ELSEVIER

Journal of Chromatography B, 690 (1997) 338–342

JOURNAL OF CHROMATOGRAPHY B

Short communication

Analysis of dipalmitoyl phosphatidylcholine in amniotic fluid by high-performance liquid chromatography

Juan G. Alvarez*, Brant Slomovic, Jack Ludmir

Department of Obstetrics and Gynecology, Beth Israel Hospital, Harvard Medical School, 330 Brookline Avenue, Boston, MA 02215, USA

Received 17 June 1996; revised 28 August 1996; accepted 6 September 1996

Abstract

A novel method for the determination of dipalmitoyl phosphatidylcholine (DPPC) in amniotic fluid by high-performance liquid chromatography (HPLC) is described. Aliquots of 50 μl of amniotic fluid were hydrolyzed with phospholipase C from *Bacillus cereus* and the resulting dipalmitoylglycerol analyzed by HPLC. Run-to-run and day-to-day precision for DPPC analysis were 4.2 and 6.1%, respectively, and analysis time was 10 min. Recoveries for DPPC ranged between 92 and 98%. In summarizing, this method provides a high precision and fast turnaround time means for the analysis of DPPC in amniotic fluid.

Keywords: Dipalmitoyl phosphatidylcholine; Lipids

1. Introduction

Current tests used for the prediction of respiratory distress syndrome (RDS) include the lecithin/sphingomyelin ratio [1], analysis of the concentration of phosphatidylglycerol in amniotic fluid by one-dimensional thin-layer chromatography [2] and latex agglutination [3], foam stability tests [4,5], surface-tension tests [6] and turbidimetric analysis of amniotic fluid at 650 nm [7]. These methods have high sensitivity at the expense of low specificity. More recently, tests that monitor the level of fluorescence polarization (TDx-FLM, Abbott Laboratories, Abbott Park, IL, USA) and the number of lamellar bodies in amniotic fluid (LBND) have been introduced for the prediction of RDS [8–15]. Although these methods are useful screening tests and have a fast turnaround time, they also have low specificity for RDS.

Torday et al. reported that the concentration of disaturated phosphatidylcholine in amniotic fluid was an accurate predictor of RDS [16]. The sensitivity and specificity of the saturated phosphatidylcholine test for RDS reported was 98 and 92%, respectively. When an *L/S* ratio of less than 2 was used to predict RDS in their study population, the sensitivity and specificity of the *L/S* ratio for RDS were 93 and 66%, respectively, thus demonstrating the saturated phosphatidylcholine test to be a more specific test than the *L/S* ratio. Its main drawbacks are that it requires relatively high volumes of amniotic fluid for the analysis; it is time-consuming, it is technically complex and it requires the use of toxic reagents, i.e. osmium tetroxide and carbon tetrachloride. For these reasons, the saturated phosphatidylcholine test cannot be made widely available to clinicians.

*Corresponding author.

PII S0378-4347(96)00415-X

Dipalmitoyl phosphatidylcholine (DPPC) is the major surface-active component of the mature fetal lung surfactant, comprising 60% of the total lamellar body lipid content and more than 90% of the saturated phosphatidylcholine species [17]. DPPC is only found in trace amounts in other biological fluids and has been suggested as an excellent marker for surfactant activity in amniotic fluid. We have recently reported a novel test that measures the concentration of DPPC in amniotic fluid by enzymatic hydrolysis and high-performance thin-layer chromatography [18]. The run-to-run precision for the DPPC test by HPTLC is 12% and the turnaround time 45 min. The performance characteristics of the DPPC test by HPTLC in the prediction of respiratory distress syndrome have been recently reported in a blind prospective study that included 174 cases [19]. In this study, when a concentration of DPPC<12 μg/ml was used to predict RDS, the sensitivity and specificity of the test were 100 and 96%, respectively.

In this report, a novel method for the analysis of DPPC in amniotic fluid by high-performance liquid chromatography (HPLC) is introduced. The DPPC test by HPLC uses standard HPLC equipment, it has a turnaround time of 10 min, a run-to-run precision of 4.2% and, therefore, can potentially be made widely available to clinicians.

