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DETECTION OF GLUCOSE BY USING IMMOBILIZED GLUCOSE OXIDASE

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Abstract

We present herein a method for analyzing glucose using an immobilized-enzyme membrane. The enzyme membrane is prepared using glutaraldehyde to couple glucose oxidase to a cellulose acetate membrane which is then attached over the teflon membrane of a Clark-type oxygen electrode. The prepared membrane is suitable for use with the Beckman Glucose Analyzer, and an evaluation of its use in this manner yielded the following results. Analytical accuracy averaged 97.86 - 99.58% by the addition method and 96.22 - 98.31% by the dilution method. Coefficients of variation averaged 1.67% for within-run precision and 3.39% for between-run precision. The present method showed good correlation (r = 0.9752) with the Certified Glucose Reagent method, and can reduce analysis costs by a factor of 100.

Introduction

The detection of glucose in biological fluids such as blood, urine and cerebrospinal fluid has challenged the clinical chemist for over a century. The determination of glucose is one of the most commonly requested blood chemistry tests, particularly because of the high incidence of Diabetes Mellitus. This is a well known chronic disease, and its diagnosis depends on blood glucose determination. Also, to assess and follow up treatment, diabetic patients need to have their blood glucose checked periodically[1]. Therefore, in such cases a reliable method for glucose determination is needed, which should ideally be accurate, precise, specific, rapid, sensitive and inexpensive.

Although many different methods have been described for the determination of glucose in biological fluids, the search for a simple, specific, accurate and inexpensive method continues. Basically, methods for determination of glucose can be classified into two categories; namely chemical and enzymatic methods[2]. Nowadays, chemical methods are generally used less because of interference by other compounds, corrosiveness of reagents, large sample size and lack of specificity[2-4]. Enzymatic methods have been developed to overcome those problems, to increase specificity and sensitivity, and to reduce the interference. However, a major problem with such methods is the high cost of the enzyme itself.

Recently immobilized enzyme technology has been used to solve the problem[5-7]. Instead of using an enzyme solution, the enzyme itself is immobilized on a supporter or placed in a more natural environment, so it is not discarded with the reaction mixture after the analysis has been completed. Consequently, the enzyme can be recovered and recycled.

The Beckman Glucose Analyzer is an instrument for determining glucose in plasma, serum and urine. Glucose is determined by means of the oxygen rate method employing a Beckman oxygen sensor. An enzyme reagent (commercial Certified Glucose Reagent) containing glucose oxidase converts the glucose in the sample combined with dissolved oxygen from the solution according to the reaction:

$$\text{Glucose} + O_2 \xrightarrow[H_2O]{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

The rate of oxygen consumption is determined by an oxygen sensor coupled to an electronic system. This is directly proportional to glucose concentration in the sample.

In this report, we describe a method for the preparation of immobilized glucose oxidase membrane which is suitable for glucose determination in the Beckman Glucose Analyzer. In addition, an evaluation of the linearity, sensitivity, accuracy and precision of glucose determination with the prepared membrane has been made and comparative studies of glucose determination using the prepared membrane and using the commercial Certified Glucose Reagent have also been carried out.

Materials and Methods

Preparation of glucose oxidase membrane. Two steps were required for the preparation of the glucose oxidase membrane: firstly the preparation of cellulose acetate membrane attached to a teflon membrane, and secondly the immobilization of glucose oxidase on the cellulose acetate membrane.

Cellulose acetate membrane was prepared by adding 833 mg of cellulose acetate powder to a mixture of 1.1 ml of isopropanol and 21.2 ml of cyclohexanone. The solution was gently stirred for several hours until all the cellulose acetate powder was dissolved. One drop of the cellulose acetate solution was dispensed onto the surface of distilled water. After the membrane had formed, it was picked up from the water with a teflon membrane prewashed with distilled water. This membrane was placed on a wax paper and allowed to air dry in a dust-free chamber.

Glucose oxidase immobilization was performed by pipetting 10 µl of glucose oxidase solution (1 mg of glucose oxidase in 10 µl of deionized water) onto the membrane, followed by 10 µl of 0.50% glutaraldehyde solution, pH 5.8. After gentle mixing and

spreading over the surface of membrane, an excess solution was removed by suction with a pipette. The membrane was allowed to air dry in dust-free environment for about 15 minutes and then stored in a moisture chamber at 5-10° C.

