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3,778,384

DIAGNOSTIC COMPOSITION FOR THE QUANTITATIVE DETERMINATION OF GLUCOSE

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No Drawing. Continuation-in-part of application Ser. No. 203,840, Dec. 1, 1971. This application July 28, 1972, Ser. No. 275,926

Int. Cl. G01n 21/06, 33/16

U.S. Cl. 252—408

3 Claims 10

ABSTRACT OF THE DISCLOSURE

An o-toluidine reagent composition containing an arsenic compound for use in the determination of glucose in biological fluids.

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of co-pending application Ser. No. 203,840 filed Dec. 1, 1971.

BACKGROUND OF THE INVENTION

This invention relates to a new and improved diagnostic composition which is useful for the quantitative determination of glucose in fluids, particularly body fluids such as urine, plasma, blood and the like.

The detection and quantitative determination of glucose in blood and urine is of great importance for diabetic patients whose diets must be controlled so as to regulate sugar intake and who must frequently be guided in this regard by regular checks on blood and urine glucose. Diabetes screening programs are dependent on the availability of rapid, inexpensive and accurate methods of glucose determination.

Procedures for the detection of sugar in blood and urine are well known in clinical chemistry. The Folin-Wu and Nelson-Somogyi procedures utilize copper reduction for the detection and determination of sugar. U.S. Pat. 3,008,879 describes a means for testing biological fluids for their glucose content which involves a combination comprising an enzyme system having glucose oxidase activity, a material having peroxidative activity, a primary indicator material which is capable of being preferentially oxidized to a colored form in the presence of hydrogen peroxide and the material having peroxidative activity and, as a secondary indicator, a material which is capable of reducing the preferentially oxidized primary indicator material while simultaneously becoming colored.

The use of o-toluidine solutions in glacial acetic acid for the determination of aldosesaccharides in serum or plasma is described by Hultman, E., *Nature*, 183, 108 (1959) and Dubowski, K. M., *Clin. Chem.*, 8, 215 (1962). Ordinarily, glucose is the only aldohexose present in clinically significant amounts in blood serum; other aldohexoses that may be present in minute quantities are mannose and galactose. This test method is based on the formation of a colored complex of glucose and o-toluidine in glacial acetic acid. The concentration of this complex, and thus the intensity of the green color developed, is directly proportional to the concentration of glucose present in the reaction mixture, and can be measured photometrically.

For the determination of glucose in blood, the Folin-Wu and Nelson-Somogyi test methods can be applied only to protein-free filtrates of blood serum or plasma. There is some loss of specificity and reliability because of saccharoids and other reducing compounds that cannot be completely removed in preparation of a protein-free filtrate with resulting values higher than "true glucose" levels. Other disadvantages include the limited stability of the test reagents, the instability of the final test color,

and critical factors in the test procedure involving temperature, heating time and alkalinity of reaction mixture.

The glucose-oxidase test is a highly specific test for glucose when correctly done with pure materials. A protein-free filtrate is required. Other disadvantages include the sensitivity and limited stability of the test reagents, the necessary purity and standardization of the glucose-oxidase enzyme, the errors arising from interfering enzyme inhibitors, and the extreme care necessary during the three minute heating process where a matter of seconds can critically affect the accuracy of results.

The o-toluidine test is a simple, rapid, reliable and economical method for glucose determination. Importantly, there is no need for protein-free filtrates although the test can be performed with such filtrates. The test can be performed directly with blood serum or plasma. Ortho-toluidine is the only reagent required. Acetic acid solutions are stable for at least six months. This stability is enhanced by the addition of thiourea. The final test color has good stability. An important disadvantage, however, of o-toluidine is its susceptibility to oxidation with its resultant effect on test sensitivity. The depressed sensitivity of the reagent results in a regression line with a lower slope when the optical densities of the final test colors are plotted against sugar concentrations, with serious loss of assay accuracy.

The object of this invention is to provide an improved diagnostic composition containing o-toluidine and an arsenic compound for the simple, rapid, inexpensive and accurate quantitative determination of glucose in the biological fluids of humans.

