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Determination of Serum Uric Acid and Glucose Using Dichlorofluorescin-Cycloheptaamylose Complex

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Dichlorofluorescin was found to be greatly stabilized against autoxidation by complex formation with cycloheptaamylose. This finding facilitated the practical use of dichlorofluorescin in the ultramicrodetermination of serum uric acid and glucose. Only 5 μl and 2.5 μl of serum were required for the determination of uric acid and glucose, respectively. This procedure is especially useful for the assay of these compounds in diluted serum, and should reduce the number and amount of samples required for chemical diagnosis.

Keywords——fluorometry, uric acid and glucose; ultramicroanalysis, uric acid; dichlorofluorescin, uric acid and glucose determination; uric acid, fluorometry; glucose, fluorometry; cycloheptaamylose; serum uric acid

The estimation of hydrogen peroxide using peroxidase (POD) is a useful tool for the assay of oxidases or their substrates that generate hydrogen peroxide. Although numerous colorimetric methods have been devised using chromogenic substrates of POD, their sensitivity has been insufficient for the assay of low serum concentrations of biological materials, such as uric acid. However, the sensitivity has recently been improved by the use of fluorescent substrates of POD. Guilbault *et al.*[2] have reported a method based on the appearance of fluorescence as a result of the oxidative dimerization of homovanillic acid (HVA). On the other hand, Keston and Brandt[3] utilized dichlorofluorescin (LDCF),[4] which is more sensitive and more readily available than HVA. Moreover, dichlorofluorescein (DCF), the oxidation product of LDCF, fluoresces at far longer wavelength than HVA dimer, and the emission of the former is less affected by co-existing materials than that of the latter. However, LDCF is susceptible to autoxidation. Diacetyldichlorofluorescin (LDADCF), which is more stable on storage than DCF, was therefore employed.[3] LDADCF was converted to LDCF by hydrolysis with ethanolic sodium hydroxide immediately before use. Nevertheless, this procedure still gave a high blank value due to autoxidation of DCF in the course of determination, and the reproducibility was greatly reduced.

Cycloheptaamylose (C7A) has been shown to stabilize various fluorescent compounds in our laboratory.[5,6] This paper describes the stabilization of LDCF using C7A, and the application of this finding to the microdetermination of uric acid and glucose in serum.

Materials and Methods

POD (80 U/mg) and glucose oxidase (GOD 20 U/mg) were purchased from Sigma Chemical Co. and Boehringer Mannheim Chemical Co., respectively. Uricase (1.1 U/mg) was a product of Seishin Seiyaku Co. C7A was obtained from Tokyo Kasei Co. LDADCF was prepared according to the method of Keston and

1) Location: *Shirokane, Minato-ku, Tokyo 108, Japan.*
2) G.G. Guilbault, P.J. Bringnac, Jr. M. Juneau, *Ana. Chem.*, **40**, 1256 (1968).
3) A.S. Keston and R. Brandt, *Anal. Biochem.*, **11**, 1 (1965).
4) The letter "L" of LDCF implies that the dye is in the leuco-form.
5) T. Kinoshita, F. Iinuma, and A. Tsuji, *Anal. Biochem.*, **61**, 632 (1974).
6) T. Kinoshita, F. Iimura, and A. Tsuji, *Biochem. Biophys. Res. commun.*, **51**, 666 (1973).

Brandt.[7] Other reagents were of reagent grade. Glass-Distilled water was used throughout the experiments. Fluorescence spectra were taken and fluorescence intensities measured with a Simadzu RF-510 spectrofluorophotometer.

Standard Solution——Uric acid standard solution was prepared by dissolving 100 mg of uric acid and of Li_2CO_3 in 20 ml of water at 60° and diluting the resulting solution to 100 ml with water. This uric acid stock solution was appropriately diluted before use.

Enzyme Solution——POD solution was prepared by dissolving 6.0 mg of POD in 25 mM phosphate buffer (pH 7.5) to make 500 ml. This solution was stable for 1 week at 4°. Uricase-POD solution was prepared by dissolving 140.0 mg of uricase and 4.0 mg of POD in 25 mM phosphate buffer (pH 8.1) to make 100 ml before use.

Fluorescence Reagent——An aliquot of 10 ml of the LDADCF stock solution was mixed with 40 ml of 10 mM NaOH and the resulting solution was mixed vigorously. Next, 150 ml of 12.5 mM phosphate buffer (pH 7.5) containing 1% C7A was added. This solution was stable for 1 week at 4°.