Glucose analysis. The procedure for glucose analysis followed that described in the operating manual of Beckman Glucose Analyzer[8], except that the teflon membrane was replaced by the prepared membrane. A drop of electrolyte gel was placed on the oxygen electrode, the prepared membrane was then press fitted over the electrode and held in place with an O-ring (as shown in Fig. 1). A 100 mM phosphate buffer, pH 7.3 was used instead of commercial Certified Glucose Reagent. The instrument was first calibrated with a 50 µl of standard glucose concentration of 150 mg/dl. Then the glucose concentration in the sample was determined.

Evaluation of the glucose oxidase membrane in glucose determination. Human serum, Moni-Trol 1 normal control serum (obtained from American Dade, Florida), and Ortho-normal and abnormal control sera (obtained from Ortho-Diagnostics, Inc., New Jersey) were used as samples in this study.

The sensitivity and linearity of glucose determinations were analyzed by using series of standard glucose concentrations ranging from 25 to 500 mg/dl. The results obtained were plotted, then the sensitivity and linearity of this method were observed from the graph.

The analytical accuracy was studied using addition and dilution techniques. In the addition technique, three sets of mixture were prepared from various concentrations of standard glucose solution, two levels of normal and one level of abnormal commercial control sera. The first set was composed of the mixture of an equal volume of paired control sera. The second set was composed of the mixture of an equal volume of paired glucose standards and the last one was composed of the mixture of an equal volume of control serum and glucose standard. The glucose concentration in each mixture was determined in quadruplicate and its recovery was calculated. For the dilution technique, glucose standard and control serum were diluted to ratios of 1 : 2, 1 : 4 and 1 : 8 with distilled water to form sets of mixture of various glucose concentrations. The glucose concentration of each sample was determined in quadruplicate and its recovery was calculated.

For the within-run precision experiments, normal and abnormal pooled sera were analyzed for glucose concentration continuously for twenty times. The between-run precision was determined by analyzing normal and abnormal pooled sera once a day for twenty consecutive days. The mean, standard deviation and coefficient of variation (CV) of the assay were computed.

Finally, a comparative study was carried out for glucose determination between the proposed method and the Certified Glucose Reagent method.

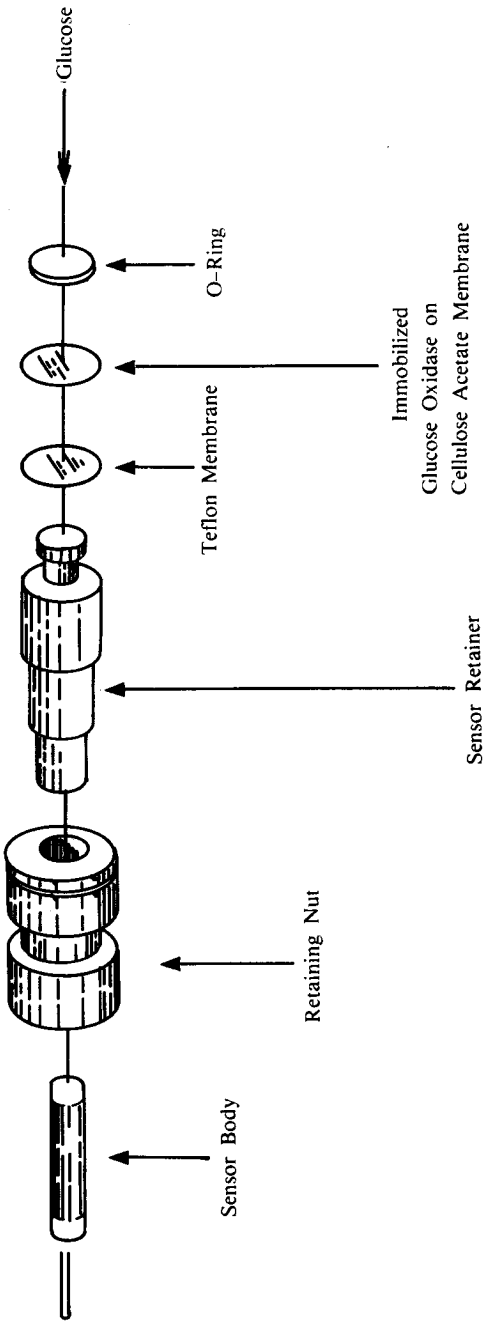

Fig. 1 Diagram of the detector part of Beckman Glucose Analyzer. Glucose oxidase immobilized on cellulose acetate membrane was attached to the teflon membrane of the oxygen electrode of Beckman Glucose Analyzer.

