Enzymatic Blood Glucose Determination by Colorimetry of 
N,N-Diethylaniline–4-Aminoantipyrine

Peter Kabasakalian, Sami Kalliney, and Anita Westcott

The hydrogen peroxide produced from glucose by glucose oxidase action at pH 7 is determined by the peroxidase oxidative coupling of N,N-diethylaniline with 4-amino-antipyrine. A 20-μl sample of plasma or serum is reacted at room temperature for 10 min. The sensitivity of the method is such that 60 μg of glucose (300 mg/dl) in a final volume of 5 ml gives an absorbance of 0.8 at 553 nm with a 1-cm cell. Absorbances are linearly related to glucose concentrations as high as 10.00 g/liter.

In the enzymatic determination of glucose in biological fluids (1, 2), the glucose oxidase reaction is combined with determination of the hydrogen peroxide produced. This report introduces an extension of this approach by the novel use of the well-known 4-aminoantipyrine method for arylamines (3). The alkaline oxidative coupling agent that is usually used, potassium ferricyanide, has been replaced with the hydrogen peroxide-peroxidase couple at pH 7. The major advantages of this method are that it is fast and uses noncarcinogenic reagents.

Materials and Methods

Apparatus

A Cary Model 15 recording spectrophotometer (Varian, Monrovia, Calif. 91016) with optically matched 1-cm cells was used to measure absorbancies.

An Eppendorf Push-Button 20-μl Pipet (Brinkman Instruments Inc., Westbury, N.J. 11590) was used to measure the serum, plasma control, and (or) standard samples.

Reagents

1. Combined reagent. Transfer 3200 U of glucose oxidase (p-glucose:O₂ oxidoreductase, EC 1.1.3.4; grade GOP; Worthington Biochemical Corp., Freehold, N.J. 07728), 3000 U of horseradish peroxidase, (donor:H₂O₂ oxidoreductase, EC 1.11.1.7; grade HPOD; Worthington), 62 mg of 4-aminoantipyrine (Eastman Organic Chemicals, Rochester, N.Y. 14650), 60 μl of N,N-diethylaniline (Aldrich Chemical Co., Inc., Milwaukee, Wis. 53233), and 100 mg of sodium azide (Matheson, Coleman and Bell, East Rutherford, N.J. 07073) to a 100-ml volumetric flask and dilute to volume with phosphate buffer (50 mmol/liter, pH 7). Make freshly each day and store refrigerated until used.

2. Glucose standard solution (1 g/liter). Transfer 1 g of dextrose (Matheson, Coleman and Bell) into a 1-liter volumetric flask and dilute to volume with aqueous 0.2% benzoic acid solution. Age for at least 24 h before using.

3. Control. “Calibrate” reference serum (General Diagnostics Division, Warner-Chilcott Laboratories, Morris Plains, N.J. 07960) grades 1, 2, and 3, was prepared to contain 75, 150, and 300 mg of glucose per deciliter, respectively, and used as serum controls in this study.

Procedure

1. Glucose analysis. Transfer 5.00 ml of the combined reagent into a 15 x 125 mm test tube. Add 20 μl of unknown (serum or plasma). Stopper, mix gently by inversion, and record the time. Let the reaction proceed for 10 min at room temperature. Read the absorbance against the combined reagent blank at 553 nm.

<table>
<thead>
<tr>
<th>Time, h</th>
<th>Absorbance at 553 nm (25°C)</th>
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<tbody>
<tr>
<td>0.0</td>
<td>0.032</td>
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<tr>
<td>1.0</td>
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<td>24.0</td>
<td>0.430</td>
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Dihydroxyphenyl-crease

Results and Discussion

We determined the optimum conditions for the analysis, with regard to pH, time, and concentrations of enzymes, 4-aminoantipyrine, and N,N-diethylaminine. The final results are incorporated into the procedure.

Absorbance is maximum at 553 nm, but the peak is quite broad, permitting the use of a colorimeter with a 20-40 nm bandpass filter. The hemoglobin background color interferes negligibly in this region of the spectra.

Under the outlined experimental conditions, a standard curve for glucose yields absorbances that are directly proportional to glucose concentrations as large as 1000 mg/dl.

In the recovery experiments, an average of 102% of the glucose was analytically accounted for. The coefficients of variation for results of eight replicate analyses at three glucose concentrations (75, 150, and 300 mg/dl) were 5, 6, and 7%, respectively.

Table 1 lists the behavior of the absorbance of the blank (the combined reagent) with time at room temperature (25 °C). A calibration curve obtained with 24-h old combined reagents is essentially superimposable on one made with a freshly prepared solution. Storing the combined reagent in a refrigerator (5 °C) decreases the blank at least twofold. No clinical appraisal or comparative studies with other methods were made.

References


Interference with Measurement of 3-Methoxy-4-hydroxymandelic Acid and 5-Hydroxyindoleacetic Acid by Reducing Metabolites

Jerome M. Feldman, Susan S. Butler, and Barbara A. Chapman

Using standard analytical techniques, we noted a decrease in the urinary excretion of 3-methoxy-4-hydroxymandelic acid and 5-hydroxyindoleacetic acid in a patient with alcaptonuria, and in subjects receiving aspirin or L-dopa [3-(3,4-dihydroxyphenyl)-L-alanine]. This apparent decrease in excretion of the two compounds is an artifact produced by metabolites in urine, such as homogentisic acid, gentisic acid, 3,4-dihydroxyphenylacetic acid, or homovanillic acid.

Additional Keyphrases: alcaptonuria • dihydroxyphenylacetic acid • homovanillic acid • homogentisic acid • diagnosis of pheochromocytoma or carcinoid tumor

Alcaptonuria, a rare hereditary metabolic disorder, is caused by a lack of the enzyme homogentisate 1,2-dioxygenase (EC 1.13.11.5). In this disorder a large quantity of the potent reducing agent, homogentisic acid, is excreted in the urine. Homogentisic acid produces an artifactual increase in the apparent urinary concentrations of glucose (1), creatinine (2), and uric acid (3). We recently performed a series of endocrine function tests on a patient with alcaptonuria. These studies showed that homogentisic acid also produces an artifactual decrease in the apparent urinary excretion of 3-methoxy-4-hydroxymandelic acid (VMA) and 5-hydroxyindoleacetic acid (5-HIAA). Of perhaps greater clinical significance is our observation that related compounds such as gentisic acid (a metabolite of salicylate) and 3,4-dihydroxyphenylacetic acid (a metabolite of L-dopa) also produce an artifactual decrease in VMA and 5-HIAA excretion.

Twenty-four-hour urine samples were collected under constant refrigeration (3 °C) from the following subjects: a 46-year-old man with alcaptonuria; four men who were ingesting 3 to 5 g of aspirin per day for osteoarthritis or rheumatoid arthritis; two men and one woman who were ingesting 1 to 6 g of L-dihydroxyphenylalanine (L-dopa) for Parkinson's disease; 57 nonalcaptonuric subjects who were not receiving aspirin or L-dopa; and a 67-year-old woman with the carcinoid syndrome. Immediately after collection, the urine samples were acidified to a pH of 2 with hydrochloric acid (12 mol/liter).

We measured VMA (4), homovanillic acid (5), and 5-HIAA (6) in the urine. In some urines 5-HIAA was also determined by the method of Mustala (7). We also analyzed for homogentisic acid, gentisic acid, and 3,4-dihydroxyphenylacetic acid, with use of the appropriate standards, by a previously described thin-layer chromatographic method (8). Urinary epinephrine and norepineph-

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