United States Patent

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[54] **GLUCOSE DETERMINATION METHOD EMPLOYING ORTHOTOLUIDINE**
9 Claims, No Drawings

[52] **U.S. Cl.** ..... **23/230 B,** 252/408
[51] **Int. Cl.** **G01n21/22,** G01n 31/22, G01n 33/16
[50] **Field of Search** ..... 23/230, 230 B, 253 TP; 252/408; 195/103.5

[56] **References Cited**

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ABSTRACT: An improved orthotoluidine reagent composition for use in determination of glucose in biological fluids effective sufficient hydrazine to provide a uniform intensity of color when the composition is reacted with a predetermined amount of glucose, such as a standard solution.

GLUCOSE DETERMINATION METHOD EMPLOYING ORTHOTOLUIDINE

BACKGROUND OF THE INVENTION

The determination of glucose in biological fluids using an orthotoluidine reagent composition is an accepted method in many hospital, industrial, reference and commercial clinical laboratories. The method is based on the color formed when glucose reacts with orthotoluidine in glacial acetic acid during a short heating period. The intensity of the resulting blue-green color is measured photometrically.

Conventional orthotoluidine reagents generally contain from about 5 to about 10 grams per 100 milliliters of orthotoluidine in glacial acetic acid and often contain a small amount of thiourea usually in a concentration of from about 0.1 to 1 gram per 100 milliliters, to prevent subsequent discoloration of the reagent.

The glucose determination is usually performed on a biological fluid such as serum or plasma; however, whole blood, cerebrospinal fluid, urine, and other biological fluids may be analyzed. A predetermined volume of sample fluid, generally 0.100 milliliter, is mixed with a predetermined volume of orthotoluidine reagent composition, generally from about 3 to about 5 milliliters in a suitable container such as a colorimeter cuvette or test tube.

A predetermined amount of a glucose standard is added to a second container containing the same volume of reagent as used for the sample. The glucose standard will often contain 100 milligrams of glucose per 100 milliliters. Both containers are capped, mixed, and heated to develop color, typically in a heating block at 100° C. or boiling water bath for a predetermined period of time, usually from 7 to 10 minutes. The tubes are simultaneously removed at the end of the heating period and cooled simultaneously, typically in a water bath at a temperature below 25° C. for 3 minutes. The intensity of blue-green color in the glucose standard and serum sample is measured photometrically in a spectrophotometer or suitable filter colorimeter at a wavelength from 590 to 625 millimicrons or nanometers. The instrument employed will have been previously adjusted to indicate zero absorbance using an appropriate blank composition, e.g. reagent without glucose. Such procedure prevents measurement of intensity of color due to the reagent along, so the measurements are made only on the color produced by the glucose-orthotoluidine reaction. In the present specification and claims, it is understood that the absorbance of the reagent composition alone is accounted for by setting the measuring instrument to read zero absorbance with reagent blank containing no glucose, and terms such as "absorbance," "final absorbance" and the like are employed to refer solely to the absorbance or color intensity produced by the reaction of the reagent composition with glucose. The concentration of glucose in the serum sample is calculated by multiplying the ratio of the final absorbances obtained with the serum sample to that obtained with standard solution by the concentration of glucose in the standard. The final absorbance obtained in a given case depends on such factors as the exact reagent and standard used and the heating time employed for color development.

As a practical matter, prepared is desirable that different batches of an orthotoluidine reagent composition give nearly the same intensity of color when reacted with the same amount of glucose standard in identical determination procedures. So long as each batch of reagent composition is prepared from identical homogeneous lots of ingredients, substantially uniform final absorbances can generally be obtained. However, in practice, different batches of orthotoluidine reagent compositions prepared with ingredients from different lots have been found to give wide variations in absorbance values when reacted with an identical glucose standard under identical conditions. Moreover, even batches of reagent composition prepared from the same homogeneous lot of orthotoluidine may differ in final absorbance if the lot of orthotoluidine is stored for extended