Results

Sensitivity, Linearity and Stability. The optimum sample volume was 50 µl. A calibration curve using series of standard glucose solutions was constructed from quadruplicated determinations at each concentration. Fig. 2 shows a straight line relationship up to a concentration of 400 mg/dl. The lowest concentration of glucose determined by the proposed method was 25 mg/dl. The durability of the prepared membranes varied from membrane to membrane: each membrane was used to construct a glucose calibration curve. The well-prepared membrane referred to the one that yielded a typical calibration curve. It was found that each well-prepared membrane remained stable enough to allow at least 200 consecutive analyses over 6 hours, and was therefore suitable for the routine service.

Accuracy and Precision for Glucose Determination. The analytical accuracy of proposed method for glucose determination were investigated by addition and dilution techniques. The results are presented in Tables 1 to 5. As shown in Tables 1, 2 and 3, the average percent recoveries for addition of control sera, glucose standards and mixtures of glucose standard and control sera were 97.86, 99.21 and 99.58, respectively. The percent recoveries for dilution of control sera and glucose standards ranged from 92.63 to 99.01 with an average of 96.22 and from 93.33 to 102.50 with an average of 98.31 respectively, as shown in Tables 4 and 5.

The within-run and between-run precisions of the proposed method for glucose determination were assessed with the results shown in Table 6. The coefficient of variation (CV) ranged from 1.45 to 1.92% with an average of 1.67% for within-run precision, and from 2.86 to 4.13% with an average of 3.39% for between-run precision.

Intermethod Comparison. A comparative study of serum glucose determination between the immobilized-enzyme membrane method and the Certified Glucose Reagent was carried out. Fig. 3 shows a highly correlated result with a correlation coefficient (r) of the linear regression line of 0.9752. The regression equation was : Y (immobilized-enzyme membrane method) is equal to 5.27 + 0.95 X. This line was constructed from data based on four determinations of each of the one hundred and eleven serum specimens. There was no statistically significant difference ($P > 0.05$).

Discussion

Determination of glucose is one of the most routinely performed clinical analyses. The most specific method for determination of glucose is glucose oxidase. Nowadays, the problem of high cost of the enzyme has been overcome by using immobilized-enzyme techniques and there have been many reports for glucose determination using immobilized-enzyme techniques[6,9-21]. We present herein a method for determination of glucose with an immobilized glucose oxidase membrane. This proposed method offers the same precision and accuracy of analysis as those reported by Chua, *et al.*[17]

