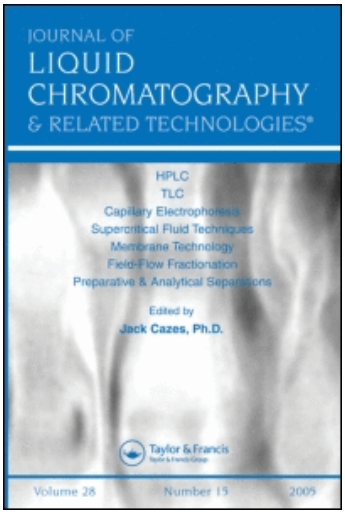

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Simultaneous Determination of Phospholipid Classes and the Major Molecular Species of Lecithin in Human Amniotic Fluid by HPLC

B. H. Klein[a]; J. W. Dudenhausen[a]

[a] Frauenklinik und Poliklinik Charlottenburg, Arbeitsgruppe Perinatale Medizin, Freie Universität Berlin, Universitätsklinikum Rudolf Virchow, Berlin, Germany

To cite this Article Klein, B. H. and Dudenhausen, J. W.(1994) 'Simultaneous Determination of Phospholipid Classes and the Major Molecular Species of Lecithin in Human Amniotic Fluid by HPLC', Journal of Liquid Chromatography & Related Technologies, 17: 5, 981 — 998

To link to this Article: DOI: 10.1080/10826079408013381

URL: http://dx.doi.org/10.1080/10826079408013381

JOURNAL OF LIQUID CHROMATOGRAPHY, 17(5), 981-998 (1994)

SIMULTANEOUS DETERMINATION OF PHOSPHOLIPID CLASSES AND THE MAJOR MOLECULAR SPECIES OF LECITHIN IN HUMAN AMNIOTIC FLUID BY HPLC

BURKHARD H. KLEIN AND JOACHIM W. DUDENHAUSEN

Freie Universität Berlin
Universitätsklinikum Rudolf Virchow
Frauenklinik und Poliklinik Charlottenburg
Arbeitsgruppe Perinatale Medizin
Pulsstraße 4
D 14059 Berlin, Germany

Abstract

Phospholipid classes in human amniotic fluid, whose quantitative determination plays an important role in obstetrics for diagnosing lung maturity in the fetus, are separated through normal phase chromatography by HPLC. In this study an already known chromatographic system is described with which not only the phospholipids, but also phosphatidylcholine partly after being combined with both its fatty acid residues, is separated. This separation implies the fatty acid residues which are of greatest interest to the obstetrician. A mass or evaporative light-scattering detector was used for the detection.

Introduction

The determination of surface active phospholipids in amniotic fluid is of great importance in obstetrics for diagnosing lung maturity in the fetus. A number of quantitative or semiquantitative methods of determination exist today [1]. Tests which measure the physical (surface) properties of lung surfactant, tests which measure the chemical components of amniotic fluid (AF) or tests which provide an index of fetal maturity and thus an approximation of fetal lung maturity are used in clinical routine. In the "chemical" methods three phospholipids in particular are measured - phosphatidylcholine or lecithin, sphingomyelin and phosphatidylglycerol.

The most versatile method of determination is chromatography [2,3], in particular HPLC which can quantitatively detect up to 11 classes of phospholipids in one analysis. This is achieved exclusively with normal phase chromatography either in silica gel columns [4] or in chemically modified silica gel, particularly the diol phase [5]. The individual phospholipids, like for example lecithin, are not chemically homogenous substances but are in fact a mixture of an homogenous basic frame with one or two fatty acid residues which can vary according to the method of production [6]. A large number of saturated and unsaturated fatty acids are attached to the lipids; however their surface active effect can be very different. Using reversed phase chromatography in ODS phases these individual lipid classes can be separated according to the single fatty acid residue and/or combinations [2,3]. In the case of lecithin the chromatogram can sometimes show more than 20 peaks [7].

In this study a HPLC separation system is described that separates not only the phospholipid classes but also the major molecular species of lecithin from each other in one chromatography run. It concerns normal phase chromatography with chemically non-modified silica gel. Detection is achieved using a mass or evaporative light-scattering detector.

