Measurement of the Ratio of Lecithin to Sphingomyelin in Amniotic Fluid by Fast Atom Bombardment Mass Spectrometry

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We describe a fast atom bombardment mass spectrometric method to determine the ratio of lecithin to sphingomyelin in amniotic fluid. Between m/z 400 and 1000, amniotic fluid extracts showed clear peaks for only sphingomyelin and lecithin. The molecular species of fatty acid in the phospholipids were analyzed quantitatively and quickly in a small volume of the extracts. The dipalmitoyllecithin/palmitoylsphingomyelin ratio, determined by the proposed method, showed a reasonable correlation to the lecithin/sphingomyelin ratio measured by thin-layer chromatography. This method allows rapid and sensitive measurement of these phospholipids.

Additional Keyphrases: phospholipids • fatty acids • fetal status • thin-layer chromatography compared

Measuring phospholipids in amniotic fluid is important for estimating fetal lung maturity (1–6). Thin-layer chromatography (TLC) has been used for this, but it is time consuming and not suitable for distinguishing molecular species in phospholipids. Mass spectrometry (MS) has recently played a considerable role in chemical measurements in hospital laboratories, especially for the diagnosis of metabolic diseases. In addition to gas chromatography–mass spectrometry for screening tests of inborn errors of metabolism, fast atom bombardment mass spectrometry (FAB-MS), with and without tandem mass spectrometry, has been used to analyze nonvolatile larger molecules. FAB-MS is a soft ionization technique used to detect signals corresponding to intact molecules. Applications of FAB-MS to clinical materials in new diagnostic procedures have increased, along with improvement of the method's sensitivity, accuracy, and operational ease. FAB-MS generates metabolic profiles from body fluids with a speed and convenience unrivaled by other methods. Indeed, the discovery and initial characterization of disease-specific acylcarnitines in urine by Millington et al. (7) was dependent on FAB-MS. We have now analyzed phospholipids in amniotic fluid by FAB-MS. This method is simpler and quicker than conventional TLC and can determine fatty-acid species in phospholipid molecules. Quantitative data regarding fatty acids in phospholipids, along with the specific numbers of carbon atoms and double bonds, may be more useful in the diagnosis of fetal lung maturity than are the data by TLC.

Materials and Methods

Reagents

The following reagents were purchased from Sigma Chemical Co., St. Louis, MO: L-dipalmitoyl phosphatidylcholine (lecithin), dioleoyllecithin, dimonoylelecithin, distearoyllecithin, 2-oleyl-3-palmitoyllecithin, and egg yolk sphingomyelin; L-lecithin/sphingomyelin standard solution, a mixture of egg yolk lecithin and bovine brain sphingomyelin; L/S-10 (L-lecithin, 1 g/L, and sphingomyelin, 1 g/L in chloroform/methanol, 1/1 by vol); L/S-20 (L-lecithin, 2 g/L; sphingomyelin, 1 g/L); L/S-30 (L-lecithin, 3 g/L; sphingomyelin, 1 g/L); and L/S-40 (L-lecithin, 4 g/L; sphingomyelin, 1 g/L).

Deuterium-labeled dipalmitoyllecithin, L-3-dipalmitoyl-16,16,16-D3-2H2phosphatidylcholine (d6-DPL), was obtained from MSD Isotopes (Merck, Montreal, Canada).

Procedures

Extraction of phospholipid from amniotic fluid: Amniotic fluid phospholipids were extracted according to the method of Gluck et al. (1). The ratio of lecithin to sphingomyelin (L/S) was measured by TLC of a cold acetone-insoluble fraction of the chloroform/methanol extract from amniotic fluid (1). We also used this fraction to measure phospholipids by FAB-MS, taking 2 mL of amniotic fluid for extraction for TLC and FAB-MS analysis. We used ~5% of the extract for FAB-MS analysis and most of the rest for TLC analysis. When we used the extract for FAB-MS analysis only, we mixed 0.3 mL of amniotic fluid with 3 μg of d6-DPL (added before extraction as an internal standard). The extract was dissolved in 10 μL of methanol, of which 1 μL was used for each FAB-MS analysis. The extraction procedure took 20 min, the FAB-MS analysis 10 min. Gestational age was determined by counting from the first day of the last menstrual period.

