Fetal Lung Maturity, as Assessed by Gas–Liquid Chromatographic Determination of Phospholipid Palmitic Acid in Amniotic Fluid

M. P. C. Ip,1,2 T. F. Draisey,1,2,4 R. J. Thibert,1,2,4 G. L. Gagneja,2,4 and G. M. Jasey3

We describe a new and specific method for measurement of lecithin palmitic acid in amniotic fluid. Dipalmitoyl lecithin, the major alveolar surfactant, has previously been estimated by measuring the lecithin–sphingomyelin ratio, total lecithin, total phospholipid phosphorus, and (or) total palmitic acid. Our method is more specific for estimation of dipalmitoyl lecithin, because nonphospholipid sources of palmitic acid are removed by solvent extraction. Using a hexane/2-propanol/sulfuric acid system, we obviated the major interferences from triglycerides and free fatty acids. The palmitic acid derived from the phospholipid fraction is measured by gas–liquid chromatography of its methyl ester. No contribution appears to be made by sphingomyelin palmitic acid—probably owing to the mild hydrolysis conditions. The measured palmitic acid therefore appears to be derived from lecithins, principally dipalmitoyl lecithin. The value for palmitic acid determined by this method correlates well with the lecithin–sphingomyelin ratio and total phospholipid phosphorus. Infants are unlikely to develop respiratory distress syndrome when the measured palmitic acid in amniotic fluid exceeds 8.0 mg/liter, which corresponds to an lecithin–sphingomyelin ratio of 2.0.

Additional Keyphrases: respiratory distress syndrome • surfactant in fetal lung • phospholipids • inter-method comparison • diagnostic aids • lipids • newborns

The lecithin/sphingomyelin ratio in amniotic fluid has been widely used for estimating fetal lung maturity ever since it became evident that part of the amniotic fluid originates from pulmonary secretions (1–3). The use and significance of this ratio method has been widely reviewed and modified (4–10). Alternatively, lecithin can also be measured as palmitic acid, because dipalmitoyl lecithin is the most predominant component of surface-active lecithin at the later stages of gestation (11).

Several proposed methods for measurement of amniotic fluid palmitic acid content by gas–liquid chromatography have been described (12–16). Chloroform/methanol extraction [Folch extract (17)] was invariably used. Experiments done in this laboratory show that “co-extracted” nonphospholipid substances, such as triglycerides and free fatty acids, which contain palmitic acid residues and are present in significant amounts in amniotic fluid, do contribute, increasing the measured value for palmitic acid.

This report describes a more specific method for estimating dipalmitoyl lecithin. Nonphospholipid fatty materials are removed by solvent extraction before the palmitic acid is measured in the extracted phospholipids. We also describe an attempt to define the relationship of results by this new method to those by the lecithin–sphingomyelin ratio method and total phospholipid phosphorus measurement which are both widely accepted as good methods for assessment of fetal lung maturity.

Materials and Methods

Samples

Sixty amniotic fluid specimens were obtained from 60 different patients by amniocentesis or at normal delivery. In five cases, delivery was effected by cesarean section. Of all the children born, two weighed less than 2500 g. Four samples were obtained at term from four patients having complications from toxemia or mild diabetes. One patient was mentally retarded; the gestational age is uncertain. Gestational age was calculated from the last day of the menstrual period and was confirmed by the obstetric data obtained at birth by the attending obstetrician. Once obtained, the amniotic fluid samples were centrifuged at 250 × g for 5 min. The supernatant fluid, which was free from cell debris or other sediment, was separated and kept frozen at −20 °C in sterile test tubes until use.

Reagents

All chemicals used were reagent grade, and they were all used without further purification. These include hexane, 2-propanol, heptane, n-nonane, absolute methanol, chloroform, petroleum ether (bp 37.5–42.9
°C) and sulfuric acid. Boron trifluoride (125 g/liter in methanol) was obtained from Matheson Coleman and Bell, Norwood, Ohio 45212; tripalmitin from Mann Research Laboratories Inc., New York, N.Y. 10006; and L-α-lecithin (β-γ-dipalmitoyl) from Calbiochem, La Jolla, Calif. 92037. Other materials required for this study (sphingomyelin, palmitic acid, and linoleic acid) were purchased from Sigma Chemical Co., St. Louis, Mo. 63178. A 0.5 mol/liter methanolic sodium hydride solution was prepared by dissolving 10.0 g of sodium hydride in 500 ml of methanol. The clear solution obtained after filtering was stable for at least three months.

