Journal of Chromatography, 276 (1983) 29—35
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 1715

ANALYSIS OF PHOSPHOLIPIDS IN HUMAN SEMEN BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

H.P. NISSEN* and H.W. KREYSEL

Department of Dermatology, Rheinische Friedrich-Wilhelms-University, D-5300 Bonn 1, Venusberg (G.F.R.)

(First received December 6th, 1982; revised manuscript received March 18th, 1983)

SUMMARY

A sensitive method for the separation of phospholipids by a high-performance liquid chromatographic procedure is described. The chromatographic separation was achieved on a 25-cm column packed with Bio-Sil HP-10 coupled with a pre-column packed with Si-100 Polyol. Phosphatidylinositol, phosphatidylglycerol, cardiolipin, phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine, sphingomyelin, lysophosphatidylethanolamine and lysophosphatidylcholine were completely separated and quantitated. The eluted phospholipids were monitored at 203 nm. The method was shown to be applicable to the analysis of phospholipids from human semen.

INTRODUCTION

Lipids are major constituents of all plant and animal tissues. Among several other lipid classes the phospholipids are of physiological importance, as they are components of biologically active membranes. In studies of lipid metabolism by human spermatozoa the phospholipids have to be extracted, separated and analysed [1].

Previous determinations of phospholipids were based on quantitative thin-layer chromatography (TLC). Multi-component mixtures from biological material have required development in two dimensions to achieve satisfactory resolution [2, 3]. High-performance liquid chromatography (HPLC) combines the separation capability of TLC with several advantages, with reductions in sample preparation, clean-up steps and time. HPLC methods using silica gel columns to separate a variety of phospholipids have been reported [4—6].

The method described here is a modification of earlier HPLC methods for the determination of the lecithin/sphingomyelin ratio in amniotic fluids [7, 8],

0378-4347/83/$03.00

and has been used to analyse phospholipids from human seminal plasma and spermatozoa.

EXPERIMENTAL

HPLC was performed with a Beckman (Munich, G.F.R.) Model 334 instrument. The chromatographic column (250 × 4 mm I.D.) was pre-packed with Bio-Sil HP-10 (10 μm) supplied by Bio-Rad (Munich, G.F.R.). The guard column (75 × 4.6 mm) was pre-packed with Si-100 Polyol, 30 μm (Serva, Heidelberg, G.F.R.) or Vydac-101 S I, 30—40 μm (Macherey, Nagel & Co., Düren, G.F.R.). Detection was effected with a Biotronik BT 3030 variable-wavelength UV detector (190—350 nm) (Biotronik, Frankfurt, G.F.R.) coupled to a Shimadzu C-R 1 A data processor (Shimadzu, Kyoto, Japan). The solvents were HPLC-grade acetonitrile and purified water. The phospholipids were obtained from Sigma (Munich, G.F.R.) and Applied Science Europe (Heidelberg, G.F.R.). Silica gel 60 F-254 thin-layer plates were purchased from Merck (Darmstadt, G.F.R.).

The chromatographic analysis was performed at ambient temperature. Mobile phase component A was acetonitrile—water (80:20) and component B was acetonitrile. The solvents were degassed prior to use. The chromatographic system was programmed for gradient elution using these two mobile phases described. A linear solvent gradient from 87.5 to 25% B between 3 and 15 min was used in all instances, delivering a gradient of water running from 2.5 to 15% water. The flow-rate was held constant at 1 ml/min. The effluent was monitored at 203 nm.

Standard solutions of phosphatidylinositol (PI), phosphatidylglycerol (PG), cardiolipin (CL), phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingomyelin (S), lysophosphatidylethanolamine (LE) and lysophosphatidylcholine (LC) were prepared at concentrations between 25 and 500 μg/ml in chloroform—methanol (2:1). A 20-μl volume of each standard solution was injected in triplicate and the areas of the peaks averaged to produce calibration graphs for each of the phospholipids. TLC was performed by the method of Darin-Bennett et al. [9].

Phosphorus analyses were carried out according to Fiske and Subbarow [10] as modified by Gentner and Haasemann [11].

Spermatozoa and seminal plasma were separated and lipids were extracted as described by Darin-Bennett et al. [9]. The treatment of the lipid extracts to separate neutral lipids and phospholipids has been described previously [12].

The phospholipid-bound fatty acid methyl esters were prepared and determined by gas—liquid chromatography [13]. Plasmalogens were hydrolysed as described by Vishwanathan et al. [14]. After removing the solvent with a stream of dry nitrogen, the phospholipids were exposed to fumes of concentrated hydrochloric acid for 3 min. The excess of hydrochloric acid was removed with a stream of dry nitrogen and the samples were dissolved in chloroform—methanol (2:1).