SUMMARY OF THE INVENTION

This invention is concerned with a diagnostic composition containing o-toluidine for the quantitative determination of glucose in such biological fluids as blood and urine. It has been found that the incorporation of an arsenic compound in the o-toluidine reagent solution increases the sensitivity of the test reagent and increases the stability of the final test color in the assay procedure.

DETAILED DESCRIPTION OF THE INVENTION

The o-toluidine test method for the quantitative determination of glucose in blood and urine involves the formation of a colored complex of glucose and o-toluidine in glacial acid which follows Beer's law. The green color, read at 630 nm., is stable (>97%) after standing one hour in air at 25° C.

o-Toluidine is relatively sensitive to oxidation. Bulk supplies of uniform quality and purity are difficult to obtain, with Eastman Chemical Company the preferred source. Freshly distilled o-toluidine is, of course, eminently suitable. The reagent composition contains from about 2 to about 10% of ortho-toluidine. Thiourea is preferably added as a color stabilizer at a concentration of from about 0.1 to about 1%, preferably 0.2%. The stabilizing effect is augmented by storage of the composition under nitrogen at a temperature range of about 25° C. to preferably refrigerator temperature (4–5° C.).

The intensity of the color formed by o-toluidine with glucose is a function of o-toluidine concentration. o-Toluidine in glacial acid is slowly acetylated under room temperature storage, with the o-toluidine concentration dropping approximately 50% in about 8 months. This acetylation is inhibited by adding water to the formulation in the amount of about 2 to about 20%, preferably 6%. The addition of 6% water to the glacial acetic acid solution of o-toluidine lowers the freezing point to about 2° C., and thus allows the preferable storage of the homogenous

reagent composition at refrigerator temperature (4–5° C.).

The addition of an arsenate salt or oxide of arsenic increases the sensitivity of the o-toluidine reagent to glucose up to 120% as reflected in the slope of the optical density curve of the final test colors. An increase in sensitivity, over solutions containing no arsenic additive, is observed with the addition of 0.01% arsenic compound. The sensitivity increase levels off in the region of about 0.5% arsenic compound additive. Sodium arsenate



is the preferred arsenic compound and is added to the reagent composition at the preferred concentration of about 0.05%. Other suitable arsenic compounds at the same concentration may be selected from the group consisting of the following:

sodium orthoarsenate: $\text{Na}_3\text{AsO}_4 \cdot 12\text{H}_2\text{O}$
 sodium mono-H-orthoarsenate: $\text{Na}_2\text{HASO}_4 \cdot 12\text{H}_2\text{O}$
 sodium di-H-orthoarsenate: $\text{NaH}_2\text{AsO}_4 \cdot \text{H}_2\text{O}$
 sodium metaarsenate: NaAsO_3
 sodium arsenite: NaAsO_2
 arsenic trioxide: As_2O_3
 arsenolite: As_4O_6
 claudetite: As_4O_6
 arsenic pentoxide: As_2O_5
 sodium thioarsenate: $\text{Na}_3\text{AsS}_4 \cdot 8\text{H}_2\text{O}$

The color formed by the o-toluidine, with and without arsenate additive, is quite stable for the first hour. However, an increase in stability is noted for the sodium arsenate additive solution after 15 hours with a 32% decrease in color retained, compared to a 38% decrease for the control solution. This increased color stability is important when large numbers of test samples are run, and assay readings cannot be immediately made.

The following examples are provided to illustrate the present invention, but not to limit its scope.

EXAMPLE I

Preparation of reagent

An o-toluidine reagent composition is prepared by mixing together the following ingredients in the following proportions by weight:

	Percent
o-Toluidine	4
Thiourea	0.2
Glacial acetic acid	90
Water	6
$\text{Na}_2\text{HASO}_4 \cdot 7\text{H}_2\text{O}$	0.05

The thiourea placed in an Erlenmeyer flask is dissolved in fresh glacial acetic acid. Freshly opened o-toluidine (Eastman Chemical Company) is added dropwise to the stirred acetic acid solution. Sodium mono-H-orthoarsenate ($\text{Na}_2\text{HASO}_4 \cdot 7\text{H}_2\text{O}$) dissolved in water is added with gentle stirring under nitrogen. The reagent composition, under nitrogen with prior nitrogen deaeration, is packaged in dark glass bottles or bottles made from high density polyethylene or polypropylene. The packaged material is preferably stored under refrigeration (4–5° C.).