periods between formulation of the different batches. Furthermore, photometric error is increased with color intensities of high absorbance. Thus, it is undesirable that a reagent give excessive color intensity with a sample containing glucose at a concentration within the normal physiological range. For example, if a reagent gives a high absorbance value with a glucose standard containing 100 milligrams glucose per 100 milliliters, then sera from untreated diabetic patients will develop even more color, thereby requiring the sample to be read in the extreme upper end of the colorimeter absorbance scale—the region of maximum photometric error. Thus, it is important to be able to control the formulation of a reagent so that final absorbances will be both predictable and optimum over a reasonable range of sample glucose concentrations. A maximum absorbance of from about 0.28 to 0.35 for a standard containing 100 milligrams of glucose per 100 milliliters is desirable.

In a typical glucose determination mixture containing about 50 parts by volume of reagent composition and one part by volume of a typical standard solution or control serum, the glucose concentration is about 15 to about 18 to about 21 to 25 micrograms glucose per milliliter most generally about 19.6 micrograms per milliliter and a final absorbance of 0.28 to 0.35 is desirable a absorbance of 0.30 to 0.34 being considered preferable. Optimum final absorbances could be obtained by changing the amount of orthotoluidine employed in formulating various batches of reagent composition, or by altering the determination procedure in the relative proportions of reagent composition and standard or sample employed; however, such modifications of formulation and procedure or both are not desirable.

There is a need for a simple improvement in orthotoluidine reagents which provides for regulation of the color developed with predetermined amounts of glucose to obtain uniformly optimum absorbance from batch to batch without necessitating changes in analytical procedure or extensive reformulation in each batch of reagent composition.

BRIEF SUMMARY OF THE INVENTION

This invention relates to an improvement in orthotoluidine reagent compositions in biological fluids. The invention gives a uniform intensity of color when reacted with a specified quantity of glucose. The intensity of color developed by the reagent with such quantity of glucose can be predetermined, adjusted or regulated whenever a new batch of reagent is produced from new or different lots of ingredients to achieve optimum final absorbances in each lot.

It has now been found that the intensity of color developed in glucose determinations carried out with a conventional orthotoluidine color reagent composition can be regulated by the addition of a minor amount of hydrazine to the reagent composition prior to mixing the reagent composition with glucose. More particularly it has been discovered that the addition of small amounts of hydrazine to an orthotoluidine reagent composition, in an amount sufficient to lower the final absorbance in glucose determinations, provides for control of the final color intensity within the optimum range for colorimetric determinations, and additionally increases the storage stability of the reagent composition thus treated.

In formulating and using orthotoluidine reagents according to the invention, the reagent composition can be formulated with the same orthotoluidine and glacial acetic acid and thiourea (if desired) in the same proportions as one typically employed, and the glucose determination can be carried out in the identical procedures, with the same proportions of reagent composition and glucose standard or sample as have been heretofore employed, with the sole modification being the addition to the reagent composition of sufficient hydrazine to provide a final absorbance after maximum color development of about 0.28–0.35 for from about 15 to about 25 micrograms glucose per milliliter of the ultimate mixture of sample, control serum or standard with the reagent composition. The

hydrazine can be added to the reagent composition at any time prior to use, and is preferably added thereto by mixing the required amount of hydrazine with the orthotoluidine prior to formulating the reagent composition, generally in amounts of from about 9 to about 15 to about 20 micromoles of hydrazine per gram of orthotoluidine.

The exact amount of hydrazine to be added to any particular batch of orthotoluidine reagent composition can vary depending upon the proportion of ingredients employed in formulating the desired composition and the exact procedure to be employed in using the same, as well as depending upon the extent to which the final absorbance obtained with a particular batch varies from the optimum range. In practice the relative proportions of ingredients in the reagents composition, and the parameters of the analytical procedure such as proportions of reagent composition and standard or sample, heating time and temperature for color development, wavelength of light at which color intensity is measured, etc. are predetermined. Consequently, the exact amount of hydrazine to be added to control color intensity for a particular batch of a predetermined composition in a predetermined procedure is dependent upon the color intensity obtained with the unmodified batch.

