


CLIN. CHEM. 34/3, 560–563 (1988)

Interference by Endogenous Glycerol in an Enzymatic Assay of Phosphatidylglycerol in Amniotic Fluid

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We investigated the possibility of interference by endogenous glycerol with the enzymatic measurement of phosphatidylglycerol in amniotic fluid. Phosphatidylglycerol is an important indicator of fetal lung maturity. The concentrations of glycerol and phosphatidylglycerol in amniotic fluid were measured by using a coupled enzymatic assay with and without phospholipase D (EC 3.1.4.4). The precision of the assay was acceptable (within-run CV = 1.2%, between-run CV = 4.8%). Endogenous glycerol content was demonstrated to be approximately 10–20 times that of phosphatidylglycerol. This high proportion of endogenous glycerol in amniotic fluid would preclude the accurate enzymatic determination of amniotic fluid phosphatidylglycerol unless the glycerol is first removed. Nor can the actual phosphatidylglycerol concentration be determined by subtracting the endogenous glycerol concentration from the total glycerol, which includes that glycerol derived from phosphatidylglycerol. With a usual range of 9 ± 7 μmol/L, the error for a given phosphatidylglycerol measurement of ± 6.6 μmol/L (± 2 SD) clearly is too high for this assay to be clinically useful. There was no correlation between concentration of endogenous glycerol or apparent phosphatidylglycerol in amniotic fluid and the lecithin/sphingomyelin ratio of the sample.

Additional Keyphrases: analytical error • phospholipase D • fetal status • lecithin/sphingomyelin ratio • centrifugal analyzer

Respiratory distress syndrome (RDS)\(^1\) is the most common complication faced by the premature newborn, leading to significant neonatal morbidity and mortality despite tremendous advances made in the care of these infants. RDS develops in many infants whose lungs are immature, i.e., lacking necessary pulmonary surfactant to decrease alveolar surface tension (to keep the alveoli open) at end expiration. Accurate, precise, rapid, and cost-effective tests are needed to determine fetal lung maturity (FLM), so that the obstetrician can plan delivery so as to maximize the chances of delivering an infant with mature lungs.

In current methods to evaluate FLM, amniotic fluid is used; because the fetal tracheobronchial tree is in constant contact with the amniotic fluid, any surfactant, once produced, will be found in the amniotic fluid. The methodologies currently in use measure either the components of the surfactant—mostly phospholipids—biochemically or measure the physical properties of the surfactant, namely its surface-tension-reducing abilities. The most widely used biochemical assay is the lecithin/sphingomyelin (L/S) ratio proposed by Gluck et al. in 1971 (1). However, this test involves thin-layer chromatography, which is labor-intensive and whose interpretation is very subjective; moreover, it yields many false predictions of fetal lung immaturity (L/S ratio <2.0), the predictive value of an immature test result being as low as 33% (2). More recently, enzymatic (3) and fluorescence polarization (4) assays have been proposed to evaluate FLM, both with claims of rapidity, precision, and accuracy. Here we comment on our experience with the enzymatic method described by Bradley et al. (3), who used a Multistat III microcentrifugal analyzer and loader to measure lecithin, sphingomyelin, and phosphatidylglycerol (PG)—the presence of PG being considered by many to be more predictive of FLM than is the L/S ratio.

Materials and Methods

We used the Cobas-Bio centrifugal analyzer (Roche Diagnostic Systems, Montclair, NJ 07042) for our analysis of the PG content of amniotic fluid. L/S ratios were determined by thin-layer chromatography, with silica gel plates from American Scientific Products, McGaw Park, IL; phospholipid standards from Sigma Chemical Co., St. Louis, MO; and phosphomolybdic acid, used for visualization, from Fisher Scientific Co., Pittsburgh, PA.

\(^1\) Nonstandard abbreviations: RDS, respiratory distress syndrome; FLM, fetal lung maturity; L/S, lecithin/sphingomyelin; G, glycerol; PG, phosphatidylglycerol.

Received September 28, 1987; accepted December 8, 1987.

