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Electric Microassays of Glucose, Uric Acid and Cholesterol Using Peroxidase Adsorbed on a Carbon Electrode

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Highly sensitive electrochemical methods for the assays of glucose, uric acid and total cholesterol in serum were established by using peroxidase adsorbed on a carbon electrode.

Glucose, uric acid and cholesterol were oxidized by glucose oxidase, uricase and cholesterol oxidase, respectively, to give a hydrogen peroxide, which was measured by means of a hydrogen peroxide sensor using peroxidase adsorbed on a carbon electrode. Each method required 5 μl of serum, and the detection limits of glucose, uric acid and total cholesterol were 0.10 μg (0.555 nmol), 0.05 μg (0.295 nmol), and 0.25 μg (0.650 nmol) per assay tube, respectively.

Keywords——peroxidase-adsorbed carbon electrode; hydrogen peroxide; glucose; uric acid; cholesterol; electrochemicalassay

In assays of clinically important compounds, oxidative enzymes which generate hydrogen peroxide have been widely used. The generated hydrogen peroxide has been determined by the various colorimetric[1,2] and fluorimetric methods.[3] It has also been assayed by the electrochemical method.[4]

In our previous report, we showed that the use of peroxidase adsorbed on a carbon electrode provided a very rapid and sensitive method for the determination of hydrogen peroxide.[5] Thus, we have developed and applied highly sensitive assay methods for clinically important compounds by the use of peroxidase adsorbed on a carbon electrode as a sensor of hydrogen peroxide. This paper describeds sensitive methods for the determination of glucose, uric acid and total cholesterol in serum.

Materials and Methods

Horseradish peroxidase (HRP), glucose oxidase (GOD), uricase, cholesterol oxidase (COD) and cholesterol esterase (CEH) were purchased from Toyobo Co., Ltd., Osaka, Japan, and albumin (bovine, Fraction V), hemoglobin and Triton X-100 from Sigma Chemical Co., St. Louis, Mo., U.S.A. Glucose, uric acid, cholesterol, cholesterol linoleate, ascorbic acid, 4-aminoantipyrine and *N,N*-dimethylaniline were obtained from Wako Pure Chemical, Ltd., Osaka, Japan, and bilirubin was from Daiichi Pure Chemical Co., Tokyo, Japan. The activities of HRP, GOD, uricase, and COD were standardized by means of the spectrophotometric method.[6-9]

Determination of Glucose——A 20 μl aliquot of 26 U/ml GOD solution was added to 0.5 ml of 0.1 M phosphate buffer solution pH 5.6. The reaction was started by addition of 5 μl of glucose sample. After incubation for 10 min at 30 °C, 10 μl of reaction mixture was taken out and injected into the measuring cell of apparatus.[5] The increase in voltage was read at 30 s of after injection of the sample.

Determination of Uric Acid——A 20 μl aliquot of 0.1 U/ml uricase solution was added to 0.2 ml of 0.1 M phosphate buffer solution, pH 7.5. The reaction was started by adding 5 μl of uric acid sample. After incubation for 10 min, at 30 °C, 20 μl of reaction mixture was taken out and injected into the measuring cell. The increase in voltage was read as in the case of glucose determination.

Determination of Total Cholesterol——A cholesterol-containing sample (5 μl) was added to a mixture of 0.2 ml of 0.1 U/ml COD solution and 0.2 ml of 0.1 U/ml CEH solution in 0.1 M phosphate buffer, pH 7.0, containing 0.05% Triton X-100. After incubation for 10 min at 30 °C, 5 μl of reaction mixture was injected into the measuring cell of the hydrogen peroxide sensor, and the increase in voltage was read.

Comparison with the Reference Methods——Glucose: The enzymatic colorimetric assay based on GOD–HRP coupled with 4-aminoantipyrine–dimethylaniline (4AA–DMA)[2] was used as the reference. Glucose was determined by measuring the absorbancy at 550 nm.

Uric Acid: As the reference, we used the uricase ultraviolet (UV) method.[10] Uric acid was determined by measuring the absorbancy at 293 nm.

Total Cholesterol: The modified Zak–Henly method[11] based on the Kiliani reaction was used as the reference. Cholesterol was determined by measuring the absorbancy at 560 nm.

