitoylphosphatidylcholine by HPLC with refractive index detection. For conditions, see Experimental section. FFA = free fatty acids; PC = phosphatidylcholine; LPC = lysophosphatidylcholine.

isocratic system. This is in any case a desirable objective for simplicity of operation. Acetonitrile–methanol–water mixtures have been used for separating phospholipid classes by several research groups[10–13]. With these solvents in the proportions 50:45:6.5 (by vol.) respectively, excellent resolution of all the compounds of interest was obtained. Fig. 1 illustrates an application to the products of phospholipase A hydrolysis of synthetic L-α-dipalmitoylphosphatidylcholine. Each component is easily collected as it emerges. It is not necessary to add to this solvent any acid or inorganic salts, which might interfere with subsequent transesterification for gas chromatographic analysis, or which might have deleterious effects on seals in the HPLC pump in the long term.

A comparison of the results of a phospholipase A hydrolysis of commercial egg phosphatidylcholine with separation of the products by either TLC or HPLC is shown in Table I. Little difference is apparent between the two sets of results.

TABLE I

FATTY ACID COMPOSITIONS (mol.-% OF THE TOTAL) OF THE PRODUCTS OF PHOSPHOLIPASE A HYDROLYSIS OF PHOSPHATIDYLCHOLINE SEPARATED BY HPLC OR BY TLC

Results are means of duplicate experiments.

Fatty acid	*HPLC separation*		*TLC separation*	
	Position 1	*Position 2*	*Position 1*	*Position 2*
14:0	0.8	0.4	0.7	0.5
16:0	62.7	5.8	63.6	5.5
16:1	0.4	1.7	0.5	1.6
18:0	28.1	3.2	27.2	2.5
18:1	7.7	46.0	7.4	46.7
18:2	0.3	29.7	0.6	29.0
20:3 (*n*-6)		0.2		0.4
20:3 (*n*-3)		0.5		0.8
20:4 (*n*-6)		9.5		10.3
22:5 (*n*-3)		1.2		1.8
22:6 (*n*-3)		1.8		0.9

This HPLC separation method has also been used in the final step of a procedure for the stereospecific analysis of triacylglycerols, in which these compounds are first hydrolysed to diacylglycerols for conversion to phosphatidylcholines which are in turn hydrolysed with phospholipase A. The products are free fatty acids from position *sn*-2, lysophosphatidylcholine containing the fatty acids of position *sn*-1 and unhydrolysed 2,3-*sn*-diacylglycerophosphorylcholine. Fig. 2 illustrates an application of this method to the final stage in the stereospecific analysis of rabbit plasma triacylglycerols. Some broadening of the peaks is apparent because of the range of different fatty acids present.

Some excellent HPLC separations of phospholipids have been achieved on columns contained bonded amine groups[14–16]. We obtained particularly good separations of phosphatidylcholine and lysophosphatidylcholine with a column packed with Nucleosil-5NH_2 with tetrahydrofuran–methanol–water (7:5:5:1, by vol.) as eluting solvent. However, it was necessary to add on ethylamine–formic acid buffer (pH

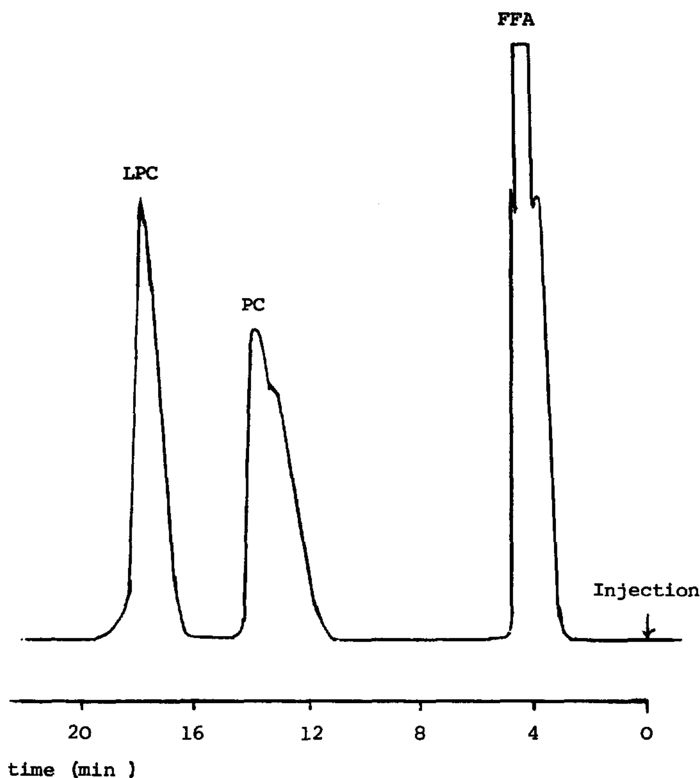

Fig. 2. Separation by HPLC with refractive index detection of the products of phospholipase A hydrolysis as the last step in the stereospecific analysis of rabbit plasma triacylglycerols. FFA = free fatty acids; PC = 2,3-*sn*-diacylglycerophosphorylcholine; LPC = lysophosphatidylcholine. For conditions, see Experimental section.

6.0, 0.5 *M*) as the aqueous component to elute free fatty acids from this column.

The HPLC procedure using the silica column described above is more rapid than TLC (20 min *versus* 2 h) and the products emerge cleanly in relatively small volumes of solvent which are easily removed by evaporation. No contamination with small amounts of silica gel or binder is possible, nor are dyes needed to visualise the components. This last point may be particularly important where the components are required for liquid scintillation counting, for example[17,18]. In addition, the making and use of TLC plates inevitably generates silica gel dust in the laboratory; the introduction of methods which avoid this is desirable.

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