Semiautomated Assay of Normal Concentrations of Urinary Glucose by an Enzymatic Fluorometric Technic

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A semiautomated, enzymatic, fluorometric method for the determination of the low glucose concentrations normally present in urine from subjects in the fasting state is described. The method is developed from a manual, enzymatic, fluorometric technic for the determination of urinary glucose using hexokinase (HK) and glucose-6-phosphate dehydrogenase (G-6-PDH). The procedure is based on measurements of the fluorescence of reduced nicotine amide adenine dinucleotide-phosphate (NADPH₂) which is formed in proportion to the glucose content of the sample. The unspecific fluorescence of urine is almost completely removed by treating the urine with a mixed ion-exchange resin prior to applying the automated procedure. The chief advantage of the method is that it may afford an opportunity for low-cost screening for detection of bacteriuria.

For a number of years there has been a need for a rapid and sensitive method for determination of glucose in urine. The glucose oxidase method for determination of urinary glucose has numerous disadvantages, mainly due to the presence of substances interfering with the enzyme system (1–6). A few reports deal with the removal of such substances by adsorption, using activated charcoal, Lloyd’s reagent, or resins (7–11). However, these trials have not yielded adequate results for the determination of the low concentrations of glucose normally present in urine. The reliability of polarimetric methods for the determination of urinary glucose is so poor at low concentrations that determinations with results below 250 mg./100 ml. are meaningless (12). Chromatographic technics are too laborious for routine use. Schmidt (13) devised a spectrophotometric method, using HK and G-6-PDH, which has proved sensitive enough for determining the low con-

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centrations of urinary glucose present in normal urine. Using this method Renschler et al. (14) and Scherstén and Fritz (15), respectively, found the upper normal limit for urinary glucose concentrations to be 30 and 20 mg./100 ml. Furthermore, Scherstén and coworkers (15, 16) demonstrated that, under standardized conditions, levels below 2 mg. of glucose per 100 ml. of urine were highly correlated with a bacteriuria of more than 100,000 organisms per milliliter. These conditions seem to offer opportunities for mass screening for undetected urinary tract infection in a general population. However, the spectrophotometric method using HK and G-6-PDH is rather expensive and too laborious for mass analyses. The fluorometric adaptation of this method (17) reduced the reagent cost per analysis 40-fold compared with that of the spectrophotometric method. However, this manual method was still too cumbersome for use in population surveys for the detection of bacteriuria.

This paper will describe an automated, enzymatic, fluorometric method based on measuring the fluorescence of NADPH₂ which is formed in proportion to the glucose content of the sample by incubation in the presence of HK, G-6-PDH, adenosinetriphosphate (ATP), and nicotine amide adenine dinucleotidephosphate (NADP). The unspecific fluorescence of the urine was almost completely removed by treating it with a mixed ion-exchange resin before the automated procedure.

Methods and Materials

Reagents

**Cation exchange resin**  
Amberlite IR-120 (20–50 mesh) standard grade in the Na⁺ form. The resin is transformed to the free acid (hydrogen) form by treatment with 5 parts (v/v) of 1 N HCl; it is then washed with deionized water until the reaction is neutral. The excess of water is filtered off by suction for 5 min. The treated resin is stored in bottles with tightly fitting caps.

**Anion exchange resin**  
Amberlite IRA-400 (20–50 mesh) standard grade in the Cl⁻ form. The resin is transformed to the acetate form by treatment with 5 parts of 1 N NaOH (v/v), followed by washing with carbon dioxide-free water until the reaction is neutral. The resin is then treated with 5 parts (v/v) of 2% (v/v) acetic acid, followed by washing with carbon dioxide-free water until the reaction is neutral. The excess of water is filtered off by suction for 5 min. The treated resin is stored in bottles with tightly fitting caps.

**Mixed ion-exchange resin**  
Equal parts (v/v) of the treated cation and anion exchange resins are thoroughly mixed for the analyses planned for 1 day.
**ATP, 0.02 M (Sigma)**   Keep frozen in suitable portions.

**NADP, 0.006 M (Sigma)**   Keep frozen in suitable portions.

**HK (Sigma), Type C-301, 2000 units/ml.**   Crystalline ammonium sulfate suspension. Keep at 4°.

**G-6-PDH (Sigma), Type V**   Diluted to 500 units/ml. Keep at 4°.

**Triethanolamine-HCl buffer**   Prepare as 0.5 M with 1.0 mM of MgCl₂; adjust to pH 8.0 with 5 N HCl; keep at 4°. For the day's use: add 1 drop of Triton X-405 (Technicon Co.) per 100 ml. of buffer.

