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10. Jg. 1972, S. 372—373

Manual and Automated Determination of Glucose in Blood with Glucose Oxidase and Molybdate/Iodide as Redox Catalyst

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(Eingegangen am 25. November 1971/20. April 1972)

A method for glucose determination with glucose oxidase is proposed in which the redox catalyst Na-molybdate-KI replaces peroxidase. Whole blood (100 µl) may be used after deproteinization; serum or plasma (10 µl) can be analyzed directly. The color is stable for at least 30 minutes after stopping the reaction.

Es wird eine Methode zur Bestimmung von Glucose mit Glucoseoxidase vorgeschlagen, bei der der Redoxkatalysator Na-Molybdat/KI Peroxidase ersetzt. Vollblut (100 µl) wird nach Enteiweißung, Serum bzw. Plasma (10 µl) direkt analysiert. Der gebildete Farbstoff ist nach Abstoppen der Reaktion mindestens 30 Minuten stabil.

The oxidation of β-D-glucose by glucose oxidase to δ-glucono-lactone which spontaneously hydrolyzes to D-gluconic acid has been widely and successfully used for the determination of glucose in clinical chemistry. The method is specific for glucose (1) and has replaced procedures employing unspecific redox systems. In the presence of peroxidase the H_2O_2 formed in the glucose oxidase reaction oxidizes a chromogen in amounts equimolar to glucose. The color is measured photometrically.

Several improvements to this method have been described. The widely used chromogen *o*-dianisidine has been replaced by noncarcinogenic substances such as ethylbenzthiazolinsulfonic acid (ABTS) (2), diethylphenylenediamine (DEPPD) (3) and the use of iodine in the presence of metallic ions has been suggested. Peroxidase has been replaced in some procedures by catalytic systems such as iodine-vanadate (5), ammoniummolybdate-iodine (3) or molybdic acid-iodine (4). Köhler and Braunfeld in a critical review (6) gave preference to the use of peroxidase over non-enzymatic redox systems.

We have developed and used in our laboratory for two years a glucose oxidase method for the determination of glucose in which peroxidase is replaced by the redox system of iodine plus Na-molybdate. Only 100 µl of blood or 10 µl of serum or plasma are needed. The procedure requires only one pipetting step. The blank is zero. The reaction takes place in a weakly acid medium and is stopped by adjusting the pH to 12.7.

Materials and Methods

Reagents

1. Na_2SO_4 30 g/l
2. $ZnSO_4 \cdot 7\,H_2O$ 50 g/l
3. NaOH 0.25 mol/l } neutralised
4. NaOH 100 g/l.
5. Na-molybdate-KI: 0.4 g $Na_2MoO_4 \cdot 2\,H_2O$ + 8 g KI to 1000 ml H_2O.
6. Glucose oxidase (Pliva, Pharmaceutical Co. Zagreb), 1 mg contains 4 I. U. of activity. 0.7 g of the enzyme are dissolved in a few milliliters of 0.2 mol/l acetate buffer pH 5.5, and diluted with the acetate buffer to 1 liter. It is filtered and kept in a dark bottle in a refrigerator. The solution is stable for two weeks.
7. *o*-Dianisidine. 0.6 g of *o*-dianisidine is dissolved in 10 ml 1 mol/l HCl and diluted to 100 ml with H_2O. In the refrigerator the solution is stable for 10 days. Every day 5 ml is diluted with 20 ml of H_2O.
8. Standard solution of glucose in 1 g/l benzoic acid.
9. Mixed reagent. To be prepared daily in the amount required; it consists of:

400 ml Na-molybdate-KI (reagent 5)
400 ml glucose oxidase (reagent 6)
40 ml diluted *o*-dianisidine (reagent 7).

For Clinomak the mixed reagent is diluted with an equal amount of water before use.

Procedure

Blood is deproteinized by adding 100 µl blood to a mixture of 1 ml of isotonic Na-sulfate, 200 µl of 50 g/l zinc sulfate and 200 µl of 0.25 mol/l NaOH. After centrifugation 200 µl of the supernatant is added to 1.5 ml of the mixed reagent.

Deproteinization is omitted with serum and plasma, 10 µl of which are directly added to 1.5 ml of the mixed reagent (reagent 9). After exactly 20 min the reaction is stopped by adding one drop of 100 g/l NaOH. Blanks and standards are treated simultaneously. Readings are made at 460 nm.

Procedure for the Clinomak autoanalyzer

100 µl of deproteinized supernatant (see above) or 10 µl of serum or plasma are aspirated. Valve 1 is set in position 4 for the mixed reagent; the reading is taken at 460 nm during the second cycle. Digital readouts are obtained with the Datamak with a standard solution of 800 mg/l glucose. In each series are included "pooled serum" and several standards of higher concentrations as controls.

