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3,362,886

COMPOSITION AND METHOD FOR THE DETECTION OF GALACTOSE

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This invention relates to improved test compositions and test devices utilizing the same. In one of its particular aspects this invention is concerned with test compositions and devices useful in the qualitative detection and quantitative determination of galactose in fluids. Another aspect of this invention is concerned with test compositions incorporated with bibulous carriers. This application is a continuation-in-part of my copending application Ser. No. 237,733, filed Nov. 14, 1962 and now abandoned which is a continuation-in-part of my copending applications Ser. No. 232,260 filed Oct. 22, 1962, now U.S. Patent 3,186,919 and Ser. No. 232,872, filed Oct. 24, 1962 now U.S. Patent 3,186,921.

Galactose is rarely in the urine, blood or other body fluids of normal individuals, since the galactose which is ingested by the human body is normally converted to glycogen by the liver. In the case of patients with liver impairment, however, galactose may appear in the bloodstream and urine of the patient. The occurrence of galactose in body fluids, however, is most meaningful in the cases of patients afflicted with certain metabolic disorders. Those who are unable to properly metabolize galactose are said to be afflicted with the metabolic disorder, galactosemia. The disease becomes evident shortly after birth and may result in physical and mental retardation and early death if not corrected. Thus, the early diagnosis and treatment of galactosemia is essential.

The presence of galactose in the blood or urine of babies evidences an abnormality in metabolism and suggests the incidence of galactosemia. It would be extremely desirable if all babies could be screened for the incidence of this disease which, if not detected and treated, leads to malnutrition, cataract formation, mental retardation and finally to an early death. If detected two or three months after birth, a dietary regime can be instituted which will insure more nearly normal development.

Previously, no adequate convenient test for galactose has been available. In those instances where galactose has been previously determined, the methods used have been cumbersome and time consuming. For example, the copper reduction test, the one most commonly used, requires the removal of glucose by treating the urine or blood specimen with either a yeast suspension or glucose oxidase. The amount of reducing substance remaining is estimated and assumed to be galactose. This is not always a valid assumption since sugars other than galactose and non-carbohydrate reducing substances may also be present.

The mucic acid and the osazone test, also previously used, are not reliable tests when applied to biological fluids such as blood and urine.

Another more specific test involves the chromatographic detection of galactose. This method, however, requires several hours to accomplish and special, expensive equipment to properly execute.

It is accordingly a principal object of the present invention to provide a test composition which may be used for the specific detection of galactose in the presence of other reducing sugars.

Another object of this invention is the provision of convenient test devices of the "dip and read" stick variety which may be used in the home or in the doctor's

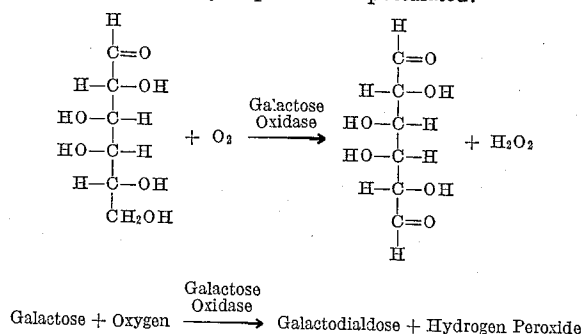
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office by relatively unskilled personnel without resorting to the use of special laboratory equipment.

It is another object of the invention to provide a method of testing for the presence of galactose in body fluids by a simple and convenient procedure.

Other objects and advantages of this invention will be obvious in view of the following detailed disclosure and description.