In a convenient procedure, a series of aliquots of the reagent composition batch are prepared containing varying concentrations of added hydrazine, and the aliquot compositions are employed in the test procedure to measure the final absorbances obtained with a standard glucose composition containing 100 milligrams of glucose per 100 milliliters, and to ascertain the concentration of hydrazine required to provide the desired final absorbance. The hydrazine concentration required to provide a particular absorbance in successive batches of reagent composition remains the same, so long as the orthotoluidine and glacial acetic acid ingredients are from identical homogeneous lots of such ingredients. Once the hydrazine concentration required to control absorbance is determined for one batch prepared from particular lots of ingredients, additional batches of reagent composition formulated from the same ingredients lots can be treated to contain the same concentration of hydraine to standardize the final absorbances obtained with any such batch. In a preferred embodiment, the required hydrazine concentration is determined for a given set of ingredient lots, and the amount of hydrazine required to provide the same concentration in subsequent batches is added to the orthotoluidine. This procedure eliminates the need for measuring additional quantities of hydrazine each time a new batch of reagent composition is prepared from the same lots of orthotoluidine and glacial acetic acid, and the addition of the hydrazine to the orthotoluidine also enhances the stability of the orthotoluidine during storage.

The hydrazine can be conveniently employed in the present invention as liquid hydrazine or as hydrazine hydrate, and a concentrated aqueous solution of hydrazine hydrate is particularly convenient. The orthotoluidine reagent composition and glucose determination procedure are conventional in determination of glucose by reaction with orthotoluidine as the essential color forming reactant. Preferably the reagent composition contains from about 5 to about 10 grams of orthotoluidine per 100 milliliters of ultimate composition and from about 0.1 to about 1 gram of thiourea per 100 milliliters, the remainder of the composition being glacial acetic acid. A particularly preferred composition is prepared by dissolving 9 grams orthotoluidine and 0.5 gram thiourea in glacial acetic acid to obtain a final volume of 100 milliliters. Such reagent composition is preferably employed in glucose determination my mixing one part by volume of a biological fluid sample or a glucose standard solution with about 30 to 50 parts by volume of reagent composition, heating the mixture in a tube in a heating block or water bath at a temperature of about 100° C. for about 7 minutes, then cooling in a cold water bath (temperature of water bath below 25° C.) for about 3 minutes before measuring color intensity with light having a wavelength of 590 to 625 millimicrons (nanometers). With such a reagent composition and method, excellent control of final absorbance is obtained with concentrations of hydrazine equaivalent to from about 3 to about 10 milligrams of hydrazine hydrate per 100 milliliters of reagent 180, to about 200 micromoles of hydrazine per 100 milliliters of composition. Compositions so prepared are stable for over 6 months at room temperature, producing substantially uniform optimum final absorbances of about 0.28 to 0.35 when color is developed in the ultimate mixture of reagent and sample or standard containing 1.5 to 2.5 milligrams glucose per 100 milliliters. The reagent compositions give excellent results in glucose determination on biological fluids, including sera from either normal or diabetic subjects.

DESCRIPTION OF PREFERRED EMBODIMENTS

The following examples are illustrative and representative of the invention.

EXAMPLE 1

A series of orthotoluidine reagent compositions is prepared by mixing together the following ingredients in the following proportions:

orthotoluidine	9.0 grams
Thiourea	0.5 grams
Glacial acetic acid	to a final volume of 100 milliliters

Each such composition is prepared in an identical procedure, with the sole variation being the use of orthotoluidine from one of two homogeneous lots, A and B, and the use of glacial acetic acid from one of three lots, 1, 2 and 3.

