Use of the *o*-Phenylenediamine Fluorescence System in the Enzymatic Assay of Serum Uric Acid

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A manual enzymatic method is described for sensitive fluorometric determination of uric acid in human serum. This method is based on an enzymatic reaction with uricase to form hydrogen peroxide from uric acid and the following oxidation of *o*-phenylenediamine with peroxidase and hydrogen peroxide for the production of a fluorescence compound. The specificity and the selectivity in the method are due to the uricase reaction and the fluorometry, respectively. The formed fluorescence in the reaction mixture is measured at 410 nm (an excitation) and 550 nm (an emission). This enzymatic method can determine uric acid at 30–1000 μ M, with a between-assay relative standard deviation of 4.35% or less. A good correlation is obtained between the present method and the colorimetric kit method.

Keywords serum uric acid; *o*-phenylenediamine; fluorometry; uricase; peroxidase; hydrogen peroxide

Studies on the metabolism of purine have recently proceeded in the field of clinical medicine. One important study is the determination of uric acid in biological samples. Basically, the methods for determining uric acid can be divided into two groups; either those that utilize the reducing activity of uric acid or the enzymatic oxidation of uric acid with uricase.

Among the former methods, colorimetry¹⁾ with phosphotungstic acid was most commonly employed. However, these colorimetric methods lacked specificity and required deproteinization because they were affected by reducing substances besides uric acid in biological samples. Recently, specificity has been increased by various combinations of uricase.²⁾ Other methods, using 1,10-phenanthroline,³⁾ 2,4,6-tripyridyl-*s*-triazine,⁴⁾ and 2-(5-nitro-2-pyridylazo)-5-(*N*-propyl-*N*-sulfopropylamino)phenol⁵⁾ required relatively large volumes (more than 50 μ l) of sample.⁶⁾

On the other hand, the latter methods using uricase can be classified into three types; the ultraviolet absorption method,⁷⁾ the uricase-catalase method,⁸⁾ and the uricase-peroxidase method.⁹⁾ The first method is not sensitive, therefore, the second or third colorimetric methods are practically available. However, these methods also require large volumes of sample. Therefore, more sensitive methods using fluorometry,^{6,10)} high performance liquid chromatography,¹¹⁾ and chemiluminescence¹²⁾ were investigated. But in these methods, some faults were involved. For example, the time-consuming deproteinization process is adopted in the fluorometric methods and special equipment is required in the other methods. Therefore, these methods are not suitable for routine mass screening.

o-Phenylenediamine (*o*-PD), a typical hydrogen donor, is converted to 2,3-diaminophenazine, a highly fluorescence compound, by oxidation with peroxidase and hydrogen peroxide.¹³⁾ We have studied the application of fluorometry with *o*-PD to determine the uric acid concentration in serum by the deproteinization process using an ultrafilter membrane.

Experimental

Materials Chemicals, Reagents, and Apparatus: Uric acid, *o*-PD dihydrochloride, uricase from yeast (*Candida utilis*) (urate oxidase; urate; oxygen oxidoreductase, EC 1.7.3.3), ascorbate oxidase from cucumber (ascorbate, L-ascorbate; oxygen oxidoreductase, EC 1.10.3.3), peroxidase from horseradish (donor: hydrogen-peroxide oxidoreductase, EC 1.11.1.7), sodium sulfite (Wako, Osaka, Japan), human serum albumin, cerulo-

plasmin (Sigma, St. Louis, Mo., U.S.A.), bilirubin, tripalmitin, and ascorbic acid (Wako) were used. A hemoglobin stock solution was prepared as previously described.¹⁴⁾ All other chemicals were of reagent grade.

The uricase solution (150 mU/ml) was prepared by dissolving uricase, authorized by Wako, in water. The ascorbate oxidase solution (50 U/ml) was prepared by dissolving authorized ascorbate oxidase powder in water. These solutions were stored by freezing at -20°C and fused on the day of use. The uric acid stock solution was prepared as previously described.⁵⁾ Standard solutions were prepared by diluting the uric acid stock solution with water to give concentrations of up to 1000 μ M. The *o*-PD solution (50 mM) was prepared by dissolving 90.5 mg of *o*-PD dihydrochloride in 10 ml of water.

