Some Aspects of Bilirubin Determination in the Newborn Using Dimethyl Sulfoxide*

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ABSTRACT

Although serum bilirubin determination is a common procedure in most clinical laboratories, the test seems to be of particular importance in the screening of neonates, especially the premature or the erythroblastotic infant. However, bilirubin determination is subject to interferences, particularly in the presence of hemolysis and lipemia, which may seriously affect the results. It is, therefore, of paramount importance to the pediatrician to be aware of these potential shortcomings of the test.

To improve the reliability of bilirubin determination, a method using dimethyl sulfoxide is proposed which is simple, rapid, and ideally-suited for small amounts of blood such as are commonly encountered in pediatrics. The effect of hemolysis and moderate lipemia are minimal, and the use of a sample blank can usually be eliminated in most circumstances.

Introduction

Bilirubin determination remains a popular test in clinical laboratories. Its importance is particularly evident when the test is applied to monitoring either the newborn, the premature or the erythroblastotic infant. In the latter case, prompt and reliable results are needed to initiate steps to prevent encephalopathy.

There are many methods for serum bilirubin determination, but by far the most popular are those based on azobilirubin formation which employ diazotized sulfanilic acid as the reagent. It is generally understood that bilirubin in plasma exists in both unconjugated and conjugated forms. The latter form, bilirubin diglucuronide, being water soluble, reacts promptly with diazotized sulfanilic acid to form azobilirubin, whereas the unconjugated form does not react at all or reacts very slowly unless either compound, which has come to be called ac-
catalysts, or an organic solvent is present. Thus, the two forms of bilirubin are differentiated on the basis of their reaction rates in an all-aqueous system, as compared to the value obtained in either a semi-aqueous medium or in the presence of accelerators. In the latter media, both conjugated and unconjugated bilirubin react rapidly. These two bilirubin forms are important because an increased level of unconjugated bilirubin in plasma is usually associated with hemolytic jaundice while a rise in conjugated bilirubin is associated with obstructive jaundice.

The use of methanol as the organic solvent for total bilirubin determination has been popular, but the presence of protein may cause a turbidity to develop in the system. To obviate the problem, the present authors experimented some years ago with organic solvents other than methanol, notably methyl cellosolve and dimethyl sulfoxide (DMSO). The latter proved satisfactory because, unlike methanol, it caused the reaction between bilirubin and diazotized sulfanilic acid to proceed almost instantaneously, and no turbidity was formed from precipitating proteins. This solvent was then adopted for total bilirubin determination in our routine laboratory about 20 years ago. Subsequently, a number of papers appeared in the literature which described the use of DMSO for bilirubin determination. However, on comparing these methods to our own methods, it was found necessary to re-examine the use of DMSO for bilirubin determination, especially when treating hemolyzed and/or lipemic specimens. Sample blanks and stability of reagents were also re-examined in order to complete the picture of testing of suitability for studies of bilirubin in the newborn. As a consequence, a method for total bilirubin determination is proposed which is relatively free of the common shortcomings of other procedures when hemoglobin and lipemia may be potential interferences.

**Reagents and Procedure**

The reagents and procedure used for the determination of total bilirubin in serum involving dimethylsulfoxide as a solubilizing and accelerating agent and a tablet form of diazo reagent have been previously described. Therefore, they will not be discussed here. What is offered in the discussion which follows is a critique of the characteristics of the proposed procedure involving some reactions of bilirubin in this diazo modification along with the minimizing of the problem of interference of hemoglobin in total bilirubin determinations. The overcoming of the latter problem has proven to be extremely useful to the neonatologists who have received this more accurate chemical information.

**Discussion and Results**

The reaction for direct bilirubin is carried out in an all-aqueous medium for exactly one minute, and then the absorbance is measured. The pink color of azobilirubin formed within this period is considered to be primarily due to conjugated bilirubin. It is generally believed that the latter reacts rapidly in an aqueous medium while the unconjugated bilirubin reacts very slowly. The differentiation between the two forms of bilirubin is based on the relative reaction rate in an all-aqueous medium as compared to total bilirubin reacted in the presence of DMSO.

The time of one minute is arbitrary, and it may not be sufficient for all conjugated bilirubin to react. It is also not certain when the reaction goes to completion or when the unconjugated bilirubin begins to react. There is undoubtedly some overlap in the reaction times of the two forms of bilirubin. The determination of direct bilirubin should be considered an estimation rather than a quantitation. This fact should not preclude its clinical usefulness, although it must be
understood that it may be difficult to interpret the results under some circumstances, notably when one encounters a combination of hemolytic and obstructive jaundice in the newborn.

