Enzymatic Colorimetry of Lecithin and Sphingomyelin in Aqueous Solution

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A procedure for the enzymatic determination of lecithin and sphingomyelin in aqueous solution is described. The phospholipids are first dissolved in chloroform:methanol (2:1 by vol), the solvent is evaporated, and the residue is redissolved in an aqueous zwitterionic detergent solution. The enzymatic reaction sequences of both assays involve hydrolysis of the phospholipids to produce choline, which is then oxidized to betaine, thus generating hydrogen peroxide. The hydrogen peroxide is subsequently utilized in the enzymatic coupling of 4-aminoantipyrine and sodium 2-hydroxy-3,5-dichlorobenzenesulfonate, an intensely red color being formed. The presence of a non-reacting phospholipid enhances the hydrolysis of the reacting phospholipid. Thus we added lecithin to the sphingomyelin standards and sphingomyelin to the lecithin standards. This precise procedure may be applicable to determination of lecithin and sphingomyelin in amniotic fluid.

Additional Keyphrases: potential procedure for evaluating fetal status · respiratory distress syndrome · enzymic methods · phospholipids · colorimetry · sensitive procedure for \( H_2O_2 \) · L/S ratio as determined by two procedures

Phospholipids in amniotic fluid have been measured by several different means. In most, the amniotic fluid is first extracted with a chloroform:methanol mixture such as that described by Folch et al. (1), the extract is then concentrated, and the phospholipids are separated by thin-layer chromatography. Measurement of the individual phospholipids may then be attempted by one of several procedures. These include digestion of the organic material, followed by phosphate determination (2), or staining (3) or charring (4) of the phospholipids, followed by densitometry (5) or planimetry (6). Perhaps the two most commonly measured phospholipids in amniotic fluid are lecithin and sphingomyelin. Information on the concentrations or ratio (L/S) of these are of some value in assessing fetal lung maturity and thus in predicting the risk of respiratory distress syndrome. As an alternative to these separation procedures, several methods have been described for the direct enzymatic determination of lecithin (7–9) and of total choline-containing phospholipids (9) in amniotic fluid.

We described here an enzymatic procedure for measuring lecithin and sphingomyelin in an aqueous solution. The procedure may be useful in determining the L/S ratio of amniotic fluid extracts. Previously described procedures for measurement of lecithin in amniotic fluid (8, 9) were somewhat more convenient than the one proposed here, in that they were direct and required no solvent extractions. Unfortunately, because concentrations of choline in the amniotic fluid are high relative to that of sphingomyelin, an extraction step must be included in this procedure, to reduce the blank reaction to acceptable levels.

The reaction sequence involved in our procedure is shown in Figure 1. Although we used pure phospholipid standards in developing this method, we also determined lecithin and sphingomyelin in several amniotic fluid samples, and the results lead us to believe that it may be readily applicable to the measurement of these phospholipids in extracts of biological specimens.

Materials and Methods

Apparatus

We used a Model 25 spectrophotometer (Beckman Instruments, Fullerton, CA 92634) and a Model 300N spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH 44074), both equipped with flow-through, thermostated microcuvettes.

Chemicals and Enzymes

Choline oxidase (from Arthrobacter globiformis; choline:oxygen 1-oxidoreductase; EC 1.1.3.17), alkaline phosphatase (from Escherichia coli; orthophosphoric-monoester phosphohydrolase, EC 3.1.3.1), and phospholipase D (from Streptomyces chromofuscus; phosphatidylincholine phosphatidohydrolase, EC 3.1.4.4) were obtained from Fermentas Biochemicals, Inc., Elk Grove Village, IL 60077.

Sphingomyelinase (from Bacillus cereus; sphingomyelin cholinephosphohydrolase, EC 3.1.4.12) was from Boehringer Mannheim Biochemicals, Indianapolis, IN 48250.

Peroxidase (from horseradish; hydrogen peroxide oxidoreductase; EC 1.11.1.7), phospholipase D (PL-D; from cabbage; phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4), Triton X-100, maleic acid, 4-aminoantipyrine, dipalmitoyl lecithin (synthetic), bovine-brain sphingomyelin, and bovine serum albumin were all from Sigma Chemical Co., St. Louis, MO 63178.