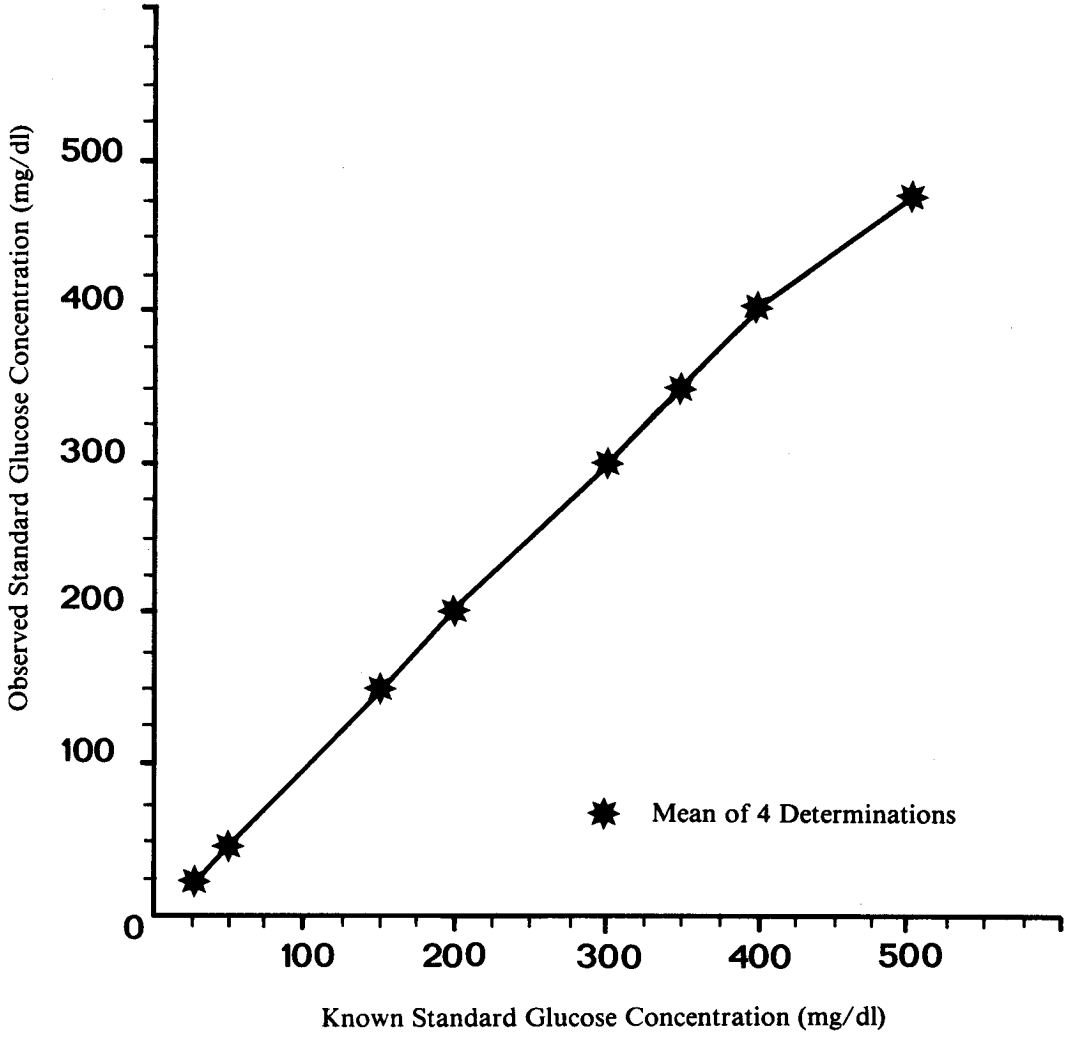

Fig. 2 Calibration Curve for Glucose Determined by Immobilized Enzyme Membrane

TABLE 1 ACCURACY OF THE PROPOSED METHOD FOR GLUCOSE DETERMINATION BY ADDITION TECHNIQUE USING CONTROL SERUM

Sample*	Glucose (mg/dl)			Recovery
	Original Value	Expected Value	Observed Value**	
KC - 1	77			
KC - 2	95			
KC - 3	304			
KC - 1 + KC - 2		86	85.5	99.42
KC - 1 + KC - 3		190.5	187.0	98.16
KC - 2 + KC - 3		199.5	191.5	95.99
			Average	97.86

* KC - 1 = Moni - Trol 1 Normal Control Serum; KC - 2 = Ortho - Normal Control Serum; KC - 3 = Ortho - Abnormal Control Serum

** Mean Value of 4 Determinations

TABLE 2 ACCURACY OF THE PROPOSED METHOD FOR GLUCOSE DETERMINATION BY ADDITION TECHNIQUE USING GLUCOSE STANDARD

Sample	Glucose (mg/dl)			% Recovery
	Original Value	Expected Value	Observed Value*	
Standard - 1	50			
Standard - 2	100			
Standard - 3	200			
Standard - 4	300			
Standard - 5	350			
STD - 1 + STD - 2		75	75.50	100.66
STD - 1 + STD - 3		125	125.50	100.40
STD - 1 + STD - 4		175	173.75	99.29
STD - 1 + STD - 5		200	200.25	100.13
STD - 2 + STD - 3		150	148.00	98.67
STD - 2 + STD - 4		200	198.00	99.00
STD - 2 + STD - 5		225	223.25	99.22
STD - 3 + STD - 4		250	243.50	97.40
STD - 3 + STD - 5		275	271.00	98.55
STD - 4 + STD - 5		325	321.00	98.77
			Average	99.21

