A Semiautomated Enzymatic Method for Urinary Glucose

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MUCH ATTENTION has been given to the development of a method for the determination of urinary glucose at clinically significant levels. A method, described by Froesch and Renold (*1*), determines glucose concentration by measuring the difference in reducing substances in the urine after incubation with glucose oxidase. A further development was the colorimetric quantitation of the hydrogen peroxide resulting from the oxidation of glucose in the presence of the enzyme. The glucose oxidase peroxidase systems, however, were primarily used for blood glucose because of substances in the urine which interfered with enzyme action.

Huggett and Nixon (*2*) and Marks (*3*) have described methods adapted for use with urine which use activated charcoal to remove inhibitory susbtances, but this technique has the undesirable effect of removing some of the glucose as well. The combination of charcoal and Lloyd's reagent used by Beach and Turner (*4*) and by Kingsley and Getchell (*5*) is somewhat more effective. However, we agree with Salomon and Johnson (*6*) who have shown that this method is not completely satisfactory. They favored ion-exchange resins as a means of removing urinary inhibitors despite a somewhat cumbersome procedure requiring special apparatus. Jacobsen (*7*) tried yet another approach, attempting to minimize the problem of inhibitors by using a high dilution of urine. Consequently, this method is limited to specimens with larger amounts of glucose.

All the methods described have some limitation. They are either affected by incomplete removal of enzyme inhibitors, too cumbersome, or unsuited for use with small amounts of glucose. Further, they do not have the advantage of speed or the potential for improved test reproducibility of an automated procedure. Hill and Kessler (*8*) succeeded in automating the glucose oxidase procedure, but they considered it suitable for blood glucose only. Our purpose, then, was to find a simple, rapid, and reliable quantitative method for urinary glucose which would be most sensitive at "normal" levels. Primary attention was given to the enzyme glucose oxidase as the specific means for measuring glucose (*9, 10*).

Both the Hill-Kessler procedure and the preliminary preparation of urine as described by Salomon and Johnson (*6*) were modified and subsequently combined to give a rapid and accurate semiautomated method for urinary glucose. Our appraisal of this method included a recovery study, replicate determinations on samples from a pool of known glucose content performed over a period of time, and the introduction of blind duplicates. These methods of assessment were applied both to the semiauto-

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mated method and to a manual reference method. The Froesch-Renold method (*1*) was used for comparison, because it differed essentially from the glucose oxidase peroxidase systems.

Materials and Methods

The Froesch-Renold method was performed as described (*1*). Modules of the AutoAnalyzer (*A*) were used to facilitate automation. The urine was treated with ion-exchange resin prior to analysis to remove inhibitors.

The reagents used were the following:

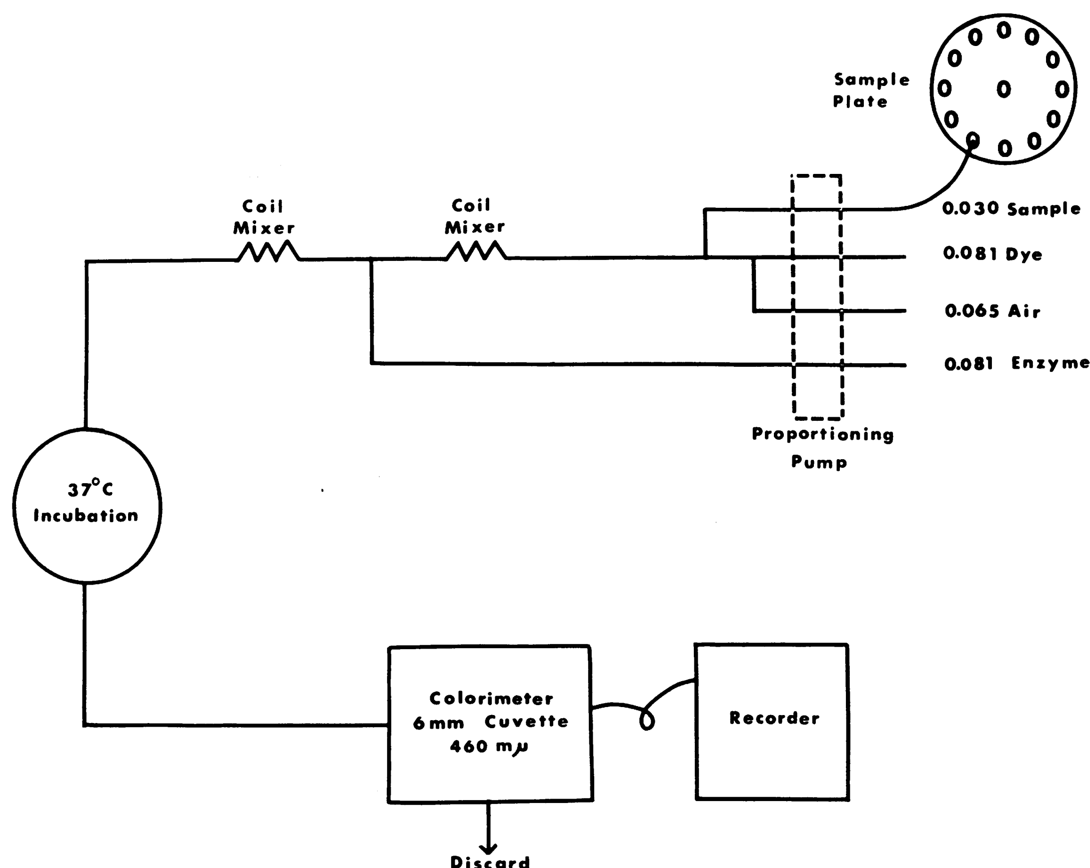
1. Glucose standards prepared in saturated benzoic acid in concentrations of 2.5, 5, 15, 25, 50, 75, and 100 mg. per 100 ml.

2. Amberlite IR-45 and IR-120 (*B*) ion-exchange resins dried overnight at 90° C. and mixed in equal volumes or weights.

3. Enzyme solution prepared by dissolving 1.0 gm. of glucose oxidase (*C*) and 50.0 mg. of horseradish peroxidase (*D*) in 1,000 ml. of 0.06 M phosphate buffer at pH 7.0. The solution is stored frozen in amber bottles and brought to room temperature immediately before use. It is diluted by 20 percent with distilled water and 5 ml. of Triton X-100 (*E*) is added. The diluted solution is good for 2 days if kept refrigerated but not refrozen.

4. Chromagen solution prepared by dissolving 400 mg. of o-dianisidine (*F*) in 100 ml. of 95 percent ethyl alcohol, adding 250 ml. of glycerine, and diluting to 1,250 ml. with distilled water. It is refrigerated until use, then brought to room temperature, filtered, and combined with 5.0 ml. of Triton X-100.

Procedure. The urine specimens with thymol added as a preservative are stored at −20° C. The urine is brought to room temperature and filtered. Clinitest (*G*), a quantitative glucose test, determines the proper dilution for each sample according to the following scale:



Flow diagram for enzymatic determination of urinary glucose

Clinitest (percent)	*Dilution*
0	None
0.25	1:5
0.5	1:10
0.75	1:20
1.0	1:25
2.0	1:50

Using a volumetric pipette, 5.0 ml. of the diluted urine is added to approximately 3.0 ml. of mixed resin, and the urine-resin mixture is refrigerated for ½ hour. After removal from the refrigerator, 5.0 ml. of distilled water is added, the tube mixed, and the resin allowed to settle. The supernatant is poured off and centrifuged at 2,000 rpm for 1 minute. The supernatant is decanted into AutoAnalyzer cups (*A*).

The supernatant is analyzed by Auto-Analyzer modules excluding the dialyzer, as shown in the flow diagram. The sampler is operated at a rate of 60 determinations per hour and the samples are alternated with air. The absorbency of the solution is measured in a 6-mm. flow cuvette at 460 mμ. Concentrations are read from a standard curve and the final results, in milligrams of glucose per 100 ml. of urine, are calculated in accordance with the original dilution of the urine.

Factors influencing the procedure. A glucose recovery study showed a ¼- to ½-hour exposure to resin to be superior to treatment with charcoal and Lloyd's reagent for the removal of enzyme inhibitors. Uric acid content of the filtrate was found to be less than 1.0 mg. per 100 ml. in all cases.