Procedure

Phospholipid was extracted from the centrifuged amniotic fluid sample (2.0 ml) with 8.0 ml of hexane, 14.0 ml of 2-propanol, and 4.0 ml of 40 mmol/liter H₂SO₄. To the 2-propanol (lower) layer, 0.4 ml of a 100 \( \text{mg/liter} \) solution of linoleic acid (40 \( \mu \text{g} \)) was added as an internal standard. The mixture was heated on a hot plate for 3 min after the addition of 4.0 ml of 0.5 mol/liter methanolic sodium hydride and then boiled for 5 min after adding 5.0 ml of boron trifluoride in methanol (125 g/liter). About 15 ml of saturated sodium chloride solution was added to the mixture and the suspension was extracted with an equal volume of petroleum ether. The petroleum ether layer was then evaporated at room temperature in a "Vapo-Vent." The residue was reconstituted with two drops of methanol and the palmitic acid ester concentration of this solution was analyzed by gas–liquid chromatography. A glass column containing 10% diethylene glycol succinate on Chromosorb W (100 mesh) was used on a L-M Series 3000 Hydro-Flow system Gas Liquid Chromatograph under an isothermal condition of 135 °C. The injector port was set at 242 °C and the detector (flame ionization) was maintained at 248 °C. The carrier (N₂) flow rate was set at 49 ml/min. The palmitic acid ester was expressed in milligrams of amniotic fluid phospholipid palmitic acid per liter, from a linear calibration curve of palmitic acid concentration (in micrograms) vs. the ratio of the palmitic acid and linoleic acid peak areas.

Calculations

The ratio of the palmitic acid/linoleic acid peak was calculated from the chromatogram. The palmitic acid value (as \( y \) micrograms) was interpreted from the calibration curve, which ranged from 0.5 to 50.0 \( \mu \text{g} \) of palmitic acid. The phospholipid palmitic acid content in amniotic fluid was expressed in mg/liter by using the following equation:

\[
\text{Concentration of palmitic acid} = \frac{y \text{ micrograms}}{2.0 \text{ ml of amniotic fluid used}} = \frac{y}{2.0} \text{ mg/liter}
\]

### Table 1. Amount of Selected Lipid Components in Human Amniotic Fluid at 40 Weeks of Normal Pregnancy

<table>
<thead>
<tr>
<th>Lipid components</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Mean value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipids ( a ) (mg/liter)</td>
<td>43.8</td>
<td>37.5</td>
<td>35.0</td>
<td>61.3</td>
<td>49.3</td>
</tr>
<tr>
<td>Mono-, di- and triglycerides ( a ) (mg/liter)</td>
<td>81.7</td>
<td>42.6</td>
<td>62.5</td>
<td>74.3</td>
<td>103.1</td>
</tr>
<tr>
<td>Free fatty acids ( a ) (mmol/liter)</td>
<td>0.73</td>
<td>0.21</td>
<td>0.18</td>
<td>0.44</td>
<td>0.37</td>
</tr>
</tbody>
</table>

\( a \) The results are the mean of two gas–chromatographic measurements made on each sample.

\( b \) Estimated as total phospholipid phosphorus × 25.

\( c \) The modified method of Royer and Ko as described by Moses et al. (18) was used, except that we used 3.0 ml of amniotic fluid instead of 0.3 ml of serum. The volume of 2-propanol and "Lipo-Flax" columns used were increased by 10-fold, to adjust for the larger sample size. The 2-propanol eluate was concentrated to 3.7 ml before color development.

\( d \) Determined by the method of Trout et al. (19).
Results

The calibration curve for palmitic acid (in micrograms added) vs. the peak ratio of the palmitic and linoleic acid esters (representing 40 micrograms of linoleic acid as an internal standard) is a straight line passing through the origin. Figure 1 illustrates a typical chromatogram of the phospholipid fatty acid esters in amniotic fluid.

The relative concentrations of phospholipids, triglycerides (triacylglycerols), and free fatty acids in seven different patients are shown in Table 1. The patients selected for this group are apparently healthy subjects with no previous history of stillbirth, diabetes, or other complications. The mean values obtained for phospholipids, triglycerides, and free fatty acids from these random selections are 51.0 ± 16.2 mg/liter, 68.6 ± 25.0 mg/liter, and 0.40 ± 0.21 mmol/liter, respectively.