* Mean value of 4 determinations

TABLE 3 ACCURACY OF THE PROPOSED METHOD FOR GLUCOSE DETERMINATION BY ADDITION TECHNIQUE BETWEEN CONTROL SERUM AND GLUCOSE STANDARD

Sample*	Glucose (mg/dl)			% Recovery
	Original Value	Expected Value	Observed Value**	
KC - 1	77			
KC - 2	95			
KC - 3	304			
Standard - 1	50			
Standard - 2	100			
Standard - 3	200			
Standard - 4	300			
Standard - 5	350			
KC - 1 + STD - 1		63.5	62.25	98.03
KC - 1 + STD - 2		88.5	87.75	99.15
KC - 1 + STD - 3		138.5	138.75	100.18
KC - 1 + STD - 4		188.5	188.25	99.87
KC - 1 + STD - 5		213.5	214.00	100.23
KC - 2 + STD - 1		72.5	73.75	101.72
KC - 2 + STD - 2		97.5	97.25	99.74
KC - 2 + STD - 3		147.5	146.50	99.32
KC - 2 + STD - 4		197.5	195.25	98.86
KC - 2 + STD - 5		222.5	220.25	98.99
KC - 3 + STD - 1		177.0	177.50	100.28
KC - 3 + STD - 2		202.0	204.75	101.36
KC - 3 + STD - 3		252.0	247.50	98.21
KC - 3 + STD - 4		302.0	302.00	100.00
KC - 3 + STD - 5		327.0	319.75	97.78
			Average	99.58

* KC - 1 = Moni - Trol 1 Normal Control Serum; KC - 2 = Ortho - Normal Control Serum; KC - 3 Ortho - Abnormal Control Serum

** Mean Value of 4 Determinations

TABLE 4 ACCURACY OF THE PROPOSED METHOD FOR GLUCOSE DETERMINATION BY DILUTION TECHNIQUE OF CONTROL SERUM

Sample*	Glucose (mg/dl)			% Recovery
	Original Value	Expected Value	Observed Value**	
KC - 2	95			
Dilution 1 : 2		47.50	45.00	94.74
Dilution 1 : 4		23.75	22.00	92.63
KC - 3	304			
Dilution 1 : 2		152.00	150.50	99.01
Dilution 1 : 4		76.00	75.00	98.68
Dilution 1 : 8		38.00	36.50	96.05
			Average	96.22

* KC - 2 = Ortho - Normal Control Serum; KC - 3 = Ortho - Abnormal Control Serum

** Mean Value of 4 Determinations

TABLE 5 ACCURACY OF THE PROPOSED METHOD FOR GLUCOSE DETERMINATION BY DILUTION TECHNIQUE USING GLUCOSE STANDARD

Sample	Glucose (mg/dl)			% Recovery
	Original Value	Expected Value	Observed Value*	
Standard - 1	200			
Dilution 1 : 2		100	102.50	102.50
Dilution 1 : 4		50	48.50	97.00
Dilution 1 : 8		25	23.75	95.00
Standard - 2	300			
Dilution 1 : 2		150	148.00	98.66
Dilution 1 : 4		75	76.00	101.33
Dilution 1 : 8		37.5	35.00	93.33
Standard - 3	500			
Dilution 1 : 2		250	248.50	99.40
Dilution 1 : 4		125	125.00	100.00
Dilution 1 : 8		62.50	61.00	97.60
			Average	98.31

* Mean Value of 4 Determinations

TABLE 6 PRECISION OF GLUCOSE DETERMINATION BY IMMOBILIZED GLUCOSE OXIDASE MEMBRANE

Precision	Sample[3]	Glucose (mg/dl)[4]	% CV[5]
Within - Run[1]	Normal	98.60 ± 1.63	1.65
	Medium	163.20 ± 2.37	1.45
	High	304.80 ± 5.88	1.92
		Average	1.67
Between - Run[2]	Normal	102.28 ± 3.25	3.18
	Medium	168.60 ± 4.82	2.86
	High	307.15 ± 12.68	4.13
		Average	3.39

[1] Each sample was analysed 20 times continuously.
[2] Each sample was analysed once a day for 20 consecutive days.
[3] Pooled sera with normal, medium or high glucose concentrations respectively.
[4] Mean ± S.D.
[5] Coefficient of variation.