The characteristics of the bilirubin di-glucuronide reaction in the described system are shown in figure 1. As demonstrated by the rise of Curve A, the reaction rate of direct bilirubin seems to proceed in three phases: (1) a very rapid phase with some reaction taking place within the first few seconds (absorbance near zero time), (2) a moderately rapid phase lasting for about eight minutes, and (3) a slow phase following the eight-minute period. It is not certain what these phases may represent, but it appears obvious that both forms of bilirubin are capable of reacting in the aqueous medium. More will be said about the remaining curves of figure 1 later in the discussion.

If the reaction is followed for a time, as shown in figure 2, Curve B, the ascending absorbance for the direct reaction may reach a plateau ultimately coinciding with the peak of Curve C which is the spectrum of the reaction taking place in a semi-aqueous medium. It is also clear that within the same time frame, Curve A of the previous figure 1 has not reached maximal absorbance, for that reading should be twice the absorbance of line F of figure 1. When these studies are carried out using different serums, the slopes vary and the plateaus may never reach maximal absorbance no matter how long the reaction is followed. There is a limit, however, to this type of study because the reaction mixture is unstable. The one-minute reaction for direct bilirubin appears to coincide with the end of the most rapid phase of the reaction, which indeed may be mostly due to conjugated bilirubin. It has been estimated that as much as 30 percent of unconjugated and only 70 percent of conjugated bilirubin can react within the first minute. In hyperbilirubinemia, both forms of bilirubin may be elevated. In obstructive jaundice, the conjugated bilirubin predominates over the unconjugated, while in hemolytic jaundice it is reversed. Reporting a ratio of direct to total bilirubin would perhaps be useful to the clinician.

**Effect of Hemolysis**

It is well recognized that the presence of hemoglobin in the reaction interferes with azobilirubin formation in a negative fashion. Watson studied the effect of hemoglobin using a number of different methods and found some methods to be more affected than others. At a bilirubin level of 0.5 mg per dl, for example, the Jendrassik-Grof method produced a 60 percent error, although the error was much smaller at higher bilirubin levels.
In view of the importance of total bilirubin determination in pediatrics and the difficulty in obtaining samples free of hemoglobin in specimens obtained from neonates, it was necessary to investigate the hemoglobin effect in the proposed method. For this purpose, a blood hemolysate was prepared from red blood cells. To eliminate the presence of residual red cells or cell debris, the final hemolysate was spun in an ultracentrifuge for 30 minutes at 30,000 RPM. The hemoglobin concentration was determined and the solution was then appropriately diluted with saline. The hemolysate was added in increasing amounts to a fixed volume of unhemolyzed serum containing an elevated concentration of bilirubin. The mixture was pipetted into diazo reagent in a fashion similar to that described in the manual\(^1\) for direct or total bilirubin determination. Either spectra or time studies were then determined using a double-beam automatic recording spectrophotometer.

Spectra of the chromogens formed in semi-aqueous medium are shown in figure 3 as graphs B and D. Spectra were displaced on the ordinate by 25 or 50 nanometers to separate coincidental peaks. Typical bell-shaped curves with maxima at 560 nm owing to azobilirubin are present, and there are also small peaks near 400 nm that are due to a low concentration of hemoglobin normally present in serum. When a certain amount of hemoglobin is added to the same serum and then the diazo reaction is carried out, peaks owing to hemoglobin become much higher (graphs A and C) while the azobilirubin peaks due to bilirubin remain unchanged. Each set of graphs was recorded using the reagent blank as a reference. Graphs E and F represent the corresponding sample blank for the A and C set. Although they contain equivalent concentrations of hemoglobin, the peaks at 400 nm in graphs E and F are lower than those in A and C. The reason for this is that

![Figure 2](image2.png)  
**Figure 2.** Reaction time studies in aqueous medium (Curves A and B). Curve C is a corresponding spectrum in semi-aqueous medium curves. A and B were obtained with duplicate samples.

![Figure 3](image3.png)  
**Figure 3.** Effect of hemoglobin on total bilirubin determination. Curves B and D are spectra obtained with unhemolyzed sera. Curves A and C are spectra obtained with the same sera when contaminated with hemoglobin. Curves E and F are the corresponding spectra for the sample blanks of A and C.
The set represented by graphs E and F does not contain the diazo reagent which by itself exhibits considerable absorbance at 400 nm.

The reagent blanks show seemingly insignificant absorbance at 560 nm; however, if the combined effect on absorbance is considered due to the diazo reagent and the yellow color of bilirubin, then the absorbance from the sample blank may become significant. Furthermore, protein is known to affect spectral characteristics of bilirubin, and a fixed concentration of bilirubin present in serum seems to exhibit higher absorbance than an equivalent concentration dissolved in DMSO. It was found by the present authors that the absorbance at 560 nm due to the sample blank is negligible when 25 µl of serum with or without hemolysis is used for total bilirubin determination in the newborn. However, with larger volumes of serum used, a sample blank should probably be included although the practice is of questionable reliability. In the suggested method, the serum specimen whether hemolyzed or not, is always added to the premixed reagent, that is, DMSO is premixed with 0.36N HCl. If a hemolyzed specimen is added to 0.36N HCl first and then the diazo coupling reagent in DMSO is added, the resulting absorbance is usually lowered as compared to the same system without hemolysis.