The "Zwittergent" detergents were from Calbiochem-Behring, La Jolla, CA 92037.

* "Zwittergent" is the registered trade name for the group of synthetic zwitterionic detergents known as sulfobetaines.

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Sodium 2-hydroxy-3,5-dichlorobenzenesulfonate (HDCBS) was obtained either from Research Organics, Inc., Cleveland, OH 44125, or Biosynthetic International, Skokie, IL 60077, or was prepared as previously described (8, 9). All other chemicals were reagent grade or better.

Reagent Preparation

Prepare a 48 mmol/L solution of Zwittergent 3-10 in deionized water. Stored at 4 °C, this reagent is stable for at least one month.

Prepare the lecithin and sphingomyelin standards by dissolving the appropriate amounts of lecithin and sphingomyelin in a small volume of 2:1 chloroform:methanol so that all lecithin standards contain 500 μmol of sphingomyelin per liter and all sphingomyelin standards contain 500 μmol of lecithin per liter. Next, evaporate the solvent in a water bath at 65 °C. (The evaporation may be speeded by blowing N2 across the mouths of the tubes.) Redissolve the residue in an appropriate volume of the 48 mmol/L Zwittergent 3-10 solution by alternately vortex-mixing and heating the solution in the 65 °C water bath several times until the solution is clear. Stored at 4 °C, these standards are stable for at least one week. Once refrigerated, the standards must again be heated in a 65 °C water bath and vortex-mixed before use.

Prepare the enzymatic lecithin reagent in a 50 mmol/L Tris maleate buffer, pH 7.0, to give the following concentrations per liter, the constituents being added in the order given here: bovine serum albumin, 1 g; PL-Dc, 10 kU; choline oxidase, 3 kU; peroxidase, 10 kU; calcium chloride, 15 mmol; SDS, 175 μmol; and 4-aminoantipyrine, 1.7 mmol. Stored at 4 °C, this reagent is stable for at least 24 h.

Prepare a 77 mmol/L solution of HDCBS in the Tris maleate buffer. Stored at 4 °C, this solution is stable for a least one month.

Prepare the enzymatic sphingomyelin reagent in Tris HCl buffer (50 mmol/L, pH 7.8) to contain, per liter, 4- aminoantipyrine, 750 μmol; HDCBS, 3.0 mmol; choline oxidase, 3 kU; sphingomyelinase, 1 kU; alkaline phosphatase, 1 kU; and peroxidase, 5 kU. This reagent is stable for at least 24 h when stored at 4 °C.

All enzyme activities are reported in international units (U), except for peroxidase and PL-Dc. One unit of peroxidase is defined as that amount of enzyme that catalyzes the production of 1.0 μmol of purpurogallin from pyrogallol per minute at pH 6.0 and 30 °C. One unit of PL-Dc is defined as that enzyme activity that will result in the hydrolysis of 1.0 μmol of choline from L-α-phosphatidycholine (14C-labeled) per hour at pH 6.5 and 30 °C. These are the definitions used by these commercial suppliers of these enzymes; other suppliers may use other definitions.

Procedures

Sample preparation. Measure lecithin and sphingomyelin in amniotic fluid as follows. Extract 2.0 mL of amniotic fluid with 6 mL of chloroform:methanol (2:1 by vol), vortex-mix for 2 min, then centrifuge (1000 × g, 5 min). Transfer all of the chloroform layer to a glass test tube and evaporate the solvent at 65 °C, in a water bath. Allow the tube to cool, add 2.0 mL of chloroform:methanol (2:1), and mix briefly to dissolve the lipid residue.

Transfer 500 μL of this solution to a glass test tube and add 500 μL of a 500 μmol/L sphingomyelin solution in chloroform:methanol (2:1). This mixture will be used for lecithin determinations.

Transfer 1.0 mL of the solution to another glass test tube and add 1.0 mL of 500 μmol/L lecithin in chloroform:methanol (2:1); use for sphingomyelin determinations.

