United States Patent [19]

Ertingshausen et al.

[11] B **3,923,459**

[45] **Dec. 2, 1975**

[54] **PROCESS FOR THE DETERMINATION OF BILIRUBIN IN FLUIDS**

[75] Inventors: **Gerhard Ertingshausen,** New York; **Diane F. Byrd,** Tarrytown, both of N.Y.

[73] Assignee: **Union Carbide Corporation,** New York, N.Y.

[22] Filed: **Dec. 14, 1972**

[21] Appl. No.: **314,977**

[44] Published under the Trial Voluntary Protest Program on January 28, 1975 as document no. B 314,977.

[52] **U.S. Cl.** ..... **23/230 B;** 252/408; 260/141

[51] **Int. Cl.**[2] **G01N 33/16;** C07C 113/00

[58] **Field of Search** ..... 23/230 B; 252/408; 260/141

[56] **References Cited**

UNITED STATES PATENTS

3,585,001	6/1971	Mast	23/230 B
3,682,586	8/1972	Ertingshausen et al.	23/230 B

OTHER PUBLICATIONS

Bartels et al. "Automated Determination of Serum Bilirubin" Anal. Abst. Vol. 22 Jan. 1972 Abstract 329.

Primary Examiner—Morris O. Wolk
Assistant Examiner—Arnold Turk
Attorney, Agent, or Firm—William Raymond Moran

[57] **ABSTRACT**

A method is provided for the spectrophotometric determination of bilirubin in fluids, particularly body fluids, such as blood serum. The process comprises reacting the bilirubin-containing fluid with a mixture of diazosulfanilic acid-1,5-naphthalene disulfonate and ethylene or propylene glycol in aqueous mineral acid to form a chromophore, and thereafter measuring the absorbance of the chromophore after the reaction is complete by means of a centrifugal analytical photometer. Inasmuch as a linear relationship exists between the bilirubin concentration and the change of absorbance, the concentration in an unknown sample can be conveniently calculated by comparison with the results obtained from the simultaneous measurement of a sample of known concentration.

12 Claims, No Drawings

PROCESS FOR THE DETERMINATION OF BILIRUBIN IN FLUIDS

This invention relates in general to a process for the determination of bilirubin in fluids, particularly body fluids. In one aspect, the invention relates to a process for the determination of bilirubin in blood serum. In a further aspect the invention relates to a process for the determination of bilirubin using a centifugal analytical photometer.

In recent years the need for more sophisticated quantitative analytical methods has increased markedly due to extensive microanalytical studies in biochemical research, routine clinical testing for physicians and hospitals, and the like. In addition to the increased demand for new methods of analysis, in certain fields it is often highly desirable that the method be simple to perform, be rapid and yet provide consistently reliable results. This is particularly important for clinical testing of body fluids where a proper diagnosis or treatment often depends upon the information provided by analyses. However, few methods are available which can rapidly and accurately handle the increasing number and varied tests desired by clinicians.

The determination of bilirubin in body fluids, such as blood serum, is illustrative of clinical testing which is assuming a steadily growing share of the clinical laboratory's work load, and for which a need for more simple and rapid quantitative analytical methods exist. The determination of bilirubin in serum is important in several diseases, in particular hepatitis and erythroblastosis fetalis, where bilirubin elevation occurs. However, despite this need, bilirubin in biological samples is still determined photometrically employing variations of a single basic procedure involving the use of diazotized sulfanilic acid which reacts with bilirubin to form a chromophore (Evelyn, K.A. and Malloy, H.T. , J. Biol. Chem., 126, 655, 1938; Jendrassik, L. and Grof, P., Biochem. Z., 297, 8, 1938). Because of the instability of the diazotized sulfanilic acid employed in this technique, however, the reagent has to be prepared immediately prior to use by reacting separate solutions of sulfanilic acid and sodium nitrite, which are themselves unstable. As a result, the process does not fulfill the needs of the clinical testing laboratory for simple and rapid testing techniques employing conveniently packaged "ready-to-use" reagents.