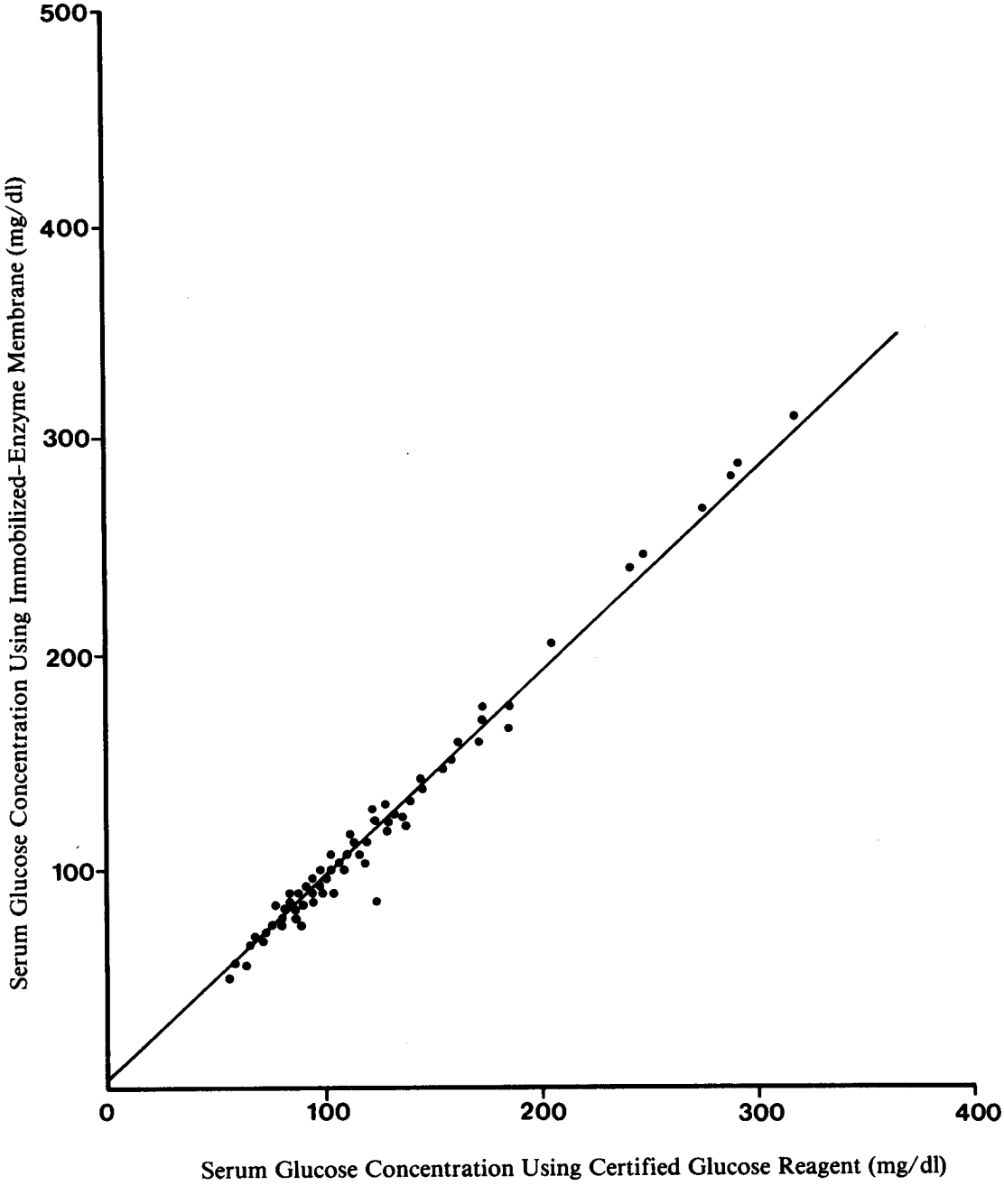

Fig. 3 Correlation of Serum Glucose Concentrations Determined by Immobilized Enzyme Membrane and Certified Glucose Reagent

and Sokol, *et al.*[21]. Although we have not investigated the interfering effects of bilirubin, hemoglobin, ascorbic acid, L-cysteine and lipid, another study[21] indicates that these substances do not interfere with the glucose determination. Routine clinical analysis requires speed, accuracy, precision and specificity, and the method presented herein meets all these critera. An enzyme membrane is used instead of the Certified Glucose Reagent which is a consumable product that requires purchase. Moreover, the teflon membrane which is attached to the enzyme membrane can be reused. A great advantage over the conventional method of glucose determination by the Beckman Glucose Analyzer is obtained, since the cost of analyses can be reduced by about 100 fold compared with those using the conventional method of the Beckman Glucose Analyzer.