Materials

Chemicals

The phospholipid standards, phosphatidylglycerol (PG, ammonium salt, from egg yolk lecithin), diphosphatidylglycerol (DPG, cardiolipin, sodium salt, from bovine

heart), phosphatidylinositol (PI, ammonium salt, Sigma: from soybean and Fluka: from bovine liver), phosphatidylserine (PS, from bovine brain), phosphatidylethanolamine (PE, from egg yolk), phosphatidylmonomethylethanolamine (PMME), phosphatidyldimethylethanolamine (PDME), lysophosphatidylethanolamin (LPE, from egg yolk), phosphatidylcholin (PC, lecithin, from frozen egg), didecanoyl- (DD-PC), dilauroyl- (DLa-PC, dihydrate), dimyristoyl- (DM-PC, monohydrate), dipalmitoyl- (DP-PC), distearoyl- (DS-PC), dioleoyl- (DO-PC), 2-oleoyl-1-palmitoyl- (OP-PC), 2-linoleoyl-1-palmitoyl-lecithin (LiP-PC), lysolecithin (LL, from soybeans), sphingomyelin (SP, from bovine brain), N-palmitoyl- (P-SP, from bovine brain) and N-stearoylsphingomyelin (S-SP, from bovine brain) stem from the firm Sigma, Deisenhofen/FRG, and also from Fluka, Neu-Ulm/FRG. Most of the substances are delivered in the form of solutions in the solvents chloroform, methanol or a mixture of chloroform-methanol.

The solvents used - chloroform, methanol, n-hexane and 2-propanol (all of grade pro analysi) - are obtained from the Merck Company, Darmstadt/ FRG, and the water "Chromasolv" from Riedel de Haen, Seelze/FRG. The charcoal activated granular about 2.5 mm is also procured from Merck, Darmstadt/FRG.

Amniotic Fluids

The amniotic fluid samples are obtained on the one hand during amniocentesis and on the other hand on delivery. They are frozen in portions of about 2 cm^3 and are stored at a temperature of between -18°C to -24°C.

HPLC Equipment

The modular equipment consists of apparatus produced by the following firms: Wissenschaftliche Gerätebau Dr. Ing. Herbert Knauer GmbH, Berlin/FRG (Programmer 50 B and two pumps type 64.00, a dynamic mixing chamber, a column oven with control unit and a recorder); Gynkotek, Germering/FRG (an injection valve with built-in reed relay and a C-R3A integrator with keyboard, monitor and floppy disk drive); Hewlett Packard, Frankfurt/Main/FRG (digital thermometer 2802A with PT 100 sensor) and Zinnser, Frankfurt/Main/FRG (mass or evaporative light-scattering detector, model ACS 750/14).

As separation columns we used Vertex-ECO-columns with dimensions of 120 mm in length and 4 mm inner diameter filled with Nucleosil 100, 3 μm, Nucleosil 120, 3 μm, and Nucleosil 50, 5 μm, supplied by Säulentechnik Dr. Ing. H. Knauer GmbH, Berlin/FRG.

Methods

When the individual standard substances are not delivered in the form of a solution with the concentration of 10 mg/cm^3, solutions are correspondingly composed with a chloroform-methanol mixture (2:1, v:v), or the concentrated solutions are diluted. Then the standard injection solvents which are listed in Table 1 are produced with these solutions.

The extraction of amniotic fluid to obtain the injection solutions has already been described [8]. 5 μl lysolecithin solution (10 mg/cm^3) was added as internal standard to the 1,5 cm^3 amniotic fluid, which was used for extraction. This lysolecithin serves to measure the reaction degree of the extraction and also to measure the peak identification of the chromatogram. The completely dried extractions are diluted in 0.2 cm^3 chloroform/methanol (2:1, v:v) and can then be injected into the HPLC equipment.

HPLC

The selection of the columns, the eluents and the composition of the gradients was made according to the studies made by Breton et al. [9], Letter [10], Dugan [11] and Geilen [12].

For the gradients a mixture of n-hexane and 2-propanal (60:40, v:v) is used as eluent A and as eluent B, a mixture of 500 cm^3 solution A with 29 cm^3 water. At first it is started in one liter of eluent A, half of which is then further used for B. Both eluents are degassed in an ultrasonic bath for at least 15 minutes. The composition of the gradient is given in Table 2. The flow amounts to 1.0 cm^3/min. A chromatogram takes either 45 or 50 minutes depending on which column is being used. The column has a temperature of 30°C in order to exclude fluctuations in the temperature in our laboratory. The detector works with compressed air purified with

TABLE 1

Standard solutions used with partial concentration for the substances (for abbreviations see Materials section Chemicals)

Solution No.	Substances	Concentration in g/cm^3
1	PG, PI, PS, PE, PC, SP, LL	0.25
1A	No. 1 + DPG, PMME, PDME, LPE	0.25
2	DD-PC, DLa-PC, DM-PC, DP-PC, DS-PC	0.25
3	DO-PC, OP-PC, LiP-PC	0.25
3A	No. 3 + DD-PC, DS-PC	0.25
4	DD-PC, DS-PC	4.00

TABLE 2

Composition of the mobile phase, eluent A: n-hexane/2-propanol (60:40) and eluent B: 500 cm^3 eluent A with 29 cm^3 water

Time in min	Portion A in %	Portion B in %
0	50	50
6	50	50
11	22	78
20	22	78
30	0	100
50	0	100
55	50	50
60	50	50

charcoal activated granular as nebulizing gas, pressure approximately 1.4 bar (20 psi) at 5 bar first pressure. The temperature in the evaporator is set at 50°C.