One widely used variation of the procedure employing diazotized sulfanilic acid as a test for bilirubin involves the use of methanol as an accelerator for the reaction between these two materials. In this method, in order to avoid precipitation of protein in the serum by the methanol, it is necessary to predilute the serum before adding the methanol. Because of this dilution, however, it is necessary to run a blank measurement of serum absorbance, thus adding an additional process step to the analysis and making the method unattractive for clinical testing purposes.

A second widely used variation of the procedure employing diazotized sulfanilic acid as a test for bilirubin involves the use of caffeine as an accelerator. While the need for serum dilution and blank measurements is eliminated by this technique, the use of caffeine requires that the reaction mixture be made alkaline in order to shift the absorbance of the resulting chromophore to a longer wavelength so that it can be determined photometrically. As a result, this process, as in

the case when methanol is used as an accelerator, is slow, tedious, complicated and incompatible with clinical testing techniques.

The use of a solution of diazo-2,4-dichloroaniline together with an ethylene glycol accelerator has been suggested as a reagent for determining the bilirubin content of biological samples (Hillmann, G. and Beyer, G., Z.Klin.Chem., 5, 92-3, 1967). However, while this procedure is rapid and does not usually require a blank measurement, the solution, like solutions of diazotized sulfanilic acid, has not been found to be sufficiently stable to fill the needs of the clinical testing laboratory for a conveniently packaged "ready-to-use" product.

Solutions of diazo-2-chloro-4-nitroaniline and several other arylamines have been successfully stabilized with various stabilizers and employed to determine the bilirubin content of biological samples (Bartels, H. and Bohmer, M., Z. Klin. Chem., 7, 444, 1969). However, as in the case of diazotized sulfanilic acid solutions which employ caffeine as an accelerator, the mixture must be made alkaline in order to shift the absorbance of the resulting chromophore to a longer wavelength to permit it to be determined photometrically.

It is, therefore, an object of this invention to provide a process wherein many of the disadvantages indicated above are eliminated or minimized. Another object of this invention is to provide a process for the determination of bilirubin which is much more rapid than those currently in use. A further object of the invention is to provide a process for the determination of bilirubin which utilizes a centrifugal analytical photometer. A still further object of the invention is to provide a process for the determination of bilirubin which employs a stable diagnostic reagent which can be packaged in a convenient "ready-to-use" form. An additional object of the invention is to provide a reagent which when added to a bilirubin-containing serum generates a chromophore whose absorbance is linearily related to bilirubin concentration, and whose use does not require dilution and a blank absorbance measurement on the test sample prior to use. Yet another object of the invention is to provide a process which does not require a blank absorbance measurement on the diagnostic reagent employed, or that the reaction mixture be made alkaline or treated with another reagent, in order to permit determination of bilirubin photometrically. These and other objects will readily become apparent to those skilled in the art in the light of the teachings herein set forth.

In accordance with the present invention, it has now been discovered thatt stable diazosulfanilic acid-1,5-naphthalene disulfonate can be prepared, and that mixtures of this material and ethylene or propylene glycol in aqueous mineral acid can be employed as a diagnostic reagent for total bilirubin in body fluids by reacting with said bilirubin to form a chromophore whhose absorbance can be measured after the reaction is complete by spectrophotometric means. The purpose of the ethylene or propylene glycol is that of an accelerator for the reaction between the diazosulfanilic acid-1,5-naphthalene disulfonate and bilirubin, but does not introduce any of the disadvantages associated with other accelerators such as methanol and caffeine. The diazosulfanilic acid-1,5-naphthalene disulfonate is stable over long periods of time and can be stored in closed vials for at least six months at room temperature. As a result, this material can be packaged and sold as a convenient "ready-for-use" diagnostic reagent.