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References

1. Zilva, J.F. and Pannall, P.R. (1983) *Clinical Chemistry in Diagnosis and Treatment, 3rd Ed.*, Lloyd-Luke, P.G. Publishing Ptd. Ltd., pp. 174-214.
2. Henry, R.J. (1968) *Clinical Chemistry, Principles and Techniques, 2nd Ed.*, Harper and Row Publishers, NY, pp. 620-698.
3. Henry, J.B. (1979) *Clinical Diagnosis and Management by Laboratory Methods, 16th Ed.*, W.B. Saunders, pp. 158-160.
4. Tietz, N.W. (1976) *Fundamentals of Clinical Chemistry, 2nd Ed.*, W.B. Saunders, pp. 242-251.
5. Zaborsky, P.R. (1973) *Immobilized Enzyme.* CRC Press, Cleveland, Ohio.
6. Change, T.M.S. (1977) *Biomedical Applications of Immobilized Enzyme and Proteins, vol. 1,* Plenum Press, NY.
7. *Chibata, I. (1978) Immobilized Enzymes,* Research Development, Halsted Press.
8. Glucose Analyzer Operating Manual. (1970) Beckman Instruments, Inc., Fullerton, California, U.S.A.
9. Updike, S.J. and Hicks, G.P. (1967) *Nature,* **214**, 986.
10. Guilbault, G.G. and Lubrano, G.J. (1972) *Anal. Chim. Acta.* **60**, 254.
11. Guilbault, G.G. and Lubrano, G.J. (1973) *Anal. Chim. Acta.* **64**, 439.
12. Kruz, H.J. (1974) *Clin. Chem.* **20**, 1018.
13. Campbell, J. Hornby, W.E. and Morris, D.L. (1975) *Biochim. Biophys. Acta.* **384**, 307.
14. Leon, L.P., Narayanan, S., Dellenbach, R. and Horvath, C. (1976) *Clin. Chem.* **22**, 1017.
15. Miller, J.N., Rocks, B.F. and Thorburns, D. (1977) *Anal. Chim. Acta.* **93**, 353.
16. Garber, G.C., Feldbruegge, D., Miller, R.C. and Carey, R.N. (1978) *Clin. Chem.* **24**, 1186.
17. Chua, K.S. and Tan, I.K. (1978) *Clin. Chem.* **24**, 150.

18. Updike, S.J., Shults, M.C. and Busby, M. (1979) *J. Lab. Clin. Med.* **93**, 518.

19. Sundaram, P.V., Blumenberg, B. and Hinsch, W. (1979) *Clin. Chem.* **25**, 1436.

20. Leon, L.P., Chua, D.K., Synder, L.R. and Horvath, C. (1980) *Clin. Chem.* **26**, 123.

21. Sokol, L., Garber, C., Shults, M.C. and Updike, S.J. (1980) *Clin. Chem* **26**, 89.

บทคัดย่อ

ได้เสนอวิธีการวิเคราะห์หาปริมาณ glucose โดยการติดตรึงเอ็นไซม์บนแผ่นเยื่อบาง ซึ่งการติดตรึงเอ็นไซม์นี้ทำโดยการเชื่อมเอ็นไซม์ glucose oxidase บนแผ่น cellulose acetate ด้วย glutaraldehyde และนำมาประกบบนแผ่น teflon ของ Clark-type oxygen electrode. วิธีการนี้มี analytical accuracy กับ precision สูง และให้ผลที่สอดคล้องกับผลที่ได้จาก Certified Glucose Reagent รวมทั้งสามารถลดค่าใช้จ่ายในการวิเคราะห์ไปได้ประมาณ 100 เท่า