Evaporate the solvent by placing the tubes in a 65 °C water bath. After the tubes have cooled, add 500 μL of 48 mmol/L Zwittergent 3-10 solution to the tube for lecithin determination and 1.0 mL of 48 mmol/L Zwittergent 3-10 to the tube for sphingomyelin determination. Stopper the tubes, vortex-mix briefly, and return to the 65 °C water bath for 5 min. Then remove the tubes and promptly vortex-mix their contents for about 1 min. When the tubes have cooled, centrifuge them at 6500 × g for 10 min. (This centrifugation step is necessary to remove any material that is insoluble in the Zwittergent solution.) Insoluble material will form a thin layer on top of the solution. The clear subnatant fluid can then be transferred to another tube and assayed.

Determination of lecithin. Pipet 1.0 mL of the enzymatic lecithin reagent and 100 μL of the HDCBS solution into a test tube and mix well. Add 100 μL of lecithin standard or sample in the 48 mmol/L Zwittergent 3-10 solution, mix, and incubate at 37 °C for 20 min. Measure the absorbance at 510 nm vs a reagent blank in which 100 μL of the 48 mmol/L Zwittergent 3-10 solution is substituted for the lecithin standard.

Determination of sphingomyelin. Pipet 500 μL of sphingomyelin reagent into a test tube, add 250 μL of sphingomyelin standard or sample in a 48 mmol/L Zwittergent 3-10 solution, mix, and incubate at 37 °C for 20 min. Measure the absorbance at 510 nm vs a reagent blank in which 250 μL of the 48 mmol/L Zwittergent 3-10 solution is substituted for the sphingomyelin standard.

The thin-layer chromatographic determination of the L/S ratio is basically a modification of the procedure of Gluck et al. (4), involving extraction of uncentrifuged amniotic fluid, no precipitation with cold acetone, use of thin-layer plates containing 5% ammonium sulfate (by weight) in the solid medium, and a mobile phase consisting of chloroform:methanol:water (75:25:2 by vol). Plates are heated at 240 °C to make the spots visible. Quantification is by transmission densitometry.

Results and Discussion

The molecular masses of lecithin and sphingomyelin differ, so we decided to compare them on a molar basis rather than a weight basis, because 1 mol of lecithin or sphingomyelin will produce 1 mol of choline or phosphorylcholine on hydrolysis with PL-Dc or sphingomyelinase. This molar basis was in turn based on the average molecular mass of bovine brain sphingomyelin, which we arrived at as follows. Solutions containing equal concentrations by weight of sphingomyelin and the synthetic lecithin were prepared and assayed with the total choline-containing phospholipids reagent previously described (10). Because the molecular mass of the synthetic lecithin is known (734.1 dalton), the average relative molecular mass (M_r) of the sphingomyelin could be calculated, which for this preparation was found to be 753. This value was used for sphingomyelin in all further calculations.

Previously (7, 8), for the enzymatic determination of amniotic fluid lecithin, it was necessary to separate the PL-Dc from the choline oxidase and peroxidase because of the differences in their pH optima—a two-step procedure. Phospholipase D from cabbage has a pH optimum of 5.4, but choline oxidase and peroxidase show optimum activity between pH 7.0 and 8.0. However, it has been reported (11) that the pH optimum of PL-Dc can be shifted to 6.6 by adding SDS. By including SDS in our reagent, we could legitimately combine the enzymes into a single reagent. Figure 2 depicts the effect of SDS on the rate of reaction of the lecithin reagent. In the presence of an optimum SDS concentration, the pH optimum for this reagent is 7.0. (We
did not examine the effects of the SDS on the individual enzymes.)

The order of addition of ingredients for the lecithin reagent is important, as already noted. Reportedly, PL-D₂ is strongly adsorbed to glass surfaces, resulting in substantial losses of enzyme activity (12). This problem has been circumvented by preparing the reagent in plastic containers (7) or by including bovine serum albumin in the reagent (8, 9, 12). An albumin concentration of 1 g/L in our reagent system suffices to prevent such adsorption. Furthermore, it is necessary to add the PL-D₂ before the calcium chloride, which is a PL-D₂ activator (12), to obviate solubility problems with the PL-D₂. The 4-aminoantipyrine is separated from the HDCBS to prevent excessive non-enzymatic coupling of the two chromogenic compounds in the reagent.

