

United States Patent

Denney et al.

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[54] BILIRUBIN ASSAY

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- [52] U.S. Cl.....23/230 B, 252/408
- [51] Int. Cl.....G01n 21/24, G01n 31/22, G01n 33/16
- [58] Field of Search23/230, 310, 253; 252/408

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[57] ABSTRACT

The use of an acid solution of hydroxylamine in the analytical determination of the quantity of direct-reacting and indirect-reacting bilirubin present in blood serum or body fluids, to block reaction of bilirubin after a certain stage of the assay and to prevent interference of substances contained in erythrocytes in the assay and to stabilize the azobilirubin color formed in the reaction, and also the use of a form of hydroxylamine in place of the unstable ascorbic acids used in certain assay procedures.

9 Claims, No Drawings

BILIRUBIN ASSAY**I. Introductory Comments as to the Significance, Nature, and Other Factors as to Bilirubin Present in Blood Serum:**

Bilirubin is an orange-colored or yellowish substance or pigment found and present in blood serum, which is formed from the hemoglobin of red blood cells and which is formed as a result of the breakdown of red blood cells normally or as a result of some bodily condition.

Physiologically it is believed the bilirubin is excreted by the hepatic or liver cells into the bile. Under normal conditions of the body, the metabolism of bilirubin is a normal process; and thus a small amount of bilirubin is usually present in blood serum.

Bilirubin occurs in the blood in two forms, first in the free form or unconjugated and secondly as bilirubin glucuronide or conjugated bilirubin. Unconjugated bilirubin is formed as a decomposition product of erythrocytes and is conjugated and excreted into the bile by the liver.

The specific determination of the two types of bilirubin yields diagnostic information which can be used to differentiate various types of disease states, particularly those relating to jaundice. For instance, since bilirubin is formed as a result of red cell destruction or hemolysis, elevated levels of unconjugated bilirubin are seen in hemolytic states. One of the most common of these is hemolytic disease of the newborn due to Rh incompatibility. Bilirubin assay is used as an aid in diagnosing this disease but more importantly is used to follow the course of the disease, since at a bilirubin level of nearly 20 mg./100 ml. of serum permanent brain damage can result. To prevent this an exchange blood transfusion is performed. However, since there is some danger to the infant's life due to the exchange blood transfusion and since brain damage is possible, it is particularly necessary that the physician have an accurate and reliable bilirubin assay upon which to base his decision.

The amount of each type of bilirubin is also used to differentiate various types of liver disease. In those liver diseases in which the liver cells are damaged the cells are unable to conjugate bilirubin, an unconjugated bilirubin accumulates in the blood. In those liver diseases characterized by obstructive processes (such as stones, tumors and other space-occupying lesions) the liver is unable to excrete bilirubin, and a larger proportion of conjugated bilirubin appears in the blood.

Conjugated bilirubin is measured as direct reacting bilirubin, and unconjugated bilirubin is quantitated by measuring total bilirubin then subtracting direct reacting bilirubin. Thus an accurate direct reaction assay is necessary both for quantitation of unconjugated and conjugated bilirubin.

As an example of the importance which has long been placed on accurate measurement of direct bilirubin Duci¹, Nosslin², and Gambino³, have independently found that one third of abnormal bilirubin problems are missed if the direct reaction is not performed. Moreover, of course it is not possible to differentiate the type of disease even when the abnormal total bilirubin level is detected, unless the overall assay yields a distinction between the direct-reacting as contrasted to indirect-reacting bilirubin.

Bilirubin assay is also performed on amniotic fluid which surrounds the fetus in the uterus and is used as an indication of the degree of erythroblastosis in the fetus. When the assay indicates severe disease, the infant is delivered prematurely and the disease process is stopped by exchange transfusion. Of course there is risk to the child's life by virtue of being prematurely born, and accurate bilirubin assay is necessary for the physician to make this decision. Amniotic fluid is a particularly demanding specimen for this assay since it may contain large amounts of hemolytic products other than bilirubin which may potentially interfere with the bilirubin assay.

II. Assaying for Bilirubin: and Disadvantages of Process Using Ascorbic Acid as A Reagent

The earliest method of quantitating bilirubin involved observing its yellow color in comparison to yellow standard solutions, by visual means. This method, although somewhat useful at higher levels, is only semiquantitative, and thus cannot

yield the desired accuracy which is needed. Furthermore, the visual observation is rendered less accurate by the fact that there are a number of yellow pigments in serum, such as carotene, the color of which the eye cannot separate from the bilirubin pigment; and since this pigment level varies between 40 and 95 percent of the yellow pigment in normal serum, the estimate by this means is extremely crude and unreliable. Moreover, differentiation cannot be made between conjugated and unconjugated bilirubin by this means.

