United States Patent [19]

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[11] **3,868,219**

[45] **Feb. 25, 1975**

[54] **DETERMINATION OF HCG GLUCOSE AND GALACTOSE IN BODY FLUIDS WITH CHROMOGENIC INDICATORS**

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[22] Filed: **May 30, 1973**

[21] Appl. No.: **365,393**

[30] **Foreign Application Priority Data**

June 14, 1972 Netherlands 7208092

[52] **U.S. Cl.** ..... **23/230 B,** 23/253 TP, 162/78, 424/12

[51] **Int. Cl....G01n 21/06,** G01n 31/32 G01n 33/16

[58] **Field of Search** ..... 23/253 TP, 230 B; 162/70, 162/78; 424/12

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[57] **ABSTRACT**

Process for the determination of a component of a reaction system, which determination is based upon the oxidation of a chromogenic compound and in which determination use is made of a test paper containing the remaining components of the reaction system. Such determinations are often disturbed by undesired compounds being present in the test fluid containing the component to be determined. The process is characterized in that the test fluid is first contacted with a material consisting essentially of cellulose or a cellulose-like substance, which material has been subjected to a treatment with an oxidizing agent.

Test papers containing or made from the above-said material are useful for detecting HCG, glucose and galactose in body fluids.

5 Claims, No Drawings

DETERMINATION OF HCG GLUCOSE AND GALACTOSE IN BODY FLUIDS WITH CHROMOGENIC INDICATORS

The invention relates to a process for the determination of a component of a reaction system, which determination is based upon the oxidation of a chromogenic compound and in which determination use is made of a test paper provided with the remaining components of the reaction system, by bringing said test paper into contact with the test fluid, in which the first-mentioned component is to be determined.

Many determinations, both in the chemical and in the biochemical field, are disturbed by the presence of undesired compounds. In order to raise the reliability of the determination the effect of a disturbing compound is often neutralized by masking. The disturbing compound can also be removed from the system in which the determination has to be carried out by adding a suitable absorbent, such as active carbon or bentonite, or ion exchanging material.

When absorbents or ion exchanging material are used for that purpose, there is a risk that not only the disturbing compound(s) will be removed from the test fluid, but also the component of the reaction system to be determined.

In the determination of, for example, glucose in urine by means of a test paper impregnated with glucose-oxidase, peroxidase and a chromogenic compound, the test results are seriously disturbed by the presence of reducing components, such as uric acid and ascorbic acid (vitamin C).

It has been found now that the reliability and the effectiveness of the determination of a component of a reaction system, which determination is based upon the oxidation of a chromogenic compound, and in which determination use is made of a test paper provided with the remaining components of the reaction system, by bringing the said test paper into contact with the test fluid, in which the firstmentioned component is to be determined, can strongly be improved by contacting first the test fluid with material, consisting essentially of cellulose or a cellulose-like substance, which material has been subjected to a treatment with an oxidizing agent.

Surprisingly said treated material proves to inactivate the disturbing compounds being present in the test fluid in a very selective way. On application of the said treated material in, for example, the determination of glucose in urine, that contains uric acid and ascorbic acid as disturbing compounds, by means of glucoseoxidase, perioxidase and an oxidizable chromogenic compound, for example o-tolidine or the reduced form of 2,6-dichloro-phenolindophenol, the said disturbing compounds do not have any detrimental effect on the result of the determination.

The said material, which consists essentially of cellulose or cellulose-like material may be treated with any suitable oxidizing agent, that does not completely destruct it. In particular, good results are obtained with perhalogenic acids, persalts and hypochlorites, for example, periodic acid, potassium permanganate and sodium hypochlorite. A convenient and also cheap treatment-method proved to be the use of commercial bleaching liquor or the concentrated form thereof.

The treatment is, of course, followed by a careful removal of any excess of the oxidizing agent from the

treated material obtained. Additionally the said treated material may be dried, if necessary.

The treated material may be used separately or in combination with a test paper any may also be the test-paper itself. The treated material lends itself particularly to application on or in the test paper used for the determination of a component of a reaction system, for example, consisting of a perioxide, peroxidase and an oxidizable chromogenic compound, or consisting of a hydrogen peroxide-supplying oxido-reductase, a suitable substrate for it, a peroxidase and an oxidizable chromogenic compound. Besides the treated material the test paper contains all components of the reaction system except, of course, the component to be determined. Moreover the test paper might contain one or more auxiliaries, for example a buffering substance, if required.