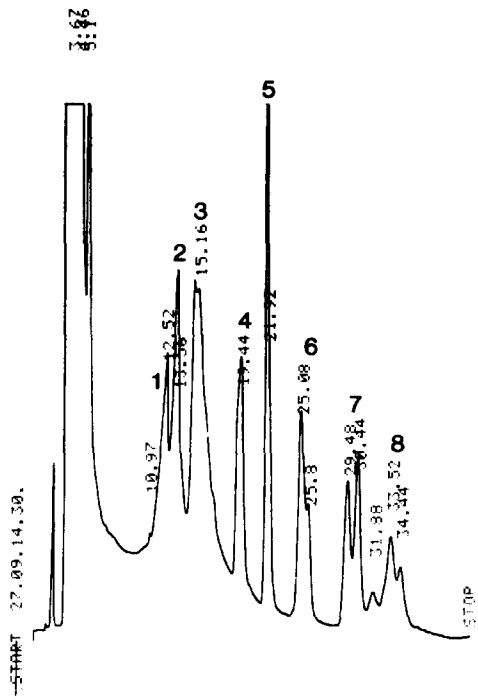

Fig. 1. Typical HPLC elution pattern for a standard mixture of eight phospholipids. Chromatographic conditions as given in the text. Peaks: 1 = CL; 2 = PI; 3 = PS; 4 = PE; 5 = LE; 6 = PC; 7 = S; 8 = LC.

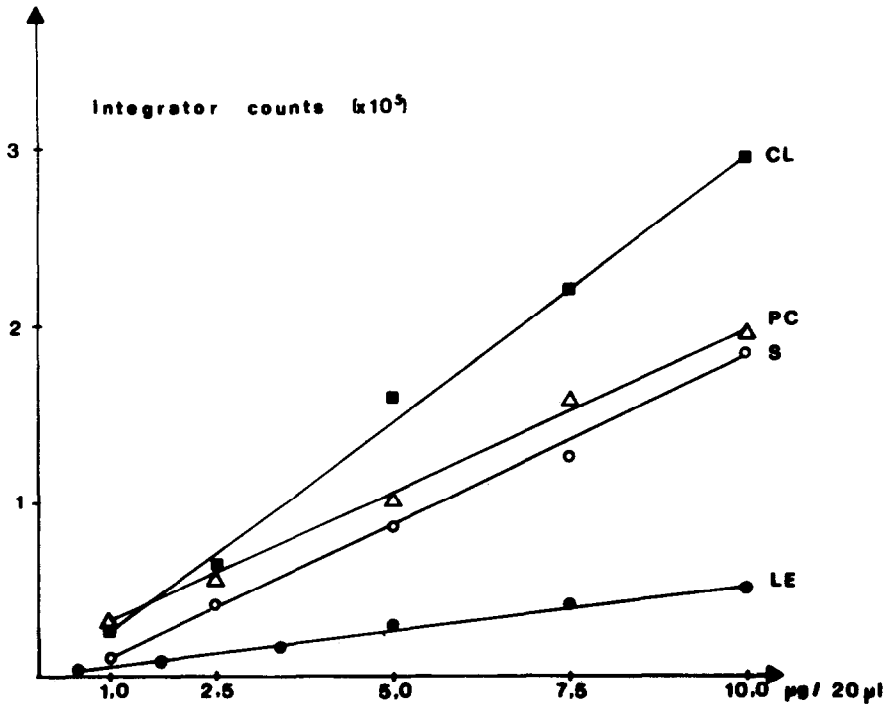

Fig. 2. Calibration graphs for CL, PC, S and LE. Each point represents the average of at least three determinations. Absorbance range: 0.08.

RESULTS

The HPLC analysis of a mixture of eight phospholipids is shown in Fig. 1. The analysis was completed in less than 41 min. PC and S appeared as a split peak in the chromatogram. The two species of S were characterized by analysing their fatty acid composition. The compositions of the two species were different. One contains long-chain fatty acids (> 20 carbon atoms) and the other fatty acids with 14—18 carbon atoms. In contrast, the two species of PC did not show any differences in their fatty acid patterns. Presumably this could account for species having an ether linkage instead of an ester linkage, as indicated by Do and Ramachandran [15] for PE.