Because a microbial sphingomyelinase with both high specificity and specific activity is now commercially available, it is convenient to measure sphingomyelin in a manner analogous to lecithin. The pH optima of all the enzymes utilized in this procedure are sufficiently similar to permit use of a combined reagent with an overall pH optimum of 7.8. Furthermore, at this pH it is unnecessary to separate 4-aminoantipyrine from HDCBS, because there is little non-enzymatic coupling of the two, especially when the reagent is stored at 4 °C. Although both Mg²⁺ and deoxycholic acid reportedly are activators of the sphingomyelinase from B. cereus (13), we found that neither substance had an activating effect with this reagent; in fact, deoxycholic acid proved to be inhibitory.

The zwitterionic detergent, Zwittergent 3-10, was chosen as the solubilizing agent for both lecithin and sphingomyelin because it is compatible with both reagents and because it solubilizes phospholipids. We tested other Zwittergents, as well as cationic and non-ionic detergents. Most of them inhibited the sphingomyelin reagent at relatively low concentrations. Using pure standards, we examined the effect of the Zwittergent 3-10 on the reaction rates of both lecithin and sphingomyelin. Figure 3 shows the results. This experiment led us to select a Zwittergent 3-10 concentration of 24 mmol/L for both standards. However, with mixtures of lecithin and sphingomyelin we noticed that the color developed in each assay failed to become constant. This we corrected by increasing the Zwittergent 3-10 concentration to 48 mmol/L, whether single phospholipid standards or mixtures of lecithin and sphingomyelin were used. Figure 4 shows the resulting curves for each reagent for mixtures of 200 μmol of lecithin and 500 μmol of sphingomyelin per liter and 30 μmol of sphingomyelin and 500 μmol of lecithin per liter as samples in 24 and 48 mmol/L Zwittergent 3-10.

When we assayed mixtures of lecithin and sphingomyelin for each phospholipid, we noticed that the presence of lecithin somehow enhances the reaction for sphingomyelin, and similarly, sphingomyelin enhances the reaction for lecithin. We thought that the PL-D₂ might be hydrolyzing sphingomyelin and sphingomyelinase might be hydrolyzing

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**Fig. 2.** Effect of SDS concentration on the rate of color development measured at 510 nm for the lecithin reagent.

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**Fig. 3.** Effect of Zwittergent 3-10 concentration on the rates of color development, as measured at 510 nm, for the lecithin (L) and sphingomyelin (S) reagents.

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**Fig. 4.** Effects of 24 and 48 mmol/L Zwittergent 3-10 on reaction rates for the lecithin (L) and sphingomyelin (S) reagents, measured in terms of absorbance at 510 nm.
lecitin, so we checked each reagent for activity towards the other phospholipid. We found that the PL-D, only hydrolyzed lecitin, and the sphingomyelinase only hydrolyzed sphingomyelin. Then, we added various amounts of lecitin to 30 \( \mu \text{mol/L} \) sphingomyelin solutions, which were then assayed for sphingomyelin, and various amounts of sphingomyelin to 200 \( \mu \text{mol/L} \) lecitin solutions, which were then assayed for lecitin. Figure 5 shows the results of this experiment: hydrolysis of sphingomyelin increased with increasing lecitin concentration up to 250 \( \mu \text{mol/L} \). For lecitin concentrations between 250 and 750 \( \mu \text{mol} \) the hydrolysis of sphingomyelin remained constant. Similarly, the hydrolysis of lecitin increased with increasing sphingomyelin concentration until a sphingomyelin concentration of 250 \( \mu \text{mol/L} \) was reached; it then was constant to 750 \( \mu \text{mol} \) of sphingomyelin per liter. Furthermore, this enhancing effect of the non-reacting phospholipids could not be duplicated by detergents such as Triton X-100 or Zwittergents of a longer chain length than the Zwittergent 3-10.