Studies similar to those illustrated in figure 3 were carried out using a random pediatric specimen first without hemoglobin (graph A of figure 4) and then a fixed amount of hemoglobin was added to the serum (graph B, figure 4). The concentration of hemoglobin in the final mixture was 85 µg per ml and 170 µg per ml for graphs A and C correspondingly in figure 3, and 100 µg per ml for graph B in figure 4. These represent 340, 680, and 300 mg of hemoglobin per dl in the original specimen.

Since a hemolyzed specimen is rarely encountered which contains more than 300 mg of Hb per dl, the maximum hemoglobin concentration in the original specimen studied here never exceeded 680 mg per dl. These studies were based on the use of a volume of serum of either 25 µl or 50 µl as applied to a method designed for neonates where blood specimens are often hemolyzed. However, it did not seem realistic to include in our studies serum specimens containing as much as 4,000 mg per dl as reported by others. Such a gross concentration of hemoglobin is not encountered under real circumstances, and if it is, bilirubin or any other constituent probably would be difficult to determine in this specimen.

The presence of hemoglobin does not appear to interfere in total bilirubin determinations in the concentrations studied, but hemolysis does seem to affect the direct reaction as seen on going back to figure 1. When the concentration of hemoglobin is increased from 0 mg per dl
(rise curve A) to 800 mg per dl (rise curve E) in the original specimen, there is a significant negative interference. The experiment was performed in the same fashion as described in the manual for direct bilirubin determination, except that varying concentrations of hemoglobin were included in the specimen. The horizontal lines, F, G, H, I, and J, represent the rise curves observed for the same specimen-Hb mixtures when they are added to the aqueous diazo first followed by dilution with DMSO.

It is clear that the presence of hemolysis may cause a significant error from an analytical standpoint, but it is uncertain whether or not the error would have any real effect on the diagnosis if the specimen was from an adult. Furthermore, it is relatively easy to obtain a hemoglobin-free specimen from adults and thus avoid the problem. The difficulty may become more serious when one must determine direct bilirubin in a highly jaundiced and hemolyzed specimen from an infant. A hemolysis-free sample is usually difficult to obtain and, therefore, one can expect direct bilirubin values to be lower under these circumstances. Furthermore, when serum is added to an aqueous acid solution, it is not uncommon to observe turbidity formation. A sample blank is often included to correct for turbidity. However, this type of correction is only approximate and, on occasion, the turbidity can be observed to appear more pronounced in the sample blank than in the sample. The sample blank also contains the yellow color of bilirubin which exhibits some absorbance at 560 nm and much more at shorter wavelengths, but it is not present in the sample because it has been converted to pink azobilirubin. Thus, it appears that direct bilirubin is in general underestimated.

In addition, there are different diazo concentrations employed in various methods. Since it is known that the concentration of the diazo reagent may affect azobilirubin formation, direct bilirubin concentration may vary somewhat on that basis from laboratory to laboratory as would the difference in the ratios of direct to total bilirubin. In consideration of the latter, the present authors investigated a large number of direct to total bilirubin ratios determined in our pediatric department and correlated them with the diagnosis. The ratios in many instances were higher than reported, but outside of known hemolytic jaundice, there was no evidence of additional obstruction or liver involvement in these cases.

It has been reported that a percent ratio of direct to total bilirubin exceeding 15 percent should be looked upon with suspicion. Since it is difficult to discern hemolysis visually in jaundiced specimens, it is not clear whether or not other authors did include hemolyzed specimens in their studies. In our laboratory, a simple system is employed to ascertain the presence of hemolysis. A simultaneous direct and total bilirubin, and a total bilirubin alone are determined as described in the manual. If the values for total bilirubin differ by more than 5 percent, it is concluded that hemolysis is present which may have suppressed the reaction for direct bilirubin. This procedure is by no means quantitative, but it is useful in some instances.

Effect of moderate lipemia does not seem to interfere in the determination of total bilirubin simply because the organic solvent, DMSO, solubilizes the lipids. This is not the case when an all-aqueous system is employed, such as is used in the direct bilirubin determination. In the latter case, a sample blank is usually used to correct for turbidity, but it is not certain that accurate results are possible under these circumstances. Very grossly lipemic sera probably should not be used either for direct or total bilirubin determination, although methods have been described in which steps are taken to cope with the problem.
References


