Determination of Bilirubin in Amniotic Fluid

A New, Simple, and Efficient Method

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A simple, speedy, and efficient method is described for the quantitative determination of unconjugated bilirubin in amniotic fluid. The method makes it possible to use an uncentrifuged sample and to detect as little as 0.00125 mg of bilirubin per 100 ml of amniotic fluid without interference by hemoglobin. The analysis can easily be completed in 30 min. Bilirubin is completely separated from all contaminants, except in samples that are grossly contaminated with meconium. If such interference is absent only one reading, at one wavelength, is necessary; a scanning spectrophotometer is therefore not required.

Diagnosis of Erythroblastosis fetalis and prediction of hemolytic disease in the newborn by the indirect Coombs test or use of other antibody titers has not proved to be entirely reliable. For this reason, it has become accepted practice in many departments of obstetrics to perform amniocentesis on patients whose indirect Coombs titers exceed one in eight, and to measure the products of hemolysis causing yellow pigmentation in the amniotic fluid. The nature of this yellow pigment was considerably doubted at first, but now many authors agree that it is largely unconjugated bilirubin. Indeed, we have confirmed that this pigment is soluble in chloroform and has the same absorption spectrum as bilirubin, both before and after diazo reaction.

Ordinarily, the concentration of bilirubin in the amniotic fluid of normal pregnant women is very low and falls with maturity from about 0.04 mg/100 ml in the 32nd week of pregnancy to about 0.01 mg/100 ml at term, but much higher concentrations are found in the amniotic fluid of abnormal fetuses.

Various methods have been used to estimate the concentration of the pigment in amniotic fluid. Broadly speaking, they have been (a) the increased absorbance of the amniotic fluid at 450 nm (1-4), (b) one of the standard azo-bilirubin methods (5-10), or (c) extraction of the bilirubin pigment in chloroform (11, 12).

Direct spectrophotometry of amniotic fluid (a) has two serious disadvantages; the high background absorption caused by other dissolved substances and the interference by a hemoglobin peak in the Soret band between 412 and 415 nm in bloodstained samples, renders both the Liley (3) and the Freda (4) methods uninterpretable. The diazo method also has the disadvantage of background absorption caused by other substances, which makes a sample blank necessary. This background absorption often presents a serious problem with amniotic fluid, which is virtually colloidal, and it is doubtful that sample blanks can correct adequately for turbidity.

All solvent-extraction methods in the past have had the disadvantage of incomplete extraction of bilirubin, necessitating multiple extractions, evaporation of the extracting solvent, and reconstitution of the residue with a small volume of the solvent before absorption of the bilirubin is measured. Thus, these solvent methods have been used largely as research procedures, since they were too tedious for routine use. Moreover, the solvent-extraction methods often allowed partial loss of bilirubin from oxidative and mechanical causes.

Our method overcomes all the disadvantages cited above. We believed a basic substance, soluble only in the nonaqueous phase, would facilitate liberation of bilirubin from its protein binding in the aqueous phase and enable its quantitative
transfer to the organic solvent phase. In this method, aniline in chloroform (1 ml/100 ml) achieves this objective; it extracts more than 99% of the bilirubin from four times its volume of amniotic fluid, thus effecting a fourfold concentration.

Materials and Methods

Spectrophotometer

A Beckman DBG spectrophotometer with 1-cm stoppered silica cuvets of approximately 3-ml capacity was used. Glass cuvets may be used instead.

Reagents

Extracting solvent, chloroform–aniline. Dissolve 1 ml of aniline (AR) in chloroform (NP) and dilute to 100 ml with chloroform.

Buffer (12). Dissolve 5.4 g of K2HPO4, 6 g of ascorbic acid, and 40 mg of sodium salicylate in about 80 ml of distilled water; neutralize with aqueous NaOH (40 g/100 ml) and dilute to 100 ml. The final pH should be 8.15.

Store under 1-cm layer of xylene in darkness in a refrigerator. The xylene serves to prevent aerobic oxidation of the ascorbic acid.

An automatic pipettor (Model S-A 400, Oxford Labs., San Mateo, Calif. 94401) is convenient for dispensing measured volumes without contamination by xylene.