The importance of the palmitic acid from both triglycerides and free fatty acids is indicated in Table 2 by comparing the present method with a selected gas-liquid chromatography method (13) where both triglycerides and free fatty acids are extracted with the phospholipids. The two methods showed a remarkable difference in the measured palmitic acid concentrations, which amounts to as much as 42.9% in patient II. The other patients studied all show a decrease of at least 19.3% by the present method as compared with the comparison method (13).

The extraction efficiencies of different solvent systems for lipids are given in Table 3. Lecithin, tripalmitin, and palmitic acid (all in 15.0 mg/liter solutions) were dissolved in either chloroform or 2-propanol, according to solvent requirements. Chloroform/methanol [Folch extract (17)] is the only solvent system with which extraction is quantitative for all three classes of lipids. With the hexane system 86.8% of the phospholipids is extracted; 96.2% and 83.7% of triglycerides and palmitic acid, respectively, are isolated.

Blood components from two different healthy subjects were added to two different specimens of amniotic fluid (Table 4). There was no appreciable increase in measured palmitic acid when serum or hemolyzed serum components were present in the amniotic fluid. The presence of whole blood (25% by volume) in amniotic fluid, however, tremendously increases values for phospholipid palmitic acid.

We studied the correlation between phospholipid palmitic acid (y) and lecithin–sphingomyelin ratio (x) with 60 different samples. The correlation coefficient is 0.96 and the regression equation is y = 1.44 x + 0.90. Phospholipid palmitic acid as determined by this method also correlates well (n = 36) with total phospholipid phosphorus; the correlation coefficient was 0.82 and the regression line equation is y = 0.20 x + 0.00.

---

**Table 2. Amniotic Fluid Phospholipid Palmitic Acid, as Determined by Two Gas-Chromatographic Methods**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Comparison method</th>
<th>Present method</th>
<th>Difference, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>10.0 ± 0.60</td>
<td>6.7 ± 0.30</td>
<td>33.0</td>
</tr>
<tr>
<td>II</td>
<td>11.2 ± 0.10</td>
<td>6.4 ± 0.10</td>
<td>42.9</td>
</tr>
<tr>
<td>III</td>
<td>10.9 ± 0.40</td>
<td>8.8 ± 0.30</td>
<td>19.3</td>
</tr>
<tr>
<td>IV</td>
<td>19.1 ± 0.50</td>
<td>12.5 ± 0.50</td>
<td>34.6</td>
</tr>
</tbody>
</table>

* Data presented represents mean of duplicates ± SD; each measurement was made in duplicate.
* Method of Warren et al. (13).

---

**Table 3. Efficiency of Different Solvent Systems for Extracting Phospholipids, Triglycerides, and Free Palmitic Acid**

<table>
<thead>
<tr>
<th>Solvent systems</th>
<th>CHCl₃/MEOH</th>
<th>CHCl₃ layer</th>
<th>MeOH layer</th>
<th>Hexane/2-propanol</th>
<th>hexane layer</th>
<th>2-propanol layer</th>
<th>Heptane/2-propanol</th>
<th>heptane layer</th>
<th>2-propanol layer</th>
<th>Nonane/2-propanol</th>
<th>nonane layer</th>
<th>2-propanol layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPA lecithin</td>
<td>100.0ᵦᵥ</td>
<td>100.0ᵦᵥ</td>
<td>100.0ᵦᵥ</td>
<td>13.2ᵦ</td>
<td>96.2ᵦ</td>
<td>86.8ᵦ</td>
<td>18.5ᵦ</td>
<td>97.5ᵦ</td>
<td>81.5ᵦ</td>
<td>9.3ᵦ</td>
<td>98.5ᵦ</td>
<td>90.7ᵦ</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>10.0ᵦᵥ</td>
<td>10.0ᵦᵥ</td>
<td>10.0ᵦᵥ</td>
<td>0.0ᵦ</td>
<td>0.0ᵦ</td>
<td>0.0ᵦ</td>
<td>0.0ᵦ</td>
<td>0.0ᵦ</td>
<td>0.0ᵦ</td>
<td>0.0ᵦ</td>
<td>0.0ᵦ</td>
<td>0.0ᵦ</td>
</tr>
</tbody>
</table>