Preparation of lecithin and sphingomyelin fractions by TLC: Amniotic fluid, 3 mL from a pregnant woman at 32 weeks of gestation, was extracted with methanol/chloroform (1/2 by vol). The cold-acetone precipitate of the extract was fractionated by TLC. After developing the chromatographic plate, we detected the lecithin and sphingomyelin fractions by staining the TLC strip. Each

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1 Nonstandard abbreviations: TLC, thin-layer chromatography; FAB-MS, fast atom bombardment mass spectrometry; DPL, dipalmitoyllecithin; PS, palmitoylsphingomyelin; d6-DPL, L-3-dipalmitoyl-16,16,16-D3-2H2phosphatidylcholine; and L/S, total lecithin/total sphingomyelin ratio.

Editor's note (added in proof): A related paper was published in this journal in 1983 (29:1349–53). The earlier paper is no longer in some current databases of the medical literature and thus may be overlooked. The two papers are complementary, each with information not available in the other. See also the related editorial in this issue (pp 1321–2).

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fraction was then collected by scraping off the appropriate areas of the plate and was extracted from the silica scrapings into chloroform/methanol. Extracts were dried under nitrogen gas and dissolved in 30 μL of methanol, with 1 μL of this being used for FAB-MS analysis.

The cold acetone-soluble fraction was also dried under N₂ gas and dissolved in methanol for analysis by FAB-MS.

Mass spectrometric analysis: The FAB-MS system used in this study was a JEOL (Tokyo, Japan) JMS DX-300 mass spectrometer, with a JMA 3100 mass data analysis system. Positive-ion FAB spectra were obtained by magnetic field scan. Xenon gas was used, the accelerating voltage was 3 kV, and the energy of the xenon atom bombardment beam was +3 keV. A range of \( m/z \) 400–1000 was scanned in a 10-s sweep, with 30 repetitions. The spectra of 30 scans were averaged for quantitative analysis. The overscaled peaks of the ions of interest were omitted from the calculation. The average value and ratio of the designated peaks were calculated by a computer algorithm provided in the JMA 300 (JEOL) mass data analysis system.

For standardization, authentic phospholipids were dissolved in methanol at 1 g/L; 1 μL of each standard was used for each analysis. We also assayed 1 μL of unaltered Sigma L/S standard solutions in each analysis. For calibration, we prepared 1 g/L methanolic solutions of DPL, palmitoylsphingomyelin (PS), and \( \delta_\mathrm{6} \)-DPL. DPL and PS solutions were mixed in several ratios to make a standard DPL/PS ratio line. We mixed DPL and \( \delta_\mathrm{6} \)-DPL solutions in several ratios to make a standard curve for the quantitative assay of DPL. We used 1 μL of the mixture for each FAB-MS analysis. A methanolic solution of authentic phospholipids or amniotic extracts was mixed with an approximately equal volume of glycerol matrix on the FAB-MS target.

Results

FAB-MS spectra of amniotic extracts showed a simple pattern. Peaks at \( m/z \) 480, 496, 703, 706, 734, 760, and 788 were prominent (Figure 1). These peaks were accompanied by ions 2 amu smaller, which corresponded to homologous molecules having one additional double bond (Figure 1). No other peaks of notable height were detected. The sphingomyelin fraction separated by preparative TLC showed a peak mainly at \( m/z \) 703 (Figure 2b). The lecithin fraction showed peaks at \( m/z \) 678, 706, 720, 732, 734, 758, 760, 768, and 788 (Figure 2a). These peaks were compared with authentic lecithin and sphingomyelin; typical spectra are shown in Figure 4. The peak at \( m/z \) 703 was the MH⁺ ion of PS. The identification of peaks corresponding to lecithin was as follows: the highest peak, at \( m/z \) 734, was the MH⁺ ion of DPL, C₁₆₀–₁₇₀, which means that two fatty acid molecules in lecithin consisted of 16 carbons with no unsaturated bond (expressed for convenience as C₉₂₀). This convention for symbols is used below. The other ions in the lecithin spectrum were \( m/z \) 678, C₃₂₀; \( m/z \) 706, C₃₀₀; \( m/z \) 720, C₃₁₀; \( m/z \) 732, C₃₂₁; \( m/z \) 760, C₃₄₁; \( m/z \) 786, C₃₆₂; and \( m/z \) 788, C₃₈₁.

The peaks at \( m/z \) 480 and 496 were fragment ions of DPL, which were detected in the spectra of the lecithin fraction of amniotic fluid and also in authentic DPL. All peaks detected in the sample before fractionation were assigned to phospholipids. Other molecules contained in the extracts showed no clear peaks because amniotic fluid does not contain high concentrations of polar substances having molecular masses in this range. The amount of phospholipid in the cold acetone-soluble fraction was low, and only unsaturated lecithin peaks were detected (Figure 2c).