The measuring data are recorded over the integrator on the diskette for later evaluation. The integrator and also the programmer are started by injection by hand through the contact point of the reed relay.

The calibrations were done according to the one point method and were stored in various files in the integrator. One calibration contains various phospholipid classes (standard solution 1), a second one the lecithins with the various fatty acids residues (standard solution 2 and 3, respectively 3a). No calibration was made with sphingomyelin on its own.

Results and Discussion

By using the mass/light-scattering detector and due to its function [3] no solution peaks and no changes in the base line through the gradients are to be found in the chromatogram. The peak occurring at the point of the solution does at least represent the proteins present in the solution, if not any other substances. The presence of the proteins could be proved by an injection of a purified protein solution with a portion of phospholipid.

The temperature of the columns has no influence on the separation as regards the retention time, the resolution and the form of the peaks. The chromatograms undergo no changes whether they are being produced at room temperature or whether the column oven has a temperature of 45°C.

Separation of the Phospholipids Classes

In figure 1 chromatograms of the standard solutions 1 and 1A can be seen. Chromatogram (A) serves as concentration calibration. The individual substances, with the exception of PI and PS are separated from each other on the base line. The PS peak is set upon the PI peak with its characteristic form, the strong fronting through the sixring sugar in the molecule. Sphingomyelin is represented by its typical double peak, here also separated on the base line.

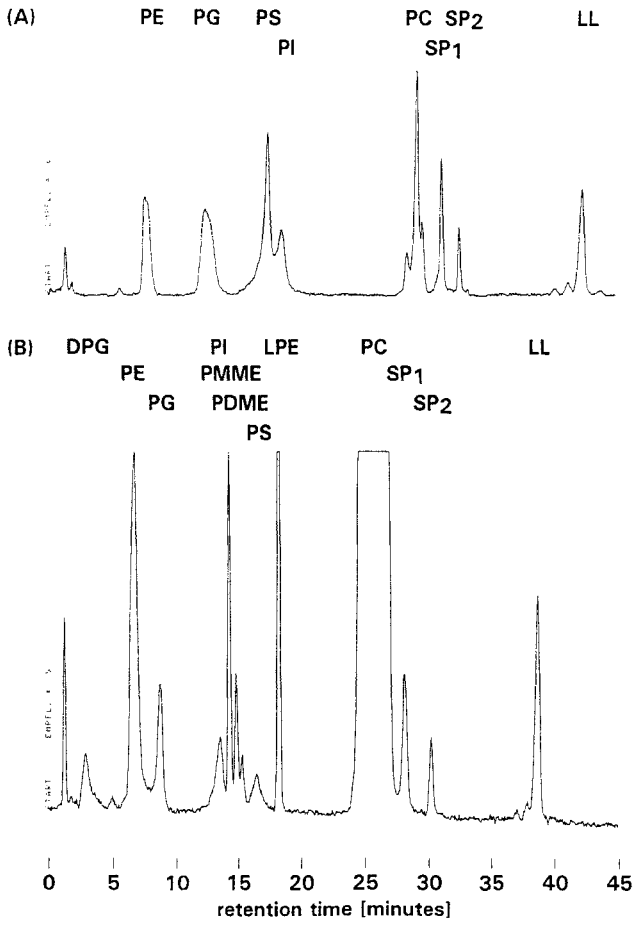

FIGURE 1: Chromatogram of the standard solution (A) No. 1 on a Nucleosil 100/3 and (B) No. 1A on a Nucleosil 120/3

Chromatogram (B) shows a separation of the extended standard solution with substances that possibly may also be present in amniotic fluid. All 11 substances are clearly separated from each other. In this column - a different one than in (A) - PI and PS are widely separated from each other. The separation of these two substances is very strongly dependent on the column and can only result in one peak or in the two forms described.