Procedure

Mix 20 ml of uncentrifuged amniotic fluid to obtain uniform suspension, then draw up with a wide-bore pipet and transfer to a 50-ml stoppered flask. Add 10 ml of buffer and 5 ml of solvent and shake the flask for 2 min. Transfer this mixture to a tube and centrifuge at 2500 to 3000 rpm for 20 min. Carefully transfer the clear solvent layer to a stoppered 1-cm cuvet with a 3-ml pipet; read the absorbance (A) at 453 nm against that of the solvent.

Calculation

If a standard rectangular cuvet with a 1-cm light path is used, then the amniotic fluid bilirubin in mg/100 ml is \( A_{453}/4 \).

The simplicity of this calculation arises from the coincidence that the numerical values for the (1-cm light path) absorbance of bilirubin at 453 nm in chloroform–aniline and the concentration of bilirubin, in mg/100 ml, were essentially identical, with fresh commercial bilirubin used as a reference. If standard 1-cm cuvets are not used, then a standard solution of bilirubin in chloroform and aniline must be used for calibration.

According to The Committee on Bilirubin Standards (13), a solution of 0.100 mg of bilirubin in 100 ml of chloroform should give a 1-cm absorbance at 453 nm of 0.104, but our commercial standard had a 1-cm absorbance of 0.097 at this wavelength and concentration. Therefore our corresponding solution of bilirubin in chloroform and aniline, from our reference bilirubin, which gave an absorbance of 0.100, should be corrected to 0.1074. For more accurate work, it would be correct to divide the answer obtained from the simple formula by 1.07. Alternatively, this correction can be included in the method by using 5.35 ml of chloroform instead of 5.00 ml, so that the simple formula will still apply.

Glassware

All glassware was washed before use with dilute ammonia, dilute acetic acid, distilled water, and finally alcohol, and air dried.

Experimental Basis for the New Method

The partition of bilirubin between four volumes of water and one volume of chloroform, with and without aniline, was studied in the presence of buffers with pH's ranging from 0 to 12. To do this, we had to reverse the process of the method and start with the reference bilirubin in the chloroform phase, because it is very poorly soluble in water and highly unstable in alkaline aqueous solutions. It is evident from Figure 1 that chloroform with aniline added can quantitatively extract bilirubin...
over a wider pH range than can chloroform alone. Furthermore, in the normal pH range of amniotic fluid (pH 7 to 8), only chloroform–aniline provides quantitative extraction. The pH of 8.15 was chosen for the extraction because interference by other substances in the amniotic fluid was least, and often nil, at this pH. At this pH, chloroform alone extracts only 70 to 75% of the bilirubin from amniotic fluid.

It was incidentally observed that the bilirubin reference in chloroform, with or without aniline, had its optical density slightly enhanced when extracted with acidic buffers in the range of pH 4 or less. Consideration has been taken of this in constructing Figure 1, which shows the percent of bilirubin recovered in the chloroform phase.

The addition of ascorbic acid to the buffer did not significantly alter the relationship between pH and partition, so ascorbic acid was included in the buffer to provide a reducing milieu, which would help prevent loss of bilirubin by oxidation during extraction. This seems to be more important with amniotic fluid than with aqueous buffers alone.

Bilirubin is relatively stable in chloroform and is even more stable when aniline is included in the solvent. Indirect and diffuse lighting in laboratory conditions does not seem to affect the results. However, the sample may be unstable before extraction so it should be kept in the dark and in the cold. After the sample and reagent mixture has been shaken, stability no longer seems to be a problem, and the mixture may be centrifuged at room temperature.

Addition of 1 g of aniline to 100 ml of chloroform does not increase the solubility of conjugated bilirubin in chloroform. Serum, diluted 10-fold and also diazo-analyzed, was extracted several times with chloroform to remove all unconjugated bilirubin. The serum was from a jaundiced patient. This sample was then extracted once with chloroform–aniline by the new method. The absorbance was only 0.005, representing less than 1% of the conjugated bilirubin in the sample.

Results

Determination of bilirubin in centrifuged and uncentrifuged samples of amniotic fluid by this method reveals the presence of approximately 25% (8 to 47%) more bilirubin in uncentrifuged samples (Figure 2). This difference probably results from the presence of bilirubin in the lipoproteins of the epithelial cells that are in the amniotic fluid, or in the suspended fat droplets of the vernix caseosa.