It has now been found that galactose may be readily and accurately detected in various fluids by means of compositions which may be used as such or preferably incorporated with a bibulous carrier for use as a "dip and read" stick. These compositions include the enzyme galactose oxidase. Galactose oxidase is an enzyme which catalyzes the oxidation of galactose in the presence of atmospheric oxygen to produce hydrogen peroxide and an oxidation product of galactose believed to be a dialdehyde. Although galactose oxidase will effect the oxidation of galactose it has practically no effect upon glucose and other simple reducing sugars. Thus, in the case of a mixture of simple sugars, galactose oxidase will catalyze the oxidation of galactose without any effect upon the other simple reducing sugars present. Galactose oxidase may be produced by various fermentation processes such as disclosed in U.S. Patent No. 3,005,714, to John A. D. Cooper and in my copending applications, Ser. No. 232,260, filed Oct. 22, 1962, and Ser. No. 232,872, filed Oct. 24, 1962. The oxidation of galactose may be illustrated in accordance with the following equation in which a dialdehyde product is postulated:



In addition to galactose oxidase the compositions of this invention contain an indicator system which is activated upon the oxidation of galactose to produce an observable phenomenon. One system which may be used utilizes an indicator material which is responsive to the presence of the hydrogen peroxide formed in the oxidation of galactose under the influence of galactose oxidase. Few indicators, however, are oxidizable by hydrogen peroxide unless there is also present a catalyst or other oxidation promoting agent such as an electron transfer agent in whose presence the oxidation of the indicator by the hydrogen peroxide readily proceeds.

For example, a second enzyme such as a peroxidase may be utilized to catalyze the oxidation of a suitable indicator material by the hydrogen peroxide formed in the oxidation of galactose. Various peroxidases are known. One which is frequently utilized commercially is horseradish peroxidase. Other sources of peroxidase include potatoes, fig-tree sap, turnips and white blood corpuscles. In addition to peroxidase as such, various other substances show a peroxidase-like or peroxidative activity. These substances include hemin, methemoglobin, oxyhemoglobin, hemoglobin, hemochromogen, alkaline hematin and other hemin derivatives.

For the purpose of convenience the peroxidases, hemoglobin derivatives and other substances effective in promoting the oxidation of oxidation-reduction indicators by hydrogen peroxide will be hereinafter referred to as

substances having peroxidative activity, although it is understood that these materials may not all function in the same way. By the term "substance having peroxidative activity" is therefore meant the above mentioned substances as well any other substance which is effective in promoting or causing a response of the indicator material to the presence of hydrogen peroxide whether their function is one of catalyst, electron transfer agent or otherwise.

A wide variety of indicator materials may be used in the test compositions of this invention. For example, gum guaiac o-tolidine, 2,7 - diamino fluorene, o-dianisidine, leucoindophenol, benzidine, benzidine derivatives and the like may be used for this purpose. The indicator preferred for the composition of this invention is o-tolidine which has been found to give better color development than any other suggested indicators.

In order to produce a test having the desired reactivity, sensitivity and stability it is essential that the aforementioned ingredients when contacted with the fluid being tested be buffered at a hydrogen ion concentration of about from pH 5.5 to pH 8.0. Preferably an approximately neutral pH should be utilized, for example, one in the range of about from pH 6.8 to pH 7.2. Although various buffers may be found which will maintain the pH of the ingredients within the desired range, it has been found that those buffers which consist of a salt of a weak monobasic or polybasic acid with tris (hydroxymethyl) aminomethane produce results which, both with respect to sensitivity and stability, are markedly superior. For example, whereas a formulation containing 0.4 M sodium citrate buffer will produce an observable color change in a 2% solution of galactose in water in 48 seconds, a 0.4 M solution of the reaction product of glutamic acid and tris (hydroxymethyl) aminomethane will produce an observable color change in about 4 seconds. Thus, the use of a buffer of this type gives preferred results, especially where it is desired to conduct a large number of tests in a sort period of time, for example, in screening patients for galactosemia, where many tests must be run on right after another.

In addition to utilizing glutamic acid with tris (hydroxymethyl) aminomethane, it has been found that numerous other organic and inorganic acids may be utilized to form the novel buffer system of the present invention. Generally those weak monobasic and polybasic acids which have a pK (negative logarithm of the dissociation constant) of from about 2 up to about 12 are preferred in forming the present tris (hydroxymethyl) aminomethane buffer salts. When a polybasic acid is utilized this pK range applies to the primary dissociation thereof. Exemplary of these acids are the monobasic acids such as gluconic acid, acetic acid and lactic acid, the dibasic acids such as malonic acid, phthalic acid, aspartic acid, maleic acid, malic acid, saccharic acid, succinic acid, glutoric acid and tartaric acid, and the tribasic acids such as citric acid, aconitic acid, isocitric acid, ortho-boric acid and ortho-phosphoric acid.