* All data included in this table were obtained by the present method.
* Percent distribution calculated by comparing the palmitic acid content present in each layer.
* Folch extract (17) CHCl₃/MEOH/H₂O = 2/1/1. "MEOH" is methanol.
* No palmitic acid detected in the MeOH layer.
* Results represent the average of duplicate determinations on each sample; each measurement was made in duplicate.
* Actual solvent composition: hydrocarbon/2-propanol/H₂SO₄ (0.04 mol/liter)/H₂O = 4/1/2/1.
* The results represent the average of two gas-chromatographic measurements on each sample.

---

CLINICAL CHEMISTRY, Vol. 23, No. 1, 1977 37
Table 4. Effect of Presence of Blood Components on Results for Phospholipid Palmitic Acid in Amniotic Fluid

<table>
<thead>
<tr>
<th>Blood component mixture added (% by vol)</th>
<th>Patient I</th>
<th>Patient II</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Palmitic acid measured, mg/liter</td>
<td>% change</td>
</tr>
<tr>
<td>Control</td>
<td>11.8</td>
<td>—</td>
</tr>
<tr>
<td>Whole blood (25%)</td>
<td>23.0</td>
<td>95.0</td>
</tr>
<tr>
<td>Serum (25%)</td>
<td>12.3</td>
<td>4.5</td>
</tr>
<tr>
<td>Serum from hemolyzed blood (25%)</td>
<td>12.7</td>
<td>8.3</td>
</tr>
</tbody>
</table>

* Results represent the average of duplicate determinations.
* Blood components used on patient I and II were from different sources.
* Blood caused to hemolyze by forcing it through a syringe.

Figure 2 shows the significance of changes in phospholipid palmitic acid contents at different stages of gestation. Of the 59 samples studied from patients with gestational age known—which include three cases of toxemia and one case of borderline diabetes—only one of the infants developed signs of respiratory distress syndrome (marked with a diamond symbol in Figure 2). However, this particular infant, who was premature, did soon regain good health. This infant, weighing 2500 g, was delivered at term; the phospholipid palmitic acid content of the amniotic fluid measured 3.5 mg/liter, its lecithin—sphingomyelin ratio was 1.01, and the total phospholipid phosphorus was 0.5 mg/liter. The other infant born weighing less than 2500 g also showed a low palmitic acid value, 3.0 mg/liter; its lecithin—sphingomyelin ratio was 1.30 and total phospholipid phosphorus 0.6 mg/liter.

In general, the phospholipid palmitic acid in amniotic fluid increases significantly after 37 weeks of gestation. Samples from patients having gestational periods of less than 35 weeks all showed phospholipid palmitic acid values of less than 4.5 mg/liter. An occasional value of 4.5 mg/liter or less was also obtained from subjects at 35 to 40 weeks of gestation. The maximum value for phospholipid palmitic acid also tends to increase, and the range of values to broaden, with duration of gestation.

Discussion

Our findings listed in Table 1 show that free fatty acids and mono-, di-, and triglycerides in amniotic fluid had mean concentrations (±SD) of 0.40 ± 0.21 mmol/liter (102.6 ± 53.8 mg/liter, expressed in terms of palmitic acid) and 68.6 ± 25.0 mg/liter, respectively. These concentrations are considerable when compared with the mean value of 51.0 ± 16.2 mg/liter for phospholipids; particularly when we are estimating phospholipids by their content of palmitic acid, which is also present in triglycerides to an extent that may reach 25% of their mean molecular weight (20).

Analytical recoveries performed with different amounts of dipalmitoyl lecithin (5 to 30 μg) added to 2.0 ml of amniotic fluid, averaged 55.6% (i.e., 44.4% losses) and ranged from 49.6 to 58.3%. Because the results given in Table 3 indicate only a 13.2% loss of lecithin during the extraction process, the remaining 31.2% losses must be incurred in the saponification step. This is comparable to the 22.7 to 29.3% recovery losses reported elsewhere (13), which we believe is not really due to the extraction losses in chloroform/methanol (see Table 3), but rather to losses at the saponification stage on using a relatively mild alkaline hydrolysis.