![Fig. 3. Direct spectrophotometry of a centrifuged and filtered specimen of amniotic fluid from a very mildly affected fetus showing interference of a large hemoglobin peak at 414 nm. This affects the position at which a tangential base line must be drawn to measure the bilirubin hump at 450 nm by either the Liley (3) or the Freda (4) method.](image)

![Fig. 4. Spectral absorbance of a x4 extract of the same specimen of bloodstained amniotic fluid as in Fig. 3, the new method completely eliminates all interference of hemoglobin and other background absorption and intensifies the bilirubin reading.](image)
The quantitative nature of the extraction by the present procedure was verified by consecutive extractions of amniotic fluid samples with pure chloroform; the cumulative quantity of bilirubin in the chloroform extracts was equal to the quantity found after a single extraction with chloroform-aniline. In these calculations we took into account the slight difference in the molar extinction of bilirubin in chloroform and bilirubin in chloroform-aniline.

The presence of blood in samples of amniotic fluid did not interfere with the determination of bilirubin by this method (Figures 3 and 4). Only gross contamination with meconium interferes with this method, and this is evidenced by the absorbance at 400 nm or 550 nm being more than 70% or 10%, respectively, of the absorbance at 453 nm. In such instances, the results may be expressed as "bilirubin and other pigments" and they may still be clinically useful if the total value is low.

The method can measure as little as 0.005 mg of bilirubin per 100 ml of the solvent phase, or 0.00125 mg/100 ml of amniotic fluid.

Smaller volumes of amniotic fluid, such as 5 ml, may be used with 2.5 ml of buffer and 5 ml (or 5.35 ml) of solvent to obtain a one-to-one extraction of bilirubin and still give an acceptable sensitivity of 0.005 mg of bilirubin per 100 ml of amniotic fluid.

Discussion

The work of Brazie et al. (14), Mandelbaum and Robinson (15), and Willis and Faulkner (16), as well as our own studies, confirm the identity of the yellow pigment in amniotic fluid as being chiefly unconjugated bilirubin. Bevis (1), Liley (9), and Freda (4) have relied on the 450 nm peak of bilirubin in centrifuged and filtered amniotic fluid as a measure of the severity of erythroblastosis fetalis. Gambino and Freda (10) correlated the change in absorbance at 450 nm of amniotic fluid with the amniotic fluid bilirubin (AFB) concentration, according to the equation

\[ \Delta A_{450} \text{ (Freda)} = (0.7413 \times AFB \text{ in mg/100 ml}) - 0.011 \] (1)
Table 1. Bilirubin–Total Protein Ratio in Amniotic Fluid as an Index to Severity of Erythroblastosis Fetalis

<table>
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<tr>
<th>Severity</th>
<th>Bilirubin (mg)/total protein (g) ratio</th>
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<tbody>
<tr>
<td>Normal or mildly affected</td>
<td>&lt;0.25</td>
</tr>
<tr>
<td>Moderate to severely affected</td>
<td>0.25–0.40</td>
</tr>
<tr>
<td>Severely affected</td>
<td>&gt;0.40</td>
</tr>
</tbody>
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By comparing the Freda and Liley values for 51 amniotic fluid samples, we have found a statistical relationship between the Freda and Liley values as follows:

\[
\Delta A_{450} \text{ (Liley)} = [1.186 \times \Delta A_{450} \text{ (Freda)}] + 0.021 \tag{2}
\]

From Equations 1 and 2 we have

\[
\text{AFB mg/100 ml} = 1.137 \times [\Delta A_{450} \text{ (Liley)} - 0.008] \tag{3}
\]

Based on the equations above, the Liley (17) method of evaluating the severity of erythroblastosis fetalis may be recalculated in terms of bilirubin levels. We have done this and then added 25% to correct for the use of uncentrifuged samples. Figure 5 is a redrawn Liley chart which may be useful for interpreting the significance of amniotic fluid bilirubin levels obtained by the present method. It may be noted that, for convenience, the Liley B zone has been divided into three equal subzones: 1, 2, and 3. The action line of Whitfield et al. (18) has also been adapted in the same way and incorporated into Figure 5.

In cases of uncertain gestation period or polyhydramnios in which there may be an unknown dilution factor, one may use the ratio, milligrams of bilirubin per gram of protein, as used by Cherry et al. (19). This has also been modified appropriately to suit the present method (Table 1), in which Liley's 450-nm peak values have been changed to bilirubin, in mg/100 ml, for interpretation of data on this ratio from the amniotic fluid of fetuses of unknown maturity. The suggested interpretations in Figure 5 and Table 1 are purely tentative and await confirmation by extensive clinical trials which have been initiated.

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References