2. Experimental

2.1. Chemicals and reagents

The lipid standards including dipalmitoyl phosphatidylcholine, 1-O-hexadecyl-2-O-methylphosphatidylcholine, 1-O-palmitoyl-2-O-palmitoleoyl phosphatidylcholine, dipalmitoylglycerol, 1-O-palmitoyl-2-O-palmitoleoylglycerol, cholesterol, *Bacillus cereus* phospholipase C (Sigma Type XI, 1000 units/0.6 ml) and Kodak X-Omat film, were purchased from Sigma (St. Louis, MO, USA). Fatty acid methyl esters were obtained from Supelco (Supelco Park, Bellefonte, PA, USA). Phosphate-based saline was obtained from Gibco-Life Technologies (Grand Island, NY, USA). Analytical and guard ChromSpher lipid columns (250×4.6 mm I.D. and 50×4.6 mm I.D., respectively) were obtained from Chrompack (Raritan, NJ, USA). Varex evaporative light scattering (ELS) detector was obtained from Alltech Associates (Deerfield, IL, USA). Solvents were EM Science chromatographic grade. Inorganic salts were from J.T. Baker (Phillisburg, NJ, USA) and of the highest purity available.

2.2. Analysis of DPPC by HPLC

Aliquots of 5 μl of a *Bacillus cereus* phospholipase C solution (1600 U/ml) in phosphate-based saline was added to 5 μl of a micro dispersion of the internal standard, 1-O-hexadecyl-2-O-methylphosphatidylcholine (0.1 mg/ml) in phosphate-based saline and incubated at 37°C for 5 min. Following incubation, 50 μl of the amniotic fluid sample was added and vortex-mixed for 5 s. Then, 200 μl of hexane–acetone (2:1, v/v) was added, the emulsion vortex-mixed for 5 s and allowed to equilibrate for 30 s at room temperature. Aliquots of 20 μl of the upper, organic phase were injected into the ChromSpher column connected to a Waters pump and a Varex ELS detector. The settings used in the Varex detector included a temperature of 65°C and a gas flow-rate of 15.17 kPa. The ChromSpher analytical and guard columns consisted of spherical silica coated with a strong cation-exchange ligand, with Ag^+ as a counter ion. This results in retention of unsaturated lipid species and elution of their saturated counterparts. Amniotic fluid components were eluted isocratically using chloroform–acetone (90:10, v/v) as the mobile phase at a flow-rate of 1.0 ml/min.

2.3. Effect of common contaminants of amniotic fluid

The effect of common contaminants of amniotic fluid on the determination of DPPC was also evaluated. Blood, meconium, semen or vaginal fluid (1 to 20%, v/v) were spiked into 10 different amniotic fluid samples with DPPC concentrations ranging between 0.2 and 30 μg/ml, and the concentration of DPPC determined as previously described. The concentration of DPPC as measured by free dipalmitoylglycerol, was obtained before and after the addition of these contaminants.

2.4. Analysis of fatty acid methyl esters by gas chromatography

The component that comigrated with the dipalmitoylglycerol standard, obtained from amniotic fluids ranging from 28 to 40 weeks of gestational age, was eluted and evaporated to dryness. The lipid residue was hydrolyzed with 0.5 ml of 0.1 *M* sodium methoxide at 40°C for 1 h. After hydrolysis, the tubes were allowed to cool to room temperature, 1 ml of chloroform and 0.25 ml of distilled water added, the emulsion centrifuged at 600 *g* for 5 min and the lower phase aspirated and evaporated to dryness. The resulting fatty acid methyl esters were dissolved in a volume of *n*-hexane to give concentrations of 1 μg/μl. The resulting fatty acid methyl esters were analyzed by gas chromatography on a WCOT capillary column (Supelco-Wax-10) with initial and final temperatures in the column oven of 150 and 250°C, respectively, programmed to increase at a rate of 10°C/min. The analysis was performed on a Varian 3700 instrument equipped with a flame ionization detector, operated at 260°C. The fatty acid methyl ester peaks were identified by comparison with the retention times of known standard mixtures, and quantified using a Hewlett–Packard 3392A integrator, using methyl heptadecanoate as the internal standard. The identity of dipalmitoyl glycerol as the component comigrating with the dipalmitoylglycerol standard was verified by the presence of methyl hexadecanoate in the gas chromatogram.