Because the enzyme reagents appear to be specific for their intended substrates, we thought that the enhancing effect of one phospholipid on the determination of the other might be ascribed to better micelle formation. Perhaps the presence of a similar but non-reacting phospholipid helps form mixed micelles from which the reacting phospholipid is hydrolyzed more completely. We also observed that the enhancing effect is more pronounced if the phospholipids are first mixed in chloroform:methanol than if they are initially dissolved in the Zwittergent 3-10 solution. The possible application of these techniques to the determination of lecitin and sphingomyelin in amniotic fluid would appear to be relatively straightforward, because the lecitin and sphingomyelin of amniotic fluid can be extracted with chloroform:methanol.

For assaying lecitin and sphingomyelin, lecitin was added to all sphingomyelin standards to give a concentration of 500 \( \mu \text{mol/L} \) and sphingomyelin was added to all lecitin standards to give a concentration of 500 \( \mu \text{mol/L} \). The resulting standard curve (four points) for sphingomyelin was linear to at least 30 \( \mu \text{mol/L} \) (\( y = 0.0144x + 0.0032; r = 0.998 \)), and that for lecitin was linear to at least 300 \( \mu \text{mol/L} \) (\( y = 0.0042x - 0.0026; r = 0.969 \)).

Table 1 summarizes our precision data for both the lecitin and sphingomyelin assays, for solutions of pure standards.

![Figure 5. Absorbance at 510 nm for the reaction of (A) 200 \( \mu \text{mol/L} \) lecitin in the presence of various concentrations of sphingomyelin and (B) 30 \( \mu \text{mol/L} \) sphingomyelin in the presence of various concentrations of lecitin](image)

| Lecithin, 50 \( \mu \text{mol/L} \) | 0.219 | 0.001 | 0.52 | 0.217 | 0.002 | 0.92 |
| Lecithin, 300 \( \mu \text{mol/L} \) | 1.300 | 0.011 | 1.85 | 1.286 | 0.014 | 1.10 |
| Sphingomyelin, 5 \( \mu \text{mol/L} \) | 0.075 | 0.001 | 1.30 | 0.076 | 0.003 | 3.90 |
| Sphingomyelin, 30 \( \mu \text{mol/L} \) | 0.448 | 0.003 | 0.87 | 0.450 | 0.008 | 1.30 |

For as discussed earlier, it was necessary to add lecitin to one portion of the extract and sphingomyelin to the other, to ensure a consistent degree of hydrolysis of the phospholipid being measured. Once the residues were redissolved in 48 \( \mu \text{mol/L} \) Zwittergent 3-10, we noticed that some solutions were slightly turbid. Centrifugation at 6500 \( \times g \) caused the insoluble material to form a thin floating layer, so that the clear subnatant could be removed for lecitin or sphingomyelin determination. The centrifugation step removes none of the lecitin or sphingomyelin.

In this preliminary study, we assayed 10 amniotic fluid samples for lecitin and sphingomyelin as described above. These results are compared to the L/S ratio as determined by a thin-layer chromatographic method (4) in Table 2. As would be expected, the sphingomyelin concentration varies over a much narrower range than does the lecitin concentration in these samples. In general, the L/S ratio as determined by these enzymatic assays parallels the L/S ratio as determined by the thin-layer chromatographic method. Unfortunately, for many of the samples we assayed,
a cutoff L/S ratio value of 4:1 was used and the L/S ratio was not quantified beyond this number.

Existing methods for determining the L/S ratio of amniotic fluid by use of thin-layer chromatography have several problems: poor resolution, due to humidity (14), variations in charring temperature (15), and differing abilities of the various phospholipid visualization sprays to detect both saturated and unsaturated phospholipids (16). Furthermore, Gluck and Kulovich (17) and others (18) have reported that L/S ratios of 2:1 as measured by thin-layer chromatographic methods correspond to actual L/S concentration ratios of from 4:1 to 6:1. If this is indeed the case, the chromatographic methods will underestimate the true L/S ratio. In spite of these methodological problems, the determination of the L/S ratio of amniotic fluid by thin-layer chromatography has proven useful due to many years of clinical correlation. However, the present enzymatic method potentially may provide not only a more rapid but also a more precise and accurate measurement of actual amniotic fluid L/S ratios, as well as a convenient tool for investigating the effects of centrifugation speed and precipitation with cold acetone, two procedural steps that have evoked considerable controversy (19).

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References
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