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METHOD AND COMPOSITION FOR TESTING BILIRUBIN IN URINE

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This invention relates to diagnostic composition for detecting and estimating bilirubin in urine.

The qualitative detection and semi-quantitative estimation of bilirubin in urine is important in the diagnosis of liver dysfunctions in which bilirubinuria occurs. Normal urine contains no appreciable amount (less than 0.05 mg./100 ml.) of bilirubin. However, when the bile duct is obstructed or the polygonal cells of the liver are damaged by anoxia, infections, chemicals, and the like, bilirubin accumulates in the body, and often times the first sign of early liver dysfunction is the appearance of bilirubin in the urine. Bilirubinuria occurs long before jaundice is apparent and it may even occur before the bilirubin level of the blood increases significantly.

The determination of bilirubin in urine is likewise important in differentiating jaundice due to obstruction or liver damage from hemolytic jaundice. Hemolytic jaundice is caused by excessive red blood cell destruction and not by specific liver dysfunction or biliary obstruction. In hemolytic jaundice, the elevated bilirubin of the blood is of a type which is not excreted by the kidney. In other words, a positive test for bilirubin in the urine of a patient with elevated blood bilirubin level and, of course, a jaundiced appearance, would suggest the presence of some hepatic dysfunction whereas a negative test for bilirubin in the urine of such a patient would suggest hemolytic jaundice.

Despite the importance of such a test, the techniques, procedures, and reagents heretofore used for the detection of bilirubin in urine have not been found entirely satisfactory. Thus, Rosenbach's test for bilirubin in the urine (a modification of the earlier Gmelin's test) consists in filtering a small amount of urine through a filter paper, and then dropping a drop of concentrated nitric acid in the cone of the paper. If bilirubin is present, there results a play of various colors (green, blue, violet, red and yellow). However, many other compounds which occur in normal urine, or in urine of patients under various therapeutic regimes may interfere with this test by giving a color with nitric acid. Furthermore, this test is sensitive only to concentrations of bilirubin of 2 mg./100 ml. or more. Furthermore, the concentrated nitric acid which is used as an oxidizing agent is a dangerous liquid reagent, particularly in the hands of a layman, and accordingly, the test is not advisedly made by unskilled persons.

Fouchet's reagent is another strongly acid solution (ferric chloride in trichloroacetic acid) used in detection of bilirubin in urine. In this test strips of filter paper are soaked in barium chloride solution and allowed to dry. Dry strips are then dipped in the urine to be tested and any bilirubin present in the urine is adsorbed on the barium sulfate which precipitates on the paper due to the combination of barium ions with sulfate ions in the urine. A drop of Fouchet's reagent is placed on the paper where it is wet with urine and the development of a green color (biliverdin) shows the presence of bilirubin.

This test is sensitive to about 0.1 mg. bilirubin/100 ml. of urine.

Another commonly used test for bilirubin in urine is the methylene blue test, wherein a solution of the methylene blue is added drop by drop to a measured amount of urine in a test tube. One drop of methylene blue solution will give a green color when added to most urines, and as more methylene blue is added, the color changes from green to blue. Urine which contains bilirubin requires more methylene blue to render it blue than does normal urine, and consequently the greater the amount of bilirubin in urine, the greater the amount of dye necessary to produce a persistent blue color. However, it requires a trained technician to read the end point since the initial green color very gradually becomes blue and no sharp color change is noted with either normal or bilirubin-containing urines. Furthermore, the sensitivity of this test is such that it only detects concentrations of bilirubin above 1 or 2 mg. per 100 ml. of urine.

It is seen then, that the common tests for bilirubinuria posses one or more disadvantages, among them being the following:

(1) They require strong acid, in the form of liquid reagents, which are inconvenient and frequently dangerous to use.

(2) They require the skill of a trained technician.

(3) They may be satisfactorily performed only in a well equipped laboratory.

(4) They may give tests with negative urines which may be confused with positive urines even by skilled technicians.

(5) Many such tests are complicated and time consuming, requiring several steps.

The principal object of the present invention therefore is to provide a simple accurate specific test for bilirubin in urine which can successfully be used even by an unskilled person to readily distinguish between positive and negative specimens.

The present invention proceeds on the discovery that urinary bilirubin may be adsorbed on a test mat and having thus been separated from the urine may be identified by means of a novel bilirubin reagent composition in dry solid form, preferably in the form of a test tablet.