Assay Procedure——An aliquot of 5 ml of the reagent prepared by mixing 1 volume of the uricase-POD solution, 3 volumes of the fluorescence reagent and 1 volume of water is added to 5 μl of serum. The resulting solution is incubated at 37° for 20 min and the fluorescence intensity is measured at excitation and emission wavelengths of 336 nm and 515 nm, respectively.

This method can be modified for the assay of diluted serum as follows. A 1 ml aliquot of 200 fold water-diluted serum is mixed with 4 ml of the reagent prepared by mixing 1 volume of the uricase-POD solution and 3 volumes of the fluorescence reagent. The resulting solution is incubated at 37° for 20 min and the fluorescence intensity is measured at the wavelengths described above. For the assay of glucose, 0.5 ml of water is added to 0.5 ml of this diluted serum, and the mixture is treated according to the modified assay procedure for uric acid, except that the incubation time is 40 min.

Results

All the concentrations in the following descriptions are the final concentrations in the reaction mixtures.

Figure 1 shows the excitation and fluorescence spectra for H_2O_2, uric acid and glucose. The excitation and emission maxima are at 336 nm and 515 nm. Figure 2 shows the increase in the fluorescence intensity caused by autoxidation of the fluorescence reagent in the presence and absence of C7A. It is clear that C7A markedly stabilizes LDCF. Figure 3 shows the

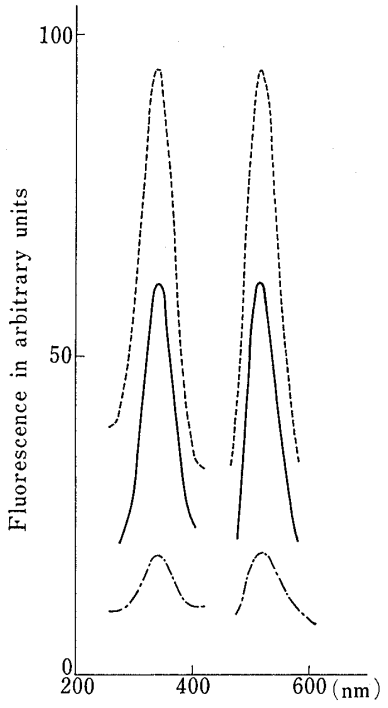

Fig. 1. Excitation and Emission Spectra of the Fluorescence Developed from H_2O_2 (----), Uric Acid (——) and Glucose (———)

Peaks are appropriately arranged for convenience of comparison.

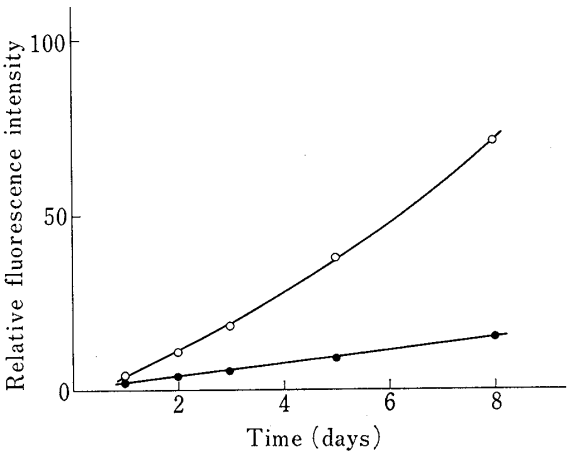

Fig. 2. Stability of LDCF–C7A Solution for the Assay of Uric Acid and Glucose in the Presence (●) and Absence (○) of C7A

7) A.S. Keston and R, Brandt, *Anal. Biochem.*, **11**, 6 (1965).

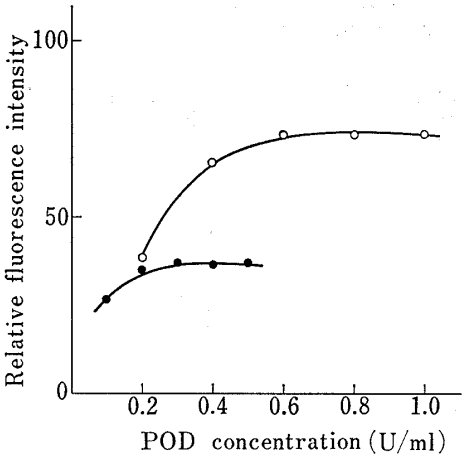

Fig. 3. Effect of POD Concentration on the Fluorescence Intensity in the Assay of Uric Acid (○) and Glucose (●)