Spectrophotometric means can be used to measure the yellow pigment, but since bilirubin itself can absorb at various wave lengths depending upon other constituents in serum such measurements are complicated without special equipment.

As long ago as 1883 Ehrlich introduced the diazo reaction for bilirubin. In this reaction azosulfanilic acid is used to form azobilirubin. Ehrlich showed that azobilirubin behaves as an indicator, appearing blue at strongly acid and alkaline pH and red near neutrality.

Van den Bergh and Muller, other early workers in the field, demonstrated in 1916 that two types of bilirubin could be distinguished in serum, a direct reaction which occurs in about 1 minute in the absence of alcohol and an indirect reaction requiring the addition of alcohol. The former is now known to measure primarily conjugated bilirubin and the latter both conjugated and unconjugated bilirubin.

The use of alcohol to cause the reaction of the unconjugated bilirubin is complicated by the fact that alcohol precipitates protein; and bilirubin may be coprecipitated with the protein, thus being subsequently unavailable for the bilirubin measurement in the supernatant liquid, thus causing a negative error in the observation. Adler^{4,5} as early as 1922, reported that many substances promote diazo coupling of unconjugated bilirubin and that many of these are water soluble. In 1937 Malloy and Evelyn⁶ introduced a procedure using methanol in a final concentration of 50 percent which eliminated protein precipitation; and although there were many disadvantages such as those due to spectral shifts, turbidity, and slow reaction, the fact that the procedure could be used with a standard in chloroform (as opposed to a difficult-to-prepare standard in serum) caused its widespread acceptance, and it is used in many if not most of the clinical laboratories today.

Jendrassik and Grof⁷ in 1938 devised a method in which the red azobilirubin color was formed as in the methods recited above, then alkali was added transforming the red azobilirubin into the blue form. The blue color is desirable because there are fewer interfering pigments in this region. However, in spite of that desirability, this procedure requires a standard in protein, which was not then commercially available and which was difficult to prepare in the laboratory.

Moreover, since unconjugated bilirubin will react even in the absence of a promoter such as alcohol when in alkaline conditions, the conversion to alkaline condition complicates the measurement of conjugated bilirubin. For this reason, ascorbic acid has been added to the mixture, before alkalinization, to prevent reaction of unconjugated bilirubin in the direct procedure.

With the development, several years ago, of commercially available bilirubin standards in serum, the reason for the wide use of the Malloy-Evelyn method since about 1945 (that is, the usability with a bilirubin standard in chloroform which could be prepared in the laboratory) is eliminated. With^{8,9} and Fog¹⁰ long ago recognized the superiority of the Jendrassik Grof method over others in the literature. Watson¹¹ commented on the lack of recognition given the favorable reports of With and Fog, and suggested that a good method, that is, the method of Jendrassik and Grof, was not being utilized. The only disadvantage he suggested as to Jendrassik Grof was a slight sensitivity to hemoglobin. Moreover, Gambino¹² in the widely circulated "Manual on Bilirubin Assay" published by the American Society of Clinical Pathologists, strongly recommended the Jendrassik Grof method over other methods including that of Malloy and Evelyn, and points out that the

hemoglobin interference of Jendrassik Grof is less than that of Malloy-Evelyn.

In spite of all these recommendations, the Jendrassik Grof procedure has not become the most frequently used method in the clinical laboratory. The reason for this is that the procedure requires ascorbic acid which is unstable and cannot be used for even as long as one working day. Accordingly, ascorbic acid, if prepared for longer than its short stability life, lends to unreliability of the test; for after becoming unstable, it does not properly or consistently block the reaction of unconjugated bilirubin when performing the direct bilirubin procedure, nor properly or consistently minimize the interference of hemoglobin. It may perhaps be used by mistake, whether carelessly or inadvertently or negligently, even after becoming unstable; and the operator would wrongly interpret the observation. Perhaps the physician would not know his conclusions were erroneous also. In fact while control specimens are used for most procedures in the clinical laboratory, there is no control serum known for direct reacting bilirubin. At best, the use of ascorbic acid requires the extra expense of the repetitive small-batch preparation methods inherently required because of its exceedingly short stability life; and it has the inherent disadvantage and expense of scrapping the unused portion of the batch after just a short period of time.

