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SIMPLIFIED ACCURATE METHOD OF
DETECTING BACTERIURIAFrank A. Finnerty, Jr., 6 Langley Place, McLean, Va.
22101, and Arnold C. Johnson, 6103 Seminole St.,
College Park, Md. 20741

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10 Claims

ABSTRACT OF THE DISCLOSURE

The method of producing a dip stick for detecting bacteriuria comprising applying to the stick an aqueous solution of sulfanilic acid and an acid selected from the group consisting of citric and oxalic acids, drying the stick, applying a second coating of an anhydrous organic solvent solution of a naphthylamine; and the dip stick produced by this method.

HISTORICAL BACKGROUND

This invention relates to the use of a combination of the Griess nitrite test (which has been relegated to a "stick procedure") and the diphenylamine test in detecting bacteriuria. This invention improves the accuracy of the Griess nitrite test by differentiation between a truly negative and a falsely negative test.

In recent years, suggestions were made as to the possibility of diagnosing and treating patients with genito-urinary infection before onset of symptoms. Recognition and treatment of asymptomatic bacteriuria in pregnant patients not only reduces the incidence of symptomatic infection in the mother but also reduces the incidence of prematurity and perinatal mortality. Although many investigators have shown that genito-urinary infection can be detected before the onset of symptoms, the importance in the pathogenesis of chronic pyelonephritis renal vascular disease or the relationship to prematurity or perinatal mortality requires documentation for accurate and positive predictions. To date, the only true reliable methods for such documentation consist of sophisticated bacteriologic techniques which are not available in the offices of most practising physicians. It is therefore of utmost importance that reliable methods of detecting asymptomatic bacteriuria be available for performance by the practising physician in his office.

In an attempt to simplify the procedure for identifying bacteriuria, several chemical methods have been developed. The Griess nitrite test, which was developed in 1879, has more recently received the most attention. It is simple to perform and relatively inexpensive. Its main disadvantage; however, continues to be the high incidence of false negative results. The Griess nitrite test for the detection of asymptomatic bacteriuria is based on the finding that nearly all of the bacterial species which cause asymptomatic bacteriuria reduce nitrate in the system to nitrite if given sufficient time. By definition, bacterial species such as *E. Coli* and *A. Aerogenes* classified in the family Enterobacteriaceae have this characteristic. It has been found that the Griess test, detects nitrite in concentrations of 0.1 µg./ml. A false negative test can result from (1) the presence of organisms which do not reduce nitrate (rare), or (2) insufficient substrate. Insufficient substrate can result from either (1) an insufficiency of nitrate in the diet or (2) utilization of both nitrate and nitrite by the rapid growth of large numbers of organisms.

Performing the Griess nitrite test on the first morning specimen of urine in asymptomatic patients who do not have nocturia and who are not undergoing catheter drainage or taking excessive amounts of fluid has insured sufficient bladder incubation time and has decreased the in-

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cidence of false negative tests. Since patients of a lower income bracket may not have adequate nitrate in the diet, a decrease in dietary nitrate could contribute to the incidence of false negative tests. Admittedly, increasing the oral intake of nitrates lessens this possibility but the accomplishment changing patients' diets is well beyond the scope of most physicians. Similarly, it has been found that the addition of nitrate to the urine was of no practical value since at least two hours of incubation time were needed to produce the amount of nitrite which could be detected. One way to simplify the standard Griess test would be to provide a nitrite indicator stick as a substitute for the liquid solutions which would be inexpensive to manufacture and which would have a relatively long shelf life and high sensitivity.

OBJECTS AND SUMMARY

It is therefore an object of this invention to provide an improved nitrite indicator stick which is highly sensitive to nitrite, is inexpensive to manufacture, has a long shelf life and can easily be utilized by physicians in their offices.

Another object of this invention is to provide an additional chemical to the Griess nitrite test which reduces the high incidence of false negative Griess tests when used for the detection of bacteriuria.

Still another object of this invention is to provide a simple test for the determining of bacteriuria which does not require elaborate laboratory facilities for its utilization.

A further object of this invention is to provide chemical tests which will permit early detection of genito-urinary infections prior to the onset of symptoms and thus reduce the incidence of prematurity and perinatal mortality.

In summary therefore, this invention has as its objects, the simplification and the increasing of accuracy of a method for detecting bacteriuria utilizing the diphenylamine test with either the standard Griess reagent or an improved nitrite indicator of the type described in this disclosure.

