Glucose in Serum and Cerebrospinal Fluid by Direct Application of a Glucose Oxidase Method

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Glucose is measured directly in 0.02 ml. of serum or cerebrospinal fluid by reaction with glucose oxidase. The hydrogen peroxide produced reacts with iodine in the presence of a catalyst to form molecular iodine. The iodine color is proportional to the glucose and is measured photometrically. The reaction is carried out directly on serum following preincubation with molecular iodine and can be completed in 15 min. The results on normal serum average 8 mg./100 ml. lower than those obtained with the AutoAnalyzer reduction method. At a glucose concentration of 150 mg./100 ml., the procedure has a coefficient of variation of 1.4%. Recovery of added glucose averaged 98%. Hemolysis, lipemia, or icterus do not interfere.

For glucose assay, glucose oxidase is an attractive analytic enzyme, because it generates an easily measured oxidant, hydrogen peroxide (1–9). The quantitation of hydrogen peroxide in biologic fluids is susceptible, however, to the presence of reducing substances which may interfere with its measurement (1, 3–5). Analysts have minimized this error by the deproteinization of serum with selected precipitants (1, 4), or in the case of urine, by removal of reducing substances with ion-exchange resins (5) or activated charcoal (6).

Malmstadt and co-workers described a glucose oxidase procedure using deproteinized serum, in which hydrogen peroxide reacts with iodide in the presence of molybdate catalyst to produce molecular iodine; the iodine is measured spectrophotometrically (7) or potentiometrically (8). The possibility occurred to the authors that preincubation of the serum with iodine would eliminate reducing substances and permit subsequent analysis of glucose by this method without deproteinization. Such a technic has been devised which permits direct analysis of glucose on serum or cerebrospinal fluid (CSF) with a minimum of technical manipulations (9).

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The technic consists of incubating a buffered iodide-iodine complex with the specimen. An initial rapid loss of iodine occurs, following which the glucose oxidase reaction is initiated, and the iodine produced is recovered without significant loss. It is necessary to make photometric readings after the initial incubation with iodine and following the addition of glucose oxidase when iodine production is completed. Under these conditions, the measurable increase in iodine is directly proportional to the glucose present.

To make this method technically feasible, it was found necessary to add polyvinylpyrrollidone (PVP) to the buffered iodine complex. This compound shifts the absorption of the iodine from the near-ultraviolet toward the blue portion of the visible spectrum. A maximal effect is produced with 0.04% (w/v) PVP which results in a two- to threefold increase in the absorption of iodine in the 400- to 470-m\(\mu\) region. This provides sufficient sensitivity for the method so that dissolved oxygen is not a limiting factor in the assay.

**Materials and Methods**

**Reagents**

Buffered-iodine reagent (color reagent) Dissolve 25 gm. of potassium dihydrogen phosphate, KH\(_2\)PO\(_4\); 12.8 gm. of potassium monohydrogen phosphate, K\(_2\)HPO\(_4\); 4 gm. of ammonium molybdate (NH\(_4\))\(_6\)Mo\(_7\)O\(_{24}\)·4H\(_2\)O; 25 gm. of potassium iodide, KI; 0.4 gm. of PVP; and 0.2 gm. of iodine, \(I_2\), in water, make up to 1 L., and adjust to pH 6.3 ± 0.1 at 25°, if necessary. Bring to a boil and add sufficient additional iodine to give an absorbance of approximately 0.3, when read at 420 m\(\mu\) with a 1-cm. light path at 37°.

This reagent is stable under refrigeration but gradually loses iodine when left for long periods of time at higher temperatures.

Glucose oxidase Purified enzyme—either Calbiochem purified enzyme, approximately 16 I.U./mg., or Fermco Fermcozyme No. 653 AM, 750 I.U./ml., can be used. Dissolve the enzyme in the color reagent, or dilute with the color reagent to produce a concentration of 190 I.U./ml. This solution is stable for at least 1 week under refrigeration. Glucose is converted at maximum rate with this concentration of enzyme.

The enzyme is selected so that, when incubated with the color reagent under the conditions of the test, it does not produce a significant change in absorbance; otherwise, a correction must be made in calculating the results.

*Reagents were supplied through the courtesy of Mr. Roy Anderson of Hyland Division, Travenol Laboratories, Inc., Los Angeles, Calif.
Glucose standard Dissolve pure glucose in 0.2% (w/v) benzoic acid.

Test Procedure

1. Combine 5 or 6 ml. of color reagent (analyst can choose volume necessary for the type of instrument used) and 0.02 ml. of test specimen in a cuvet. Warm to 37° in a heating block.
2. In 5 min. or more, read absorbance at 37° by transferring to a photometer and reading immediately.
3. Return to the 37° heating block and add 0.2 ml. of glucose oxidase reagent. Mix.
4. Read absorbance again after 15 minutes, maintaining the temperature at 37°. (Absorbance readings remain constant up to 25 min. and then decrease gradually.)
5. Subtract absorbance reading obtained at Step 2 from that obtained at Step 4. The difference represents iodine produced from glucose in the test specimen.

Alternate Procedure

1. Add color reagent (room temperature) to each of 2 cuvets. To each tube add 0.02 ml. of specimen. To 1 tube add 0.2 ml. of glucose oxidase reagent. Mix both tubes and place in 37° heating block for 15–25 min.
2. The tube without glucose oxidase serves as a blank. Set the instrument at zero absorbance with the blank tube and read the absorbance of the second tube. The result represents iodine produced from glucose in the test specimen.
NOTE: In Step 2 both tubes may be read against distilled water or other convenient reference. In this event the blank reading must be subtracted.

Calculation

The results obtained by either method may be converted to glucose concentration by comparing the results to those obtained with glucose standards treated in the same manner.