Results and Discussion

Establishment of the Standard Method for Glucose Determination

The necessary concentration of GOD for determination of glucose was examined. Constant voltage was obtained at concentrations greater than 26 U/ml of GOD. The optimum pH for the measurement in this system was 5.6, and the reaction was completed within 10 min. From these results the standard method was established as mentioned above.

Standard Curve——The standard curve was obtained with glucose solutions of various concentrations. A linear relationship existed between the voltage and the concentration of glucose in the range of 0.02 mg to 2.0 mg/ml (0.10 μg to 10 μg/tube). The detection limit of glucose was 0.1 μg at the signal-to-noise ratio of 3.0.

The precision of the present method for glucose determination was examined. The coefficient variation (CV) was 4.9, 4.8 and 3.5% ($n=10$ each) for 0.5, 1.0 and 2.0 mg/ml of glucose.

Interfering Compounds——As shown in Table I, albumin, bilirubin, uric acid and ascorbic acid did not affect the value of glucose at concentrations of less than approximately 7.5 g, 5.0 mg, 5.0 mg and 5.0 mg/dl, respectively, but the coexistence of higher concentrations of these compounds caused an apparent decrease of the glucose value.

Recovery Test——To determine the recovery of glucose in serum, 1 ml of 0.1 M phosphate buffer (pH 5.6), containing 6 or 12 mg glucose, was added to 9 ml of serum, which contained 0.6 mg/ml glucose. The recoveries of glucose were 101.0±4.5 and 100.1±3.5% (mean ± standard deviation (SD), $n=5$), respectively.

Comparison with the Reference Method——The comparison with the GOD–HRP–4AA

TABLE I. Effect of Various Compounds on the Determinations of Glucose, Uric Acid and Total Cholesterol

Compounds	Concentration (mg/100 ml)	Recovery (%)		
		Glucose[a]	Uric acid[b]	Cholesterol[c]
Albumin	5000	100.0	100.6	101.6
	7500	99.4	100.2	100.2
Bilirubin	5	102.0	99.4	99.3
	10	98.5	98.3	98.9
Ascorbic acid	5	100.0	95.0	100.0
	10	97.4	90.2	99.6
Uric acid	5	101.0	—[d]	100.0
	10	100.6	—	100.0
Glucose	100	—	100.2	100.0
	200	—	99.5	100.0
Hemoglobin	50	—	—	97.3
	100	—	—	91.2

a) Glucose 100 mg/100 ml. *b*) Uric acid 5 mg/100 ml. *c*) Cholesterol linoleate 200 mg/100 ml. *d*) Not determined.

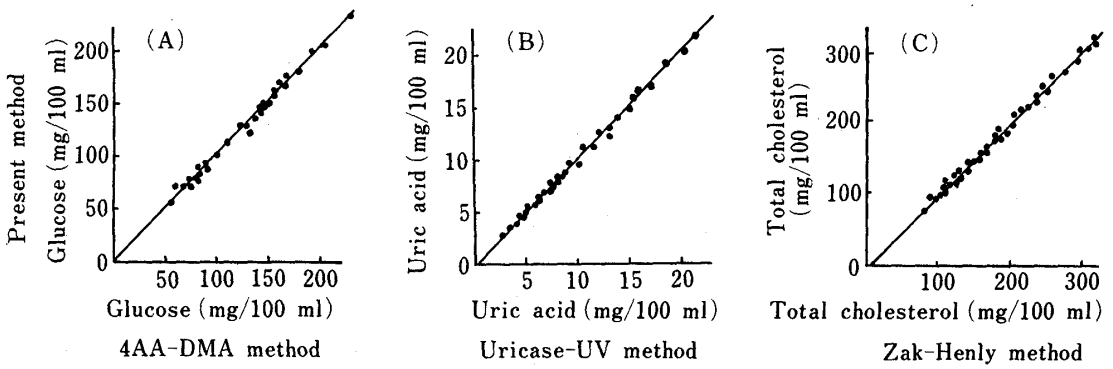

Fig. 1. Correlations between the Values of Serum Glucose (A), Uric Acid (B) and Total Cholesterol (C) Obtained by the Present Methods and the Reference Methods

colorimetric method[2] showed a correlation coefficient of 0.990 ($n=38$), and the linear regression equation for the present method (Y) against the colorimetric method (X) was $Y=0.990X+0.45$. This indicates that the present method gives values virtually identical to those obtained by the colorimetric method.