While the major types of bilirubin assay methods have been discussed for illustrative purposes, there are a number of what might be referred to as minor variations of these methods, differing mainly in the type compound used to promote the reaction of unconjugated bilirubin with an azo compound and also in the type azo compound used. After Adler¹³ reported in 1922 that many substances promote diazo coupling of unconjugated bilirubin, a variety of substances were used, such as urea, caffeine, sodium benzoate and others.

III. The Present Invention

It has been found that an assay process involving the addition of an acidic hydroxylamine solution stabilizes the azobilirubin color after its formation, eliminates interference of hemolysis, and prevents reaction of unconjugated bilirubin after addition of alkali. The disadvantageous ascorbic acid is wholly eliminated, and all its disadvantages are avoided.

While an hydroxylamine compound in just equimolar concentration as ascorbic acid when substituted for the ascorbic acid used in bilirubin assay does not achieve the effect of inhibiting completely the reaction of unconjugated bilirubin after the addition of alkali in the procedure, it has been found that greatly increased hydroxylamine concentration, that is, an hydroxylamine concentration of several times that of the generally used 0.24 molar ascorbic acid, does achieve the desired effect.

Moreover, while both ascorbic acid and hydroxylamine are reducing agents under some conditions, they are considerably different compounds, ascorbic acid being an organic compound while hydroxylamine is an inorganic salt. Moreover, other reducing agents are either without effect or only partially effective, or, even if effective in inhibiting the reaction of unconjugated bilirubin, have other undesirable properties such as developing an interfering color which might be mistakenly quantitated as bilirubin.

The overall combination of several properties is achieved by the use of hydroxylamine compounds as herein set forth. That is, the properties of being compatible with acid conditions in the first stage of the direct reaction, being effective in the strongly alkaline pH of the second stage of the reaction, not forming colored complexes with azo compounds or upon exposure to air during the test, being stable upon storage in solution, and being inexpensive to purchase, are unique to hydroxylamine compounds in solution when stored under acid conditions in a concentration of at least 1.3 molar when used in the same proportions as would be the amount of ascorbic acid in the Jendrassik-Grof procedure.

Although hydroxylamine may be advantageously used in bilirubin assays in conjunction with other promoters and in conjunction with other azo compounds, the present inventive concepts are set forth illustratively in what might be referred to as a Jendrassik type method.

III.A. Reagents

Hydroxylamine Reagent: The hydroxylamine compound used is hydroxylamine hydrochloride in a 1.6 molar concentration.

Caffeine Reagent: 20 gm. caffeine, 30 gm. sodium benzoate, and 50 gm. sodium acetate are dissolved in 400 ml. of distilled water at 50° to 60° C.

Sulfanilic Acid Reagent: 15 ml. of concentrated HCl and 5 gm. sulfanilic acid are added to 500 ml. distilled water, and a quantity of distilled water sufficient to make 1 liter is added.

Sodium Nitrite: 100 gm. sodium nitrite is dissolved in 1 liter of distilled water.

Fehling II Reagent: 100 gm. sodium hydroxide and 350 gm. sodium potassium tartrate are dissolved in 1 liter distilled water.

Preparation of Diazo Reagent: 20 microliters of the above sodium nitrite solution is added to 3 ml. of the sulfanilic acid reagent. The solution is to be used in the following assay procedures within 1 hour after its preparation.

III.B. ASSAY PROCEDURES:

(It will be assumed that the user will have marked two tubes, one marked "direct" and the other marked "total.")

a. Macro Procedure

1. Add 2.4 ml. 0.05 HCl to tube marked "direct."
2. Add 2.4 ml. Caffeine Reagent to tube marked "total."
3. Add, and mix with the contents of each tube, 0.3 ml. serum or plasma.
4. Add, and mix with the contents of each tube, 0.2 ml. Diazo Reagent. Allow to stand 2 min.
5. Add, and mix with the contents of each tube, 0.1 ml. Hydroxylamine Hydrochloride Reagent.
6. Add, and mix with the contents of each tube, 1.5 ml. Fehling II Reagent. Read each tube against a water blank at 600 mu.

b. Micro Procedure

(This procedure is designed for spectrophotometers having a minimum readout volume of no more than 2 ml., such as the Coleman Jr. using a 12 x 75 mm. cuvette.)