2.5. HPTLC–autoradiographic analysis

Aliquots of 10 μl of [^{14}C]DPPC (30 μCi/ml) solutions in PBS were spiked into term amniotic fluid samples, hydrolyzed with *Bacillus cereus* phospholipase C, and the resulting [^{14}C]DPG extracted and redissolved in 6 μl of hexane–acetone (2:1, v/v). Aliquots of 4 μl of both the lower and upper phases were applied, on separate lanes, to Whatman HP-K plates and developed in chloroform–methanol (1:1, v/v). Following development, the plates were dried for 10 s using a hair dryer and exposed to Kodak X-Omat film for 12 h at room temperature. The resulting bands were scanned with a Shimadzu CS-9000 spectrodensitometer in the reflectance mode at 400 nm. The extent of DPPC hydrolysis and the recovery of DPG were calculated by dividing the integration areas obtained for [^{14}C]DPG in the upper phase by the integration areas of [^{14}C]DPPC and [^{14}C]DPG in the lower phase, respectively.

2.6. Precision analysis

Twenty amniotic fluid samples with concentrations of DPPC ranging between 0.1 and 40 μg/ml, were analyzed by the HPLC procedure described above. The relative standard deviation, corresponding to the dipalmitoylglycerol values obtained herein, was utilized to calculate the run-to-run and day-to-day precision. The lower limit of detection was calculated based on a signal-to-noise ratio above 3.

3. Results and discussion

The chromatogram of amniotic fluid before hydrolysis is shown in Fig. 1. A major component eluted at 6.2 min, corresponding to the retention time of the cholesterol standard, and a minor component eluted at 1.20 min, corresponding to an unretained solute. Following enzymatic hydrolysis with phospholipase C from *Bacillus cereus*, two additional components eluted at 3.01 and 4.20 min, corre-

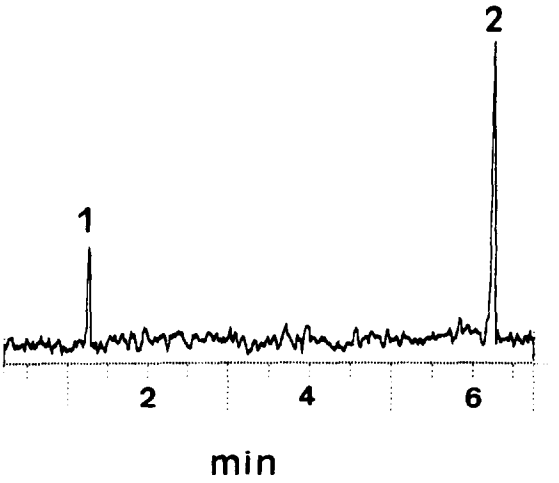

Fig. 1. Chromatogram of amniotic fluid in the absence of phospholipase C hydrolysis. An aliquot of 50 μl of a term amniotic fluid was treated with PBS and processed as indicated in Section 2. Peaks: 1=unretained component; 2=cholesterol.

sponding to the retention times of dipalmitoylglycerol and 1-O-hexadecyl-2-O-methylglycerol standards, respectively (Fig. 2). Fig. 3 shows a chromatogram of amniotic fluid from an infant that developed respiratory distress syndrome.

The identity of the component that co-eluted with dipalmitoylglycerol was further confirmed by gas chromatographic analysis of the fatty acid methyl esters released after alkaline methanolysis. Gas chromatographic analysis of these fatty acid methyl esters indicated that methyl tetradecanoate, methyl hexadecanoate, methyl hexadecenoate, methyl octadecanoate and methyl octadecenoate comprised

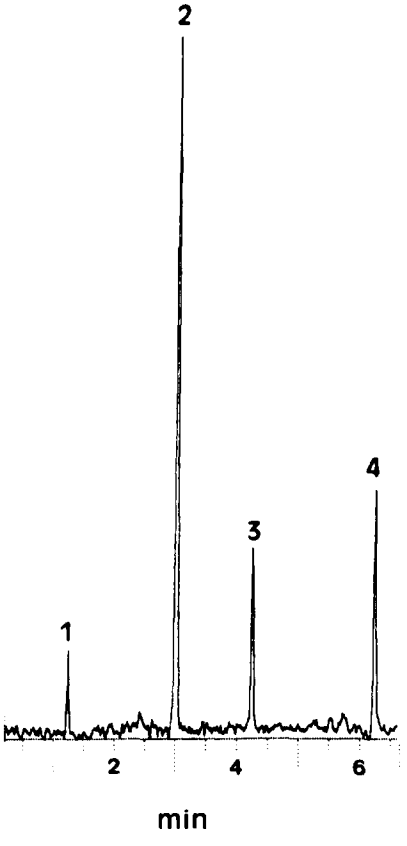

Fig. 2. Chromatogram of amniotic fluid after phospholipase C hydrolysis. An aliquot of 50 μl of the same amniotic fluid used in Fig. 1 was hydrolyzed with phospholipase C, as described in Section 2. Peaks: 1=unretained component; 2=dipalmitoylglycerol; 3=1-O-hexadecyl-2-O-methylglycerol; 4=cholesterol. The dipalmitoylglycerol peak corresponded to a concentration of 40 μg/ml and the 1-O-hexadecyl-2-O-methylglycerol peak to 10 μg/ml.