**Reagent solution**   Prepare daily. Composition per 20 ml.: 1.0 ml. of 0.02 M ATP, 1.0 ml. of 0.006 M NADP, 18 ml. of 0.5 M triethanolamine-HCl buffer, 3 µl. of HK, and 5 µl. of G-6-PDH. The reagent solution is kept on ice during the analyses.

**Standards**   Working standards used are between 5 and 25 mg. of glucose per 100 ml. of water in saturated benzoic acid.

**Procedure**

**Urine Collection**

From 6 to 10 ml. of urine are collected in nonsterile test tubes containing 20-50 µl. of a preservative (8 gm. of sodium propyl-paraoxybenzoate and 12 gm. of sodium ethyl-paraoxybenzoate per 100 ml. of water). The samples may be stored at room temperature up to 4 days with this preservative, or longer if frozen.

**Resin Treatment**

The optimal conditions for the resin treatment of the urinary specimens have been described in a previous communication (17). According to this, 0.2 ml. of urine are diluted with 3 ml. of water, and a "spoon" of the mixed resin (2 ± 0.25 gm.) is added to the test tube which is then thoroughly shaken. The test tubes are allowed to stand for 10 min., then shaken again, and centrifuged. The supernatants are transferred to the AutoAnalyzer specimen cups and analyzed according to the automated procedure. The standards must be treated the same as the urinary specimens.

**Instrumental Considerations and Operating Procedure**

The manifold and the flow diagrams for use with the AutoAnalyzer are shown in Fig. 1. Sampler II is fitted with a 40-specimen-per-hour (2:1) sampling cam. As seen in Fig. 1, the sample is mixed with the reagent solution, and the air-segmented donor stream is incubated for about 7 min. in a double mixing coil. The donor stream is then diluted and mixed with water in a single mixing coil. The fluorescence of NADPH₂ is measured in a filter fluorometer (Technicon) equipped with a 15-mm. tubular flow cell, a 7-60 primary filter (360 mµ, narrow
pass), and a No. 8 secondary filter (485 mμ, sharp cut). To determine the concentration range, 0–25 mg. of glucose per 100 ml. of urine, slit 4, and light aperture 3X are used.

The procedure is started by pumping water through the sample line, and water with added Triton (1 drop/100 ml.) through the reagent line (for adjusting the base line on the recorder at 2 or 3 scale units). Then the buffer solution is pumped through the reagent line. After a few minutes Sampler II is started in order to record the remaining non-specific fluorescence of the resin-treated urinary specimens. After the last sample has been aspirated, the buffer solution is pumped for a few more minutes, and then is replaced by the reagent solution which is pumped for a few minutes while the reagent base line is recorded. Sampler II is then started again for the first specimen cup, and the fluorescence obtained after incubation is recorded for each sample. After the analyses for the day, the equipment is rinsed by pumping deionized water, to which Triton has been added, for about half an hour.

Calculation

A calibration curve is constructed from the peaks of the standards. The values for the urine blanks (remaining unspecific fluorescence) and the samples are obtained directly by using the ordinary chart reader. The urinary glucose concentrations are then calculated by subtracting the blank values from the corresponding sample values.
Results and Discussion

The evaluation of the optimal composition of the reagent solution for the enzymatic fluorometric assay of urinary glucose has been reported in a previous communication (17). In the method described here, this composition had to be changed to suit the automated technic. The amounts of HK, G-6-PDH, and NADP per analysis could also be reduced by half, compared with those used for the manual fluorometric method. The reagent cost per analysis was not changed, however, as continuous pumping of the reagent solution was required.

With the automated technic, a concentration range of 0–25 mg. of glucose per 100 ml. of urine was chosen. Incubation time suitable for the AutoAnalyzer system was studied within this concentration range and with the reagent composition chosen. The fluorescence generated for each concentration tested was not significantly changed when the incubation time was reduced from 30 to 7 min.

Blank

The blank fluorescence obtained from the buffer solution, and from the reagent solution were stable for the duration of the day's analyses,

Fig. 2. Frequency of remaining unspecific fluorescence of 272 randomly selected urinary specimens after treatment with a mixed ion-exchange resin. Unspecific fluorescence is expressed as milligrams of glucose per 100 ml. of urine.
and corresponded to about 0.2 and 0.4 mg. of glucose per 100 ml. of urine, respectively. However, slight variations in the blank level of the reagent solution may occur when new batches of the reagents are used. The remaining unspecific blank fluorescence of urine varied after resin treatment. In a series of 272 urinary specimens selected at random from apparently healthy subjects and from hospitalized patients, the median blank fluorescence corresponded to 0.3 mg. of glucose per 100 ml. of urine (Fig. 2). In 90% of the samples, the blank fluorescence corresponded to a glucose concentration of less than 1.0 mg./100 ml. of urine. All the samples in which the blank fluorescence was equivalent to more than 1.5 mg. of glucose per 100 ml. of urine were collected from hospitalized patients undergoing treatment for a variety of medical complaints. It may be mentioned that, in some rare instances, the blank fluorescence was found to correspond to 8.4–12.9 mg. of glucose per 100 ml. of urine. These urinary specimens were collected from patients suffering from monocytic leukemia and the specimens also had a high concentration of lysozyme (18). It is suggested that this high blank fluorescence remaining after the resin treatment was due mainly to the fluorescence of the lysozyme present (19).