Fluorescence spectra and fluorescence intensity were measured with a fluorescence spectrophotometer (model 650-40, Hitachi, Tokyo, Japan), setting at 10 mm slit-width in the excitation and emission monochrometers.

Methods Deproteinization: Filter 100 μ l of sample or uric acid standard solution through a ultrafilter membrane (molecut II GC, Millipore, Bedford, Mass., U.S.A.).

Enzymatic Reaction: To the mixture of 3 ml of 0.1 M phosphate buffer (pH 8.0), 10 μ l of 150 mU/ml uricase solution, and 10 μ l of 50 U/ml ascorbate oxidase solution, add 20 μ l of the deproteinized serum or uric acid standard solution, and mix well. After the addition of 100 μ l of 100 mU/ml peroxidase solution and 100 μ l of 50 mM *o*-PD solution, incubate at 37°C for 15 min. Cool the reaction mixture in water, add 0.5 ml of 50 mM sodium sulfite solution, and mix well. Measure the fluorescence of the reaction mixture at 410 nm (an excitation) and 550 nm (an emission) within 60 min, using a blank solution in which deproteinized serum has been replaced with water.

For comparison, we also determined the uric acid concentration in serum or artificial samples by a colorimetric method¹⁵⁾ with uricase, peroxidase, and 4-aminoantipyrene, using an available kit (uric acid C-test Wako, Wako).

Results and Discussion

Reaction Conditions In the presence of uric acid, uricase, and peroxidase, *o*-PD is oxidized and then exhibits fluorescence in the reaction mixture (Fig. 1). The fluorescence-producing reagent, *o*-PD, was used as an indicator of hydrogen peroxide produced by the oxidation of uric acid with uricase. This fluorescence substance was a dimer of *o*-PD produced by oxidation in the presence of hydrogen peroxide with peroxidase.¹⁶⁾ An appropriate pH of 0.1 M phosphate buffer (pH 6.0–8.6) was 8.0, because of its high fluorescence production. The combination of 150 mU/ml uricase, 50 U/ml ascorbate oxidase, and 100 mU/ml peroxidase activities gave almost maximum fluorescence intensity when they were tested at the range of 0–1000 mU/ml, 0–500 U/ml, and 0–1000 mU/ml, respectively. The optimal concentrations of both *o*-PD and sodium sulfite were 50 mM when they were tested between

Fig. 1. Excitation and Emission Spectra of the Fluorescent Product in the Reaction Mixture

Fig. 2. Time-Course of the *o*-Phenylenediamine Oxidation Reaction at Different Temperatures

Uric acid concentration in sample: 1000 μM . Reaction temperatures: 25 ($\blacktriangle$), 37 ($\bullet$), and 45 ($\blacksquare$) $^{\circ}\text{C}$.

0 and 300 mM and 0 and 1000 mM.

Figure 2 shows the time-course for the *o*-PD oxidation reaction. The reaction proceeded more quickly at 37 $^{\circ}\text{C}$, and reached its maximum within a period of 15 min. Therefore, the oxidation reaction was completed within 15 min at 37 $^{\circ}\text{C}$. However, the fluorescence produced decreased gradually without a stabilizer. But the adoption of sodium sulfite after the reaction, produced fluorescence that was stable and gave a constant value for at least 60 min.

Effect of Interfering Substances The fluorometric methods^{6,10} reported formerly involved the deproteinization process with heating at 95 $^{\circ}\text{C}$ in their procedures because the proteins in sample plasma or serum may act as acceptors for hydrogen peroxide in the presence of peroxidase,¹⁷ or as the effect of catalase.¹⁸ In our method, we adopted the deproteinization process by ultrafiltration for simplicity and time-saving. Table I shows the recovery of uric acid in the serum sample containing each interfering substance, albumin, bilirubin, lipids, hemoglobin, ceruloplasmin, glucose, or ascorbic acid.