The components of the reaction system can either be used as free substances or bound to a suitable carrier, the bound form can be obtained in various ways, for example, by adsorption or by a covalent bond to the carrier. The latter form is applied preferably, since it prevents the relative component from being easily rinsed from the test paper during the determination and, moreover, it offers the opportunity to determine the quantity of carrier material indirectly, if desired. Thus, for example, the test paper for determination of peroxidase may contain hydrogen peroxide that is bound to a phosphate buffer, while peroxidase itself may also be bound to a suitable carrier, for example, to human chorionic gonadotrophin (HCG). In the latter example the quantity of peroxidase found is simultaneously a measure for the amount of HCG.

The invention is illustrated further by the following examples.

EXAMPLE I

Strips of filter paper (Schleicher und Schüll 2316) measuring 20 × 4 cm, were immersed in a solution of 4 g of periodic acid in 600 ml of water, for about 16 hours (leave to stand overnight). Then the strips were washed with water until negative reaction on periodic acid and finally dried.

EXAMPLE II

Strips of filter paper (S & S 589/3; 83–87 g of paper/sq.m.) measuring 20 × 5 cm, were laid for 3 hours in a solution consisting of 300 ml of 0.1 N potassium permanganate and 30 ml of 4 N sulphuric acid. Then the strips were washed with 0.1 N sulphuric acid till no purple colour was visible any more. After that the strips were treated with a 3% solution of hydrogen peroxide till all the manganese dioxide the paper contained, had completely dissolved. Finally the strips were washed with water until negative reaction on hydrogen peroxide, and dried.

EXAMPLE III

Strips of Whatman 3 MM-paper (185 g of paper/sq.m.) were laid in a sodium hypochlorite solution (5 g of active chlorine per 100 ml) for 5 hours. After being thoroughly washed with water until negative reaction on chlorine (KI-starch paper), the strips were dried.

EXAMPLE IV

Strips of Whatman 3 MM-paper (15 × 6 cm) were

immersed, for about 16 hours, in a solution consisting of 200 ml of sodium hypochlorite (10 g of active chlorine per 100 ml) and 200 ml of 4 N sodium hydroxide. After being washed with water until negative reaction on chlorine, the strips were dried.

EXAMPLE V

One hundred grams of cellulose powder were treated with 500 ml of the solution from example IV for 5 hours and then washed with water until negative reaction on chlorine and neutral reaction of the washing-water. Finally the cellulose powder was air dried.

EXAMPLE VI

a. A strip of filter paper, width 6 cm, prepared in the way as described in example IV, was washed twice in a phosphate buffer solution (consisting of 13.6 g of potassium dihydrophosphate dissolved in 800 ml of water, to which enough 4 N sodium hydroxide solution had been added to obtain a pH of 6.8, after which the volume had been completed with water to 1,000 ml) and then dried. The buffer solution was kept.

b. One hundred mg of ascorbic acid were dissolved in 5 ml of water. After that a solution was added of 100 mg of 2,6-dichlorophenolindophenol (DCPIP) and 400 mg of polyvinylpyrrolidon (PVP) in 35 ml of absolute ethanol to obtain a light coloured solution. (Any resulting precipitate can be removed by centrifugation).

c. Then a lengthwise stroke (width abt. 5 mm) was made in the middle of the strip of paper obtained in a) with the reduced DCPIP-solution from (b), after which the strip was dried.

d. To 200 ml of the phosphate buffer solution mentioned in a) 16 ml of a 30% hydrogen peroxide solution were added. The DCPIP stroke made in c) was dried and after that a stroke (width abt. 5 mm) was made with the phosphate buffer-hydrogen peroxide solution (Ph-H) at some distance beside it, which stroke was also dried.

e. After being dried, the prepared paper was cut into strips of 60 × 7 mm, and that crosswise, so that in each strip there was a DCPIP-zone and a pH-H-zone.

f. To 0.1 ml of a sample of urine from a woman suspected to be pregnant, in which urine the presence of human chorionic gonadotrophin (HCG) had to be demonstrated, 0.1 ml of antiserum (rabbit-anti HCG) was added, and that enough to bind 0.2 units of HCG.

After a wait of about 2 minutes 0.1 ml of a solution of HCG-peroxidase conjugate with a potency of 0.2 units of HCG per 0.1 ml was added.

The mixture was left to stand for 2 minutes and shaken now and again during this time. After that a suspension of sheep antirabbit-γ-globulin coupled to cellulose was added.

Then the suspension was left to stand for 5 minutes and shaken now and again during this period. After that the supernatant fluid was allowed to be absorbed by a strip of paper as described in (e). The strip was kept in the fluid in such a way that the Ph-H stroke was below the DCPIP stroke. A marked blue colouring showed the presence of HCG in the relative sample of urine.