The possibility of interference from protein in the urine was discounted when protein, added in concentrations up to 1 percent, had no effect on glucose recovery. The optimum pH of the system appears to be 7.0. Although the pH differs little between samples (approximately 5.0), any marked change is buffered by the potassium phosphate in the enzyme solution. The standard curve adheres to Beer's law at the diagnostically significant range of 50–150 mg. per 100 ml., although there is a slight deviation from the straight line at higher or lower concentrations.

Carryover between samples was minimized by the following modifications of the Hill-Kessler method: (*a*) replacing the 10-mm. flow cuvette with a 6-mm. cuvette, (*b*) using a 20 percent dilution of reagents, (*c*) alternating samples and standards with air, and (*d*) increasing the sampler speed to 60 determinations per hour. This decreased contamination and reduced the amount of reagents used.

Results

The semiautomated method was appraised in four ways: (*a*) glucose recovery, (*b*) analysis, over a 6-month period, of replicates from a control pool of known glucose content, (*c*) introduction of blind duplicates, and (*d*) routine comparisons with the Froesch-Renold manual method, which was used as the reference method.

Glucose recovery. This study compared the capabilities of each method to recover glucose from pools of urine which ranged in glucose content from 0 to 2,000 gm. per 100 ml. The results are shown in table 1. The mean values, which represent five determinations at each

Table 1. Recovery of urinary glucose by semiautomated (SA) and by Froesch-Renold (F-R) methods

Glucose added (mg./100 ml.)	Number of determinations	Glucose expected (mg./100 ml.)		Glucose observed (mg./100 ml.)		Percent recovered	
		SA	F-R	SA	F-R	SA	F-R
None	5			3. 1	0. 8		
30.4	5	33. 5	31. 2	35. 7	29. 0	107	93
70.5	5	73. 6	71. 3	68. 2	61. 8	93	87
100.2	5	103. 3	101. 0	114. 2	98. 0	111	97
490.8	5	493. 9	491. 6	552. 8	504. 0	108	103
2,000.0	5	2, 003. 1	2, 000. 8	2, 292. 0	2, 088. 0	114	104

level, show the average recovery for the semiautomated method to be 107 percent as compared with 97 percent for the manual method.

Control pools. To assess the reproducibility and recovery of the method over a period of time, a control pool was prepared, containing a measured 20 mg. of glucose per 100 ml. of urine. The pool was divided into aliquots containing thymol as a preservative and stored at −20° C. An aliquot was included with each set of determinations for a period of 6 months for the semiautomated method and 2 months for the Froesch-Renold method. The results are shown in table 2. The mean for the semiautomated method is 21.2 mg. per 100 ml. as compared with 21.6 mg. per 100 ml. for the reference method. The spread about these mean values is quite comparable for both methods.

Blind duplicate study. Sixty-seven pairs of duplicates were mixed with the samples routinely arriving from the clinic and submitted for analysis by the semiautomated method. The mean difference between these blind duplicates was 2.0 mg. per 100 ml. with a standard deviation of ±2.16 mg. per 100 ml. The glucose levels of the duplicates ranged from 2.8 to 87.0 mg. per 100 ml.

Routine comparisons. The semiautomated method was developed primarily for use with specimens in the "normal" clinical range. However, several glycosuric samples were obtained for analysis from diabetic and potentially diabetic patients. The results of their analysis by both methods is shown in table 3, grouped according to glucose level. The difference between methods ranged from 2.2 mg. per 100 ml. for the 71 specimens that would be arbitrarily defined as normal to 79.1 mg. per 100 ml. for those with high glucose content.