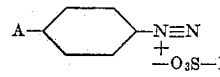
Generally, the procedure, according to the present invention, of determining bilirubin in urine, comprises moistening a test mat formed of bibulous, bilirubin-adsorbing material, and then placing a test tablet having a composition to be described hereinafter, on the spot where the urine has moistened the test mat. The tablet is then "flooded" with a few drops of water. If the urine which is being tested contains bilirubin, the area of the test mat surrounding the test tablet will assume a purple coloration. We have found that this coloration is highly characteristic and specific for bilirubin-positive urine specimens, even with urines containing as little as 0.05 mg. of bilirubin per 100 ml.

The test mat used in the practice of our invention is a fibrous material such as cellulose, and may contain associated therewith, material such as asbestos or other "filter aids" such as Celite and the like which have a high adsorption affinity for bilirubin in urine. The composition of the test mat may be varied from pure cellulose to mixtures of cellulose fibers and filter aids containing preferably 11% and as much as 20% of materials like Celite, asbestos, clays and the like. A very satisfactory test mat is of the type commercially available in sheet form as "Hy-Speed AAA–1 Quality Filter Sheets, Density 69" and available from the Alsop Engineering Corp., of Milldale, Connecticut.

The bilirubin reagent composition, which, as hereinbefore stated is preferably in the form of a tablet, con-

tains a stable diazonium salt which under acid conditions produces with the bilirubin a bright purple color. Among the stable diazonium salts which we have found useful in the practice of our invention are p-nitro benzene diazonium p-toluene sulfonate, and pu-sulfobenzene diazonium 4-nitrotoluene-2-sulfonate.

The diazonium salts which can be used as components of our novel bilirubin reagent composition are represented by the following generic formula:



wherein A is a radical selected from the group consisting of carboxyl, sulfo and nitro, and B is a member selected from the group consisting of benezene, p-toluene, and 4-nitrotoluene-2-.

Specific representative additional examples of such diazonium salts are p-carboxybenzene diazonium benzene sulfonate; p-carboxybenzene diazonium p-toluene sulfonate; p-corboxybenzene diazonium 4-nitrotoluene-2-sulfonate; p-nitrobenzene diazonium 4-nitrotoluene-2-sulfonate; and p-sulfobenzene diazonium p-toluene sulfonate.

As indicated above, acid conditions are necessary before the reaction between the adsorbed bilirubin on the test mat and the diazonium salt component of our reagent takes place. The acid we prefer to use for this purpose is sulfosalicylic acid. This is a strong solid acid which is readily soluble and which has a pH (approximately 1.0) which is suitable for the reaction. Other solid acids such as citric acid, tartaric acid, or maleic acid may be used but they are generally not as satisfactory as sulfosalicyclic acid.

Sodium bicarbonate is another component of our bilirubin reagent composition. It facilitates the solution of the reagent composition particularly when the reagent composition is in tablet form, by providing, with the acid component hereinbefore mentioned, an effervescent couple. The flooding technique used in carrying out our test is not completely satisfactory unless such an effervescent couple is present. Of course, equivalent quantities of other soluble bicarbonates or carbonates may be substituted for sodium bicarbonate, but the latter is preferable since it gives excellent results and is readily available commercially.

Diluents such as boric acid, starch, lactose and the like may be used in considerable amounts for the purpose of facilitating the tabletting operation, as is well known in the art.

The proportions of the diazonium salt, the acid and carbonate or bicarbonate are not critical within broad limits, and these ingredients may be used within a rather wide range of proportions.

When p-nitrobenzene diazonium p-toluene sulfonate is used as the diazonium salt component of the reagent composition, it should not comprise over approximately 2% by weight of the total bulk of the mixture and should represent at least 0.015% of the total bulk thereof. The preferred concentration is 0.15%; this gives very satisfactory sensitivity but the amount is still low enough that it does not give any color development with normal urines. When p-sulfobenzene diazonium 4-nitrotoluene-2-sulfonate is used in the reagent composition, it should not comprise over approximately 25% by weight of the total mixture and should represent at least 0.15% of the total bulk thereof, the preferred concentration being 0.5%.