In its broad aspect the invention is directed to a process for the determination of bilirubin in fluids. The process comprises the steps of:

a. forming a mixture of a bilirubin-containing fluid and a mixture of diazosulfanilic acid-1,5-naphthalene disulfonate and ethylene or propylene glycol in aqueous mineral acid;

b. measuring by means of a centrifugal analytical photometer an absorbance reading of the mixture at 550 nm (nanometers) after the reaction between the bilirubin and the diazosulfanilic acid-1,5-naphthalene disulfonate is complete;

c. comparing the absorbance reading with the absorbance reading obtained simultaneously under the same conditions from a mixture containing a known concentration of bilirubin; and

d. determining the amount of bilirubin in the bilirubin-containing fluid.

The reaction between bilirubin and diazosulfanilic acid-1,5-naphthalene disulfonate in an aqueous mineral acid solution results in the formation of a purple colored bilirubin diazonium salt complex. By comparing the intensity of the color of the resulting complex to that of a complex prepared in like manner from a fluid containing a known concentration of bilirubin, it is possible to quantitatively determine the amount of bilirubin present. Absorption of this complex occurs in the visible range and can be quantitatively measured at 550 nm (nanometers). In view of the fact that fluids containing bilirubin concentrations of up to 20 milligrams per 100 milliliters exhibit a linear increase in absorbance with increasing concentration when reacted with the diagnostic reagent, it is possible to accurately determine the amount of bilirubin present over a relatively wide concentration range.

As indicated above, the absorbance of the purple colored complex produced by the reaction of bilirubin and diazosulfanilic acid-1,5-naphthalene disulfonate in an aqueous mineral acid solution can be measured in accordance with the instant invention by means of a centrifugal analytical photometer, and the amount of bilirubin present can be quantitatively determined by a comparison of this absorbance with that of a complex prepared in like manner from a fluid containing a known concentration of bilirubin. Analytical photometers which utilize a centrifugal field have recently become available for the rapid microanalysis of a wide variety of liquids, such as body fluids, e.g., blood serum, food products, and the like. Since numerous analyses can be performed rapidly and simultaneously, these devices are of particular interest wherein a large number of samples is involved or a variety of tests on one sample is desired. Moreover, since these devices allow the use of relatively small volumes of reagents, i.e., in the microliter range, the use of expensive reagents can be minimized.

One such device which utitlizes a centrifugal field in microanalytical studies is described in U.S. Pat. No. 3,555,284. This device employs the principle of double-beam spectrophotometry wherein absorbances of a liquid sample and a reference solution are intercompared. The system is basically a series of cuvettes arranged around the periphery of a rotor so that when it is spun, centrifugal force transfers reagents and samples to the cuvettes where the concentration is measured spectrophotometrically. A sample loading disk is provided which consists of rows of cavities arranged concentrically. Reagents are placed in the innermost cavity and serum samples in the center cavity of the sample loading disk which is then indexed and positioned in the rotor with each reagent and serum sample having its respective cuvette. As the rotor is accelerated, centrifugal force moves the reagents and sample to the outermost cavity where they are transferred through a small channel to the curvette. During the transfer, the reagent and sample mix. The filled cuvettes rapidly spin past the fixed light beam and the transmission of light is measured.

For the analysis of bilirubin by the process of this invention a centrifugal rotary photometer supplied by Union Carbide Corporation under the trademark "CentrifiChem" was utilized. In this instrument, a Teflon disk containing samples and reagents is inserted into a rotor with **30** radially arranged cuvettes. When the rotor starts spinning, the reagent rises from every individual reagent well up to individual sample cavities, and the sample reagent mixture is transferred into the single cuvettes within 1.5 seconds. One cuvette containing water is used as a reference when the cuvettes spin past the stationary light beam of a spectrophotometer which measures the absorbance and displays it on an oscilloscope. Two sets of digitized absorbance readings of each cuvette can be stored simultaneously and the difference between them processed in a computer. A reading is taken of the first absorbance after start and the time interval after which the second measurement occurs also noted. A number of readings can be taken after the initial one. In the rate mode the absorbance change per interval is expressed in ΔA/min. In the end point mode the absorbance differences between initial and every individual subsequent set of readings is measured. This approach allows automatic compensation for cuvette to cuvette variations and for serum and reagent blanks if readings are taken before the reaction has started to a measurable degree. Also, a blank reading recorded in a preceding run can be used as initial reading in a subsequent run. After a selected time interval has elasped, the readings are printed out. The last set of data stored in the memory can be multiplied by means of the computer which permits direct print-out in concentration units. The rotor is thermostated within ± 0.1°C. in an air bath.