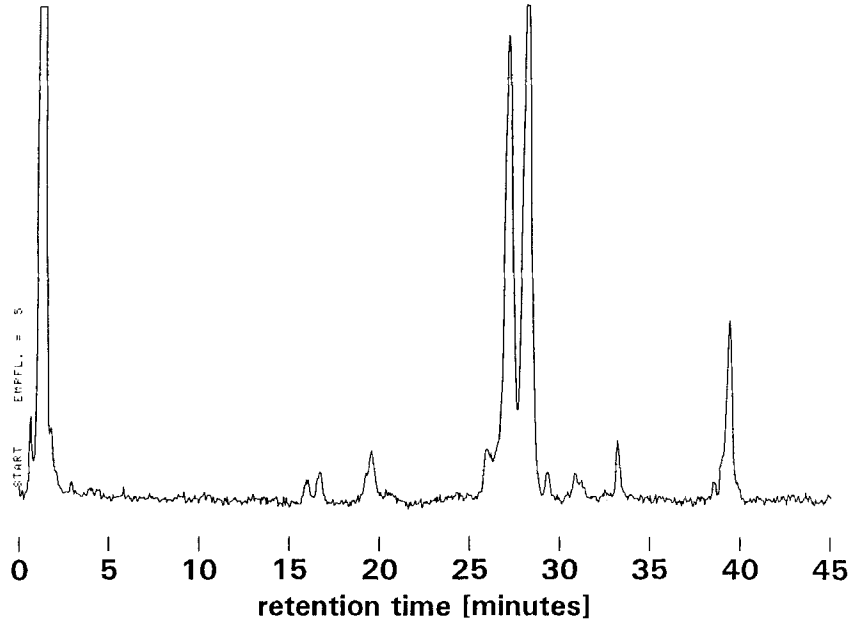

FIGURE 2: Phospholipid separation of the extract of amniotic fluid (AF 244, week of gestation 39/1) on Nucleosil 100/3

An exemplary chromatogram of an extract of mature amniotic fluid is given in figure 2. The last peak at about 39 minutes is lysolecithin, the "internal standard". Lecithin with approximately 70 to 80% proportion of the complete phospholipid content of amniotic fluid [13] forms the "main" peak between 26 and 29 minutes. Furthermore in this amniotic fluid, SP (31 and 33 minutes) and PI (19.5 minutes) and PG (16 minutes) are clearly detected. PE was not found in this sample. These other phospholipids together form only 20 to 30% of the total and are therefore partially very difficult to determine since the mass detector has a very high detection limit at 0.5 to 1 μg of the injected quantity.

Separation of the Molecular Species of the Lecithin

If we consider the lecithin peak in the previous chromatogram and in figure 3 (frozen egg lecithin), then, in contrast to the study made by van der Meeren et al.

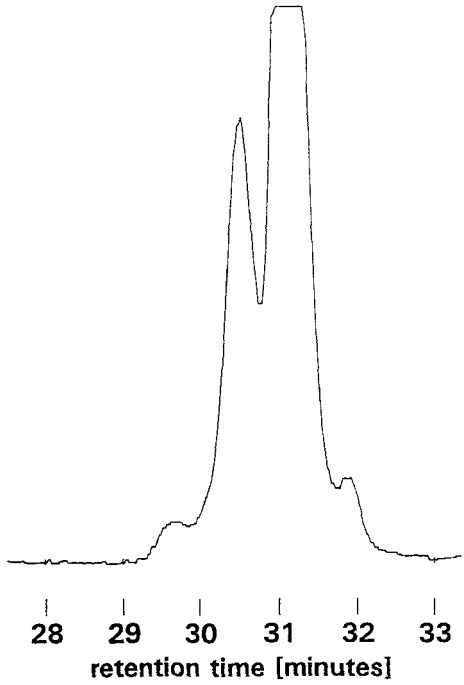

FIGURE 3: Part of a chromatogram of lecithin (from frozen egg) from Sigma, 5 μg injected

[14], who found a deformed lecithin peak, a clear separation into up to 4 peaks is striking. It is assumed that here a separation is to be seen according to the individual combinations of both the fatty acid residues of the lecithin. To prove this the PC standard solutions 2 and 3 were composed and injected. In order to detect retention time fluctuations due to the charge-like insertion of the eluents and - if necessary - to correct these, solution 3 was modified in 3A after making certain that the substances were separated enough from each other, to accurately measure the retention times of the maxima. Figure 4 shows the result of the dependence of the retention time and of the capacity factor as natural logarithm from the total of the number of the carbon atoms in the fatty acid residues. It can be seen that both dependencies are to be described as straight lines in mathematical terms. As a result of this for later calculation of the amount of carbon, only the relationship retention time to the