Test procedure

(1) Using a suitable pipette add 0.05 ml. of blood serum or other sample to be tested to a 150 mm. x 15 mm., or similar, Pyrex test tube. If serum or plasma is used, deproteinization is not necessary.

(2) Add 4.0 ml. of o-toluidine reagent to the tube. Mix sample and reagent thoroughly, preferably with a vortex mixer.

(3) In a similar manner, prepare a reagent blank using 0.05 ml. of distilled water instead of sample, and prepare a glucose standard using 0.05 ml. of standard glucose solution instead of serum or other sample.

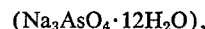
(4) Place the test tubes in a boiling water bath for exactly 10 minutes.

(5) Cool the solutions to room temperature, and determine optical densities at 630 nm.

(6) Determine the concentration of glucose in the sample from a previously prepared curve of optical density vs. glucose concentration, using a series of at least three different glucose concentrations in the range of expected results (50–400 mcgs. percent).

EXAMPLE II

The preparation of the o-toluidine test reagent of Example I is repeated in turn with sodium orthoarsenate



sodium mono-H-orthoarsenate ($\text{Na}_2\text{HASO}_4 \cdot 12\text{H}_2\text{O}$), sodium di-H-orthoarsenate ($\text{NaH}_2\text{AsO}_4 \cdot \text{H}_2\text{O}$), sodium metaarsenate (NaAsO_3), sodium arsenite (NaAsO_2), arsenic trioxide (As_2O_3), arsenolite (As_4O_6), claudetite (As_4O_6), arsenic pentoxide (As_2O_5) and sodium thioarsenate ($\text{Na}_3\text{AsS}_4 \cdot 8\text{H}_2\text{O}$) in place of sodium mono-H-orthoarsenate ($\text{Na}_2\text{HASO}_4 \cdot 7\text{H}_2\text{O}$), with comparable test results.

EXAMPLE III

An o-toluidine reagent composition is prepared by the method of Example I by mixing together the following ingredients in the following proportions by weight:

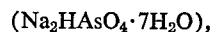
	Percent
o-Toluidine	2–10
Thiourea	0.10–1.0
$\text{Na}_2\text{HASO}_4 \cdot 7\text{H}_2\text{O}$	0.01–0.5
Water	2.0–20
Glacial acetic acid	96–68.5

What is claimed is:

1. A diagnostic composition for quantitatively determining glucose in the biological fluids of humans which comprises and aqueous acetic acid solution of o-toluidine, thiourea and an arsenic compound selected from the group consisting of sodium orthoarsenate ($\text{Na}_3\text{AsO}_4 \cdot 12\text{H}_2\text{O}$), sodium mono-H-orthoarsenate ($\text{Na}_2\text{HASO}_4 \cdot 7\text{H}_2\text{O}$), sodium mono-H-orthoarsenate ($\text{Na}_2\text{HASO}_4 \cdot 12\text{H}_2\text{O}$), sodium di-H-orthoarsenate ($\text{NaH}_2\text{AsO}_4 \cdot \text{H}_2\text{O}$), sodium metaarsenate (NaAsO_3), sodium arsenite (NaAsO_2), arsenic trioxide (As_2O_3), arsenolite (As_4O_6), claudetite (As_4O_6), arsenic pentoxide (As_2O_5) and sodium thioarsenate ($\text{Na}_3\text{AsS}_4 \cdot 8\text{H}_2\text{O}$).

2. The diagnostic composition of claim 1 wherein said arsenic compound is sodium mono-H-orthoarsenate ($\text{Na}_2\text{HASO}_4 \cdot 7\text{H}_2\text{O}$).

3. The diagnostic composition of claim 1 wherein said composition comprises by weight about 4% toluidine, 0.2% thiourea, 0.05% sodium mono-H-orthoarsenate.



6% water and 90% glacial acetic acid.

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U.S. Cl. X.R.

23–230 B