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[54] **DIAGNOSTIC METHOD FOR THE DETERMINATION OF URIC ACID IN BLOOD**

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[51] **Int. Cl.**.....**G01n 33/16**, G01n 31/22, G01n 21/24

[58] **Field of Search**.....23/230 B, 253 TP; 252/408; 424/7

[56] **References Cited**

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[57] **ABSTRACT**

A diagnostic method for the determination of uric acid in the blood of humans by detecting the color change of a copper neocuproine complex in the presence of N-ethylmaleimide.

2 Claims, No Drawings

DIAGNOSTIC METHOD FOR THE DETERMINATION OF URIC ACID IN BLOOD

BACKGROUND OF THE INVENTION

This invention relates to a novel diagnostic test. More particularly, it relates to an improved diagnostic method for the determination of uric acid in the blood of humans, which is based on the detection of a color change of a copper neocuproine complex in the presence of N-ethylmaleimide.

The determination of uric acid is of particular importance in screening individuals for certain disease states. Uric acid is the metabolic end product of purines. The purines may be derived from nucleic acids, nucleotide cofactors, or from nucleic acids in food. In gout, the pool of uric acids is increased and urates are deposited in and around joints producing pain and swelling. Thus in gout, or in gouty arthritis, the blood uric acid level increases. High uric acid values are also found in nephritis, eclampsia, leukemia and other conditions affecting the excretory function of the kidneys.

Uric acid has been determined in several different ways. Two of the most common methods are by the reaction of uric acid with alkaline phosphotungstate and by the enzyme uricase. In the first method, uric acid is estimated by reducing alkaline phosphotungstate to tungsten blue and measuring the colored product in a colorimeter. The second method utilizes the property of uric acid of absorbing light at 290–293 Mμ. The products of the uricase reaction do not absorb at this wavelength. The decrease in absorbance is proportional to the amount of uric acid initially present.

Bittner, in U.S. Pat. No. 3,282,649, discloses a method for the determination of uric acid in deproteinized blood utilizing a preformed copper chelate of cuproine, neocuproine or bathocuproine at a pH of 5–6 and measuring the amount of copper reduced to the cuprous state by the uric acid present in the blood.

The new and novel features of the present invention over this prior art procedure will be obvious as the invention is further disclosed.

SUMMARY OF THE INVENTION

This invention broadly comprises the steps of:

a. adding a solution of N-ethylmaleimide to a predetermined quantity of serum,

b. adding to said solution a predetermined volume of a neocuproine solution, followed by the addition of a predetermined quantity of a copper solution in the cupric state,

c. measuring the amount of copper reduced to the cuprous state by reference to the optical density of the solution and determining the amount of uric acid by comparing the optical density of a standard at a wavelength of from about 450 to 460 μ.

Experimentally, it is preferred to use the following amounts of materials when carrying out the aforedescribed diagnostic test.

Serum	0.2 ml.
Buffered N-ethylmaleimide solution (0.4 % w/v).	1.0 ml.
Buffered Neocuproine solution (0.04% w/v)	1.0 ml.
Copper sulfate solution (0.02% w/v)	2.0 ml.

Of course, it is to be understood that any equivalent volume proportions may be used in lieu of these above; however, the above amounts are preferred since the total resulting volume is appropriate for the subsequent optical density measurements.

With regard to the wavelength at which the optical density measurements are carried out, it is preferred to use a wavelength of 445 Mμ; however, a range of from about 450 to 460 Mμ is equally suitable.

This invention is an improvement over prior art methods in that it can be run directly on serum and eliminates the necessity of deproteinizing the blood to produce protein free serum. This results in a saving of valuable laboratory time and is a more convenient method. This important feature is attributable to the use of N-ethylmaleimide which increases the sensitivity of the copper-neocuproine reduction complex and makes the reaction more specific for uric acid which therefore allows ti to be run on serum.

DETAILED DESCRIPTION OF THE INVENTION

The herein disclosed diagnostic method determines the amount of uric acid in human serum wherein said amount is measured by means of a change in optical density of a copper-neocuproine complex in the presence of N-ethylmaleimide.

This new method is an advance over prior art methods in that the new method is run directly on serum whereas prior art procedures are run on protein-free filtrates. The new method utilizes N-ethyl maleimide which

a. increases the sensitivity of the copper-neocuproine reduction complex,

b. enhances the linearity of the reaction, and

c. increases the accuracy of recovery experiments when two serums of divergent values are mixed.

In the new method, the serum is treated successively with the N-ethyl maleimide, neocuproine and finally with a separate cupric solution.

Experimentally the diagnostic procedures may be carried out in the following manner: A sample of blood serum, from about 0.1 ml. to about 0.8 ml., with the preferred amount being 0.2 ml., is mixed with about 0.5 to about 4.0 ml., the preferred amount being 1.0 ml. of a buffered N-ethylmaleimide solution having a concentration of about 0.4 percent w/v. The pH of said buffered solution is from about 7.0 to 8.0, the preferred pH being 7.5.