We measured the ratio of the MH⁺ form of DPL, \( m/z \) 784, to the MH⁺ ion of PS, \( m/z \) 703, in the extract from amniotic fluid and calculated the mass ratio of DPL/PS by reference to a standard curve. DPL is the main molecular species of lecithin in amniotic fluid, especially late in gestation. Palmitoylsphingomyelin is also a major molecular species of sphingomyelin in amniotic fluid. Therefore, the DPL/PS ratio is not exactly the
same as the ratio of total lecithin to total sphingomyelin (L/S) determined by TLC. The values of these ratios were different but correlated, as shown in Figure 3.

**Standard curves and linearity:** The FAB-MS analysis with the Sigma standard solution L/S-10 showed peaks at m/z 703, 731, 734, 758, 760, 786, 788, and 813, and cluster ions of these peaks (Figure 4c). The peaks were identified by comparing the relative peak heights of different L/S standard solutions and by the m/z values. The highest peak in L/S-10 was at m/z 760, which corresponded to lecithin (C_{16:0-18:1} or C_{16:1-18:0}). The second highest peak was at m/z 731, which corresponded to stearoylsphingomyelin (C_{18:0}) and was about 55% of the height at 760 (Figure 4c). The ratio of the peak heights for m/z 760/731 (γ) was plotted against the L/S ratio (x) of each standard solution (data not shown). Linear regression of the results gave y = 0.165 + 1.808 x (S_γ = 0.154, r = 0.998).

The ratio (γ) of the peak height of the MH⁺ ion of DPL, m/z 734, to the MH⁺ ion of PS, m/z 703, was measured by FAB-MS with the DPL/PS standard solutions we prepared; the standard curve is shown in Figure 5 (top). Linear regression gave y = 0.213 + 2.423x (S_γ = 0.240, r = 0.997).

The ratio of the peak height of the MH⁺ ion of DPL, m/z 734, to the MH⁺ ion of d₄-DPL, m/z 740, was measured by FAB-MS with the DPL/d₄-DPL standard solutions (Figure 5, bottom); linear regression gave y = 0.05 + 1.895x (S_γ = 0.07, r = 0.999).

**The coefficient of variation (CV) of the assay:** In each FAB-MS scan, marked changes in the appearance of the spectra were observed. The difference between the lowest and highest values of an ion peak was about 3% to 15%, depending on the condition of the apparatus and the sample concentration. The peak height for an ion varied, with an irregular amplitude and a high frequency around a constant value. Still, the average height of 30 repeated scans was constant. We measured the mixture of DPL and d₄-DPL in the mass ratio of 2.0.

**Fig. 3.** Comparison of the L/S ratio as measured by TLC and the DPL/PS ratio as measured by FAB-MS from extracts of amniotic fluid. Broken lines indicate cutoff values for fetal lung maturity.

![Fig. 3](image)

**Fig. 4.** FAB-MS spectra of authentic dipalmitoyllecithin (a), sphingomyelin from egg yolk (b), and Sigma L/S standard solution, L/S-10 (c).

The ratio of the MH⁺ ion of DPL, m/z 734, to the MH⁺ ion of d₄-DPL, m/z 740, was calculated from the average of 30 scans of each measurement; the CV of the averages was 1.5% in continuous assays (n = 10) and 2.1% in assays on different days (n = 10). The variation of DPL/d₄-DPL in assays of amniotic fluid extracts were similar to those in assays of the standard mixture. In amniotic fluid, d₄-DPL was added before extraction. We converted the DPL/d₄-DPL ratio to the concentration of DPL in amniotic fluid by using a standard curve. The concentration of DPL in a sample of amniotic fluid was 48.8 mg/L (CV 2.3%), which is the mean of 10 measurements made on one day. The data from different days had a mean of 47.0 mg/L (CV 2.0%, n = 10). With a sample of low concentration, the intra-assay mean was 61 mg/L (CV 3.0%); the inter assay values were mean 5.8 mg/L, CV 3.9% (n = 10 in each assay). The CV of the peak ratio of DPL/PS without a stable isotope was greater than that of molecular isotopes. The DPL/PS ratio of a sample extracted from amniotic fluid had an intra-assay mean of 4.5 (CV 15%, n = 10), whereas the interassay value was 4.7 (CV 16%, n = 10). The DPL/PS ratio of a sample at a lower concentration had an intra-assay mean of 0.95 (CV 14%, n = 10) and the interassay value was 1.02 (CV 17%, n = 10). To assess the FAB-MS method, we determined precision for repeated measurements of a single amniotic fluid extract. The variation caused by the extraction procedure was not included in these experiments.