9. The same test with a strip of paper that had not been pretreated by oxidation, however, gave only a very faint change of colour.

EXAMPLE VII

To 1 ml of the sample of urine mentioned in example VI (f) 50 mg of cellulose powder, pre-treated as described in example V, were added. The mixture was shaken for 1 minute, after which 0.1 ml of this fluid was tested for the presence of HCG in accordance with example VI, but using a test paper that had not been oxidized. A marked blue colouring was formed.

EXAMPLE VIII

The test described in example VI can also be performed with a test paper provided with all reagents required. This is done as follows:

In 28.6 ml of distilled water 11.43 g of $CuSO_{4.5}H_2O$ are added while heating to 95°C. The solution is cooled down to some 50° to 60° C. Then 22.86 ml of 25% ammonia are added to form a precipitate of $Cu(OH)_2$, which dissolves again on adding more ammonia. Now the solution is cooled down further to 0°–5° C, after which 800 mg of sucrose and 1,000 mg of m-aminobenzyloxymethylcellulose is added. For the preparation of this cellulose reference is made to: Methods in Immunology and Immunochemistry, Vol. 1, eds. C. A. Williams and M. W. Chase (Academic Press, New York, London, 1967, p. 378). Then 5.7 ml of 10 N NaOH are added, after which the cellulose derivative completely dissolves.

A strip of Whatman 3 MM filter paper, measuring 20 × 8 cm, pretreated as described in example IV, soaked in a 0.5 M solution of KH_2PO_4 and dried, is provided with a stroke of the relative Cu cellulose solution (width 15 mm). Through the presence of the primary phosphate the pH is lowered so much that the dissolved cellulose precipitates in the paper. A blue band is visible. Now the paper is washed with cold 0.1 N H_2SO_4 for some time, during which process the washing-liquid is regularly replaced by a fresh one. The whole process, which serves to remove all the traces of copper, may last up to 24 hours.

Then 14 g of $NaNO_2$ are dissolved in 1,000 ml of 1.6 N H_2SO_4. The paper is treated with this solution for 30 minutes (replace the solution three or four times by a fresh one). Then the solution is washed with distilled water until neutral and then in a borate buffer, prepared by dissolving 3.1 g of H_3BO_3 and 20 ml of 1 N NaOH in distilled water, adjusting the pH at 8.6 with 4 N NaOH and completing the solution to 1 l with distilled water. This borate buffer is replaced by a solution of sheep anti-(rabbit-γ-globulin) (2.5 mg/ml in borate buffer, pH 8.6) and the paper is kept in this solution overnight maintaining the pH of the paper and buffer at 8.6. Then the globulin solution is decanted and the paper finally treated with a saturated solution of β-naphtol in borate buffer of pH 8.6. This latter treatment is performed at room temperature. The paper is finally washed with an 0.1 M phosphate buffer of pH 6.8 as mentioned in example VI, and dried. Then this paper is provided with the other reaction components, viz. a stroke of a reduced DCPIP solution, a stroke of urea hydrogen peroxide (starting from a 1.6% solution in ethanol) below the stroke of cellulose sheep anti-(rabbit-γ-globulin) and a stroke of HCG-peroxidase conjugate and below the latter a stroke of antiserum (rabbit-anti-HCG). The last two components are applied in equivalent quantities and that in such a way that urine containing 2 U of HCG/ml can exactly bind the quantity of antiserum. For this purpose the suction capacity of each lot of paper must be determined again and again. When the paper has been pro-

vided with all the components and dried, it is cut into strips (width 7 mm).

When urine is allowed to be absorbed by the paper, and if enough HCG is present, which indicates the existence of preganancy, the absorbed urine will bind the antiserum, transport the applied HCG-peroxide through the paper, releasing on its way sufficient hydrogen peroxide from the urea hydrogen peroxide, and finally the peroxidase and the hydrogen peroxide will react with the reduced DCPIP giving a blue colour.

If no pregnancy exists the antiserum will react with the HCG-peroxidase conjugate and the complex formed will be retained by the sheep anti(rabbit-γ-globulin) zone. Consequently the peroxidase does not reach the redox indicator and the paper will remain uncoloured.