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Seventeen amniotic-fluid samples, obtained by amniocentesis, were centrifuged at 500 × g for 5 min to remove particulate matter, then frozen at −15 °C before analysis by thin-layer chromatography and enzymatic means.

L-α-Phosphatidyl-DL-glycerol, phospholipase D (EC 3.1.4.4; Type VI, from Streptomyces chromofuscus), calcium chloride dihydrate, magnesium chloride, adenine 5′-triphosphate, glycero kinase (EC 2.7.1.30, from Escherichia coli), hydrazine dihydrochloride, α-glycerophosphate dehydrogenase (EC 1.1.1.8, from rabbit muscle), β-nicotinamide adenine dinucleotide, disodium EDTA dihydrate, glycine, and glycerol were all from Sigma Chemical Co.

Triton X-100 surfactant and Tris buffer were purchased from Fisher Scientific Co.

PG standards were prepared by evaporating 1 mL (10 mg) of PG and reconstituting the residue in 50 mL of 1 g/L Triton X-100 solution to yield a 200 mg/L stock solution. Dilutions of this stock solution were made with 1 g/L Triton X-100 to yield solutions containing 10, 20, 30, 60, and 100 mg of PG per liter. Refrigerated at 4 °C, these standards were stable for one month. This method is similar to that of Bradley et al. (3).

We also prepared PG reagents similar to those of Bradley et al. (3). "Phase one" reagent consists of phospholipase D (5000 U/L), calcium chloride dihydrate (10 mmol/L), magnesium chloride (5 mmol/L), adenosine triphosphate (750 μmol/L), and glycero kinase (500 U/L) dissolved in Tris HCl buffer (50 mmol/L, pH 7.6). We froze this in 4-mL aliquots and stored them at −80 °C.

"Phase two" reagent was prepared by dissolving 10.5 g of hydrazine dichloride, 18.75 g of glycine, and 5 g of sodium EDTA in 250 mL of 1 mol/L sodium hydroxide solution, pH 9.5. We stored this buffer at 4 °C and checked and adjusted the pH weekly. Daily, we removed 5-mL aliquots and dissolved 20 mg of NAD⁺ and 100 U of glycerophosphate dehydrogenase in the buffer before each day's assay.

The enzymatic reaction for the assay of PG is as follows.

**Phase one**

\[ \text{PG} \xrightarrow{\text{phospholipase D}} \text{phosphatidic acid + glycerol} \]

\[ \text{Glycerol + ATP} \xrightarrow{\text{glycerokinase}} \text{glycerol 3-phosphate + ADP} \]

**Phase two**

\[ \text{Glycerol 3-phosphate + NAD⁺} \xrightarrow{\text{glycerol 3-phosphate dehydrogenase}} \text{glycerone phosphate + NADH (λ_max = 340 nm)} \]

Glycerol standards were prepared as molar equivalents to the PG standards. We suspended 20 μL of glycerol in 1100 mL of 1 g/L Triton X-100, then diluted this stock solution with 1 g/L Triton X-100 to yield standards equimolar to the 10, 20, 30, 60, and 100 mg/L PG standards.

We performed the assays with the Cobas-Bio. In the first phase, the instrument mixes 76 μL of standard or amniotic fluid with 95 μL of phase one reagent and incubates this for 15 min at 37 °C. After incubation, 50 μL of phase two reagent is added and six absorbance readings at 340 nm are taken at 10-s intervals. The absorbance of a Triton X-100 reagent blank is subtracted from the change in absorbance (ΔA) during this 60 s for each standard and sample. We assayed both sets of standards and amniotic fluid in the phase one reaction, with and without phospholipase D in the reagent. The rationale for omitting the enzyme was to determine the amount of endogenous glycerol in amniotic fluid, Rosenthal and Tocci (5) having suggested that endogenous glycerol is present in sufficient quantity to interfere with the accurate measurement of PG. Standard curves were fit by a gradient method to a four-parameter logistic model (6, 7), as performed with the Ligand-Data Calculator program (Rhoads Software/David G. Rhoads Assoc, Inc., Kennett Square, PA). Paired t-tests were used to evaluate differences between the results with and without phospholipase D.