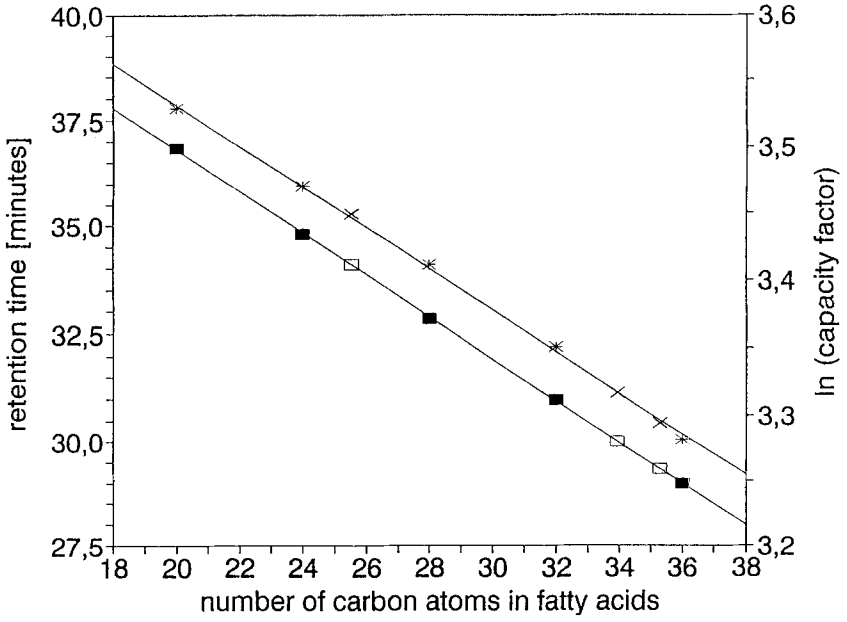

FIGURE 4: Dependence of the retention (time - continuous line and capacity factor - broken line) from the total of the carbon atoms of the saturated (filled in square, star) and unsaturated (empty square, plus sign) fatty acid residue of the lecithin

amount of carbon was always used. Also in the case of lecithin with unsaturated residues, the retention time was calculated back to the amount of carbon. Table 3 gives the numerical values for all the Nucleosils used. Within the framework of the measuring accuracy of the equipment used for example, the amounts of carbon given for the oleoyl residue (18:1 = number of carbon atoms: double bond amounts in the chain), for DO-PC and PO-PC correlate very well. As can be seen in figure 5, this is also true of all the PCs used with unsaturated residue.

Lecithin in Amniotic Fluid

As very large variations in the concentration of lecithin occur in amniotic fluid a calibration was performed with 6 concentrations. As can be seen in figure 6, the

TABLE 3

Calculated number of carbon atoms in unsaturated fatty acids of lecithin from retention times for the three Nucleosil used

Fatty acid residues		Number of carbon atoms of the residues					
		Nucleosil 120/3		Nucleosil 100/3		Nucleosil 50/5	
1	2	1	2	1	2	1	2
18:1	18:1	17.3	17.3	17.6	17.6	17.4	17.4
16:0	18:1	16.0	17.7	16.0	17.9	16.0	17.5
16:0	18:2	16.0	9.7	16.0	9.5	16.0	8.9

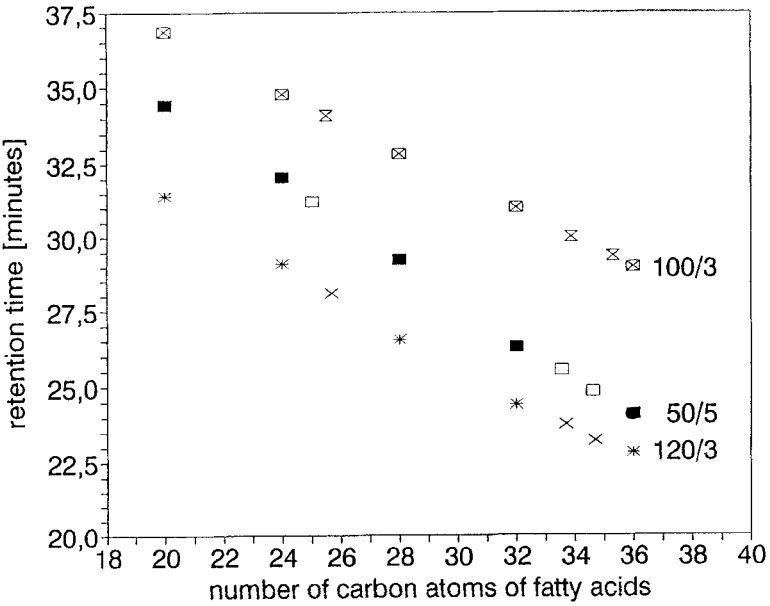

FIGURE 5: Separation of the lecithin with saturated (filled in square, square with X inside and star), and unsaturated (empty square, sand glass) on the three Nucleosils used