To this is then added from about 0.5 ml. to about 4.0 ml., the preferred amount being 1.0 ml. of a buffered neocuproine solution having a concentration of about 0.04 percent w/v. Said mixture is then allowed to incubate at ambient temperature for about 15 to about 30 minutes. The preferred time, however, is 20 minutes. Copper sulfate solution having a concentration of about 0.02 percent w/v is then added. The usual amount is from about 0.5 ml. to about 4.0 ml., with the preferred amount being 2.0 ml. The mixture is then allowed to incubate for an additional time period of from about 10 to about 20 minutes, the preferred time being 15 minutes. The optical density is determined at a wave length of from about 450 to 460 Mμ; however 455 Mμ is the preferred wave length. The quantity of uric acid present is then determined from a standard curve, determined by measurements made on samples made up to known uric acid concentrations.

PREPARATION OF REAGENTS

A. Buffered N-Ethylmaleimide Solution

To a 2-liter volumetric flask is added 8.0 g. of N-ethylmaleimide, 1.03 g. of sodium dihydrogen phosphate, and 3.19 g. of disodium hydrogen phosphate. Distilled water (800 ml.) is added and the mixture stirred until the solids are in solution. This solution is adjusted to a pH of 7.5 using 1 N NaOH. Sufficient distilled water is then added with mixing to give a 2-liter solution.

B. Buffered Neocuproine Solution

Neocuproine hemihydrate (0.720 g.) is added to 200 ml. of distilled water. Some solid remains undissolved. Sufficient 5 N HCl is added to adjust the pH to 2.0. At this point all of the solids are in solution. Sufficient 5 N NaOH is then added in order to adjust to a final pH of 5.0.

Glycine (15 g.) is added to 200 ml. of distilled water and mixed until dissolved. Sufficient 5 N NaOH is then added to adjust the pH to 9.0.

To a 2-liter volumetric flask containing 600 ml. of distilled water is added the neocuproine solution and glycine solution described above. The pH of the combined solution is then adjusted to 9.0 using 5 N NaOH. Sufficient distilled water is then added to give a 2-liter solution.

C. Copper Sulfate Solution

To 60 ml. of distilled water in a 2-liter volumetric flask is added 15.2 ml. of 2-amino-2-methyl propanol. The pH of the solution is then adjusted to 9.0 with 5 N HCl or 5 N NaOH.

Anhydrous copper sulfate 0.39 g. is dissolved in 10 ml. of distilled water.

The copper sulfate solution is then added to the solution of 2-amino-2-methyl propanol. The pH is then adjusted to pH 9.0 with 5 N NaOH. Sufficient distilled water is added to give a 2-liter solution.

D. Uric Acid Stock Solution

To 150 ml. of distilled water in a 1-liter volumetric flask is added and dissolved 0.6 g. of lithium carbonate and 1.0 g. of uric acid. The pH is adjusted to 12.0 with 1 N NaOH in order to dissolve the uric acid. Formalin 40 percent (20 ml.) and 330 ml. of distilled water is then added. The pH is readjusted to pH 2.9 with 1 N H_2SO_4. Sufficient distilled water is added to give a 1-liter solution.

EXAMPLE I

1. Reagent A, 1.0 ml. is pipetted into each of two 95×15 mm. test tubes labeled "sample" and "reagent blank."

2. The serum to be tested, 0.2 ml. is added to the "sample" tube and 0.2 ml. of distilled water to the "reagent blank" blank tube.

3. Reagent B, 1.0 ml. is added to each tube.

4. The tubes are then allowed to remain for 20 minutes at room temperature.

5. Reagent C, 2.0 ml. is then added to each tube, followed by an additional 15 minutes waiting period.

6. Spectrophometric readings are then made at 455 Mμ by adjusting the zero point of the spectrophotometer with the "reagent blank" and determining the absorptivity of the "sample."

Serum blanks are determined by adding 4.0 ml. of distilled water to each three 95×15 mm. test tubes. To each tube is added 0.2 ml. of serum. Spectrophometric determinations are then made by using distilled water to adjust the zero point and determining the absorptivity of each serum blank. The results are then averaged and the following optical density ("OD") calculation made:

$$OD_{sample} - OD_{sample\ blank} = OD_{uric\ acid}$$

The uric acid concentration is then read from a standard curve which is obtained by treating a series of dilutions of Reagent D in the same manner and plotting the resulting optical densities vs. uric acid concentration.

EXAMPLE II

The procedure of Example I is repeated wherein the following amounts of serum sample and reagents are used:

I.	Sample	0.4 ml.
	Reagent A	2 ml.
	Reagent B	2 ml.
	Reagent C	4 ml.
II.	Sample	0.8 ml.
	Reagent A	4 ml.
	Reagent B	4 ml.
	Reagent C	8 ml.

Equivalent results are obtained.

What is claimed is:

1. A method for determining uric acid concentration in human blood serum which comprises:
   a. adding aqueous N-ethylmaleimide solution to a measured quantity of said serum,
   b. adding to the thus-treated serum aqueous neocuproine solution, followed by an aqueous solution of cupric salt,
   c. and comparing the optical density of the resulting solution with that of the standard.

2. The method of claim 1 wherein said optical density is determined at a wavelength of from about 450 to 460 mμ.

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