In formulating the compositions of this invention it has also been found desirable to utilize polyvinyl alcohol as a conveyor or thickening agent for the compositions. The polyvinyl alcohol has also been observed to contribute certain protective qualities to these compositions. Other thickening agents, such as gelatin, bovine serum albumin, polyvinyl pyrrolidone (PVP), starch or sodium alginate may also be used, but polyvinyl alcohol is preferred because of its unexpected protective properties.

Wetting agents or surface active agents may be used in the compositions of this invention to assure an even distribution of the ingredients upon the test sticks, and, after drying, a uniform wetting of the test stick when used. Various types of wetting agents may be used for this purpose including cationic, anionic and non-ionic varieties. Exemplary of the wetting agents which may be used are dioctyl sodium sulfosuccinate (Aerosol®OT)

and polyoxyethylene sorbitan mono-oleate (Tween®81). Wetting agents are not essential, but their use contributes desired elegance to sticks made from the compositions of this invention.

The compositions described above may be readily prepared by adding the polyvinyl alcohol, wetting agent, if desired material having peroxidative activity, galactose oxidase and oxidation-reduction indicator to a solution of the buffer at the desired pH. The compositions may then be used as a dip for strips or sticks of bibulous carrier material. In each instance the impregnated bibulous carrier may be dried either at room temperature or at elevated temperatures depending upon considerations of time and ease of manufacture.

The compositions of this invention and the methods of using them will be better understood by reference to the following examples which are included merely for purposes of illustration and are not to be construed as in any way limiting the scope of this invention which is defined in the claims appended hereto.

Example 1

A solution is made by adding to 100 ml. of tris (hydroxymethyl) aminomethane glutamate (0.4 M in respect to glutamate) at pH 5.8, 250 mg. of bovine serum albumin, 0.5 g. potassium chloride, 2.5 ml. of 5% Tween 81 in water, 150 mg. of horseradish peroxidase, 10 ml. of o-tolidine (free base, 100 mg. in 10 ml. of 95% ethanol). The pH was readjusted from pH 6.2 to pH 5.8 with 1 N hydrochloric acid. To a 10 ml. aliquot of this mixture was then added 500 mg. of galactose oxidase assaying 5,000-8,000 units per gram. The unit of galactose oxidase activity is defined as that quantity of galactose oxidase that will give the activity equivalent to one unit of glucose oxidase as defined in D. Scott, Journal of Agriculture and Food Chemistry 1, 727 (1953). Sticks of bibulous cellulose were dipped into the resulting mixture and dried at room temperature. These sticks were found to be capable of detecting the presence of 0.08% of galactose in water within 60 seconds and reacted with 2.5% galactose in urine within 3 minutes, turning from a light buff color to blue.

Example 2

The following ingredients were mixed in the order indicated:

Tris (hydroxymethyl) aminomethane glutamate, 0.4 M (pH 7.0) -----ml---	4
Galactose oxidase -----units---	6,000
D and C Yellow No. 1 -----mg---	30

This mixture was then added to a mixture of:

Tris (hydroxymethyl) aminomethane glutamate (0.4 M in respect to glutamate) -----ml---	2
Peroxidase -----mg---	2

There were then added to the resulting mixture:

Polyvinyl alcohol, 15% -----ml---	2
Dioctyl sodium sulfosuccinate (Aerosol®OT), 5% -----ml---	2
Gum guaiac, 5% -----ml---	2

Sticks were prepared as in Example 1. These sticks were reactive to 0.05% galactose in water within 4 seconds and to 0.1% galactose in urine within 8 seconds.

Example 3

The procedure of Example 2 was followed except that in place of tris (hydroxymethyl) aminomethane glutamate there was used a solution of tris (hydroxymethyl) aminomethane malonate (0.4 M in respect to malonate). The sticks containing the resulting formulation were found to be capable of detecting 0.05% of galactose in water within 44 seconds.