**Results**

We assayed calibrators in the two-phase reaction system with and without phospholipase D in the first reagent, and plotted the change in absorbance against the molar concentrations of glycerol and PG (Figure 1). In the absence of phospholipase D, the ΔA of the PG standards was essentially zero (Figure 1). This indicated that the endogenous glycerol signal was independent of the presence of the lipase.

To test for the presence of interfering endogenous glycerol in amniotic fluid, we tested 17 amniotic fluids—12 in...
quadruplicate and five in triplicate, all with glycerol calibrators, with and without phospholipase D in the first reagent phase (Figure 2). The mean concentration in each fluid assayed with and without phospholipase D was 70.7 (SD 18.7) μmol/L and 66.2 (SD 19.4) μmol/L, a significant difference (P < 0.01). The within-run CV was 1.2% (n=10) and the between-run CVs of 12 samples analyzed eight times ranged from 2.7% to 6.4% (mean 4.8% calculated as the square root of the mean variance of the assays).

Comparison of the glycerol concentration in amniotic fluid with the L/S ratio determined by thin-layer chromatography (Figure 3A) showed no significant correlation. Finally, we compared the apparent PG concentration in amniotic fluid, obtained by subtracting the results with phospholipase D from those without phospholipase D to the same L/S ratios as above (Figure 3B); again, there was no correlation.

Discussion

Previous investigators found the mean concentration of PG in amniotic fluid, as determined by an enzymatic colorimetric method, to be 20 (SD 13) μmol/L (n = 14) (8) and 9 (SD 7) μmol/L (n = 44) (9). They recognized the possibility that endogenous glycerol, a reaction intermediate, might interfere with the measurement of PG, and therefore they used a Folch-type extraction to separate phospholipids from glycerol, effectively removing this interference.

The PG concentration in amniotic fluid reported by Bradley et al. (3) was roughly 80 μmol/L, far greater than in the previous two reports. However, this value agrees closely with reported values for glycerol in serum: 82 ± 25 μmol/L (n = 15) (10) and 119 ± 65 μmol/L (n = 57) (11). Glycerol is a small, water-soluble molecule, so it would be expected to be in dynamic equilibrium between the plasma and amniotic fluid compartments. With no provision for removal of this endogenous glycerol from amniotic fluid, apparently Bradley et al. (3) measured the total endogenous glycerol in amniotic fluid and any glycerol derived from the PG in the fluid.

The new quantitative enzymatic assay for PG in amniotic fluid that Rosenthal and Tocci (5) evaluated involves pre-treatment of the fluid with enzymes to remove endogenous glycerol (personal communication). As we have shown above, there is a substantial amount of glycerol in amniotic fluid (roughly 10–20 times the PG concentration) and this glycerol can be reliably quantified by enzymatic methods. However, the concentration of PG in amniotic fluid cannot reliably be determined without prior removal of these high concentrations of endogenous glycerol. The endogenous glycerol and the glycerol generated by enzymatic reaction of PG with phospholipase D both contribute to the formation of the NADH with the concomitant change in absorbance at 340 nm. Nor can the actual PG concentration be determined by subtracting the endogenous glycerol concentration from the total glycerol, which includes that glycerol (G) derived from PG. The CVs of the assay with and without phospholipase D (PLD) averaged 4.8%, the mean [G with PLD] was 70.7 μmol/L and the mean [G without PLD] was 66.2 μmol/L. (Brackets signify "concentration.") Thus the mean [apparent PG] = mean [G with PLD] – mean [G without PLD] = 4.5 μmol/L. Using these data, we calculated a predicted SD for [apparent PG] as 3.3 μmol/L. Thus, with an expected usual range of PG in amniotic fluid of 9 ± 7 μmol/L (9), the potential error for a given PG measurement of 6.6 mmol/L (± 2 SD) clearly is too great for this assay to be clinically useful. From these observations, we conclude that the enzymatic measurement of amniotic fluid PG is not feasible unless endogenous glycerol in amniotic fluid is removed before assay for PG, as demonstrated by Rosenthal and Tocci (5).