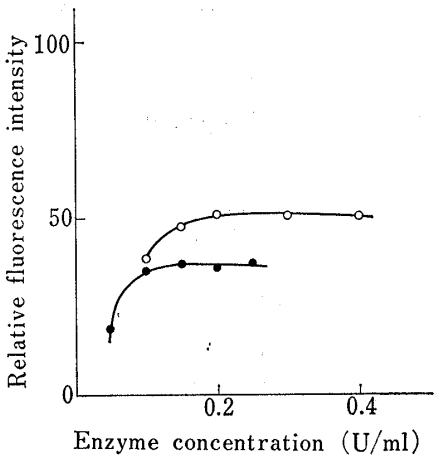

Fig. 4. Effect of Uricase (○) and GOD (●) Concentrations on the Fluorescence Intensity

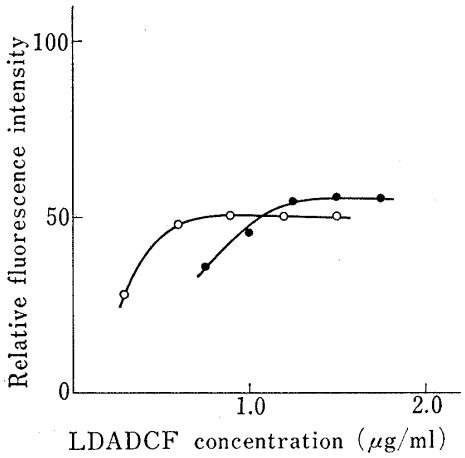

Fig. 5. Effect of LDADCF Concentration on the Fluorescence Intensity in the Assay of Uric Acid (○) and Glucose (●)

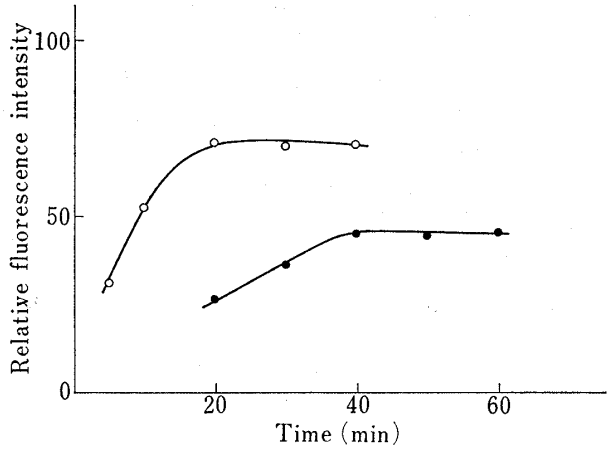

Fig. 6. Effect of the Reaction Time of Uric Acid (○) and Glucose (●) on the Fluorescence Intensity

effect of POD concentration on the fluorescence intensity. The fluorescence intensity reached plateaus at POD concentrations of 0.6 U/ml and 0.3 U/ml for uric acid and glucose, respectively. Figure 4 shows the effects of uricase and GOD concentrations in the assays of their substrates. Plateaus of fluorescence intensity were reached at 0.2 U/ml of uricase and 0.4 U/ml of GOD. Figure 5 shows the influence of LDADCF concentration on the fluorescence intensity. Maximum fluorescence was observed at concentrations of 1.0 μg/ml and 1.5 μg/ml for uric acid and glucose, respectively. Figure 6 shows the effect of reaction time at 37°. Incubation for 20 min was sufficient for uric acid whereas glucose required 40 min. Optimum pH values in the reaction mixtures were 6.6 and 7.2 for the assay of uric acid and glucose, respectively. The reaction conditions were selected in the light of these results.

The standard curves were linear in the range of final concentration of 16 ng/ml to 200 ng/ml for uric acid and 0.16 μg/ml to 1.2 μg/ml for glucose. The coefficients of variation for 20 ng/ml of uric acid and 0.2 μg/ml of glucose 1.5% (n=8) and 0.7% (n=7), respectively. Recoveries of uric acid (0.125 μg/sample) and glucose (0.5 μg/sample) added to serum were 1.0% (n=8) and 1.8% (n=8), respectively.

Table I. Recoveries of 0.5 μg/sample of Uric Acid in the Presence of Various Compounds

Compound	Amount of compound added (μg/sample)	Recovery of 0.5 μg/sample of uric acid (%)
NaCl	50	98.7
$CuSO_4$	50	100.8
	5	31.5
$FeCl_3$	5	101.6
	50	93.1
Urea	50	102.0
Glucose	50	101.0
Leucine	50	100.6
Bilirubin	5	98.6
	0.5	52.5
Ascorbic acid	0.5	140.7

Interference by various compounds was examined next. Leucine, urea, and NaCl did not interfere with the reaction. Glucose and uric acid did not interfere with the assay of the other. $CuSO_4$ affected the reaction at 5 μg/sample whereas $FeCl_3$ did not at the same concentration (Table I). Bilirubin had no effect on the reaction at 5 μg/sample, which is about 100 times the normal serum concentration. A higher level of bilirubin (50 μg/sample) showed interference. Although 0.5 μg/sample of ascorbic acid, which is about 5 times the normal serum concentration, interfered with the assay, it could be eliminated by heating the sample at 60° for 40 min prior to the analysis.

