[Chem. Pharm. Bull. 27(2) 568—570 (1979)]

UDC 547.457.1.08 : 577.158.08

Determination of Glucose in Blood using Glucose Oxidase–Peroxidase System and 8-Hydroxyquinoline–*p*-Anisidine[1]

Toshio Kinoshita, Yayoi Hiraga,[2a] Noriko Nakamura,[2b] Akiko Kitajo,[2a] and Fumio Iinuma[2c]

School of Pharmaceutical Sciences, Kitasato University,[2a] Kanagawa Prefectural College of Medical Technology[2b] and Faculty of Pharmaceutical Sciences, Teikyo University[2c]

(Received October 11, 1978)

An equimolar mixture of 8-Hydroxyquinoline and *p*-anisidine was found to be oxidized with hydrogen peroxide in the presence of peroxidase giving intense blue color. Application of this mixed reagent to the assay of glucose using glucose oxidase and peroxidase provided a sensitive and reproducible method which did not require deproteinization. Ascorbic acid did not interfere with the color development after pre-incubation of the serum for a short period. Satisfactory correlation between the present method and the conventional phenol-4-aminoantipyrine method was observed.

Keywords——glucose oxidase; peroxidase; glucose determination; 8-hydroxyquiniline; *p*-anisidine; colorimetry of blood sugar; blood glucose

Glucose, uric acid or cholesterol in biological fluid have been assayed specifically by oxidizing these compounds with oxidases and measuring generated hydrogen peroxide using peroxidase (POD) and an appropriate substrate which yields color on oxidation. Since benzidine derivatives,[3] which have widely been employed as effective substrates of POD, were found to be carcinogenic, alternative substrates have been investigated. Although the coupling reaction of 4-aminoantipyrine with phenols[4] was most frequently employed for this purpose, the products show absorption at shorter wavelengths and the readings are liable to be affected in the case of hemolysis. On the other hand, Gochmen and co-workers[5] employed the reaction of 3-methyl-2-benzothiazolinone hydrazone (MBTH) with dimethylaniline giving blue color absorbing at 600 nm. However, MBTH reacts with various compounds such as aldehydes and amines, and dimethylaniline is readily autoxized yielding colored substances. Miskiewicz and co-workers[6] found that 2,2,-azino-di-[3-ethyl-benzothiazolinone sulfonic acid] diammonium salt (ABTS) afforded blue color on oxidation with hydrogen peroxide in the presence of POD. However, the absorption spectrum of the blue pigment exhibits extremely broad peak. Although 4-methoxynaphthol[7] was proved to be a reagent of excellent sensitivity,[8] this reagent is unstable in solution. The substrate which can completely substantiate benzidine dyes has therefore not been obtained.

We found that an equimolar mixture of 8-hydroxyquinoline (8HQ) and *p*-anisidine (PA) showed intense blue color on reaction with hydrogen peroxide in the presence of POD. This fact was applied to the assay of blood glucose using glucose oxidase (GOD).

1) This work was supported by a grant-in-aid for scientific research No. 221220 from the Ministry of Education, Science, and Culture.

2) Location: *a) Shirokane, Minato-ku, Tokyo 108, Japan; b) 50–1, Nakao-cho, Asahi-ku, Yokohama 241, Japan; c) Sagamiko, Kanagawa 199–01, Japan.*

3) G.F. Domagk and H.H. Schlicke, *Anal. Biochem.*, **22**, 219 (1968).
4) P. Kabasakalian, S. Kalliney, and A. Westcott, *Clin. Chem.*, **19**, 522 (1973).
5) N. Gochman and J.M. Schmitz, *Clin. Chem.*, **17**, 1154 (1971); *idem, ibid.*, **18**, 943 (1972).
6) S.J. Miskiewicz, B.B. Arnett, and G.E. Simon, *Clin. Chem.*, **19**, 253 (1973).
7) G.G. Guilbault and D.N. Kramer, *Anal. Chem.*, **36**, 2494 (1964).
8) T. Kinoshita, F. Iinuma, M. Yamanoha, A. Tsuji, and M. Kato, *Bunseki Kagaku*, **23**, 1217 (1974).

Materials and Methods

Materials——POD, GOD and glucose were purchased from Sigma Chemical Co., Tokyo Kasei Co. Ltd. and Wako Pure Chemical Co., respectively. 8HQ and PA were of reagent grade recrystallized from ethanol. Consara® standard serum was purchased from Nissui Seiyaku Co. Glucose measuring kit using phenol-4-aminoantipyrin reagent (Anaserum®) was obtained from Daiichi Chemical Co. Ltd.

Color Reagent——Nine milligrams of 8HQ and 7 mg of PA were dissolved in McIlvaine buffer solution (pH 5.0) to make 100 ml, and to this solution are added 145 U of POD and 1300 U of GOD before use. This color reagent is stable for 1 week in a refrigerator.

Assay Procedure——To 20 μl of serum is added 4 ml of the color reagent and the resulting mixture is incubated at 37° for 20 min. The absorbance is measured at 600 nm against a reagent blank by a Hitachi 200-20 spectrophotometer.