Example 4

The procedure of Example 2 was followed except that for the buffer there was used a solution of tris (hydroxymethyl) aminomethane phthalate (0.4 M in respect to phthalate). The sticks containing the resulting formulation were found to be capable of detecting 0.05% of galactose in water within 8 seconds.

Example 5

The procedure of Example 2 was followed except that the buffer used was a solution of tris (hydroxymethyl) aminomethane citrate (0.4 M in respect to citrate). The sticks containing the resulting formulation were found to be capable of detecting 0.05% of galactose in water within 48 seconds.

Examples 6 to 9

A series of compositions were each prepared as follows: a mixture of 200 mg. of galactose oxidase (equivalent to 7000 units, defined as in Example 1), 20 mg. of sodium alginate and 10 mg. of catalase was dissolved in 5.0 ml. of water. This solution was then composited with 8 ml. of a buffer prepared from 0.5 molar solutions of tris (hydroxymethyl) aminomethane and the acid indicated in Table I, 2 ml. of a 1 mg./ml. aqueous solution of peroxidase, 1 ml. of a 20% solution of dioctyl sodium sulfosuccinate in a 1 to 1 water-ethyl alcohol mixture, and 4 ml. of a 2% solution of gum guaiac in ethyl alcohol and thoroughly mixed. Bibulous filter paper strips were then dipped into the mixture and dried.

These test strips were then contacted with a series of 0.1% galactose solutions having pH values of 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5. Table I shows the time in seconds for a color to develop when the test strips were contacted with the various galactose solutions.

TABLE I

[Response time in seconds for color to develop]

Example	Buffer Acid	pH of 0.1% Galactose Solution					
		5.0	5.5	6.0	6.5	7.0	7.5
6	Glutamic	1.0	1.0	1.0	1.0	1.0	1.0
7	Ortho-phosphoric	1.0	1.0	1.0	1.0	1.5	1.5
8	Acetic	1.0	0.5	1.5	3.0	3.5	0.5
9	Ortho-boric	0.5	1.0	2.0	3.0	2.5	1.0

Test strips identical to those prepared in these examples with the exception that a conventional 0.5 molar acetic acid-sodium acetate buffer system was used, exhibited a response time up to about 5.0 seconds which demonstrates the superiority of the buffer system of the present invention.

The above examples illustrate that various compositions prepared in accordance with this invention may be utilized for the detection of galactose in fluids including urine. Test sticks impregnated with these compositions may be used to give a quantitative estimate of the galactose present in the fluid being tested by comparing the color produced upon dipping the stick into the test fluid with a previously prepared color chart at a fixed time after dipping.

Although the invention has been described with respect to the use of sticks of bibulous cellulose, it is also anticipated that the various reagents can be selectively adsorbed onto other forms of cellulose including cellulose ion exchangers. The use of the reagents in solution is also included, for example, for use in the testing of urine-wet diapers. Similarly, the ingredients may be combined into tablets or fibers used as bibulous carriers for the reagents.

Although in general it is unnecessary to pretreat a urine or salt solution suspected of containing galactose in order to remove ions which may interfere with the test for galactose, it may sometimes be desirable to do so. For this purpose, the urine or other solution may be con-

tacted with an ion exchange resin previous to testing with the diagnostic compositions of this invention.

The concentrations of the various reagents utilized in this invention may be varied widely to suit the circumstances of use. In general, however, the following operable ranges given in terms of 100 milliliters of solution may be considered preferred ranges of concentration:

Galactose oxidase ----- units-- 10,000 to 30,000
 Substance having peroxidative activity --mg-- 1 to 10
 Polyvinyl alcohol -----g-- 1 to 3
 Oxidation-reduction indicator -----mg--10 to 50
 Buffer (pH 6.8-7.2) -----M-- 0.2 to 1
 Wetting agent (if used) -----g-- 0.1 to 0.5

In summary, this invention provides compositions for the detection of galactose in fluids, especially body fluids such as urine. The compositions include galactose oxidase, a substance having peroxidative activity, an indicator material which is responsive to hydrogen peroxide, a buffer which is a salt of a weak acid with tris (hydroxymethyl) aminomethane and, preferably, polyvinyl alcohol. These compositions may also include other thickening agents such as gelatin or albumin and wetting agents as well as other ingredients desired to contribute elegance to the compositions. These test compositions may be used in various forms such as in solutions, tablets, fibers and adsorbent columns. An especially preferred test device incorporates the test composition as an impregnation upon a stick or strip of bibulous cellulose.