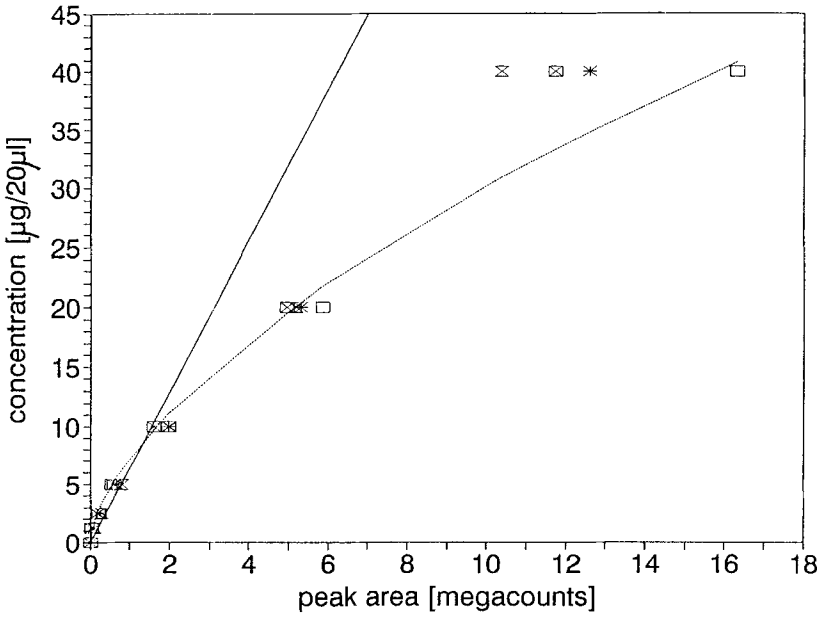

FIGURE 6: Calibration curve for the mass detector with PC (first - square with X inside and second measurement - empty square) and DD-PC, (first - sand glass and second measurement - star) one point method of the integrator (continuous line) and exposed function (broken line)

one point calibration with the help of the integrator (continuous line) can be used for the lower concentrations in the first approximation. Above an amount of 5 μg injected substance however increasing fluctuations occur. The broken line is used to calculate the portions of the total of the individual PC peaks. With the exception of the concentration 40 μg/20 μl the peak surface is independent of the substance (PC or DD-PC) and is easily reproducable. To calculate the curve the median value of the peak surfaces on 40 μg/20 μl is used.

Figure 7 shows standardized chromatograms of two samples of amniotic fluid. The addition of the standard solution 4 - peak at 24 respectively 32 minutes in chromatogram (A) - serves to calculate the carbon atom count n(C) from the retention times of the lecithin peaks. The results of the calculations for this amniotic fluid are given in Table 4. The margin of error of the carbon count amounts to a maximum of plus minus 0.5 atoms.

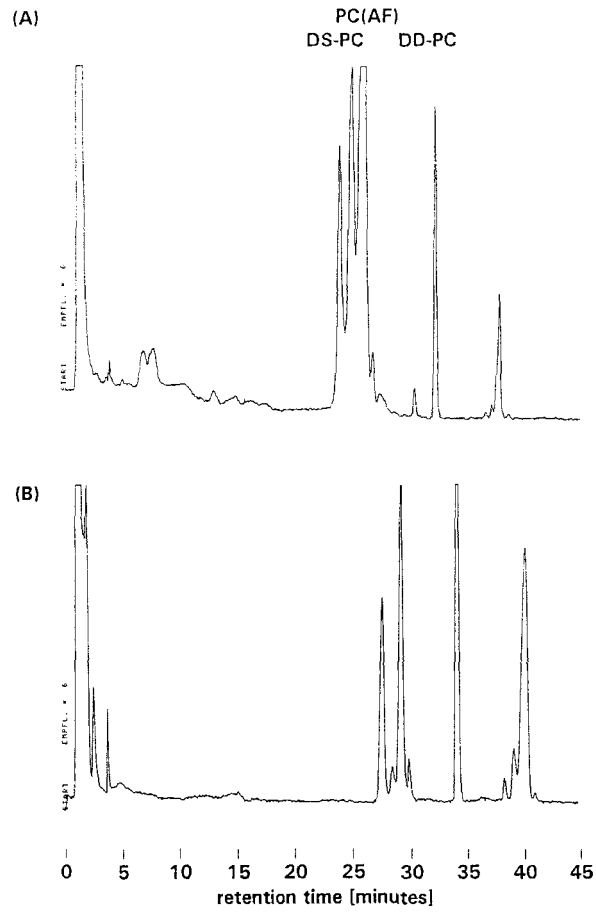

FIGURE 7: Chromatogram of (A) AF 432 (40/0 weeks) and (B) AF 491 (40/5 weeks), each with addition of standard solution 4