TABLE I. Recovery of 100 mg/dl of Glucose in the Presence of Various Compounds

Compound	Amount of compound (mg/dl)	Recovery (%)
None		100.0
+D-Ribose	400	97.7
Glycine	500	100.0
β-Alanine	500	100.0
Bilirubine	5	104.7
	10	107.0
Creatine	30	101.1
Creatinine	600	102.3
Urea	17000	102.3
Uric acid	100	101.1
	200	90.9
Vitamine C	60	95.7
Vitamine B_1	7000	99.0
NaCl	33000	100.0
$FeCl_3$	1	103.4
$CuSO_4$	70	101.2
NaF	5000	102.2

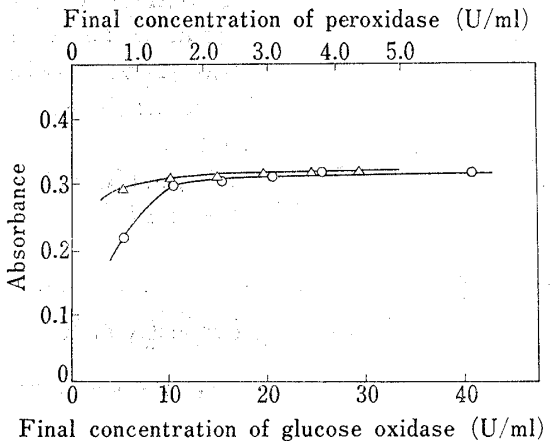

Fig. 1. Effect of Glucose Oxidase (○) and Peroxidase (△) Concentration on the Absorbance of Glucose(50 μg/sample)

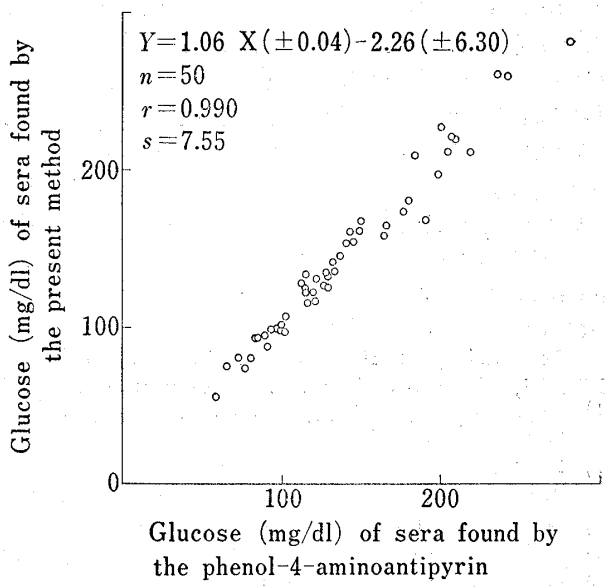

Fig. 2. Correlation between the Result by the Present Method and That by the Phenol-4-aminoantipyrine Method

Results and Discussion

The concentration of samples or reagents indicates their final concentration in the reaction mixture unless otherwise stated.

Both the absorption spectrum obtained from hydrogen peroxide and that from glucose showed the same maxima at about 600 nm. This wavelength is far longer than that of phenol-4-aminoantipyrine condensation product.

Most intense color was observed when the molar ratio of 8HQ and PA was 1:1. The absorbance increased with the increase in the concentration of the mixed reagent of 8HQ and PA (1:1), and the maximum absorbance was observed at the concentration of 0.15 mg/ml. Addition of excess PA resulted in the shift of the absorption maximum to a shorter wavelength and excess addition of 8HQ increased the absorbance of reagent blank.

As Fig. 1 exhibits the absorbance increased with the increase in the enzyme concentration and reached a plateau at 1.44 U/ml of POD and 13.1 U/ml of GOD. The highest absorbance was shown in the pH range of 4.2 to 5.2.

The absorbance reached a plateau after incubation for 20 min at 37° whereas the absorbance continued to increase even after 1 hr at 20°.

The standard curve for glucose was linear in the range of 50 mg/dl to 1000 mg/dl of sample (10 μg/ml to 200 μg/ml in the reaction mixture) and passed through the origin. The color developed was stable for 24 hr. Coefficients of variation for 100 mg/dl and 200 mg/dl of glucose were 1.7% (n=12) and 1.4% (n=10), respectively.

Table I lists various compounds added to 100 mg/dl of glucose solution in order to examine for interferences. Creatine, creatinine, urea, glycine, D-ribose, sodium chloride, copper ion, ferric ion and sodium fluoride did not affect the reaction at about 100 fold the normal serum concentration of these materials. Interferences were observed when about 20 fold (10 mg/dl) the normal serum concentration of bilirubin or about 70 fold (200 mg/dl) of uric acid were added to the sample.

Ascorbic acid did not affect the present method at the normal serum concentration (2 mg/dl), but the absorbance decreased to 50% when 60 mg/dl of this substance was added. However, ascorbic acid of this concentration was found to be completely removed by preincubation of the sample at 37° for 40 min prior to the assay.

Recovery of 133 mg/dl glucose from human serum (Consera®, containing 137 mg/dl glucose) was 102.7% and the coefficient of variation was 0.90% (n=10). Recovery of 303 mg/dl glucose from human serum (Consera®A, containing 304 mg/dl glucose) was 101.8% and the coefficient of variation was 0.79% (n=10). An excellent correlation between the present method and the conventional phenol-4-aminoantipyrine method was observed as indicated in Fig. 2.

The present method does not require deproteinization and highly sensitive and reproducible and is expected to be applicable to the assay of various biological materials using oxidases.

Acknowledgement The authors gratefully acknowledge the invariable advices of Prof. Shizuo Katagiri, Kitasato University. They express appreciation to Miss Hitomi Tashiro for the assay by phenol-4-aminoantipyrine method. They also thank Miss Etsuko Ohto and Masako Takashio for skillful assistance.