

# New automated method for measuring glucose by glucose oxidase

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**SYNOPSIS** An economical, very sensitive and precise automated method for the determination of plasma glucose by glucose oxidase is presented. At 60 determinations per hour the method required 0.05 ml. of plasma and can conveniently determine glucose levels in the range of 0 to 500 mg/100 ml. Recovery experiments gave mean yields of 99% and, for 100 specimens analysed in duplicate, the precision was 1.24 mg/100 ml.

The introduction of the coupled glucose oxidase-peroxidase-chromogen reaction was a distinct advance in the study of carbohydrate metabolism. Manual methods based on the system (Huggett and Nixon, 1957; Middleton and Griffiths, 1957; Marks, 1959) have been adapted for automated analysis (Winney and Marks, 1961; Discombe, 1963; Marks and Lloyd, 1963a; Robin and Saifer, 1965; Faulkner, 1965). The present method differs significantly from these in that the reaction takes place in the recipient dialysis stream, which also contains excess chromogen to overcome competitive oxidation of naturally occurring oxidizable substances. Colour development takes place in a jacketed cooling coil after the addition of acetate buffer. No heating bath or delay coil is required. The alcohol soluble o-tolidine is replaced by the water soluble o-tolidine hydrochloride.

## MATERIALS AND METHOD

### REAGENT SOLUTIONS

*Isotonic sodium sulphate* Dissolve 13.2 g. anhydrous sodium sulphate or 30 g. sodium sulphate decahydrate in a litre of distilled water.

*Magnesium sulphate* Dissolve 40 g. magnesium sulphate heptahydrate in a litre of distilled water. Add a few drops Brij-30.

*Acetate buffer pH 5.0, 0.5M* Sodium acetate trihydrate 47.5 g. and 9.1 g. glacial acetic acid are dissolved in about 800 ml. of distilled water. Adjust the pH to 5.0  $\pm$  0.1 with a glass electrode if necessary and make to 1 litre with distilled water.

*Glucose oxidase* A commercially available solution, Fermcozyme 653 AM<sup>1</sup>, with an activity of 750 units/ml. is convenient to use.

*Peroxidase* Peroxidase RZ 1.0, 10 mg. in 10 ml. distilled water. This solution will maintain activity for four weeks at 4°C.

*Chromogen* A stock solution of 1 g. ortho-tolidine hydrochloride is made up in 100 ml. distilled water. This solution keeps indefinitely at room temperature stored in a dark bottle.

*Combined enzyme-chromogen reagent* A sodium sulphate solution containing 5 ml. Fermcozyme solution (3,750 units), 5 ml. peroxidase, and 5 ml. o-tolidine solution in each litre is used as recipient solution. The o-tolidine should be added just before use.

*Glucose standards* A range of standards from 25 to 500 mg./100 ml. is made up from 1 g.% stock solution with benzoic acid (0.24%) as diluent. As glucose oxidase is specific for  $\beta$ -D-glucose only, the standards should stand for 12 hours to ensure that mutarotation has achieved a state of equilibrium before use.

**PROCEDURE** The automated system consists of conventional AutoAnalyzer modules, with manifold and flow diagram as shown in Figure 1. The sampler II is run at 60 samples an hour with water as 'wash' solution and a 1 : 2 sample to wash ratio cam. A single dialyser with Cuprophane membrane and colorimeter with a 15 mm. tubular flow cell and interference filters of wavelength 660 m $\mu$  are used.

In these studies plasma samples were used; blood was collected by venepuncture and placed into heparinized bottles and centrifuged at 4°C. If preservation is necessary, specimens are collected into fluoride or iodoacetate bottles (Marks and Lloyd, 1963b) and centrifuged before analysis. With the heparinized or iodoacetate samples isotonic sodium sulphate is used as diluent. If fluoride is used as glycolytic inhibitor isotonic magnesium sulphate is used as diluent to remove fluoride as the insoluble magnesium salt (Discombe, 1963). The sample diluent stream which contains a surface active