This invention reduces false negative Griess tests by detecting the presence of or absence of nitrate in urine found to be Griess test negative. Establishing the presence or absence of nitrate in urines which are Griess test negative permits differentiation between a truly negative and a falsely negative test. A truly negative test (nitrate positive, colony count negative) will show that there is nitrate in the diet which has not been reduced to nitrite by actively growing organisms. In the prior art, falsely negative Griess tests would fail to show the presence of nitrite and would suggest (1) either a lack of substrate, or (2) that the substrate had been completely utilized by rapidly growing organisms. Although diphenylamine reagent has long been used for the purposes of detecting the presence of nitrates and nitrites it has not to our knowledge been used in conjunction with the standard Griess test. We have found that the diphenylamine test for nitrites is less sensitive than the Griess test and therefore validates the Griess test results by combining the diphenylamine test therewith in order to differentiate false negatives from true negative results to thereby decrease the incidence of false negative tests.

To further simplify the method of testing, the diphenylamine test is used in conjunction with a nitrite indicator stick made in accordance with the following procedural steps.

METHOD OF PREPARING THE STICK

The unprepared stick for use in the test can be strips of paper, wood, or plastic having absorptive qualities and filter paper strips have been found to be quite suitable. The "dip sticks" of filter paper, for ease in handling and

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in detection as well as storage, should preferably be approximately three inches in length and a quarter of an inch in width, $\frac{3}{4}$ of an inch in thickness and of a fine mat. It is obvious that considerable latitude can be utilized as far as size of the dip stick is concerned. When prepared, the end of the dip sticks will be covered with two relatively thin and porous superimposed coatings.

In preparation of the sticks, individual sticks can be prepared by the method herein set out or large sheets of paper, etc., can be prepared which after treatment will subsequently cut up into small stick sizes for ease in handling. To this end, an aqueous solution from about 0.25% to about 2% of sulfanilic acid prepared in approximately 10% citric acid is utilized at room temperature. About $\frac{1}{2}$ inch of the end of strips or sheets of filter paper, etc. is dipped into the solution and dried in a relatively high vacuum at 40° C. until substantially all of the moisture has been removed. Drying by heating over 40° C. is not advised because additional heat causes deterioration of the chemical composition which has been applied to the paper.

A second solution of alpha-naphthylamine is prepared in n-heptane in a concentration of approximately 0.25% to 2% (Table 1).

TABLE 1.—CONCENTRATION OF SULFANILIC ACID IN 10 PERCENT CITRIC ACID

Concentration of alpha-naphthylamine in n-heptane	2%	1%	.5%	.25%	.12%	.06%	.03%
2%-----	+	+	+	+	±	—	—
1%-----	+	+	+	+	±	—	—
.5%-----	+	+	+	±	—	—	—
.25%-----	+	+	+	±	—	—	—
.12%-----	±	—	—	—	—	—	—
.06%-----	—	—	—	—	—	—	—
.03%-----	—	—	—	—	—	—	—

Tests conducted with 0.1 µg./ml. KNO₃.

The previously treated portion of the strips of filter paper which have been dried are now dipped into the solution of alpha-naphthylamine and subsequently dried at approximately 40° C. It has been found that a repeat dipping and drying of this step in the alpha-naphthylamine solution increases sensitivity. The optimum concentrations of the sulfanilic acid and the alpha-naphthylamine in their solutions is approximately 1%.

It is most important that the moisture (H₂O) content of the paper treated in the first step be held to a minimum because any moisture remaining in the strip will have an adverse effect when the strip is subsequently treated with the second solution causing discoloration and thus make noticeable color change undetectable. In order to keep moisture to a minimum, it is suggested that some type of desiccant such as calcium chloride be utilized in the n-heptane solution. N-heptane is preferably used as a solvent for the alpha-naphthylamine because it is commercially quite dry and does not absorb a sufficient amount of moisture from the air to seriously affect the sulfanilic acid treated strips by discoloration once they have been dipped into the n-heptane solution. Solvents in which the alpha-naphthylamine have limited solubility and little water sorbtivity such as other heptanes, octanes, or hexanes or the like may be used but in general these solvents are more expensive and not nearly as hygroscopically stable.

Upon drying of the strips which have been treated in both solutions, the strips, if they have not already been cut, are cut to proper size and placed in a dark colored bottle such as a brown glass bottle in which a small amount of desiccant has been placed. The bottles are then covered for storage. Shelf life has been found to be excellent and little discoloration of the strips has been noted even after six months storage.