In the preferred form of our invention, sulfosalicyclic acid comprises the main bulk of the mixture being conveniently, for example about 77% by weight, although this amount may vary from 20% to 90%. The acid not only gives an acid pH to the reaction mixture, but also acts as a component of the effervescent couple (with the carbonate or bicarbonate) to facilitate the solution

of the tablet and thus speed the reaction. Enough carbonate or bircarbonate must be added to produce good effervescence but not enough to neutralize much of the acid. In the case of sodium bicarbonate, which is the preferred material, the optimum range is about 5% to 20% of the total bulk. The preferred concentration is about 7.7%.

Suitable diluents (either neutral or acidic in character) may be added to the mixture to facilitate tablet making as mentioned heretofore, in concentration as high as 70%.

According to a presently preferred form of our composition, the composition of our diagnostic reagent may be as follows:

	Mg.
p-Nitrobenzene diazonium p-toluene sulfonate	.02
or	
p-Sulfobenzene diazonium 4-nitrotoluene-2-sulfonate	0.6
Sodium bicarbonate	10.0
Sulfosalicyclic acid	100.0
Diluent (boric acid)	20.0

In testing urine specimens for bilirubin according to our invention, five drops of the urine to be tested are placed on a test mat. Conveniently, the test mat may be approximately 1 inch square, and the test tablet used therewith may weigh approximately 2 grains. Obviously, either larger or smaller mats or tablets could be used. Next a tablet of our reagent composition, having the composition heretofore set forth, is placed on the moistened spot on the test mat, and 2 drops of water are put on top of the tablet, the water being allowed to go down over the side of the tablet. The area of the test mat surrounding the tablet is then observed. If bilirubin is present in the urine, a purple color varying proportionately in intensity with the amount of bilirubin present will appear within 15 seconds. Urine containing 0.05 mg. or more bilirubin/100 ml. will give a definite purple color. With negative urines, no color development occurs.

Alternatively, our composition in the form of a mixture of the stable diazonium salt and an acid constituent may be, by suitable means, applied to splinters, sticks or strips made of wood, fiber, paper, glass, metal or plastic using conventional adhesive materials for effecting adhesion. Such a "stick" will turn color when moistened with a bilirubin containing wood.

While the tablet form of our composition is quite satisfactory for carrying out bilirubin detection, we wish to point out that, if desired, other forms of the composition may be used. Thus the test can be carried out by contacting the fluid to be tested for bilirubin with a bibulous material such as paper, wood, fiber, in the form of a strip or stick, which has been contacted with a solution containing one of the stable diazonium salts mentioned together with an acid, preferably one that can exist in dry solid form.

Such a "strip" or "stick," after being dipped in the foregoing solution and dried, will undergo a distinct color change when contacted with bilirubin-containing material.

The present invention is much more sensitive than any other test known for bilirubinuria. Thus with our invention, it is possible to detect 0.05 mg. bilirubin/100 ml. urine using only 5 drops of specimen. Most other methods are able to detect only 1 mg. or more bilirubin/100 ml. as the minimum, and require the use from 1 to 10 ml. of urine. The bilirubin-in-urine determinations made in accordance with our invention are accurate. Using our method, the investigator definitely knows whether or not bilirubin is present since false positives and false negatives are not obtained. Another advantage of our invention is that the test can be done so rapidly that it is possible to use it in large screening operations where thousands of tests must be made in one day by one operator. Furthermore, it is so simple that even an unskilled

person may quickly and easily determine the bilirubin content of a urine specimen. No laboratory equipment is necessary to use the invention, the only equipment needed, other than the diagnostic composition herein described, being a dropper. For this reason bedside tests for bilirubin in urine may be readily and easily performed.

A further advantage of our invention is that the reagent composition thereof is stable, which is a great advantage since in other techniques and methods, fresh reagents must be prepared daily for good results. Since the bilirubin reagent composition is in dry form, and preferably in tablets, storage in the laboratory is facilitated, and the physician is enabled to carry the test equipment in his bag. Furthermore, since no liquid acids or alkalis or other liquid materials are used, the danger of spilling and resultant damage to clothing, furniture, etc. or burning of the skin for example is eliminated. Another advantage is that a composition of our invention contains no materials which will give off noxious fumes. This is of particular advantage to a technician carrying out screening tests where many, many tests may be made in one day—often in a small room without adequate ventilation.

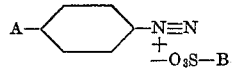
A further, and important advantage of our invention is that the test is specific for bilirubin. Normal constituents of urine and commonly used drugs may interfere with many of the other tests for bilirubinuria (for example salicylates give a purple color with Fouchet's reagent; urobilin gives a reddish-orange color and iodides give a violet color with nitric acid used in Rosenbach's test). We have determined by extensive tests that such normal constituents and common drugs do not develop any kind of color with the diagnostic reagent of our invention.