In practice it has been found convenient to employ 25 microliters of sample having a bilirubin concentration within the range up to 20 milligrams per hundred milliliters with 50 microliters of diluent and 250 microliters of the diagnostic reagent solution. The amount of sample and reagent can be varied depending upon the concentration of reagent, amount of bilirubin present and the particular analyzer employed. For example, ratios of microliters of sample to microliters of diagnostic reagent solution of from 1:5 to 1:25 can be employed.

As has been indicated, the absorbance is measured after reaction between the bilirubin and diazosulfanilic acid-1,5-naphthalene disulfonate is complete, and compared to the absorbance obtained simultaneously from a mixture containing a known concentration of bilirubin. In practice, it has been found that 4 or 5 seconds are required to complete transfer and mixing of the sample and reagent due to differences in viscosity between the accelerator employed (ethylene glycol or propylene glycol) and serum/ water. Once transfer and mixing has been completed, the color develops rapidly during the first two minutes and is completely developed after 5 minutes.

The mixture of diazosulfanilic acid-1,5-naphthalene disulfonate and ethylene or propylene glycol in aqueous mineral acid employed as diagnostic reagent for bilirubin according to the present invention can be conveniently prepared by dissolving from 125 milligrams to 400 milligrams of diazosulfanilic acid-1,5-naphthalene disulfonate in mixtures containing from 3 milliliters to 25 milliliters of 0.5 N to 1.0 N HCl and 75 milliliters of ethylene or propylene glycol. Specifically, a suitable reagent can be produced by dissolving 150 milligrams of diazosulfanilic acid-1,5-naphthalene disulfonate in a mixture of 75 milliliters of ethylene glycol and 3.6 milliliters of 1.0 N hydrochloric acid.

The diazosulfanilic acid-1,5-naphthalene disulfonate employed in the diagnostic reagent was prepared as follows: 1.73 grams (0.01 moles) of p-sulfanilic acid were added to a well stirred mixture of 3 milliliters of distilled water, 0.1 grams of a dispersing agent (Ultravon) and 3 grams of an aqueous solution containing 33–38% hydrogen chloride. After a good suspension was obtained, five grams of ice were added and the mixture was placed in an ice bath until the temperature reached −5°C. One and seven-tenth milliliters (1.7 ml.) of a 40 percent by weight aqueous solution of $NaNo_2$ were then rapidly added under the surface of the suspension. The temperature of the suspension was maintained at 5°–7°C. for 15 minutes, and then at room temperature for another 15 minutes. The addition of the $NaNo_2$ solution caused most of the solid present to dissolve, and on further stirring, a new solid began to precipitate. A solution of 2.90 grams (0.01moles) of 1.5-naphthalene disulfonic acid in 1.5 milliliters of water was then added to the mixture which was then successively stirred for another 15 minutes, chilled in an ice bath and filtered. The solid diazosulfanilic acid 1,5-naphthalene disulfonate produced in this manner was then washed successively with water, acetone and dried under vacuum.

A standard bilirubin reference solution can be prepared by dissolving 20 milligrams of bilirubin in 100 milliliters of 0.1 N Na_2CO_3.

The following example is illustrative:

EXAMPLE 1

A blank value was determined for each cuvette of a CentrifiChem Automatic Analyzer and stored in the analyzer until the actual test was performed. These values were obtained by loading the reagent cavities of the transfer disc sample holder with 400 microliters of water in each position, spinning it into the cuvettes, and allowing the instrument to record the absorbance of each cuvette after three seconds on a data channel. The value at three seconds was stored in the analyzer by turning the "write/store" switch on the analyzer.