The data in Table 2 further indicate that palmitic acid does come from other nonphospholipid sources. The major part of the discrepancies between the results of the two methods listed, which amount to 42.9% in
patient II (although this includes 13.2% extraction losses in our present method), must represent the palmitic acid in the nonphospholipid fraction.

The fatty acid compositions of different amniotic fluid phospholipids have been reported in detail (21). Either linoleic or heptadecanoic acid can be used as the internal standard. No isopropyl esters of the fatty acids listed in Figure 1 were detected, although esterification with boron trifluoride/methanol was done in a 2-propanol medium.

From Table 3, it is apparent that solvent systems that included heptane, hexane, or nonane all resulted in some phospholipid losses. However, these solvent systems are effective for the separation of triglycerides. We were unable to reach a 99.9% (mean value) separation of triglycerides as described in a recent report (22) by using a nonane/2-propanol/sulfuric acid solvent mixture despite the fact that we used the same solvent composition and ratio to amniotic fluid. In our hands, the nonane system was also not as good as reported (23) for the separation of phospholipids, because about 9.3% of the phospholipids was lost in the nonane layer. We recommend the use of hexane/2-propanol/sulfuric acid, because this is the solvent system that best separates phospholipids from the nonphospholipid fraction.

Whole-blood components severely interfere with the measurement of palmitic acid (Table 4); the large increase in measured palmitic acid is attributable to the presence of large amounts of palmitic acid in components of cellular constituents, and these must be eliminated from the sample before analysis.

For the evaluation of fetal lung maturity, results by the present method do correlate very well with those by the lecithin–sphingomyelin ratio method (r = 0.96), with palmitic acid being 8.0 mg/liter when the lecithin–sphingomyelin ratio equals 2.0; 8.0 mg of palmitic acid per liter corresponds to 11.5 mg of dipalmitoyl lecithin per liter. The lecithin value, when adjusted for a 13.2% extraction and a 31.2% hydrolysis loss, will be 20.7 mg of dipalmitoyl lecithin per liter of amniotic fluid. Bhagwani et al. (24) reported that at a concentration of 35.0 mg/liter or more for total lecithin, an infant is unlikely to develop the respiratory distress syndrome. Since about 65% of the total lecithin is surface active (25, 26), which represents 22.8 mg of dipalmitoyl lecithin per liter, this is very close to our reported value of 20.7 mg/liter. The purpose of the extraction process is to isolate phospholipids. Collectively, amniotic fluid phospholipids are found to contain a large variety of different phospholipids (27). The more important ones, such as sphingomyelin, which are reported to be stable in base (27) were not hydrolyzed under the mild hydrolyzing conditions we used (14). We observed no fatty acid peak when sphingomyelin, after hydrolysis and methylation, is used in the gas–liquid chromatographic procedure for measurement of palmitic acid.

The palmitic acid values also correlated well with total phospholipid phosphorus (r = 0.92). The 8.0 mg/liter concentration is equivalent to 1.6 mg of total phospholipid phosphorus per liter, which is close to the 1.4 mg/liter value reported by Nelson and Lawson (25), in which they recommended that a total phospholipid phosphorus of 1.4 mg/liter or greater be considered indicative of pulmonary maturity.

It would seem reasonable to assume that our present method actually measures of dipalmitoyl lecithin. A recent finding with primates (rhesus monkey) shows that dipalmitoyl lecithin synthesis (choline incorporation pathway) increases abruptly by at least twofold at approximately 90% of term (28). The significance of this sharp increase might perhaps be reflected in humans, as indicated in the scatter diagram given in Figure 2. Although occasional individual low values of <8.0 mg of palmitic acid per liter can still occur after 37 weeks of gestation, no example of respiratory distress syndrome was found when the measured value of palmitic acid exceeded 8.0 mg/liter.

Our method appears to be a reliable index of fetal pulmonary status. We are uncertain, from the available data, how the palmitic acid values are affected by toxemia, diabetes, or other disease states such as polyhydramnios, in which the overall volume of the amniotic fluid is affected. Nevertheless, our results are clinically significant. Further studies on palmitic acid and on creatinine, insulin, and cortisol changes in amniotic fluid in the later stages of gestation are being done in this laboratory; we hope eventually to draw up a maturity profile for use in high-risk pregnancies.

We thank Mr. E. Olivero and Mr. G. Moses for their assistance.

References