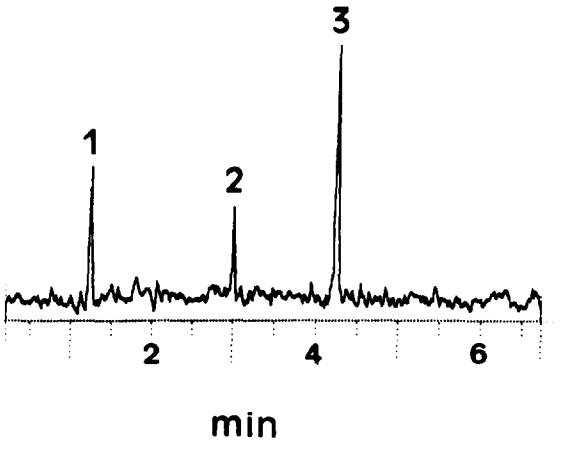

Fig. 3. Chromatogram of amniotic fluid components after phospholipase C hydrolysis. An aliquot of 50 μl of amniotic fluid was hydrolyzed with phospholipase C, as indicated in Section 2. This amniotic fluid specimen was obtained from a pregnant woman whose newborn child developed respiratory distress syndrome. Peaks: 1=unretained component; 2=dipalmitoylglycerol; 3=1-O-hexadecyl-2-O-methylglycerol. The dipalmitoyl glycerol peak corresponded to a concentration of 3 μg/ml and the 1-O-hexadecyl-2-O-methylglycerol peak to 10 μg/ml.

0.5, 96, 1, 1, and 1.5% of the total fatty acid content, respectively. Based on this fatty acid profile, should other 1,2-disaturated diglyceride species comigrate with dipalmitoylglycerol, their contribution to the dipalmitoylglycerol value obtained would be equal to or less than 4%. Therefore, the HPLC method herein described can be reliably used to determine the concentration of DPPC in amniotic fluid.

In order to ascertain whether phosphatidylcholine was the sole phospholipid source of the component comigrating with dipalmitoylglycerol, phosphatidylcholine was isolated by amino-bonded silica gel column chromatography [20], from amniotic fluids obtained by amniocentesis (gestational ages ranging from 28 to 40 weeks). The purified phosphatidylcholine was then hydrolyzed with phospholipase C and the resulting diacylglycerols analyzed by HPLC, as indicated above. The results obtained were then compared to the values of the corresponding raw amniotic fluid. The differences in dipalmitoylglycerol values for both sets of samples were not statistically significant ($P>0.5$). In a separate set of experiments, phosphatidylethanolamine, phosphatidylserine phosphatidylinositol and sphingomyelin,

isolated from amniotic fluid by amino-bonded silica gel column chromatography, were incubated with phospholipase C. Hydrolysis of these phospholipids by phospholipase C was negligible. Therefore, analysis of dipalmitoylglycerol by HPLC, following enzymatic hydrolysis of amniotic fluid with phospholipase C, can be safely utilized to determine the concentration of DPPC in amniotic fluid.

The rate of hydrolysis of the internal standard, 1-O-hexadecyl-2-O-methylphosphatidylcholine, was dependent upon temperature, phospholipase C concentration and time of hydrolysis. In addition, 1-O-hexadecyl-2-O-methyl-phosphatidylcholine hydrolysis was dependent on the concentration of DPPC in amniotic fluid. For this reason, 1-O-hexadecyl-2-O-methyl-phosphatidylcholine was preincubated with phospholipase C at 37°C for up to 5 min in the absence of amniotic fluid. Under these conditions, quantitative hydrolysis of the internal standard was obtained. Following hydrolysis, 50 μl of the amniotic fluid sample was added followed by vortex-mixing for 5 s and equilibration for 30 s. This resulted in quantitative hydrolysis of DPPC from amniotic fluid.