Applications of the coatings may be by dipping, spraying, brushing, immersing, etc.

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ALTERNATE METHOD

Instead of utilizing alpha-naphthylamine, the more stable alpha-naphthylamine hydrochloride may be used by dissolving the same in absolute ethyl alcohol which has calcium chloride therein. The calcium chloride is maintained in the ethyl alcohol in order to maintain the alcohol as dry as possible since absolute alcohol tends to pick up moisture from the air with relative ease. Other alcohols can be used such as methyl or propyl. As alpha-naphthylamine hydrochloride is difficultly soluble in the ethyl alcohol, a concentration exceeding one percent is difficultly obtainable. The optimum concentration of the alpha-naphthylamine hydrochloride in ethyl alcohol is about 0.5% and the effective range runs from approximately 0.25% to about 1% (Table 2).

TABLE 2.—CONCENTRATION OF SULFANILIC ACID IN 10 PERCENT CITRIC ACID

Concentration of alpha-naphthylamine hydrochloride in absolute ethyl alcohol	2%	1%	.25%	.5%	.12%	.06%	.03%
1%-----	+	+	+	±	—	—	—
.5%-----	+	+	+	±	—	—	—
.25%-----	+	+	+	±	—	—	—
.12%-----	±	—	—	—	—	—	—
.06%-----	—	—	—	—	—	—	—
.03%-----	—	—	—	—	—	—	—

Tests conducted with 0.1 µg./m. KNO₃.

When the dip stick is used for the detection of nitrites in urine, the acidity must be great enough to bring the acidity of the "dip stick" to a pH of about 3.8. In practical application, 10% citric acid or oxalic acid was found to be sufficient for the detection of nitrites in urine. It is felt that from about 5% to 20% of oxalic or citric acid will be sufficient to push the pH of the dip stick when testing urine to a pH of approximately 3.8. It is to be noted that a coloration change actually takes place at a pH of 5.2 but peak color development is reached approximately at a pH of about 3.8. A minimum pH therefore for testing would be more acid than 5.2.

TESTS CONDUCTED

More than five hundred first morning urine samples were tested with the nitrite indicator stick and compared with the standard liquid Griess reagent for the determination of nitrites. In all instances, the sensitivity of the dip sticks was felt to be substantially that of the standard liquid Griess test or approximately one part in a million with a sensitivity of .1 to .01 microgram per milliter in aqueous solution.

In conjunction with the nitrite indicator test, the diphenylamine procedure has been used to study over 600 asymptomatic pregnant patients (Table 3)

TABLE 3.—COMPARISON OF CHEMICAL AND BACTERIOLOGICAL DATA IN 624 ASYMPTOMATIC PATIENTS

	Chemical tests, voided urines		Colony count, clean caught urines		Organisms isolated
	Griess	Diphenylamine	Negative	Positive	
55	(—)	(+)	564	0	None.
	(+)	(—)	0	7	6 <i>Escheriachia coli</i> .*
60	(+)	(+)	19	16	1 <i>Aerobacter aerogenes</i> . 12 <i>Escherichia coli</i> .*
	(—)	(—)	11	7	4 <i>Aerobacter aerogenes</i> . 3 <i>Escherichia coli</i> . 3 <i>Aerobacter aerogenes</i> . 1 <i>Proteus vulgaris</i> .

* One formerly *Paracolonbactrum* species.

The finding of a temperature above one hundred degrees (without obvious extra genito-urinary etiology), symptoms of a current genito-urinary tract infection, or the history of taking any antibiotic during the past two weeks excluded the patient from the study. A history of prior genito-urinary tract infection did not exclude patients from the study.

The first morning voided urine was collected by the patient at home and brought for testing. The only instruction given to the patient was to collect the urine in

a nitrite-nitrate free bottle supplied to the patient. On the same day, a mid-stream sample of urine was collected by a nurse. The patient was cleansed with 5% aqueous green soap and the urine was collected while the patient was in standing position. Both the patient's bottle and the one collected by the nurse were maintained refrigerated until tested.