We thank Drs. James C. Boyd for help with statistics, and David E. Bruns for a critical reading of the manuscript.

References


CLIN. CHEM. 34/3, 563-567 (1988)

Anodic Stripping Voltammetry Procedure Modified for Improved Accuracy of Blood Lead Analysis

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In evaluating the accuracy and reliability of blood lead (PbB) measurements with the Environmental Science Associates Model 3010A Trace Metal Analyzer, intralaboratory comparison demonstrated that use of the operating conditions recommended by the manufacturer resulted in consistently underestimated PbB concentrations <400 µg/L and overestimated PbB values >400 µg/L. At PbB concentrations <50 µg/L, measured concentrations were often registered as negative results. However, these negative values could be replicated to within ±10 µg/L, indicating good precision of the method, but obviously not good accuracy. In addition, lower-than-expected lead (Pb) values were measured in samples containing increased concentrations of copper (Cu), such as may occur in pregnant women. We modified the procedure to eliminate these inaccuracies by substituting manual peak-height measurements for reliance on the integrator and digital display of the instrument. We established the accuracy of the modified procedure by using calibration standards previously quantified by isotope dilution–mass spectroscopy. A quality-control program for monitoring PbB analysis is also described.

Additional Keyphrases: variation, source of · isotope dilution–mass spectroscopy compared · copper · pregnancy · toxicology · quality control · trace elements

The toxic effects of lead (Pb) on biological systems have been well documented (1), and measurement of blood lead (PbB) concentrations is generally accepted as the best index to current Pb exposure (2). Numerous analytical methods are available for the measurement of PbB (3, 4). However, it is important that accuracy and precision be well established and frequently monitored, to ensure the adequacy of the method used. Here we report our evaluation of the accuracy and precision of PbB measurements by anodic stripping voltammetry (ASV) with a commercially available instrument.

In ASV, metal ions are plated onto a test electrode by using a negative potential, then stripped off the electrode by reversing to an anodic potential, which is varied linearly. The current generated during this removal can be displayed as a gaussian peak on a strip-chart recorder and is a function of the amount of metal plated during an already known period of time. Consequently, peak area or height can be related to those for standards of known concentrations to quantify the amount of Pb present in a blood sample.

Within the ASV instrument we routinely use, an electronic module controls the analytical cycles, including a staircase stripping voltage function during which peak signals are integrated. The integration technique used in calculating results from peak current is called "trapezoidal baseline calibration" (5). The area under the peak is quantified by registering the current at the beginning and end of a peak to construct a baseline for the peak integral, then summing the signals from each of the steps within the integration zone. The resulting value should be proportional to the concentration of the sample.

In our experience, analysis for PbB with this ASV analyzer, operated according to the manufacturer's operating manual and conditions, consistently resulted in underestimations of low PbB concentrations and overestimations of higher PbB concentrations (see Results). The most likely cause of these inaccuracies was thought to be limitations of the instrument's integrator, in that computation begins and ends at pre-set invariable points, regardless of where the ionization potential of a metal might initiate current changes. Moreover, because the width of the peak varies as the peak height varies, establishment of the baseline by the integrator may be erroneous. As the instrument is currently set up, when the metal concentration deviates from the range for which the arbitrary integration zone was established, more or less area is subtracted from the area under the peak, potentially leading to under- or overestimation of concentrations. At low PbB (<50 µg/L) a measured concentration can even be registered as a negative result.

Because ASV has been previously shown, by comparison with a definitive method (6), to be a precise and accurate technique for PbB measurements, we took these preliminary observations of instrument performance as an indication of the need to evaluate and modify the recommended operating conditions to improve the accuracy of PbB measurements. Accordingly, we altered the recommended procedure, and related the modified ASV Pb procedure to the definitive method for Pb (isotope dilution–mass spectroscopy, ID-MS) (6) by using calibration standards whose Pb concentrations had been previously determined by ID-MS.

Materials and Methods

Apparatus

We used the Environmental Sciences Associates (ESA, Bedford, MA 01730) Model 3010A Trace Metal Analyzer,