Determination of Glucose in Biologic Fluids with an Automated Enzymatic Procedure

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An automated procedure for the enzymatic determination of "true" glucose with the glucose oxidase-peroxidase system is described. A threefold increase in sensitivity was obtained through measurement of the stable, pink chromogen obtained at high acid concentration. Good reproducibility and stoichiometry was obtained over the range of 50–400 mg./100 ml. at a sampling rate of 40/hr. The normal range (70–105 mg./100 ml.) and the error of the method (±2.0%) are about the same as that obtained with the manual method of Saifer and Gerstenfeld (7).

The automated method for blood glucose recommended by the manufacturer of the AutoAnalyzer* is based on the nonspecific reduction of the yellow color of alkaline ferricyanide. It was primarily for its specificity toward glucose that the glucose oxidase-peroxidase enzymatic system was subsequently employed for this purpose by a number of investigators (1, 2). Because of the high cost of the reagents for the enzymatic method, in comparison with the ferricyanide procedure, innovations to reduce the enzyme concentration per sample were reported by Discombe (3) and by Getchell et al. (4). The former (3) used MgSO₄ solution to precipitate the fluoride used as a preservative, and a Tris-maleate buffer to avoid precipitation of the excess magnesium. His method employs double dialysis, incubation at 37° in a 40-ft. heating coil, and reading of the amber color at about 460 mμ in a 10-mm. flow-cell cuvet. Samples were run at 30/hr., i.e., 60/hr. with alternate empty spaces, but the calibration curve was not linear above 150 mg./100 ml. glucose. The latter investigators (4) combined the o-dianisidine dye with the enzyme reagent, and the mixture was added directly to oxalated plasma containing...

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Aided by a grant from the John A. Hartford Foundation, New York, N. Y.

The authors wish to acknowledge the generosity of Frank Hammer, of the Fermeo Laboratories, in providing gratis the enzyme reagents used in this study. We are also grateful to Miss Francine Altman for technical assistance with this project, and to Mrs. Lillian Salowitz for the editing and typing of the manuscript.

Received for publication Mar. 19, 1965; accepted for publication May 22, 1965.

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fluoride as a preservative. The reaction mixture also contained a detergent, Tween 20, to prevent precipitation of the dye. In addition to the omission of the dialysis step, incubation was carried out in 2 stages, i.e., at 37° for 5 min. and at 50° for 10 min. Samples could be run at 60/hr. but better separations between peaks were found if a water wash was incorporated between each sample at this speed, i.e., an effective sampling of 30/hr. The amber color formed was also recorded at 460 m\(\mu\) in a 10-mm. path flow cell with good stoichiometry up to 400 mg./100 ml. glucose.

The presently described procedure is similar to that of Discombe (3) except that only a single dialysis is required and incubation is carried out at 50° in a 40-ft. heating coil to increase enzymatic activity. A further threefold increase in sensitivity is also achieved by the incorporation of strong acid into the flow system, subsequent to the enzymatic reaction, which converts the amber color to a clear, stable pink color with a maximum absorbance at 540 m\(\mu\) (5, 6) and the use of a 15-mm. flow cell. Samples can be run at 40/hr. with good separation between peaks. These changes reduce the cost of each determination to less than 3 cents per blood sample.

**Method**

The apparatus is illustrated in Fig. 1. The specimen cups on the wheel are sampled at 40/hr. in either the Sampler I or II unit (Technicon) with no spaces between them. No water wash was employed between sampling when the Sampler II unit was used. To remove any sediments which deposit during a run, all lines and the dialyzer should be washed through with 1% (v/v) \(\text{H}_2\text{SO}_4\) after each run.

**Reagents**

1. **Magnesium sulfate (diluent) solution, 4.0% (w/v)** Dissolve 40 gm. of reagent grade MgSO\(_4\)·7H\(_2\)O in a liter of distilled water.

2. **Sodium sulfate (recipient) solution** Dissolve 16.1 gm. of reagent grade Na\(_2\)SO\(_4\) in about 800 ml. of distilled water. Add 0.5 ml. of 7X detergent (Linbro Chemicals) and dilute to a liter with distilled water.

3. **Buffer solution (pH 7.0)** a) **Stock solution:** Dissolve 23.3 gm. of maleic acid (Matheson, Coleman & Bell) in about 800 ml. of hot distilled water and then add 24.2 gm. of Tris-(hydroxymethyl)-amino methane. The solution is then cooled to room temperature and diluted to a liter with distilled water. (b) **Working solution:** To 250 ml. of the above stock buffer, in a liter volumetric flask, is added 48 ml. of 1N NaOH. The mixture is then diluted to mark with distilled water and
mixed. The pH of the solution is read in a pH meter and adjusted to 7.0 ± 0.1.