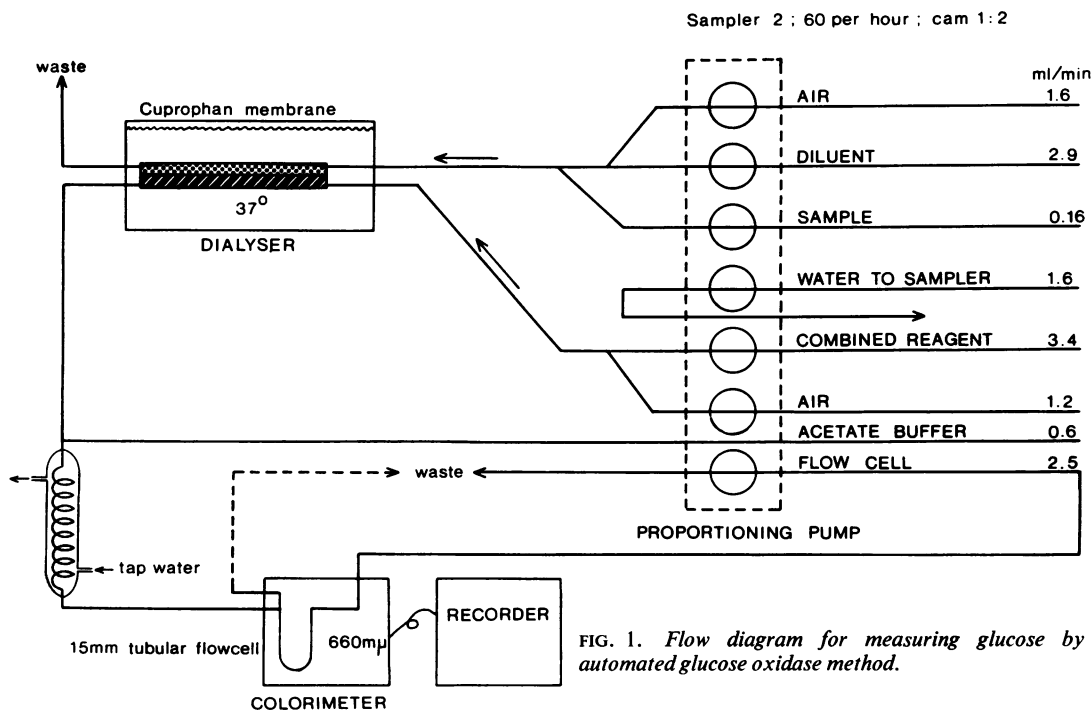


FIG. 1. Flow diagram for measuring glucose by an automated glucose oxidase method.

agent (detergent) is segmented with air and dialysed at 37°C. into the combined enzyme-chromogen solution recipient stream. The dialysate stream is joined by a 0.5M acetate buffer stream before entering a jacketed double mixing coil (cooled by tap water) for colour development before entering the flow cell.

Glucose in cerebrospinal fluid and urine can also be measured; urine is treated by shaking for a few minutes with several grams of activated charcoal, filtered through a fine paper then diluted 1 in 10 before analysis. This process removes uric acid and similar inhibitors of the system.

### RESULTS

**COMPLIANCE WITH BEER-LAMBERT'S LAW** The range of the method is 0 to 500 mg./100 ml. The calibration curve is reproducible, linear up to 150 mg./100 ml., then curves slightly becoming gradually more non-linear (Fig. 2).

**VARIABILITY** The analytical precision of the method was checked by performing duplicate analyses of 100 samples, and applying the formula  $S = \sqrt{\frac{\sum d^2}{2N}}$ , where S = precision, d = difference between paired samples, and N = the number of pairs. S = 1.24 mg./100 ml., over the range 0 to 500 mg./100 ml.