Utility of Ascorbate Oxidase Table II shows the effect of ascorbic acid at concentrations of 0, 41.8, 83.7, 167.5,

TABLE I. Effect of Some Interfering Substances on Serum Uric Acid Determination

Substance (concentration)		Uric acid ^{a)} Recovery range (%)
Albumin	(22.5—90 g/l)	95.9—105.2
Bilirubin	(0.156—0.625 g/l)	94.6—101.7
Tripalmitin	(0.9—7.5 g/l)	99.3—104.9
Hemoglobin	(1.25—2.50 g/l)	99.1—105.9
Ceruloplasmin	(0.125—1.000 g/l)	95.2—102.8
Glucose	(1.8—30 mM)	96.5—103.0
Ascorbic acid	(41.8—500 μM)	94.3—105.5

a) Uric acid concentrations of samples were 194, 485, and 776 μM .

TABLE II. Effect of Ascorbic Acid on Serum Uric Acid Determination with or without Ascorbate Oxidase

Ascorbic acid (μM)	Ascorbate oxidase (U/ml)	Uric acid ^{a)}	
		Found (μM)	Recovery (%)
None	None	486	100.2
41.8	None	463	95.4
83.7	None	436	89.8
167.5	None	408	84.1
355.0	None	297	61.2
500.0	50	487	100.5

a) The concentration of uric acid in each sample was 485 μM .

TABLE III. Precision Data

Sample	<i>n</i>	Uric acid (μM)		RSD ^{b)} (%)
		Mean	S.D. ^{a)}	
Within-assay				
Serum I	5	194	3.4	1.75
Serum II	5	485	15.6	3.21
Serum III	5	776	26.0	3.35
Between-assay				
Serum I	10	194	8.4	4.32
Serum II	10	485	14.5	2.98
Serum III	10	776	19.6	2.52

a) Standard deviation. b) Relative standard deviation.

335.0, and 500.0 μM in the absence or presence of 50 U/ml ascorbate oxidase. At the ascorbic acid concentration of 335.0 μM , the recovery of uric acid was 61.2% against the additional uric acid (485 μM). Generally, the concentration of ascorbic acid in the serum of a healthy subject is between 10 and 100 μM ,¹⁹ although values up to 250 μM are clinically important. Therefore, in our method, the effect of ascorbic acid (less than 500 μM) was eliminated by oxidation with ascorbate oxidase.

Accuracy The calibration graph was linear up to 1000 μM . The lower limit of detection was 30 μM , at a sample-to-blank relative fluorescence intensity ratio of 2.0. The typical regression equation of the curve was represented by $y = 1.057x + 31.386$ ($n = 11$, $r = 0.998$); the standard error of the slope was 0.064 ($n = 10$).

The normal concentration range of uric acid in healthy human serum was 178—416 μM for males and 118—356 μM for females.²⁰ These levels may vary in several diseases with abnormal purine metabolism. For example, the concentra-

Fig. 3. Correlation between the Results Obtained by the Kit Method and the Proposed Method

tion is higher in the case of gout, Lesch–Nyhan syndrome, and chronic kidney disorders, and lower in the Fanconi syndrome. Therefore, in the proposed method, a determinable range of up to 1000 μM was investigated.

Precision Table III gives the results in five repeated within-assays of samples containing 194, 485, and 776 μM of uric acid and the results in ten repeated between-assays of frozen samples for daily analysis of the same uric acid concentrations for 10 d. The relative standard deviation (RSD) in the former assays was 1.75–3.35% and the latter RSD was 2.52–4.32%.

Comparison with the Other Method Figure 3 show the uric acid concentrations in sera from healthy subjects and artificial sera by the proposed method and the kit method. The correlation between these two methods was good ($n=61$, $r=0.994$); the regression equation of the graph was represented by $y=1.001x+0.923$.

Therefore, the method presented is expected to be useful in clinical practice.

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