1. Add 1.1 ml. Caffeine Reagent to "total" tube.
2. For a measurement of a direct reacting bilirubin, add 1.1 ml. of 0.05 N HCl to "direct" tube.
3. To each tube add 0.05 ml. (50 microliters) plasma or serum.
4. Add, and mix with the contents of each tube, 0.1 ml. Diazo Reagent. Allow to stand 2 minutes.
5. Add, and mix with the contents of each tube, 0.1 ml. Hydroxylamine Hydrochloride Reagent.
6. Add, and mix with the contents of each tube, 0.7 ml. Fehling II Reagent. Read against water blank at 600 mu.

An assay according to the novel concepts of the present invention thus provides multiple advantages: (a) blocking the reaction of bilirubin after a certain stage of the assay so that it is possible to distinguish the measurements of conjugated and unconjugated bilirubin; (b) prevent interference of substances contained in erythrocytes in the assay, thus, for example, suppressing the interference of hemolysis of the assay; and (c) stabilizing the azobilirubin color formed in the reaction, making less critical the time of the observation. These are all accomplished without the instability of the ascorbic acid formerly used to achieve those goals. The ascorbic acid is so unstable that it was not widely adopted for use in any of the bilirubin assays except the Jendrassik-Grof procedure, in which it was used in spite of its instability because the Jendrassik-Grof procedure was not workable at all in measuring con-

jugated bilirubin without some means of blocking the continued reaction of conjugated bilirubin in the alkaline step.

Accordingly, it will thus be seen from the foregoing description of the invention according to the embodiments of the invention herein set forth, that the present invention provides a new and useful assay yielding quantitative determination of both total and conjugated bilirubin in serum, plasma, amniotic fluid, or other biological material to be tested, and provides a method and reagents therefor, all having desired advantages and characteristics, and accomplishing the objects of the invention including the objects and advantages hereinbefore pointed out and others which are inherent in the invention.

It will be understood that modifications and variations of the general and specific concepts of the overall assay may be effected without departing from the novel concepts of this invention; accordingly, the invention is not to be considered limited to the specific form or embodiments set forth herein for the purpose of disclosing and illustrating the inventive concepts discovered and herein applied.

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What is claimed is:

1. In a method for colorimetric or spectrophotometric bilirubin assay, in which the color of the azobilirubin formed from the coupling reaction of an azo reagent with bilirubin is measured, the improvement comprising adding an acid solution of hydroxylamine to the reactant solution after the azobilirubin has formed therein.
2. The method as set forth in claim 1, in which the acid solution of hydroxylamine is prepared by adding hydroxylamine hydrochloride to water.
3. The method as set forth in claim 1, in which the bilirubin assay is an alkaline azobilirubin procedure.
4. The method as set forth in claim 1, wherein the hydroxylamine is added to the reactant solution in place of ascorbic acid.
5. The method as set forth in claim 1, in which the acid hydroxylamine solution is prepared by dissolving an acid hydroxylamine salt in water.
6. In a method for colorimetric or spectrophotometric bilirubin assay, in which the color of azobilirubin formed from the coupling reaction of an azo reagent with bilirubin is measured, the improvement comprising using hydroxylamine in the reactant solution after the azobilirubin has formed therein, the hydroxylamine having been maintained stable prior to such use by being acidic.
7. The method as set forth in claim 6, in which the bilirubin assay is an alkaline azobilirubin procedure.
8. The method as set forth in claim 6, wherein the hydroxylamine is used in place of ascorbic acid.
9. In a method of bilirubin assay involving the measurement of the blue color of azobilirubin formed by the reaction of bilirubin and an azo reagent, the improvement comprising using hydroxylamine in a concentration which gives the same final concentration of hydroxylamine as when a solution which is 13 molar or greater of the hydroxylamine is substituted for ascorbic acid in a Jendrassik-Grof procedure.

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UNITED STATES PATENT OFFICE
CERTIFICATE OF CORRECTION

Patent No. 3,652,222 Dated March 28, 1972.

Inventor(s) Jerry W. Denney et al.

It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

Column 1, line 40, "an" should read -- and --. Column 3, line 56, "compounds" should read -- substances --; lines 56 and 57, "compound" should read -- substance --; line 57, "salt" should read -- substance --. Column 5, line 24, the reference numeral "14" should read -- 3 --. Column 6, line 36, the molarity number "13" should read -- 1.3 --.

Signed and sealed this 20th day of February 1973.

(SEAL)
Attest:

EDWARD M. FLETCHER, JR.
Attesting Officer

ROBERT GOTTSCHALK
Commissioner of Patents