Sample cavities 1 and 2 of the transfer disc sample holder of the CentrifiChem Automatic Analyzer were then loaded with 25 microliters of a bilirubin standard (containing 20.3 milligrams of billirubin per 100 milliliters of 0.1 N $Na_2 CO_3$) and each sample was diluted with an additional 50 microliters of distilled water. The remaining sample cavities, i.e., cavities 3–29, were each loaded with 25 microliter samples of human sera diluted with 50 microliters of distilled water. Each reagent cavity of the sample holder, i.e., 1–29 was loaded with 250 microliters of a mixture containing 25 milligrams of diazosulfanilic acid-1,5, -naphthalene disulfonate per 13.1 milliliters of a mixtuer of ethylene glycol and 1.0 N HCl in a 20:1 volume ratio. The reference position contained 400 milliliters of distilled water in the innermost cavity.

The loaded disc was placed in the rotor and covered. Because of differences in viscosity between ethylene glycol and serum/water, the solutions were given an extra mixing by simultaneously pressing the "spin" and "mix" buttons for 4 seconds (pressing the "mix" button draws a stream of bubbles through each cuvette to allow proper mixing of the contents thereof).

After 5 minutes, the instrument read the absorbance of each cuvette, and automatically substracted from it the corresponding stored blank value. Absorbance was measured with a 550 nm interference filter.

The absorbance of the samples compared to the standard was used to determine bilirubin concentrations. According to Beer's Law, the absorbance of a solution is proportional to the concentration of the solution's chromophore as long as the concentration is low. Accordingly, the concentration of bilirubin complex formed in the reaction is at any time proportional to the absorbance it generates. In turn, the concentration of the bilirubin complex is proportional to the original concentratiton of bilirubin.

In order to convert absorbance into concentration , the absorbance has to be multiplied with a factor F (Absorbance × F = Concentration). In the analyzer employed this is achieved by setting the appropriate factor on a digital switch which automatically multiples the absorbance whereupon concentration units will be printed out directlty.

During the analytical run, a standard of known bilirubin concentration is always run with the samples. If the results for the standard show slight variations from the correct concentrations, a setting of a digital switch is changed until the correct value is printed out. The same correction is then automatically applied to the results from the unknown sample.

Although the invention has been illustrated by the preceding disclosure, it is not to be construed as being limited to the particular embodiments or materials disclosed therein. Rather, the invention encompasses the generic area hereinbefore disclosed. Various modifications and embodiments thereof can be made without departing from the spirit and scope thereof.

What is claimed is:

1. A process for the determination of bilirubin in a bilirubin-containing fluid which comprises the steps of:

a. forming a mixture of said bilirubin-containing fluid and a mixture of diazosulfanilic acid-1,5-naphthalene disulfonate and an accelerator selected from the group consisting of ethylene glycol and propylene glycol in an aqueous mineral acid;

b. measuring by means of a centrifugal analytical photometer an absorbance reading of the mixture at 550 nanometers after reaction between the bilirubin and the diazosulfanilic acid-1,5-naphthalene disulfonate is complete;

c. comparing the absorbance reading with the absorbance reading obtained simultaneously under the same conditions from a mixture containing a known concentration of bilirubin; and

d. determining the amount of bilirubin in said bilirubin-containing fluid.

2. The process of claim **1** where the aqueous mineral acid is hydrochloric acid.

3. The process of claim **1** where the accelerator is ethylene glycol.

4. The process of claim 3 where the aqueous mineral acid is hydrochloric acid.

5. The process of claim 1 wherein said fluid is a body fluid.

6. The process of claim 5 where the aqueous mineral acid is hydrochloric acid.

7. The process of claim 5 where the accelerator is ethylene glycol.

8. The process of claim 7 where the aqueous mineral acid is hydrochloric acid.

9. The process of claim 1 wherein said fluid is blood serum.

10. The process of claim 9 where the aqueous mineral acid is hydrochloric acid.

11. The process of claim 9 where the accelerator is ethylene glycol.

12. The process of claim 11 where the aqueous mineral acid is hydrochloric acid.

* * * * *