Results and Discussion

Only 10 µl of serum or plasma, which may be obtained from capillary blood, are needed for the determination of glucose. The interference by glutathione, cysteine and some other substances which are present in blood in higher concentrations than in serum or plasma is thereby diminished.

The stability of glucose is ensured by using the same preservative (see below) for plasma as for whole blood. The influence of uric acid and creatinine was checked using the method both with and without deproteinization. When added to blood, serum or plasma up to 160 mg/l of uric acid and up to 30 mg/l of creatinine were without noticeable effects on the final results.

Preservatives

For blood and plasma we use Na-fluoride (1 part) + EDTA (3 parts) at a concentration of 20 mg/ml of blood.

Deproteinisation

SOMOGYI reagent is used because acid agents such as trichloroacetic acid or perchloric acid are not suitable in the further steps of the reaction. Zinc sulfate and sodium hydroxide must be neutralised titrimetrically.

Glucose oxidase

Glucose oxidase from *Aspergillus niger* was obtained from Pharmaceutical Co. Pliva Zagreb. Comparative analyses of the same samples with glucose oxidase of equivalent activity from "Pliva" and "Merck" gave identical results.

Na-molybdate-KI

This system was a suitable replacement for peroxide. Na-molybdate permits a homogeneous solution and there was no interference with the chromogen.

o-Dianisidine

o-Dianisidine reagent, prepared by dissolving the compound in hydrochloric acid, shows less tendency to subsequently form precipitates than the reagent solutions in acetone or alcohol. Benzidine and ABTS did not react under these circumstances. With *m*-phenylendiamine a green color develops, which is of lower intensity compared to *o*-dianisidine.

Absorbance

The extinction, measured from 400 to 540 nm has a maximum at 460 nm.

LAMBERT-BEER *law*

The standard curve is linear up to 3.00 g/l. The line has an ideal angle of 45 degrees. A slight turbitity develops with concentrations exceeding 30.00 g/l (Fig. 1).

Precision of the method

20 samples of a specimen with a mean value of 960 mg/l gave S. D. = ± 2.2.

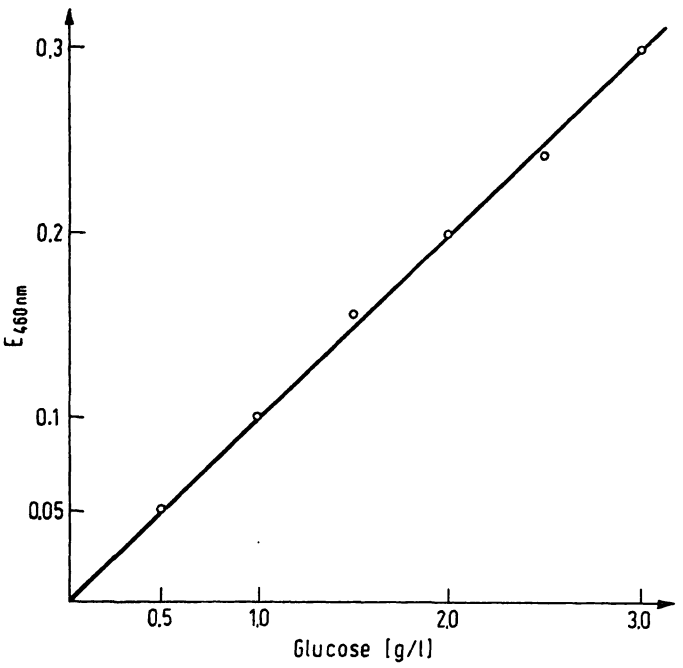

Fig. 1
Standard curve for the determination of glucose

Comparison of the method

The proposed method has been compared with the glucose oxidase method of Boehringer Mainheim Co. In 60 parallel determinations the values ranged from 600 to 2560 mg/l. Statistical evaluation of these results by STUDENT's test gave no significant differences of the results ($t = 1.18$, $t_{theor.} = 1.68$).

Test recovery

Recovery of added glucose was nearly 100% (Tab. 1).

Tab. 1
Recovery test

Results glucose g/l	Added glucose g/l	Concentration measured (g/l)	Theoretical concentration (g/l)	% Recovery
1.02	0.50	1.50	1.52	98
1.02	1.00	1.98	2.02	98
1.02	2.00	3.02	3.02	100
1.02	3.00	4.05	4.02	100.7

Normal values

In 40 persons in good healthy conditions a mean value of 740 mg/l with a range from 510 to 970 mg/l was found. Blood was used to determine the normal values.

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