**Fig. 3.** Calibration curve relating glucose concentration to fluorescence intensity using standards of from 0.5 to 25.0 mg. glucose per 100 ml. water.
Calibration Curve

Figure 3 shows a typical calibration curve with glucose concentrations of 0.5, 1.0, 1.5, 2.0, 2.5, 5, 10, 15, 20, and 25 mg./100 ml. of water. In Fig. 4 are presented the strip-chart recordings on which the calibration curve is based. It is obvious that the calibration curve is linear to the upper concentration limit. This was also true when glucose concentrations up to 50 mg./100 ml. of water was tested, but sensitivity was then reduced. Moreover, Fig. 4 shows the agreement between duplicate samples, as well as that the standards analyzed at the end and at the beginning of a series coincide. For comparison, the continuous sampling patterns of the standards 5, 10, 15 and 20 mg. of glucose per 100 ml. of water are also shown. These patterns demonstrate a high correlation between fluorescence intensity at the sampling rate and that when continuous sampling is applied.

Experimental Errors

Precision

The precision of the method was calculated as the variability between values obtained when repeating the analyses. The experimental error for 21 duplicate samples, within the range of 2.8–23.3 mg. of glucose per 100 ml. of urine, was ± 0.11 mg./100 ml. (S.D.).

Sensitivity

It is apparent from the calculation of precision that urinary glucose concentrations below 0.2 mg./100 ml. had to be regarded as indistinguishable from zero.
Table 1. Recovery of Glucose

<table>
<thead>
<tr>
<th>Specimens (No.)</th>
<th>Urinary glucose (mg./100 ml.)</th>
<th>Glucose added* (mg./100 ml.)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>1.9–13.0</td>
<td>5.0</td>
<td>99.7 ± 1.4</td>
</tr>
<tr>
<td>15</td>
<td>2.0–13.0</td>
<td>10.0</td>
<td>100.3 ± 2.1</td>
</tr>
</tbody>
</table>

* Refers to concentration after dilution in urine.

Accuracy

The accuracy of the method was determined by the results of recovery experiments, and also by parallel determinations with the manual method (17). Table 1 shows that the recovery (97–106%) was satisfactory. When plotting the results of the automated method against those of the manual method, a straight-line relationship was obtained (Fig. 5). The results indicate that the automated method has a high degree of accuracy.

Specificity

This aspect of the method was studied for unspecific urinary fluorescence. As the resin treatment of urine did not completely remove the unspecific fluorescence, the latter had to be determined in order for the

Fig. 5. Correlation between manual, enzymatic, fluorometric method and the automated, enzymatic, fluorometric method for determination of urinary glucose when using the same enzyme system with HK and G-6-PDH. Equation for the regression line: y = a + bx = 0.1 + 0.998x where a = 0.1 ± 0.1, b = 0.998 ± 0.012 (S.D.). Coefficient of correlation: r = 0.997.
method to have a sufficiently high degree of specificity. This was further studied with regard to the interference caused by fructose, as the HK and the G-6-PDH preparations are usually contaminated by phosphohexose isomerase (PH1). In the manual, enzymatic, fluorometric method (17), this interference was equivalent to 0.002 mg. of glucose per 100 ml. for each milligram of fructose present per 100 ml. of urine, and per minute of incubation time. When fructose and fructose-glucose solutions—with a concentration of 20 mg. of fructose per 100 ml. of water—were analyzed by this method, the interference was equivalent to 0.3 mg. of glucose per 100 ml. of water. When the incubation time for the automated method (7 min.) was taken into consideration, the interference caused by fructose was found to be the same as that for the manual method.

Conclusion

The present semiautomated assay of urinary glucose—applying an enzymatic, fluorometric technic—has been shown to be sufficiently sensitive for the determination of the low concentrations of glucose normally present in urine from subjects in the fasting state. The chief advantage of the method is that it affords opportunities, at a low cost, for large-scale screening for subnormal urinary glucose concentrations which have been shown to be a reliable indication of significant bacteriuria (15, 16).

References