The average results of the measurements of 40 amniotic fluid samples are illustrated in figure 8 in an example with a Nucleosil 100 column. In all the amniotic fluid peaks were found, to which a total of 5 carbon atom counts could be attributed. Three of these five values (36.0, 33.9 and 32.1) are congruent with the established standards (DS-PC, PO-PC and DP-PC). These three fatty acids occur in amniotic fluid with at least 8 others [15,16]. The determination of further lecithins with

TABLE 4

Results of composition of lecithin in two amniotic fluids, AF 432 [40/0 weeks, figure 7 (A)] and AF 491 [40/5 weeks, figure 7 (B)]

Peak No.	AF 432		AF491	
	n(C)	Portion in %	n(C)	Portion in %
1	33.9	36.2	34.0	16.8
2	32.1	51.9	32.0	67.6
3	30.5	9.2	30.5	15.5
4	29.3	2.7		

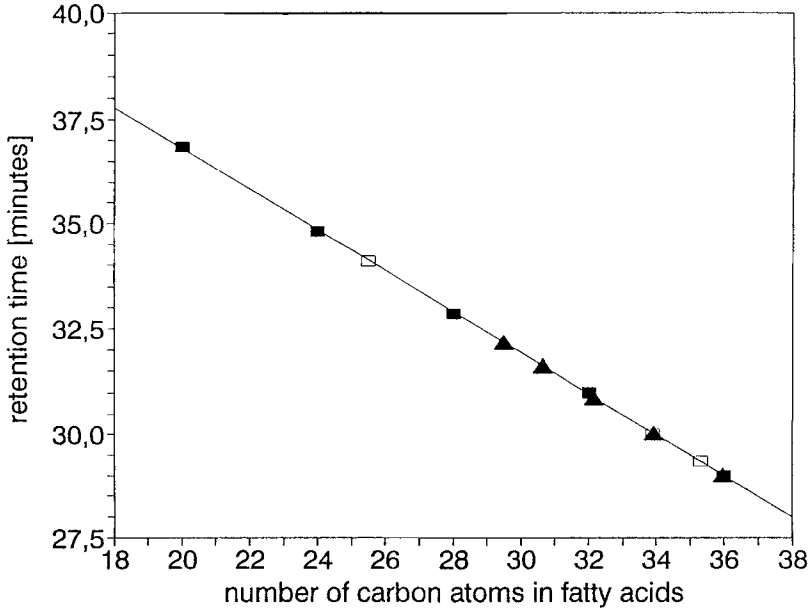

FIGURE 8: Retention of the lecithin with saturated (filled in square) and unsaturated (empty square) fatty acid residues as the peaks of amniotic fluid-lecithin (filled in triangle) in an example of a Nucleosil 100 column

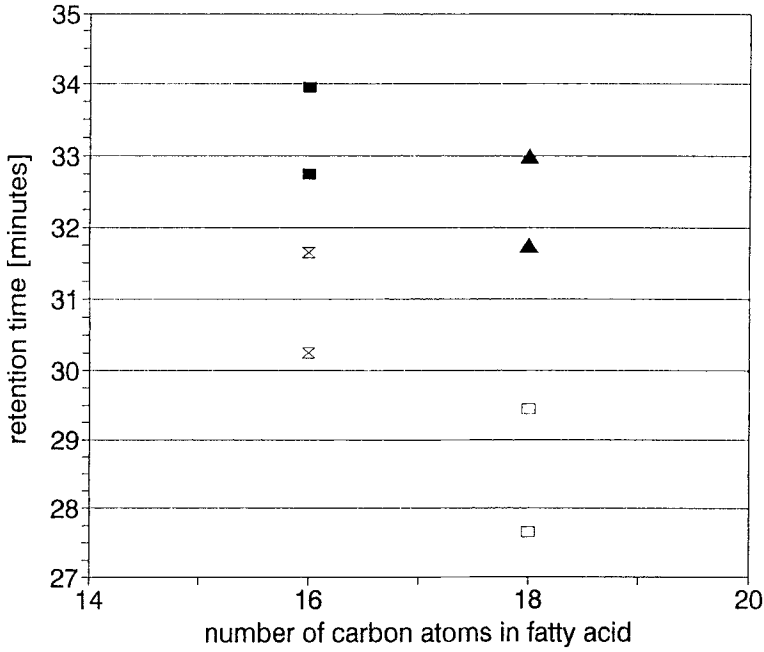

FIGURE 9: Retention times of the double peak of the sphingomyelin with a defined fatty acid residue on Nucleosil 100/3 (filled in square, or triangle) and Nucleosil 120/3 (sand glass, empty square)

unsaturated fatty acid residues (18:2, 18:3, 20:4 and 22:6) is hardly possible as they also elute in the range of sphingomyelin. Furthermore they are to be found in very small quantities in amniotic fluid so that they lie below the detection limit. The other two carbon counts determined to the peaks (30.7 and 29.2) only allow speculation which is not carried out here, on the possible combinations of the fatty acids in PC.

