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[54] **REAGENT AND METHOD FOR BILIRUBIN DETERMINATION**

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[51] **Int. Cl.** **G01n 33/16**

[58] **Field of Search** ..... 23/230 B; 252/408

[56] **References Cited**

UNITED STATES PATENTS

2,854,317	9/1958	Free et al.	23/230 B
3,511,607	5/1970	Green	23/230 B
3,585,001	6/1971	Mast	23/230 B

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[57] **ABSTRACT**

A reagent and method for the determination of total serum bilirubin by coupling of bilirubin with a stable diazonium salt, Fast Red PDC or diazotate-N'-butyl-4-methoxy-metanilamide, at an acid pH is disclosed.

14 Claims, No Drawings

REAGENT AND METHOD FOR BILIRUBIN DETERMINATION

BACKGROUND OF THIS INVENTION

1. Field of this Invention

This invention relates to a novel reagent and method for the colorimetric determination of total serum bilirubin; and more particularly, to a novel method for the determination of bilirubin by coupling with a specific diazonium salt, diazotate-N'-butyl-4-methoxymetanilamide at an acid pH.

2. Prior Art

The determination of bilirubin is frequently performed in clinical laboratories, as the determination has value in the diagnosis of cirrhosis and other hepatic diseases.

There are three basic colorimetric approaches to measurement of bilirubin: (1) direct spectrophotometric measurement of bilirubin at 450 nm; (2) diazo-coupling with diazotized sulfanilic acid; and (3) coupling with stable diazonium salts, particularly p-nitrobenzene-diazo-p-toluenesulfonate. The first approach suffers from serious interference by non-bilirubin pigments and complicated mathematical calculations are required to obtain accurate results. The second approach suffers from the instability of reagents and requires that the diazotized sulfanilic acid be prepared fresh daily. The third approach has heretofore suffered from insensitivity and all methods using stable diazonium salts have been qualitative and semi-quantitative at best, rather than quantitative. There is a need for a method to determine bilirubin that is specific and not subject to interference, and which utilizes a reagent that is reasonably stable and does not require daily preparation.

BROAD DESCRIPTION OF THIS INVENTION

It is the primary objective of this invention to provide a colorimetric method and reagent for the determination of bilirubin.

Another object is to provide a direct method without the need to remove proteins and possible interfering substances.

Still another object is to provide a method that utilizes a stable reagent that need not be prepared fresh daily.

Yet another object is to provide a method that is highly sensitive and specific, yielding quantitative determinations.

It has been found that a low pH (of 0.5 to 2.5, preferably about 1.8) in a solution of high molarity diazonium salts such as Fast Red RC, Fast Red GG, Fast Red B, Fast Red TR, Fast Blue B, Fast Bordeaux BD, etc., couple with bilirubin to give a color change that can be used to assy bilirubin. Of the innumerable diazonium salts tested, Fast Red PDC (diazoate-N'-butyl-4-methoxymetanilamide) proved to have significantly greater sensitivity, specificity, and extinction than the others. In addition, it was found that assays with Fast Red PDC were quantitative and not merely qualitative or semi-quantitative. So the preferred diazonium salt is Fast Red PDC. It has further been found that maleic acid is superior as a buffer in such bilirubin determinations to other acids that buffer in the desired pH range of about 0.5 to about 2.5. Such other acid buffers include oxalic acid, trihydroxybenzoic acid, dihydroxy-

tartaric acid, citric acid, fumaric acid, succinic acid, hydrochloric acid, sulfuric acid, butyric acid, dichloroacetic acid, hihydroxymalic acid, malonic acid, lactic acid, boric acid, etc.

The first embodiment of this invention involves a reagent which is a diazonium salt and maleic acid buffer, the solution having a pH between 0.5 and 2.5.

The reagent, in a more specific sense, contains 0.01 to 10 gram percent of a diazonium salt or Fast Red PDC, and 5 to 50 gram percent of a suitable acid buffer or maleic acid buffer (preferably 22 percent maleic acid buffer). The reagent preferably has a pH of 1.8.

This invention is practised by mixing a small sample of biological fluid with a reagent, in a ratio of 1/100 to 1/25. The absorbance is read, using a colorimeter, at an appropriate wavelength or with a suitable color filter. With Fast Red PDC, the absorbance is read between 490 and 650 nm, preferably at 590 nm. The absorbance is then converted to bilirubin concentration, such conversion can be done by means of preparing a conversion chart from known examples as illustrated in Example 1.

The reagent of this invention does not have to be prepared daily and can be in effect freeze-dried for storage of up to one year before use. The method of this invention is highly sensitive and specific, and yields quantitative determination.

Any suitable colorimeter or spectrophotometer can be used to measure the absorbance. Examples of useful colorimeters are: Coleman, Model 44; Perkin-Elmer, Model 124; the colorimeter disclosed in U.S. Ser. No. 224,457, applicants: Raymond W. Kiess and Peter H. Stewart, filed: Feb. 8, 1972, assignee: Kiess Instruments, Inc., 8768 S. W. 131st Street, Miami, Florida, 33156; and the direct reading colorimeter disclosed in U.S. Pat. No. 3,561,878, inventor: R. W. Kiess.