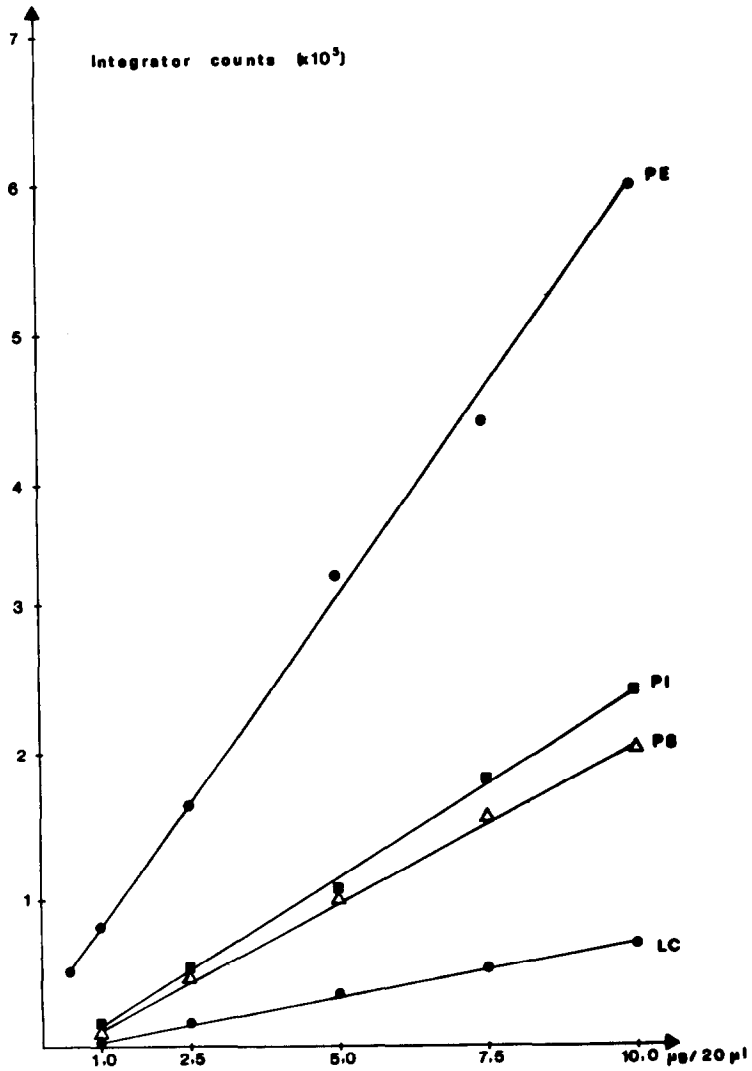

Fig. 3. Calibration graphs for PE, PI, PS and LC. Each point represents the average of at least three determinations. Absorbance range: 0.08.

PS was heterogeneous, producing broad split peaks in accordance with Briand et al. [5].

The primary difficulty was the separation of PI from PG. The use of a pre-column resulted in PG eluting prior to PI. PG and CL eluted together.

Figs. 2 and 3 show that a linear UV response, measured in terms of peak area, could be obtained for amounts of 0.5—10 μg of the different phospholipids. The linear regression correlation coefficients for each of the phospholipid calibration graphs was better than 0.98. Phosphatidalcholine and phosphatidalethanolamine were determined by the decrease in the amounts of PC and PE and the increase in the amounts of LC and LE after exposure to hydrochloric acid fumes.

A representative chromatographic separation of phospholipids from human semen is depicted in Fig. 4.

Some semen samples were analysed by both TLC and HPLC. The correlation between the results of the two methods was good (Table I).

The sensitivity of HPLC is reported to be dependent on the degree of unsaturation in the phospholipids [4], and if the degree of unsaturation varies quantitation by UV absorption would not be accurate. To reinvestigate the correlation of unsaturation and UV absorbance we analysed two commercial PE samples with different fatty acid compositions, especially with respect to the content of the polyunsaturated fatty acids (Table II).

The remarkable result of this investigation was that equal concentrations of the different PE samples produce nearly identical UV absorbances. We

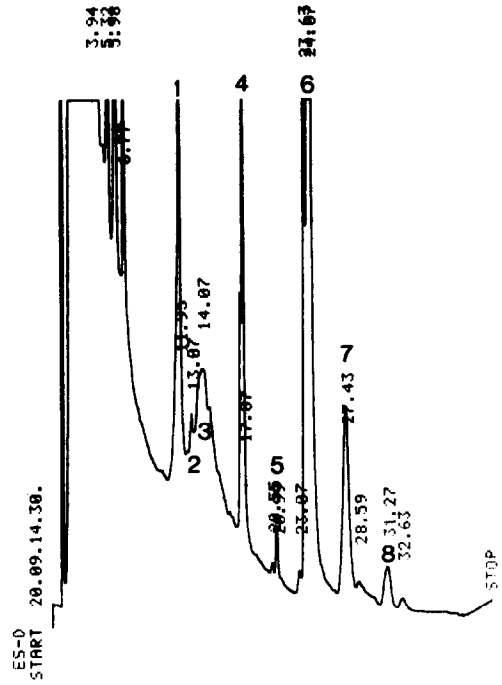

Fig. 4. HPLC trace for a spermatozoa lipid extract after exposure to hydrochloric acid fumes. Chromatographic conditions as given in the text. Peaks: 1 = CL; 2 = PI; 3 = PS; 4 = PE; 5 = PLE (phosphatidalethanolamine); 6 = PC; 7 = S; 8 = PLC (phosphatidalcholine).