The first morning urine sample was tested in accordance with this invention utilizing the nitrite indicator test and diphenylamine test. The appearance of a pink color on the stick constituted a positive test for nitrites. The diphenylamine procedure was performed as a ring test. This test is conducted by placing approximately five-tenths ml. (0.5 ml.) urine in a 1 ml. pipette and layering the urine over the diphenylamine reagent with a tube held at a forty-five degree angle and the tip of the pipette about one-quarter of an inch ($\frac{1}{4}$) above the surface of the reagent. Urine was allowed to flow slowly down the side of the tube and over the diphenylamine reagent. Care was taken not to mix the reagent with the urine. The tube was placed on a rack and read after one minute against a white background. The appearance of a deep blue color constituted a positive test for nitrates and/or nitrites. The appearance of a deep brown color which masked the blue color suggested that the urine and reagent had been mixed and in these cases a repeat test was necessary. No color change or the appearance of a slight violet constituted a negative test. In order not to overlook a delayed color change, the tubes were not discarded for ten minutes.

The control tests were run with the clean caught urines which were diluted into nutrient broth. From the dilutions, dilution pour plates were made and incubated at 36° C. and kept overnight and the colonies which developed were counted. Urines with a colony count of eighty thousand bacteria per ml. or greater were cultured on blood agar etc. The bacteria were identified biochemically. When colony count data was compared to chemical tests, in no instance was there noted a false positive or false negative chemical result. If only the standard Griess nitrate test had been used for screening, approximately 25% of the tests would have been misdiagnosed. Thus this invention clearly provides greater accuracy than heretofore afforded.

The diphenylamine solution may vary from about 0.15% to about 0.01% diphenylamine in concentrated sulfuric acid. The preferred concentration is 0.05% diphenylamine in sulfuric

While the invention has been described, it will be understood that it is capable of further modifications and this application is intended to cover any variations used or adaptations of the invention following in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice in the art to which the invention pertains, and as may be applied to the essential features hereinbefore set forth and as fall within the scope of the invention or the limits of the appended claims.

Having thus described our invention, what we claim is:

1. The method of preparing a stick material for detecting bacteriuria in urine comprising:

- (a) applying to said stick material an aqueous solution of from about 0.25% to about 2% of sulfanilic acid and from about 5% to about 20% of an acid selected from the group consisting of citric and oxalic acids to obtain a first coating on said stick material,
- (b) drying said first coated stick material at a temperature not exceeding 40° C. until substantially moisture free,
- (c) applying to said first coating an anhydrous organic solvent solution of from about 0.25% to 2% of an amine selected from the group consisting of alpha-naphthylamine and alpha-naphthylamine hydrochloride, said organic solvent being selected from the

group consisting of methyl, ethyl, propyl alcohols and hexane, heptane and octane to obtain a second coating superimposed on said first coating,

(d) and drying said second coated stick material at a temperature not exceeding 40° C.

2. The method of claim 1 and wherein:

(a) said amine is alpha-naphthylamine, said organic solvent is heptane and said alpha-naphthylamine in said heptane is approximately 1%.

3. The method of claim 1 and wherein:

(a) said amine is alpha naphthylamine hydrochloride, said organic solvent is ethyl alcohol, and said alpha naphthylamine hydrochloride in said ethyl alcohol is approximately 0.5%.

4. A method of preparing stick material as in claim 1 and including the step of:

(a) applying at least one additional coating of said anhydrous organic solvent solution to said stick, and

(b) drying said stick with at least said one additional coating at a temperature not exceeding 40° C.

5. The product produced by the method of claim 1.

6. The method of testing for bacteriuria which comprises:

(a) testing a urine sample with a Griess nitrite test composition, and

(b) subsequently testing said sample with from about 0.15 to about 0.01% diphenylamine in concentrated sulfuric acid solution by layering the urine over said acid solution and checking for a color change at the interface after approximately one minute.

7. The method of claim 6 and wherein:

(a) said concentration of diphenylamine in concentrated sulfuric acid is about 0.05%.

8. The method of claim 6 and wherein:

(a) said Griess nitrite test composition is in a stick form and said stick includes a first moisture free coating consisting essentially of sulfanilic acid and an acid selected from the group consisting of: oxalic acid and citric acid in a quantity sufficient to acidify urine in contact therewith when testing to a pH of less than 5.2, and including

(b) a second moisture free dry coating superimposed on said first dried coating and consisting essentially of an amine selected from the group consisting of: alpha-naphthylamine and alpha-naphthylamine hydrochloride, and

(c) said coatings being relatively thin and permeable.

9. The method of claim 8 and wherein:

(a) said amine is alpha-naphthylamine.

10. The method of claim 8 and wherein:

(a) said amine is alpha-naphthylamine hydrochloride.

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MORRIS O. WOLK, Primary Examiner

S. MARANTZ, Assistant Examiner

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