Table 2. Urinary glucose in control pools

Procedure	Measured value (mg./100 ml.)	Number of determinations	Mean (mg./100 ml.)	Standard deviation
Semiautomated (6 months)	20	136	21.2	±3.15
Froesch-Renold (2 months)	20	25	21.6	±2.35

Table 3. Comparison of 197 duplicate urinary glucose determinations by semiautomated (SA) and Froesch-Renold (F-R) methods

Urine glucose level (mg./100 ml.)	Number of duplicates	Difference (SA-F-R) mean ±S.D.	Median difference
≤100	71	−2.2± 5.3	−2
100–249	25	−3.8± 28.1	−1
250–499	15	7.3± 35.8	7
500–999	31	33.8± 68.3	35
≥1,000	55	79.1±297.6	13

Discussion

The evaluation of the semiautomated method for urinary glucose determinations used four essentially different approaches. The accuracy of the method in contrast to the manual Froesch-Renold method was shown by the glucose recovery study. The higher average recovery of glucose for the semiautomated method—107 percent compared with 97 percent for the reference method—is particularly satisfactory since the major difference is contributed by urines with high glucose content. The method was designed to give maximal accuracy with values in the critical clinical range of 100 mg. per 100 ml. The impaired recovery at higher levels, therefore, can be attributed in part to the technical inadequacies common to colorimetric determinations at the measurement extremes. In addition, intersample contamination is assumed to be greatest at these levels. At the level of 100 mg. per 100 ml. or less, however, the average recovery for the semiautomated method was 105 percent while that for the Froesch-Renold method was 93 percent.

The urine control pool, which measured accuracy and reproducibility over a period of time, showed both methods to be remarkably similar. For a pool containing 20.0 mg. per 100 ml., the mean recovery for the semiautomated method was 21.2 mg. per 100 ml. and 21.6 mg. per 100 ml. for the Froesch-Renold method. The third evaluative approach, the introduction of blind duplicates, further supported the reproducibility of the semiautomated method.

Finally, a set of comparisons was performed on urines likely to contain substantial amounts of glucose to test the method in the ranges where

it is least sensitive. It can be expected that the accuracy and reproducibility of both methods will be impaired with increasing glucose content, a fact reflected by the increasing difference and range of differences. Clinically, however, these are minor considerations in relation to the degree of glycosuria.

Much attention has been given in the literature to the removal of substances that interfere with enzyme action. Thymol, in large quantities, has some adverse effect but the excess is adequately removed by filtration. Uric acid is demonstrably eliminated by resin treatment. The necessity of using special equipment to recover the urine from the resin (*6*, *11*) has been avoided by dilution and centrifugation. Ascorbic acid is a problem only when present in the quantities that occur during therapy. The removal of the dialyzer further simplifies the procedure and better standard curves were obtained by segmenting the samples with greater amounts of air. This also alleviated the intersample contamination.

Contamination can be further minimized by careful maintenance of the equipment. The viscid character of the reagents necessitates more frequent and thorough cleaning of the tubing, coils, cuvette, and waterbath. It is probable that the combination of a recently available flow cuvette and a sampler, which automatically segments specimens with water, will further increase the speed of the procedure and further reduce contamination.

This appraisal of the accuracy and reproducibility of the semiautomated method supports its use as a routine method offering the advantages of simplicity, speed, and lower cost.

Summary

The semiautomated method for determining urinary glucose is a simple, rapid, and specific procedure. It is based on use of the enzyme glucose oxidase, removal of inhibitors by the simplified application of ion-exchange resin, and adoption of AutoAnalyzer modules for automation.

The method proved satisfactory following four separate appraisals: (*a*) a glucose recovery study, (*b*) analysis of replicates from a control pool which were run over a 6-month period, (*c*) the introduction of blind duplicates, and (*d*) comparison with an alternative, specific method for glucose determination that had essential methodological differences.

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EQUIPMENT REFERENCES

(*A*) AutoAnalyzer: Technicon Instruments Corporation, Chauncey, N.Y.

(*B*) Amberlite IR-45 and IR-120: Rohm and Haas Company, Philadelphia, Pa.

(*C*) Glucose oxidase, purified, type II: Sigma Chemical Company, St. Louis, Mo.

(*D*) Horseradish peroxidase: Worthington Biochemical Corporation, Freehold, N.J.

(*E*) Triton X-100: Worthington Biochemical Corporation, Freehold, N.J.

(*F*) O-dianisidine, 3–3′ dimethoxybenzidene: Eastman Organic Chemicals, Rochester, N.Y.

(*G*) Clinitest: Ames Company, Elkhart, Ind.