Figure 7 shows the correlation of uric acid assay results obtained by the LDCF–C7A method and the phosphotungstic acid method.

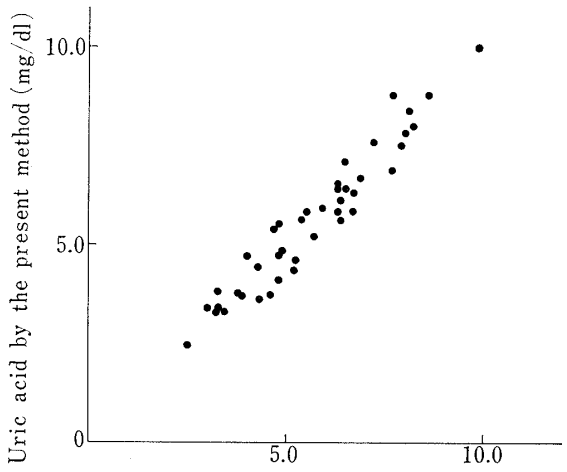

Fig. 7. Correlation between the Results of the Present Method and the Phosphotungstic Acid Method

Calculated Regression Line
$Y=0.9792(\pm 0.09)X+0.006(\pm 0.51)$
$N=45$
$r=0.961$
$s=0.4853$

Discussion

LDCF solution was markedly stabilized by the addition of C7A. This may be due to the inclusion of LDCF in the hydrophobic region of C7A. This interaction, however, had little effect on the oxidation of LDCF with peroxide and POD to produce fluorescence. Consequently, LDCF–C7A solution facilitated the practical ultramicrodeterminations of uric acid and glucose.

Uric acid has been measured by the phosphotungstic acid method,[8] the uricase-catalase method,[9] and the uricase-peroxidase method.[10] These methods require at least 0.5—1.0 ml

8) W.T. Caraway and H. Marable, *Clin. Chem.*, **12**, 18 (1966).
9) N. Kageyama, *Clin. Chim. Acta*, **31**, 421 (1971).
10) R. Richterrich, *Clinical Chemistry, Academic Press.*, **1969**, 279.

of serum because colorimetric measurements are involved. The method of measuring the decrease of uric acid by utilizing the absorption at 293 nm is more sensitive (0.3 ml), but gives only poor accuracy and precision.[11] On the other hand, the LDCF–C7A method required only 5 μl of serum and deproteinization was not necessary. The results showed an excellent correlation with those obtained by the conventional phosphotungstic acid method (Fig. 7).

The present method was also applicable to the assay of glucose, and the simultaneous determination of uric acid and glucose using diluted serum was easier. The reaction of 4-aminoantipyrine with phenols[12] has been most frequently employed for the assay of serum glucose using GOD and POD. Since this reaction required absorbance measurement at shorter wavelengths and the readings may be affected by hemolysis, a number of other methods[13–15] have been reported. However, the sensitivity of colorimetry is limited. There is a growing demand for the reliable ultramicrodetermination of biological materials using diluted serum, since this minimizes both the number of samples and the amount of serum required. This is especially important in the field of pediatrics where the assay of many items simultaneously using microamounts of blood is required.

The present method is very sensitive and is suitable for the assay of diluted serum. It seems likely that other substances which can be estimated with the oxidase-POD system can also be effecively assayed employing the LDCF–C7A reagent.

Automatic determination of serum components using the present reagent is now under investigation and the results will be reported shortly.

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11) F. Klein and G.J. Lafeber, *Clin. Chim. Acta*, **14**, 708 (1966).
12) P. Kabasakalian, S. Kalliney, and A. Westcott, *Clin. Chem.*, **19**, 522 (1973).
13) N. Gochman and J.M. Schmitz, *Clin. Chem.*, **17**, 1154 (1971); *idem*, *ibid.*, **18**, 943 (1972).
14) S.J. Miskiewicz, B.B. Arnett, and G.E. Simon, *Clin. Chem.*, **19**, 253 (1973).
15) T. Kinoshita, Y. Hiraga, N. Nakamura, A. Kitajo, and F. Iinuma, *Chem. Pharm. Bull.* (Tokyo), **27**, 568 (1979).