What is claimed is:

1. A test composition for the detection of galactose in fluids comprising:

(A) galactose oxidase;

(B) a substance having peroxidative activity;

(C) an indicator material which is responsive to the presence of hydrogen peroxide in the presence of said substance having peroxidative activity; and,

(D) as a buffer, a salt of tris (hydroxymethyl) aminomethane and a weak acid, said buffer being effective to maintain the above ingredients at a pH range of from about pH 5.5 to pH 8.0 when contacted with the fluid being tested.

2. A test composition as in claim 1 wherein said weak acid has a pK of from about 2 to about 12.

3. A test composition as in claim 1 wherein said weak acid is selected from the group consisting of citric, malonic, phthalic, glutamic, ortho-phosphoric, acetic, ortho-boric, gluconic, lactic, aspartic, maleic, malic, saccharic, succinic, tartaric, glutaric, aconitic and isocitric acids.

4. A test composition as in claim 1 wherein said weak acid is glutamic acid.

5. A test composition as in claim 1 wherein said weak acid is malonic acid.

6. A test composition as in claim 1 wherein said weak acid is phthalic acid.

7. A test composition as in claim 1 wherein said weak acid is citric acid.

8. A test composition as in claim 1 wherein said weak acid is ortho-phosphoric acid.

9. A test composition as in claim 1 wherein said material having peroxidative activity is peroxidase.

10. A test composition as in claim 1 wherein said indicator material is selected from the group consisting of gum guaiac o - toluidine, 2,7 - diamino fluorene, o - dianisidine, leucoindophenol and benzidine.

11. A test composition as in claim 10 wherein said indicator is o - toluidine.

12. A process for the detection of galactose in fluids which comprises contacting the fluid to be tested in the presence of oxygen with a composition comprising:

(A) galactose oxidase;

(B) a substance having peroxidative activity;

(C) an indicator material which is responsive to the presence of hydrogen peroxide in the presence of said substance having peroxidative activity; and,

(D) as a buffer, a salt of tris (hydroxymethyl) amino-methane and a weak acid, said buffer being effective to maintain the above ingredients at a pH range of from about pH 5.5 to pH 8.0 when contacted with the fluid being tested.

13. A test device for the detection of galactose in fluids which comprises a bibulous carrier having incorporated therewith a composition comprising:

- (A) galactose oxidase;
- (B) a substance having peroxidative activity;
- (C) an indicator material which is responsive to the presence of hydrogen peroxide in the presence of said substance having peroxidative activity; and,
- (D) as a buffer, a salt of tris (hydroxymethyl) amino-methane and a weak acid, said buffer being effective to maintain the above ingredients at a pH range of from about pH 5.5 to pH 8.0 when contacted with the fluid being tested.

14. A test device as in claim 13 wherein said weak acid has a pK of from about 2 to about 12.

15. A test device as in claim 13 wherein said weak acid

is selected from the group consisting of citric, malonic, phthalic, glutamic, ortho-phosphoric, acetic, ortho-boric, gluconic, lactic, aspartic, maleic, malic, saccharic, succinic, tartaric, glutaric, aconitic and isocitric acids.

5 16. A test device as in claim 13 wherein said weak acid is glutamic acid.

17. A test device as in claim 13 wherein said weak acid is malonic acid.

10 18. A test device as in claim 13 wherein said weak acid is phthalic acid.

19. A test device as in claim 13 wherein said weak acid is citric acid.

15 20. A test device as in claim 13 wherein said weak acid is ortho-phosphoric acid.

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ALVIN E. TANENHOLTZ, *Primary Examiner.*