**Comparison of FAB-MS and TLC:** The data obtained by the two methods are compared in Figure 3. There was a linear relationship between the DPL/PS mass ratio
measured by FAB-MS and the L/S ratio determined by TLC. Linear regression gave $y = -0.656 + 0.772x$ ($r = 0.963$, $n = 29$). The standard deviation about the regression line ($S_y$) was 0.460.

Correlation of DPL/PS with gestational age: In Figure 6, spectra with mature and immature surfactant values are shown. Figure 6a shows a typical FAB-MS spectrum of immature surfactant and Figure 6b shows a spectrum of mature surfactant. The m/z 734/703 ratio and the peak height of lecithin with unsaturated fatty acids (e.g., m/z 760, 786, 814) clearly showed mature and immature patterns. In Figure 7, the relationship between DPL/PS and gestational age is plotted. The ratio gradually increased with age and this trend accelerated after 33 weeks.

Discussion

FAB-MS spectra with chloroform/methanol extracts from amniotic fluid showed only a few peaks, all of which were assigned to phospholipids. By FAB-MS, fatty acid species in phospholipids could be measured quantitatively with a small sample. There was a good linear relationship between the peak-height ratios measured by FAB-MS and the mass ratios in standard solutions. Because we used a stable isotope as an internal standard, the CV of the assay was excellent, as shown by the DPL data (<3%). The CV of the DPL/PS ratio measured by FAB-MS without a stable isotope may be similar to, or somewhat worse than, that by TLC. With a stable isotope of PS, which is not available at present, we might improve the CV of the PS assay and thus also the CV of the DPL/PS ratio.

Differences between the DPL/PS ratios measured by FAB-MS and the L/S ratios measured by TLC may have been caused by differences in the contents of fatty acid species in each sample. A review of the literature shows that an L/S ratio >2 is associated with mature fetal lung. An L/S ratio of 2.0 roughly corresponds to a DPL/PS of 1.0. The dashed line in Figure 3 represents a tentative cutoff point.

DPL, a major molecular species in mature surfactant, is a better indicator of lung maturity than is total lecithin. We measured the DPL concentration in amni-
otic fluids by FAB-MS with $d_4$-DPL as an internal standard, which allowed precise quantitative analysis. The DPL values of 10 amniotic fluid samples ranged from 2 to 65 mg/L and correlated with gestational age and DPL/PS. However, the correlation between DPL concentration and gestational age was weaker than that between DPL/PS and gestational age. The best indicator of lung maturity may well be DPL/PS as measured by FAB-MS with stable isotopes of DPL and PS.

The slope of the DPL/$d_4$-DPL standard curve was 1.896. The discrepancy from the theoretical predicted slope of 1.0 may have arisen from the effects of moisture or impurities in the stable isotope or from disintegration of the isotope after the solution was made. Nevertheless, if samples of unlabeled DPL of high purity are weighed accurately, the standard curve for quantification of DPL thus obtained is reliable, even if its slope is greater than predicted.

Serum and blood cells contain considerable amounts of lecithin. The main molecular species of lecithin in blood consists of one C_{16} and one C_{18} fatty acid with an unsaturated bond, and two C_{18} fatty acids with an unsaturated bond. Dipalmitoylecithin is a minor molecular species of lecithin in blood. In vaginal exudate, concentrations of phospholipids were lower than in blood; this material also contained lecithin with larger fatty acid molecules with unsaturated bonds. The percentage of sphingomyelin was low in blood and also in vaginal exudate. Contamination by these materials would not greatly influence the DPL/PS data from amniotic fluid. However, a large contamination by blood or vaginal exudate could lower the sensitivity of the measurement by interfering with ionization. In such cases additional cleanup procedures might be necessary.

Phosphatidylglycerol, which is also an important indicator of lung maturity (8), showed no or only small peaks on FAB-MS analysis. Consistently detectable phosphatidylglycerol peaks would probably be measured by tandem mass spectrometry.

In the near future, various nonvolatile substances with low and high molecular masses will be analyzed by FAB-MS for clinical purposes, and the required apparatus will become easier to use. A lower-priced apparatus would be very useful, even if its applications were limited.

References