EXAMPLE IX

For the demonstration of glucose in body fluids a strip of filter paper was pre-treated as described in example III. After that the relative paper was saturated with a sloution containing the following reagents:

glucose-oxidase	200 mg
horse-radish peroxidase	5 mg
o-tolidine	100 mg
citric acid	600 mg
sodium citrate 2 aq	1,275 mg
water	20 ml

After being dried, the strips of paper prepared in this way were used successfully in the determination of glucose in body fluids in the presence of normally disturbing reducing substances.

The same good result was obtained using the reduced form of 2,6-dichloro-phenol indophenol instead of o-tolidine (see example X).

EXAMPLE X

Test paper as mentioned in example IX for the demonstration of glucose was prepared in the following way:

a. To 100 mg of vitamin C, dissolved in 5 ml of water, a solution was added of 100 mg of DCPIP and 400 mg of PVP in 35 ml of absolute ethanol.

b. Filter paper, pre-treated in accordance with example IV, was soaked in a 0.1 M phosphate buffer (pH 5.6) and then dried. Then a stroke (width about 5 mm) of the reduced DCPIP solution [according to (a)] was made on the dried paper.

c. Under this stroke a stroke was made of a solution of 200 mg glucose-oxidase and 5 mg of horse-radish peroxidase in 20 ml of water.

d. The thus obtained paper was dried, after which the fluid to be tested was allowed to be sucked up into strips of the relative paper, and that in such a way that the fluid first had to pass the enzyme system to reach the redox-indicator. The intensity of the blue colour that was formed was many times greater than the intensity of the blue colour in the same test performed with untreated paper.

EXAMPLE XI

For the demonstration of very small amounts of galactose in body fluids (the presence of reducing substances being in the nature of things very disturbing) paper pre-treated in the way described in example I, afterwards soaked in a solution of the composition given hereinafter and dried, was used with very good result.

Composition of reagents:	
0.1 M phosphate buffer(pH 6.8)	60 ml
galactose oxidase (8000 U/mg)	2 mg
horse-radish peroxidase	20 mg
o-tolidine	200 mg in 20 ml of ethanol

Example XII

For the demonstration of bacteriuria advantage was taken of the circumstance that small amounts of glucose, which are always present in urine, are degradated by microorganisms. Thus, if no glucose can be demonstrated this points to the presence of a microorganism. Particularly for the test conditions prevailing here, it is essential that reducing components in the urine to be tested should be absent. The paper pre-treated in a way as described in example II and provided with the required reagents in accordance with examples IX or X can be used successfully.

What is claimed is:

1. In a process for the detection and determination of a member of the group consisting of human chorionic gonadotrophin, glucose, and galactose present in a fluid sample, the detection and determination being based upon a visual color change produced by the oxidation of an oxidizable chromogenic compound as a component in a test reaction system including other test reaction components, the improvement which comprises inactivating any undesired substances which may be present in said fluid sample by first contacting the sample with a purified cellulose which has been further subjected to treatment with an oxidizing agent, and then contacting the sample with said chromogenic compound.

2. The process of claim **1** in which said cellulose is in the form of a test paper.

3. A test paper for the detection and determination of a member of the group consisting of human chorionic gonadotrophin, glucose, and galactose in a fluid sample to be tested, the detection and determination being based upon a visual color change produced by the oxidation of an oxidizable chromogenic compound in a test reaction system including other test reaction components, said test paper consisting essentially of a purified cellulose which has been further subjected to treatment with an oxidizing agent, and is impregnated with said chromogenic compound in a reduced form and with said other test reaction components.

4. A test paper for the detection and determination of human chorionic gonadotrophin in a fluid sample, the detection and determination being based upon a visual color change produced by the oxidation of an oxidizable chromogenic compound in a test reaction system including other test reaction components, said test paper consisting essentially of a purified cellulose which has been further subjected to treatment with an oxidizing agent, and being impregnated with said oxidizable chromogenic compound in a reduced form and with said remaining test reaction components including antibodies against human chorionic gonadotrophin, a conjugate of human chorionic gonadotrophin and peroxidase, insolubilized anti-antibodies, and a substrate for the peroxidase.

5. A test paper for the detection and determination of human chorionic gonadotrophin in a fluid sample, the detection and determination being based upon a visual color change produced by the oxidation of an oxidizable chromogenic compound in a test reaction system including other test reaction components, said test paper consisting essentially of a purified cellulose which has been further subjected to treatment with an oxidizing agent, and being impregnated with said oxidizable chromogenic compound in a reduced form and with said remaining test reaction components including antibodies against human chorionic gonadotrophin, a conjugate of human chorionic gonadotrophin and a hydrogen peroxide-supplying oxido-reductase, a substrate for said oxido-reductase, insolubilized anti-antibodies, and peroxidase.

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