Establishment of the Standard Method for Uric Acid Determination

In order to determine the optimum concentration of uricase for the standard method, the effect of uricase concentration on voltage was examined in the range of 0 to 1 U/ml at 0.2 mg/ml uric acid. Maximum and constant voltage were obtained by the use of uricase at concentration greater than 0.1 U/ml, and was maintained for 10 min. The optimum pH for this system was 7.5.

On the basis of these results, the optimum conditions for determination of uric acid were established as described in Materials and Methods.

Standard Curve——The standard curve obtained by the present method was linear in a range of 0.01 mg to 0.50 mg/ml (0.05 to 2.5 μg/tube) of uric acid. The limit of determination for uric acid was 0.05 μg.

The CV was 4.5, 3.6, and 3.3% ($n=10$ each) for 0.025, 0.05 and 0.10 mg/ml of uric acid.

Interfering Compounds——As shown in Table I, albumin, bilirubin, and ascorbic acid did not interfere with the assay at concentrations of less than 7.5 g/dl, 10 and 5 mg/dl, respectively, but higher concentrations caused a decrement of the measuerment values.

Recovery Test——To determine the recovery of uric acid in serum, 1 ml of 0.06% lithium carbonate solution, containing 0.3 or 0.6 mg/ml uric acid, was added to 9 ml of serum, which contained 0.04 mg/ml. The recoveries of uric acid were 97.0 ± 3.7 and 104.0 ± 3.0 (mean $\pm$ SD, $n=5$), respectively.

Comparison with the Reference Method——To compare the results, parallel tests with the uricase UV method[10] and the present method were carried out on 35 serum samples. The results gave a linear regression of $Y=0.996X-0.076$, with a correlation coefficient of 0.989.

Establishment of the Standadrd Method for Total Cholesterol Determination

The optimum concentrations of CEH and COD for the determination of total cholesterol were examined. Constant voltage was obtained at concentrations greater than 0.1 U/ml of CEH and 0.1 U/ml of COD in the reaction medium. The optimum pH for the reaction was 7.0 and the reaction was completed within 10 min. From these results, the standard method for the determination of total cholesterol was established as mentioned above.

Standard Curve——The standard curve was made with cholesterol standadrd solutions of various concentrations. A linear relationship existed between the voltage and the concentration of cholesterol in the range of 0.05 mg to 5.0 mg/ml (0.25 to 25.0 μg/tube).

The precision for the determination of total cholesterol was examined. The CV was 4.5, 3.5 and 3.1% ($n=10$ each) for 0.5, 1.0, and 2.0 mg/ml, respectively.

Interfering Compounds——The efects of albumin, bilirubin, uric acid, ascorbic acid and hemoglobin on the determination of serum total cholesterol contents were examined. These compounds did not affect the value of total cholesterol at concentrations of less than approximately 7.5 g, 10 mg, 10 mg, 10 mg and 50 mg/dl, respectively, but the coexistence of higher concentrations of these compounds decreased the total cholesterol value.

Recovery Test——To determine the recovery, 0.2 ml of Triton X-100 solution containing 10 mg or 20 mg of cholesterol were added to 9.8 ml of serum which contained 1.4 mg/ml cholesterol. The recoveries were $101.8 \pm 3.5\%$ and $100.0 \pm 3.0\%$ (mean $\pm$ SD, $n=5$), respectively.

Comparison with the Reference Method——Comparison with the modified Zak–Henly method,[11] showed a correlation coefficient of 0.990, and the linear regression equation for the present method (Y) against the reference method (X) was $Y=1.01-1.9$ for 40 serum samples.

Stability and Durability of the Electrode

The response of the electrode was decreased by 15 to 20% after 20 reactions in serum, but the response was stable up to 100 cycles after that. These results agreed well with our previous fidings.[5]

These observations indicated that the oxidation system of enzymes (such as GOD, uricase and COD) coupled with the peroxidase adsorbed on a carbon electrode are highly sensitive and should be useful for biological investigations in clinical laboratories. This electrode may also be used as sensor for a hydrogen peroxide detector in high-performance liquid chromatography. Such an application is being investigated, and the results will be published in the future.

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