Addition of up to 20% blood, meconium, vaginal fluid or semen did not result in any significant differences in the rate of hydrolysis ($P>0.5$). No significant differences were found in the values of DPPC obtained with the fresh samples compared to those obtained with the frozen specimens subjected to three freeze–thaw cycles.

The efficiency of DPPC hydrolysis and extraction was determined by spiking amniotic fluid samples with known amounts of [^{14}C]DPPC and measuring the concentration of [^{14}C]DPG in the lower and upper phases. The recoveries obtained ranged between 92 and 98% as determined by HPTLC–autoradiographic analysis.

The lower limit of detection of DPPC in amniotic fluid, as determined by the method herein described, was 0.1 μg/ml with linear detector response extended to 24 μg/ml ($r=0.991$). The run-to-run and day-to-day precision was 4.2 and 6.1%, respectively, and analysis time was 10 min.

In summary, analysis of DPPC by enzymatic hydrolysis and HPLC provides a high-precision and fast turnaround time method for the determination of the concentration of DPPC in amniotic fluid. Further studies are now in progress to determine the efficacy of this method in the prediction of RDS.

Acknowledgments

We are indebted to Dr. Gary Horowitz, Director of the Chemistry Laboratory at Beth Israel Hospital for agreeing to provide aliquots of amniotic fluid for this study. We would also like to thank Ms. Bernie Williams and Mr. Corey Ferreira for coordinating sample collection and for helping in the processing of the amniotic fluid samples.

References

[1] L. Gluck, M.W. Kulovich, R.C. Borer, P.H. Brenner, G.C. Anderson and W.N. Spellacy, Am. J. Obstet. Gynecol., 109 (1971) 440.
[2] M. Hallman, M.W. Kulovich, E. Kirkpatrick, R.G. Sugarman and L. Gluck, Am. J. Obstet. Gynecol., 125 (1976) 613.
[3] Amnxostat-FLM, Irvine Scientific, Irvine, CA.
[4] J.A. Clements, A.C.G. Platzker and D.F. Tierney, N. Engl. J. Med., 286 (1972) 1077.
[5] G. Sher and B.E. Statland, Obstet. Gynecol., 61 (1983) 444.
[6] A.H. Moawad, M.A. Ismail and L.P. River, J. Reprod. Med., 36 (1991) 228.
[7] V.A. Khouzami, J.C. Beck, H. Sullivant and J.W. Johnson, Am. J. Obstet. Gynecol., 147 (1983) 552.
[8] S.B. Dubin, Clin. Chem., 35 (1989) 612.
[9] E.R. Ashwood, R.G. Oldroyd and S.E. Palmer, Obstet. Gynecol., 75 (1990) 289.
[10] L.J. Bowie, J. Shammo, J.C. Dohnal, E. Farrell and M.V. Vye, Am. J. Clin. Pathol., 95 (1991) 781.
[11] E.S. Pearlman, J.M. Baiocchi, J.A. Lease, J. Gilbert and J.H. Cooper, Am. J. Clin. Pathol., 95 (1991) 778.
[12] M. Shinitzky, A. Goldfisher, A. Bruck, B. Goldman, E. Stern, G. Barkai, E. Stern, G. Barkai, S. Mashiach and D.M. Serr, Br. J. Obstet. Gynaecol., 38 (1976) 38.
[13] E.R. Ashwood, S.E. Palmer and R.R. Lenke, Obstet. Gynecol., 80 (1992) 1048.
[14] E.R. Ashwood, J.F. Tait, C.A. Foerder, R.W. Franklin and T.J. Benedetti, Clin. Chem., 32 (1986) 260.
[15] J.C. Russell, Clin. Chem., 33 (1987) 1177.
[16] J. Torday, L. Carson and E.E. Lawson, N. Engl. J. Med., 301 (1979) 1013.
[17] P.M. Farrell and M.E. Avery, Am. Rev. Resp. Dis., 111 (1975) 657.
[18] J.G. Alvarez, B. Slomovic and J. Ludmir, J. Chromatogr. B, 665 (1995) 79.
[19] J.G. Alvarez, D.K. Richardson and J. Ludmir, Obstet. Gynecol., 87 (1996) 429.
[20] J.G. Alvarez and J.C. Touchstone, J. Chromatogr., 577 (1992) 142.