Each composition is employed in a glucose determination procedure by mixing 5 milliliters of reagent composition with 0.1 milliliter of an aqueous glucose standard solution containing 100 milligrams glucose per 100 milliliters of standard solution, heating the mixtures for 7 minutes in a tube heating block set at 100° C., cooling the mixtures for 2 to 3 minutes in a cold water bath, and determining the absorbance of light by the blue-green reaction product in a colorimeter with light having a wavelength of 595 millimicrons. The colorimeter is adjusted to read zero absorbance with a reagent blank, that is, the reagent composition to which no glucose has been added, prior to measuring absorbance of the corresponding mixture of standard solution and reagent composition. The final absorbances obtained with the compositions reacted with the glucose standard are found to vary from 0.34 to 0.408.

Each reagent composition is then divided into aliquots and varying amounts of hydrazine are added to each aliquot, the hydrazine being added as an 85 percent aqueous solution of hydrazine hydrate. The aliquot compositions are then reacted with glucose and the final absorbances of the products are determined in duplicate operations carried out as described above. In a representative operation carried out with aliquots of the reagent composition prepared from Lot A orthotoluidine and Lot 3 glacial acetic acid, the following final absorbances are found with aliquots having the following amounts of added hydrazine

Added Hydrazine (grams of hydrazine hydrate per 100 milliliters)	Final Absorbance		
	Run I	Run II	Average
0.0	0.410	0.405	0.408
0.004 (80)*	0.362	0.360	0.361
0.005 (100)	0.330	0.330	0.330
0.006 (120)	0.325	0.325	0.325
0.008 (160)	0.310	0.315	0.313

Figures in parenthesis () indicate equivalent hydrazine concentration in micromoles hydrazine per 100 milliliters reagent composition.

EXAMPLE 2

The compositions of example 1 containing various amounts of added hydrazine sufficient to lower the final absorbances to a desired value are prepared and compared with the compositions without added hydrazine by the procedure described in example 1, Duplicate runs are carried out in each case. 0.1 milliliter of an identical glucose standard solution containing 100 milligrams glucose per 100 milliliters of glucose standard is employed in each determination. The results are set forth in following table.

Ortho-toluidine lot	Glacial acetic acid lot	Hydrazine (milligrams hydrazine hydrate) per 100 milliliters	Average final absorbance times 100
A	1		40.1
A	1	[1] 5 (100)	34.6
A	2		40.2
A	2	4 (80)	32.1
A	3		40.8
A	3	5 (100)	33.0
B	1		40.5
B	1	6 (120)	34.3
B	2		34.0
B	2	4 (80)	28.9
B	3		39.1
B	3	6 (120)	32.8

[1] Figures in parentheses ( ) indicate equivalent hydroazine concentration in micromoles of hydrazine per 100 milliliters of reagent composition.

EXAMPLE 3

A series of seven orthotoluidine reagent compositions is prepared using the same lot of orthotoluidine in each case and one of seven lots of glacial acetic acid (a)–(g) in each case. The final absorbances with and without added hydrazine are determined as set out above in example 1, The average results obtained in duplicate determinations are set out in the following table.

Glacial acetic acid lot	Hydrazine [1]	Average final absorbance times 100 [2]
a		40.5
a	6	34.3
b		34.0
b	4	28.9
c		39.1
c	6	32.8
d		36.8
d	6	34.3
e		37.4
e	5	34.5
f		40.0
f	5	34.5
g		40.8
g	6	34.2

[1] Milligrams hydrazine hydrate per 100 milliliters of reagent composition.
[2] Obtained with 0.1 milliliter of glucose standard containing 100 milligrams glucose per 100 milliliters.

EXAMPLE 4

The reagent compositions of examples 1–3 containing from about 80 to about 160 micromoles of hydrazine per 100 milliliters and giving final absorbances between 0.28 and 0.35 when 5 milliliters of the glucose standard solution (equivalent to glucose concentration of 1.96 milligrams of glucose per 100 milliliters of ultimate mixture) are employed in various operations to determine glucose in biological fluids according to the procedure described above. Excellent results are obtained in determination of glucose concentrations in 0.1 milliliter samples of serum containing known glucose concentrations ranging from 60 to over 190 milligrams per 100 milliliters of serum, with excellent precision between and within runs.