The term acid buffer, as used herein, means that a salt thereof is included. An example is maleic acid buffer, which includes maleic acid and a salt or ester thereof, e.g., sodium maleate, potassium maleate, barium maleate and lithium maleate.

Examples of useful diazonium salts are Fast Red PDC (preferred), Fast Red RC, Fast Red GG, Fast Orange GR, Fast Red B, Fast Orange R, Fast Red TR, Fast Red base AL, Fast Blue B, Fast Red B base, Fast Bordeaux BD, Fast Red GG base, Fast Black B base, Fast Red GL base, Fast Blue base, Fast Red 3 GL base, Fast Blue Red 0 base, Fast Red RL base, Fast Scarlet G base, and fast Scarlet R base.

The biological fluids tested can be those of man or animal.

DETAILED DESCRIPTION OF THIS INVENTION

The following examples illustrate the invention but they do not limit it.

EXAMPLE 1

The reagent is prepared by dissolving 2 grams of Fast Red PDC in 800 ml of deionized water. 220 grams maleic acid are added and dissolved, and the reagent is brought with water to 1 liter. To a series of tubes is added 2.5 ml of reagent. A set of standards are prepared and made to contain 0.1, 0.5, 0.8, 1.0. 2.0, 4.0, 12.0 and 15.0 mg percent of a specified level of bilirubin. The tubes are incubated at 37° C. for 5 minutes. A stable purple color develops and the absorbance is read at 590 nm using a colorimeter (Coleman Model 44).

There is a linear proportion between the bilirubin level and the absorbance.

EXAMPLE 2

The reagent is prepared and tubed as in Example 1. A set of standards are prepared as in Example 1, except that they are prepared in pooled human serum. The test procedure of Example 1 is followed. Again, a stable purple color develops within 5 minutes. The absorbance is linear in proportion to the bilirubin concentration.

EXAMPLE 3

The reagent is prepared as in Example 1. The reagent is stored for a month in the dark in a refrigerator at 5° C. The procedure of Example 2 is followed on the stored reagent. The absorbance is linear in proportion to the bilirubin concentration.

EXAMPLE 4

The reagent is prepared into two components. The first component is prepared by dissolving 10 grams of Fast Red PDC in 1 liter of deionized water, dispensing 0.5 ml to a set of ampules, freeze-drying the dye in the ampules, and sealing the ampules under nitrogen. The second component is prepared by dissolving 220 grams of maleic acid in a liter of deionized water. The two components are stored at room temperature for one year. At the end of a year, 2.5 ml of the second component is added to the tubes of the freeze-dried first component. The reagent is then tested as in Example 2. The results are the same as in Example 2.

What is claimed is:

1. A reagent for determining total bilirubin in biological fluids comprised of an aqueous solution of Fast Red PDC, which is diazoate-N′-butyl-4-methoxymetanilamide, and an acid buffer, said solution having a pH between 0.5 and 2.5.

2. A reagent according to claim **1** wherein the acid buffer is maleic acid buffer.

3. A reagent according to claim **1** wherein the Fast Red PDC is present at a level of 0.01 to 10 gram percent.

4. A reagent according to claim **1** wherein the acid buffer is present at a level of 5 to 50 percent by weight or volume.

5. A reagent according to claim **1** wherein the Fast Red PDC is present at a level of 0.01 to 10 gram percent, and the acid buffer is present at a level of 5 to 50 percent by weight.

6. A reagent according to claim **5** wherein the acid buffer is maleic acid buffer.

7. A reagent according to claim **6** wherein the maleic acid buffer includes maleic acid and a member selected from the group consisting of sodium maleate, potassium maleate, barium maleate, and lithium maleate.

8. A method for determining the total bilirubin in biological fluids which comprises admixing a sample of biological fluid with the reagent of claim **19,** the ratio of the biological fluid to the reagent being between 1:100 and 1:25, and determining the concentration of bilirubin by means of measuring the absorbance.

9. A method according to claim **8** wherein the acid buffer is maleic acid buffer.

10. A method according to claim **8** wherein the Fast Red PDC is present at a level of 0.01 to 10 gram percent.

11. A method according to claim **8** wherein the acid is present at a level of 5 to 50 percent by weight or volume.

12. A method according to claim **8** wherein the Fast Red PDC is present at a level of 0.01 to 10 gram percent, and the acid buffer is present at a level of 5 to 50 percent by weight or volume.

13. A method according to claim **12** wherein the acid buffer is maleic acid buffer.

14. A method according to claim **13** wherein the maleic acid buffer includes maleic acid and a member selected from the group consisting of sodium maleate, potassium maleate, barium maleate, and lithium maleate.

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