TABLE I

CONSTITUENT PHOSPHOLIPIDS OF POOLED HUMAN SEMEN

Percentage of total phospholipids.

Sample	Method	PC	PE	PS	PI	PLC	PLE	S	Other phospho-lipids	Ref.
Sperma-tozoa	TLC	38.3	29.7	3.7		2.1	10.1	12.4	3.4	[1]
	TLC	28.8	21.6	6.6		2.7	9.4	21.4	9.5	[16]
	TLC	42.1	24.1	2.3	1.1	3.0	8.9	18.4	—	This work
	HPLC	48.9	18.3	3.6	2.0	1.8	8.7	16.6	—	This work
Seminal plasma	TLC	7.8	8.5	12.9		0.8	12.3	44.0	13.7	[1]
	TLC	17.6	11.0	1.1	4.8	1.3	14.6	49.6	—	This work
	HPLC	30.7	9.1	1.6	1.1	0.3	14.9	42.2	—	This work

TABLE II

FATTY ACID COMPOSITION OF TWO DIFFERENT PHOSPHATIDYLETHANOLAMINES

Results as % of the total peak area.

Fatty acid (carbon atoms/number of double bonds)	Bovine*	Pig*
13	1.34	
14	1.51	1.83
14/1		0.94
16	7.17	6.31
16/1	1.72	0.10
18	11.48	23.00
18/1	34.66	33.05
18/2	9.14	
18/3	0.57	
20	0.84	
20/1	4.26	
20/2	1.29	2.24
20/3	17.83	13.50
20/5		1.78
22	2.41	
22/1	0.49	
22/5		1.90
22/6	2.05	6.69
22/4	3.25	8.66
Integrator counts ($\times 10^5$)	23.66 ± 0.01**	25.83 ± 0.01**

*Mean values.
**20 μg.

therefore believe that the UV absorbance was not due only to the presence of carbon double bonds, but also to functional groups such as carbonyl, carboxyl, phosphate, amino and quaternary ammonium [17].

In conclusion, the results demonstrate the successful application of HPLC in the separation of naturally occurring phospholipids.

ACKNOWLEDGEMENTS

The authors thank Mrs. E. Tiemeier and Mrs. U. Houska for assistance in the experiments. This work was supported by Deutsche Forschungsgemeinschaft grant No. KR. 429/7.

REFERENCES

1 A. Poulos and I.G. White, J. Reprod. Fertil., 35 (1973) 265.
2 G. Rouser, S. Fleischer and A. Yamamoto, Lipids, 5 (1970) 494.
3 Y.C. Jain and S.R. Anand, J. Reprod. Fertil., 47 (1976) 255.
4 F.B. Jungalawala, J.E. Evans and R.H. McCluer, Biochem. J., 155 (1976) 55.
5 R.L. Briand, S. Harold and K.G. Blass, J. Chromatogr., 223 (1981) 277.
6 V.L. Hanson, J.Y. Park, T.W. Osborn and R.M. Kiral, J. Chromatogr., 205 (1981) 393.
7 J.R. Yandrasitz, G. Berry and S. Segal, J. Chromatogr., 225 (1981) 319.
8 S.-H. Chen and A.Y. Kou, J. Chromatogr., 227 (1981) 25.
9 A. Darin-Bennett, A. Poulos and I.G. White, Andrologia, 8 (1976) 37.
10 C.H. Fiske and Y. Subbarow, J. Biol. Chem., 66 (1925) 375.
11 P.R. Gentner and A. Haasemann, Fette-Seifen-Anstrichm., 9 (1979) 357.
12 H.P. Nissen, H.W. Kreysel and C. Schirren, Andrologia, 13 (1981) 444.
13 H.P. Nissen and H.W. Kreysel, Andrologia, (1983) in press.
14 C.V. Vishwanathan, F. Philips and W.O. Lundberg, J. Chromatogr., 35 (1968) 66.
15 V.H. Do and S. Ramachandran, J. Amer. Oil Chem. Soc., 56 (1979) 185A.
16 R. Jones, T. Mann and R. Sherins, Fertil. Steril., 31 (1979) 531.
17 W.S.M. Geurts van Kessel, W.M.A. Hax, R.A. Demel and J. de Gier, Biochim. Biophys. Acta, 486 (1977) 524.