**CROSS CONTAMINATION** With the 1 : 2 sample wash

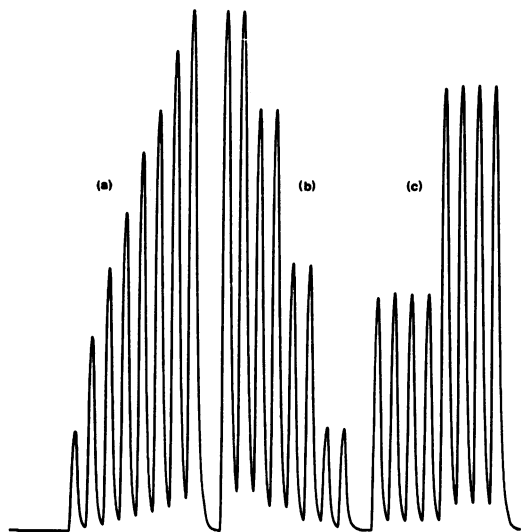


FIG. 2. Recordings showing (a) calibration curve with 50, 100, 150, 200, 250, 300, 400, 500 mg. standard peaks; (b) reproducibility of duplicates of standard solutions; (c) replicated plasma samples.

ratio there is negligible carry-over and no cross contamination of samples (Fig. 2), giving a high degree of reproducibility. This is enhanced by the use of a jacketed cooling coil which stabilizes the colour complex during colour development. The water soluble dye ortho-tolidine dihydrochloride also appears to increase this stability.

**SENSITIVITY** The sensitivity of the method is high, particularly in the low glucose range where accuracy is needed; using approximately 0.05 ml. of plasma, and with the concentration of reactants described, colour development is sufficient to give a recorder deflection of about 30 transmission lines for the 100 mg. standard.

**RECOVERY** Recovery of known added amounts of glucose averages 99% over the range 0-500 mg./100 ml. (range 98-100%).

**INTERFERING SUBSTANCES** The effects of uric acid, glutathione, fluoride, bilirubin, fructose, galactose, xylose, and mannose on the system were studied. The four sugars and bilirubin up to the highest concentration tested, *i.e.*, 6 mg./100 ml., has no effect upon the recovery of glucose. The inhibitory effect of fluoride on the system is eliminated by using magnesium sulphate as diluent as suggested by Discombe (1963). Uric acid up to 10 mg./100 ml. has no effect upon glucose recovery over the range of the method. Glutathione also appears to have no effect even with grossly haemolysed samples.

**OPTIMAL CONDITIONS** Optimal conditions are obtained by using the concentration of reactants suggested at a pH of  $5 \pm 0.1$ .

The proposed method has been compared with the modified Hoffman ferricyanide reduction method (Hoffman, 1937) as adapted to the AutoAnalyzer. Analysis of 80 specimens by both methods gave higher results by the Hoffman method, the mean difference being 8.7 mg./100 ml. (S.D. = 4.8 mg./100 ml.). These results are similar to the figures quoted by Marks and Rose (1965).

#### DISCUSSION

There is still a wide preference in clinical laboratories for glucose estimation by the Hoffman method,

probably on grounds of economy and ease of preparation of reagents. The method, however, is non-specific and tends to be insensitive in the low ranges. Of enzymatic methods specific for glucose, the glucose oxidase system can be automated simply. The use of such methods for blood or plasma glucose analysis has been unacceptable to many, either because of possible inhibition of enzymes in the system, or because of competition between the chromogen and naturally occurring oxidizable substrates, particularly uric acid, ascorbic acid, and reduced glutathione. However, dialysis, high sample dilution, and a high concentration of ortho-tolidine in the recipient stream have significantly reduced the false lowering of blood glucose by these substances.

It is felt that the use of plasma rather than blood for analysis contributes greatly to the degree of precision obtained. This eliminates variation due to uncontrollable changes in the red cell plasma ratio in the sample, change in erythrocyte permeability and water content (Mager and Farese, 1965), and variation caused by settling of red cells in the sample cups which cannot be overcome even by using the Technicon mixing device. For routine clinical use the method is, however, suitable for blood samples, especially for micro samples collected by skin puncture into a sample cup containing anticoagulant and glycolytic inhibitor. This obviates the need of a separate 'micro' method.

The procedure has been in daily use in this department for over one year and has proved to be reliable, precise and economical in use, three samples being analysed for approximately 1d.

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