While our invention is particularly useful in determining the bilirubin content of urine, it is likewise applicable to other aqueous bilirubin-containing materials such as, for example, blood serum and the like.

This application is a continuation-in-part of our copending application, Serial No. 178,344, filed August 8, 1950, now abandoned.

We claim:

1. A diagnostic composition in dry form for determining bilirubin comprising (*a*) a compound having the formula



wherein A is a radical selected from the group consisting of carboxyl, sulfo and nitro, and B is a member selected from the group consisting of benzene, p-toluene, and 4-nitrotoluene-2-; and (*b*) an effervescent couple whose acid component is in sufficient excess to provide an acid pH when said composition is dissolved in water.

2. A diagnostic composition in dry form for determining bilirubin comprising a compound selected from the group consisting of p-nitrobenzene diazonium p-toluene sulfonate and p-sulfobenzene diazonium 4-nitrotoluene-2-sulfonate, and effervescent couple whose acid component is in sufficient excess to provide an acid pH when said composition is dissolved in water.

3. A diagnostic composition in dry form for determining bilirubin comprising p-nitrobenzene diazonium p-toluene sulfonate and an effervescent couple whose acid component is in sufficient excess to provide an acid pH when said composition is dissolved in water.

4. A diagnostic composition in dry form for determining bilirubin comprising p-sulfobenzene diazonium 4-nitrotoluene-2-sulfonate and an effervescent couple whose acid component is in sufficient excess to provide an acid pH when said composition is dissolved in water.

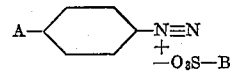
5. A diagnostic composition in tablet form for determining bilirubin comprising 0.2 mg. p-nitrobenzene diazonium p-toluene sulfonate, 10.0 mg. sodium bicarbonate, and 100.0 mg. of sulfosalicylic acid.

6. A diagnostic composition in tablet form for determining bilirubin comprising 0.6 mg. p-sulfobenzene diazonium 4-nitrotoluene-2-sulfonate, 10.0 mg. sodium bicarbonate, and 100.0 mg. sulfosalicylic acid.

7. A method of determining bilirubin which comprises the steps of wetting a bibulous mat with an aqueous liquid, being tested for bilirubin content, contacting the mat with a tablet containing p-nitrobenzene diazonium p-toluene sulfonate a member of the group consisting of alkali metal carbonates and alkali metal bicarbonates, and sufficient dry soluble acid to provide an acid pH, and then applying to the mat-contacting tablet sufficient water to dissolve said tablet and wet the said mat with the resulting solution.

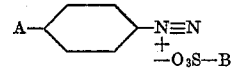
8. A method of determining bilirubin which comprises the steps of wetting a bibulous mat with an aqueous liquid being tested for bilirubin content, contacting the mat with a tablet containing p-sulfobenzene diazonium 4-nitrotoluene-2-sulfonate, a member of the group consisting of alkali metal carbonates and alkali metal bicarbonates, and sufficient dry soluble acid to provide an acid pH, and then applying to the mat-contacting tablet sufficient water to dissolve said tablet and wet the said mat with the resulting solution.

9. A method of determining bilirubin which comprises the steps of wetting a bibulous mat with an aqueous liquid being tested for bilirubin content, contacting the mat with a tablet containing (*a*) a compound having the formula



wherein A is a radical selected from the group consisting of carboxyl, sulfo and nitro, and B is a member selected from the group consisting of benzene, p-toluene, and 4-nitrotoluene-2-; (*b*) a member of the group consisting of alkali metal carbonates and alkali metal bicarbonates, and (*c*) sufficient dry soluble acid to provide an acid pH; and then applying to the mat-contacting tablet sufficient water to dissolve said tablet and wet the said mat with the resulting solution.

10. A test indicator for detecting bilirubin which comprises a bibulous material which contains therein a mixture comprising a diazonium salt having the following formula



wherein A is a radical selected from the group consisting of carboxyl, sulfo and nitro, and B is a member selected from the group consisting of benzene, p-toluene and 4-nitrotoluene-2-; and a dry solid acid.

11. The article of claim 10 wherein the bibulous material is paper.

No references cited.