We claim:

1. In a method for determination of glucose comprising the steps of mixing together predetermined quantities of a color reagent composition comprising orthotoluidine as the color forming reagent and a sample composition comprising glucose, mixing together an additional quantity of said color reagent composition and a standard composition comprising glucose at a predetermined concentration in said standard composition, and developing color in the mixtures resulting from both said mixing steps under substantially identical conditions whereby the intensity of said developed color in each such mixture is proportional to the concentration of glucose therein;

the improvement which comprises the step of adding hydrazine to the color reagent composition prior to the step of developing color, the hydrazine being added in an amount sufficient to provide a maximum intensity of said developed color equivalent to an absorbance of about 0.28 to about 0.35 for light of a wavelength between 590 and 625 millimicrons when the concentration of glucose in the ultimate mixture in which color is developed is from about 1.5 to about 2.5 milligrams of glucose per 100 milliliters of ultimate mixture.

2. The method of claim 1 wherein the hydrazine is added in an amount sufficient to provide a hydrazine concentration of from about 60 to about 200 micromoles of hydrazine per 100 milliliters of reagent composition.

3. The method of claim 1 wherein the hydrazine is added in the form of hydrazine hydrate.

4. The method of claim 1 wherein the reagent composition is prepared by mixing together orthotoluidine, thiourea and glacial acetic acid in predetermined amounts, and wherein the hydrazine is mixed with the orthotoluidine prior to preparation of the reagent composition.

5. The method of claim 4 wherein the hydrazine is employed in an amount sufficient to provide from about 9 to about 20 micromoles of hydrazine per gram of orthotoluidine.

6. In a reagent composition useful for determination of glucose by measuring the intensity of color produced by the reaction of glucose with orthotoluidine comprising a solution of orthotoluidine in glacial acetic acid, the concentration of orthotoluidine being from about 5 to about 10 grams of orthotoluidine per 100 milliliters of said solution; the improvement wherein the reagent composition further comprises from about 9 to about 20 micromoles of hydrazine per gram of orthotoluidine, said hydrazine being sufficient to decrease the intensity of the color produced when said composition is reacted with glucose.

7. The composition of claim 6 further comprising from about 0.1 to about 1 gram of thiourea.

8. The composition of claim 6 wherein the composition consists essentially of about 9 grams of orthotoluidine and about 0.5 gram of thiourea per 100 milliliters of composition and contains from about 80 to about 180 micromoles of hydrazine per 100 milliliters.

9. The composition of claim 8 further comprising from about 1.8 to about 2.1 milligrams of glucose per 100 milliliters, and wherein said hydrazine is present in an amount sufficient to decrease the intensity of color obtained by reaction of said glucose with said orthotoluidine to provide an intensity of said color equivalent to an absorbance of from about 0.30 to about 0.34 for light having a wavelength of from 590 to 620 millimicrons.

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UNITED STATES PATENT OFFICE
CERTIFICATE OF CORRECTION

Patent No. 3,615,228 Dated 26 October 1971

Inventor(s) Bernard J. Thiegs

It is certified that error appears in the above-identified patent and that said Letters Patent are hereby corrected as shown below:

In line 2 of the Abstract delete "ef-"; in line 3 delete "fective" and insert --comprises--.

In column 1, line 44, delete "along" and insert --alone--; in line 61 delete "prepared" and insert --it--.

Column 2, line 43, between "compositions" and "in" insert: --used for determination of the glucose concentration--.

Column 3, line 69, delete "my" and insert --by--.

Column 4, line 5, between "reagent" and "180," insert: --composition or from about 60 to about 80, to about--.

Column 5, line 72, between "milliliters" and "of" insert: --of reagent composition are mixed with 0.1 milliliter--.

Signed and sealed this 23rd day of May 1972.

(SEAL)
Attest:

EDWARD M.FLETCHER, JR.
Attesting Officer

ROBERT GOTTSCHALK
Commissioner of Patents