4. **Enzyme-dye reagent** Measure 400 ml. of reagent grade glycerol into a liter graduated cylinder. Add 10.0 ml. of concentrated glucose oxidase solution (Fermcozyme 952-DM, Fermco Laboratories) and 600 mg. of o-dianisidine dihydrochloride (Fermco). Dilute to 1 L. with the working buffer solution and mix well. If not clear, this solution should be filtered through glass wool.

The Fermcozyme solution should be stored at 4° when not in use. The o-dianisidine powder causes violent sneezing, and undue exposure to its “dust” should be avoided. This can be achieved by using small plastic vials as weighing vessels, e.g., AutoAnalyzer cups, inserting the bottom end into a piece of metal pipe and filling them by pushing the container into the bottle and capping. If necessary, the volume of prepared enzyme-dye reagent can be adjusted to the weight of the dye.

**Results and Discussion**

Increased sensitivity over previously reported automated enzymatic procedures (1–4) for glucose was obtained by the use of a longer path flow cell (15 mm.), a higher incubation temperature (50°), and high acid

**Fig. 1.** Flow diagram of automated glucose oxidase-peroxidase system for “true” glucose determinations, in which pink color produced by high acid concentration is measured at 540 ma in 15-mm. flow cell. Pulse suppressors: (a) indicates orange-green, 0.015 Tygon; (b), orange-yellow, 0.020 Tygon.
concentration (30% [v/v] H₂SO₄) to convert the orange color to the more sensitive and stable pink chromogen. The use of acid also eliminates any turbidity in the system, as previously proposed by Saifer and Gerstenfeld (7), and, consequently, the need for introducing detergents as recommended by Getchell et al. (4). While the latter investigators favored the direct analysis of glucose in diluted plasma with the enzymatic procedure without dialysis, all other investigators (1–3) employed this step. Previous work from this laboratory (7) indicates that in patients undergoing intensive drug therapy, these substances can act as enzyme inhibitors unless removed by protein precipitation or dialysis. The increased sensitivity of the procedure presented here eliminates the need for double dialysis as was proposed by Discombe (3). We have retained his use of MgSO₄ as a diluent to eliminate the effect of fluoride in the system, the use of glycerol to stabilize the enzyme, as was suggested both by Discombe (3) and by Washko and Rice (6), and the convenient use of the concentrated liquid glucose oxidase-peroxidase enzyme preparation (Fermco). We have, however, utilized the water-soluble salt, o-dianisidine dihydrochloride, in place of the organic solvent soluble dye.

These innovations have resulted in both increased reproducibility of the enzymatic procedure and linear results over the range from 50 to 400 mg./100 ml. While the curves illustrated in Fig. 2 show stoichiometric values up to 400 mg./100 ml., those above 400 mg./100 ml. should be diluted for accurate results. We have chosen a sampling rate of 40 determinations per hour with no water wash between samples, since, as is illustrated in Fig. 2, the peak values closely approach that of the steady state value at a given glucose concentration.* Changes in the manifold, other than those shown in Fig. 1, result in noisy base lines and skewed peaks.

The results obtained for 84 plasma samples with this automated procedure and the manual enzymatic method of Saifer and Gerstenfeld (7) are given in Table 1. Good correlation was obtained for the means of the grouped values for the same samples, over a wide range of glucose concentration. The normal range (70–105 mg./100 ml.) and the reproducibility for the same sample, with an error of ± 2.0%, are about the same for the automated procedure as for the manual method except for slightly increased accuracy in the higher range of glucose values with the former.

The method has been applied with good results to the determination of cerebrospinal fluid glucose in exactly the same manner as for serum or

*The sampling rate can be increased to 60/hr. although this increases the amount of carryover between samples and necessitates rerunning low-glucose samples.
Fig. 2. Typical AutoAnalyzer curves showing reproducibility and stoichiometry of glucose oxidase method for glucose standards ranging from 50 to 400 mg./100 ml. These results were obtained with new Sampler II by using a 40/hr. disc, with no water wash between samples.

Table 1. Average Values Obtained for 84 Plasma Samples by the Automated and Manual Enzymatic Procedures

<table>
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<th>No. of specimens</th>
<th>Range</th>
<th>Manual</th>
<th>Automatic</th>
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<td>50–70</td>
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<tr>
<td>6</td>
<td>400–500</td>
<td>448</td>
<td>456</td>
</tr>
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plasma. The analysis of urine samples for glucose may require pre-treatment of the sample with Lloyd's reagent and activated charcoal as described by Getchell et al. (4), although the interference of uric acid and other metabolites with a dialysate would be less than that encountered with a direct enzymatic method.
References