Results

Typical results of the effect of adding serum and glucose oxidase to the color reagent are represented in Fig. 1. The addition of serum alone causes a decrease in absorbance, which reaches a plateau within a few minutes. This is apparently the result of interaction of iodine with protein, lipid, bilirubin, and other reducing substances. The
decrease in absorbance is variable but rarely exceeds 0.1 unit, and it reaches a plateau within a few minutes. This reading serves as a blank and of course includes any absorbance contributed by the serum itself (hemoglobin, turbidity, etc.).

Fig. 1. Change in absorbance (A) of reaction mixture vs. time at 37°. Serum + GO = 0.02 ml. of serum + 0.2 ml. of glucose oxidase added simultaneously to 6 ml. of color reagent. GO alone = 0.2 ml. of glucose oxidase added to 6 ml. of color reagent. Serum alone = 0.02 ml. of serum added to 6 ml. of color reagent.

Following a short interval after the addition of glucose oxidase, the absorbance increases owing to the production of iodine. The reaction reaches completion in 10–15 min. as indicated by a constant absorbance reading (Fig. 1). When this reading is corrected for the blank, the difference represents the iodine production from glucose. This, of course, assumes that the glucose oxidase alone does not change the absorbance.* After 25 min. the absorbance tends to decrease gradually, probably as a result of slow reduction of iodine by protein. Therefore, for best accuracy the readings should be made between 15 and 25 min. However, in the alternate procedure in which the blank and test are incubated for the same amount of time, the 25 min. can be extended without significantly changing the results.

Photometric readings made with a Klett-Summerson filter photometer (42 filter) or a Coleman Jr. spectrophotometer (470 mµ, 19-mm. cuvet) give increases of approximately 100 Klett units or 0.1 absorbance unit with a glucose standard of 100 mg./100 ml. The readings follow Beer's law up to concentrations of 350 mg./100 ml. with a Klett-Summerson photometer and up to 250 mg./100 ml. with the Coleman instrument (Fig. 2). Above these levels, the specimen should be diluted

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*This may be tested by adding glucose oxidase alone to the color reagent. There should not be a significant difference between the absorbance after 15–25 min. and the color reagent alone when both are read at 37°. If a significant difference exists, the final result must be corrected for the difference.
and the test repeated, correcting the result for the dilution. If added accuracy is required for low glucose levels, the test may be repeated using double quantities (0.04 ml.) of the unknown, with appropriate correction in the calculation.

**Fig. 2.** Net change in absorbance ($A$) of test reagents reacting with varying concentrations of glucose.

**Precision**

Good precision with the technic is dependent upon rigid temperature control since the absorbance of the iodine-PVP complex varies approximately 1% with a temperature change of 1° (absorbance varies inversely with temperature). Temperature is controlled in the test simply by making photometric readings immediately after the cuvet is removed from the 37° environment. Precision studies made in this manner indicate coefficients of variation of 2.8 and 1.4% in the ranges of 70 and 150 mg./100 ml., respectively. These variations correspond to 95% limits of ±6% and ±3% at the 2 levels ($N = 15$). By using double quantities of serum (0.04 ml.), the 95% limits at low glucose levels is approximately ±2 mg./100 ml.

**Accuracy**

The accuracy of the technic was assessed by comparison with results obtained with the AutoAnalyzer technic ($10$), employing the reduction of ferricyanide. Results showed an average difference of 8 mg./100 ml. (range 2–14) in 25 normal serum specimens, the AutoAnalyzer always giving the higher results. This difference agrees well with that reported by Hill and Kessler ($3$) of 6.5 mg./100 ml. Normal CSF showed an average difference of 5 mg./100 ml. (range 0–8) on 10 specimens.
The differences noted above between AutoAnalyzer results and this glucose oxidase technic are not altered significantly by the presence of abnormal amounts of bilirubin, hemoglobin, or lipids. However, in the presence of azotemia, larger differences were noted which, on occasion, amounted to as much as 50 mg./100 ml. In seeking the cause of this difference, it is apparent that it is due to retention of nonglucose reducing substances, since the glucose oxidase results were confirmed using the method of Saifer and Gerstenfeld (1), as well as the orthotoluidine method of Dubowski (II). Further investigation demonstrated that creatinine and uric acid account for the major part of the difference.

In one serum, in which the difference was 50 mg./100 ml. the creatinine was 22 mg./100 ml., and the uric acid was 18 mg./100 ml. We found that creatinine reduces ferricyanide in the AutoAnalyzer procedure precisely the same as does glucose; uric acid reduces about ⅓ as much as glucose. The combined effect of these 2 reducing substances, which is additive, accounted for a 28 mg./100 ml. false elevation of glucose using the AutoAnalyzer technic. The remaining 22 mg./100 ml. difference is apparently due to other reducing substance(s) which accumulate in the blood in uremia. It is possible that the false elevation in glucose levels obtained by reduction methods may account for some of the reported decreased tolerance to glucose noted in uremic patients (12).

Recoveries of Glucose

Recoveries of glucose (100 mg./100 ml.) added to normal serum averaged 98% (range 95–101; N = 8) with this glucose oxidase technic; recoveries from uremic serum (100 mg./100 ml.) averaged 96% (range 93–99; N = 4).

Application to Urine

Application of this technic to urine was not successful, since reducing substances present appear to react unpredictably with the iodine reagent. There is, however, no apparent reason that reliable results could not be obtained from urine after removing the reducing substances with ion-exchange resin as described by Logan and Haight (5).

Normal Range

Serum glucose values, obtained from 20 normal adults in the fasting state, average 85 mg./100 ml. with a standard deviation of ±7.7. This results in a normal range for serum glucose of 69–101 mg./100 ml. (95% limits).
References