This chromatographic system can and ought not to replace the separation through reversed phase chromatography [15] to determine the combinations of the fatty acid residues particularly in lecithin. However the obstetrician is also especially interested in DP-PC, since its concentration gives information on lung maturity [15]. In addition to the total concentration of lecithin just the portion of this particular species is clearly measurable. After closer study of the chromatogramms given in the

literature, for example in Breton et al. [9], describing the analogous systems, the separation of lecithin can be observed in the same way. However this, with the exception of van der Meeren et al. [14] who presumably observed a peak widening with scatter formation due to a high water content, has apparently not been researched further.

The described separation into several peaks does not only apply to lecithin. Only in phospholipids that elute earlier can a visible peak widening be effected. On the other hand more than one peak is observed in SP and LL that elute later on.

Separation of Sphingomyelin

The double peak that is almost always observed in SP allows one to presume the separation according to various fatty acid residues is the cause. This should be examined by injecting P-SP and S-SP. In figure 9 the results are given on a Nucleosil 100 and a Nucleosil 120 column. The sphingomyelins also with a defined fatty acid residue each form a double peak. So - in contrast to the descrip-tion by Christie [3] - it cannot be a separation according to the chain length of the fatty acids. A much more probable solution seems to be that the separation is due to nonhydroxy and hydroxy fatty acids of equal chain length [17].

Conclusion

Also using normal phase chromatography by means of which the individual phospholipid classes in human amniotic fluid can be separated from each other, phosphatidylcholine can at least be separated in several particular fatty acid combinations which are of interest to the obstetrician. The obvious conclusion that the double peak of sphingomyelin is based on the same effect could not be confirmed.

Acknowledgements

We wish to express our hearty thanks to the Maria-Sonnenfeld-Gedächtnis-Stifung, Berlin for financing the mass or evaporative light-scattering detector.

Further thanks go to the company Säulentechnik Dr. Ing. H. Knauer GmbH in Berlin for the umcomplicated help given when problems arose with the columns. We wish to thank Dr. J. Reusch, the managing director of the company, for the stimulating and helpful discussions.

References

1) C. E. Parkinson and D. Harvey, "Amniotic fluid and fetal pulmonary maturity", in Amniotic fluid and its clinical significance, M. Sandler eds., Marcel Dekker New York 1981, pp. 229-252

2) W. W. Christie, Z. Lebensm. Unters. Forsch. 181: 171-182 (1985)

3) W. W. Christie, HPLC and Lipids - A practical guide, Pergamon Press Oxford 1987

4) P. R. Redden and Y.-S. Huang, J. Chromatogr. 567: 21-27 (1991)

5) T. Heinze, G. Kynast, J. W. Dudenhausen, C. Schmitz and E. Saling, Chromatographia 25: 497-503 (1988)

6) R. L. Sanders, "Introduction to lipid biochemistry" in Lung development: biological and clinical perspectives - volume 1 - biochemistry and physiology, P. M. Farrell Eds., Academic Press New York 1982, pp. 167-178

7) N. Sotirhos, C. Thörngren and B. Herslöf, J. Chromatogr. 331: 313-320 (1985)

8) G. Kynast and C. Schmitz, Z. Ernährungswiss. 27: 203-212 (1988)

9) L. Breton, B. Serkiz, J.-P. Volland and J. Lepagnol, J. Chromatogr. 497: 243-249 (1989)

10) W. S. Letter, J. Liq. Chromatogr. 15: 253-266 (1992)

11) L. L. Dugan, P. Demediuk, C. E. Pendley and L. A. Horrocks, J. Chromatogr. 378: 317-327 (1986)

12) C. C. Geilen, Freie Universität Berlin, Institut für Biochemie, personal communication

13) F. Possmayer, "Biochemistry of pulmonary surfactant during fetal development and in the perinatal period", in Pulmonary surfactant, B. Robertson, L. M. G. Van Golde and J. J. Batenburg, eds., Elsevier Science Publishers Amsterdam 1984, pp. 295-355

14) P. Van der Meeren, J. Vanderdeelen, G. Huyghebaert and L. Baert, Chromatographia 34: 557-562 (1992)

15) L. Gluck, M. V. Kulovich, R. C. Borer, P. H. Brenner, G. G. Anderson and W. N. Spellacy, Am. J. Obstet. Gynecol. 3: 440-445 (1971)

16) S. J. Robins and G. M. Patton, J. Lipid Res. 27: 131-139 (1986)

17) R. S. Lutzke and J. M. Braughler, J. Lipid Res. 31: 2127-2130 (1990)

Received: June 24, 1993
Accepted: September 9, 1993