Fluorometric Determination of Uric Acid with 4,5-Dimethyl-*o*-phenylenediamine

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A new sensitive fluorometric method for the determination of uric acid is presented. Uric acid reacted with 4,5-dimethyl-*o*-phenylenediamine (DMPD) after the oxidation using *N*-bromosuccinimide (NBS) to give a green fluorescence with excitation maxima at 350 nm and 400 nm, and an emission maximum at 500 nm. By the use of the fluorescence reaction, uric acid in aqueous solution was determined in the range of 0.01–5.0 $\mu\text{g ml}^{-1}$, and the relative standard deviation for 1.0 $\mu\text{g ml}^{-1}$ uric acid was 0.51% ($n=10$). The proposed method could be applied to the determination of uric acid in serum.

Keywords Fluorometric analysis, uric acid, 4,5-dimethyl-*o*-phenylenediamine, *N*-bromosuccinimide, purine, serum

Uric acid is excreted as the main end product of purine metabolism. There are some clinical disorders associated with uric acid in serum, plasma and urine, and many methods have been developed for the determination of uric acid. Colorimetric methods based on the formation of tungsten blue from phosphotungstic acid by the reduction of uric acid have been utilized as a chemical process in clinical investigation.^{1,2} These methods are influenced by reducing substances, *e.g.*, ascorbic acid, glutathione and protein. The enzymatic process with uricase is based on the estimation of the decrease of absorbance for uric acid at 293 nm after uricase is added.^{3–5} The methods are not always reproducible. The process using an uricase–peroxidase⁶ system was also developed. The method based on the color reaction of *o*-dianisidine and the hydrogen peroxide produced by an uricase–peroxidase system is affected by reducing substances. A colorimetric determination using uricase and catalase⁷ does not need a deproteinization process. The methods, however, are affected by bilirubin. Further, there is an enzymatic fluorometry based on the oxidation of 3,5-diacetyl-1,4-dihydrolutidine⁸ or *p*-hydroxyphenylacetic acid⁹ by uricase–peroxidase system. These methods are affected by bilirubin, glutathione and ascorbic acid. A method using peroxidase absorbed on a carbon electrode is also reported.¹⁰ It has been known that uric acid is oxidized to alloxan^{11,12}, and the alloxan reacts with *o*-phenylenediamine to give a fluorescent product.¹³ In this paper, on the basis of these reactions, the procedure for the determination of uric acid was studied. 4,5-Dimethyl-*o*-phenylenediamine (DMPD) was used as a fluorescence reagent and the procedure was applied to the microdetermination of uric acid in serum.

Experimental

Reagents and apparatus

Uric acid solution (1000 $\mu\text{g ml}^{-1}$) was prepared by dissolving 100 mg of uric acid (E. Merck Co.) and 80 mg of lithium carbonate (Wako Pure Chem. Co.) in 30 ml of water at 60°C and diluting to 100 ml with water. DMPD solution (0.01 mg dl^{-1}) was prepared by dissolving 4,5-dimethyl-*o*-phenylenediamine (Tokyo Kasei Co.) in ethanol. This solution was stable for 3 d in the dark. *N*-Bromosuccinimide (NBS) solution (0.02 g dl^{-1}) was prepared by dissolving NBS (Tokyo Kasei Co.) in water. Sodium thiosulfate solution (200 mg dl^{-1}) was prepared by dissolving sodium thiosulfate in water. This solution was stable for a week in the dark. Purine (Nakarai Chem. Co.; Table I) solutions were prepared by dissolving 10 mg each in 1.0 ml of 0.1 M sodium hydroxide solution and diluting to 100 ml with water. Controlled sera (Moni-Trol® I and Moni-Trol® II containing uric acid of 4.1 and 7.1 mg dl^{-1} , Q-PAK®-Control I and Q-PAK®-Control II containing uric acid of 4.9 and 8.2 mg dl^{-1}) were purchased from Dade Co. and Hyland Co. UA Shinotest® kit (uricase–peroxidase system) was purchased from Shinotest Shoji Co. Other reagents were of reagent grade.

Excitation and emission spectra and relative fluorescence intensity were measured with a Hitachi 650–10s fluorescence spectrophotometer.

Procedure

One milliliter of sample solution containing 0.01–5.0 $\mu\text{g ml}^{-1}$ of uric acid and 1.0 ml of 0.4 M acetate buffer (pH 4.6) were placed in a light resistant test tube

with ground stopper and mixed. The mixture was cooled in an ice bath for 10 min. To the solution, 0.2 ml of NBS solution was mixed. After 1 min, 0.2 ml of sodium thiosulfate solution and 0.2 ml of DMPD solution were mixed and the mixture was kept at 30°C for 20 min. The fluorescence intensity of the reaction solution obtained was measured at excitation and emission wavelengths of 350 nm and 500 nm. The sensitivity of the fluorescence spectrophotometer was adjusted with uranine solution of 0.5 or 1.0 $\mu\text{g ml}^{-1}$.

Results and Discussion

Effect of pH on the oxidation of uric acid

The effect of pH for the oxidation of uric acid using NBS on the fluorescence intensity was examined (Fig. 1). The oxidation reaction using NBS was carried out in buffer solution with acetic acid-sodium acetate, hydrochloric acid-sodium acetate, citric acid-disodium hydrogen phosphate or citric acid-sodium citrate buffer solution. After all, the highest constant fluorescence intensity was obtained using 0.2–0.8 M acetic acid-acetate buffer of pH 4.2–5.0. Therefore, 0.4 M acetate buffer of pH 4.6 was selected.

Effects of oxidation time and temperature

NBS, *N*-chlorosuccinimide, sodium nitrite and iodine were examined for use as an oxidizing agent in the procedure. It was found that NBS was a useful reagent for the determination of uric acid. The oxidation time using NBS was varied from 1 to 60 min and the temperature was varied from 0 to 30°C in the procedure. The results are shown in Fig. 2. The highest constant fluorescence intensity was obtained by standing for 0 to 15 min at 0° to 2°C. Therefore, standing for 1 min in an ice bath was selected for the procedure.

Fig. 1 Effect of pH on the fluorescence intensity. Uric acid ($1.0 \mu\text{g ml}^{-1}$) was reacted at various pHs. Buffer solutions used: a, 0.4 M acetic acid-sodium acetate; b, 0.4 M citric acid-0.2 M disodium hydrogen phosphate; c, 0.4 M hydrochloric acid-sodium acetate; d, 0.4 M citric acid-sodium citrate.

Effects of NBS and sodium thiosulfate concentration

NBS concentration in the range 8–40 mg dl^{-1} gave the highest constant fluorescence intensity. Therefore, 10 mg dl^{-1} NBS was used.

It was found that the fluorescence intensity had been enhanced by the addition of sodium thiosulfate after oxidation of uric acid using NBS. The addition of sodium thiosulfate more than 0.1 g dl^{-1} was advantageous in the procedure. Consequently, 0.2 g dl^{-1} sodium thiosulfate solution was used.

Effects of reaction time and temperature with DMPD

The reaction time of uric acid with DMPD was varied from 1 to 60 min and temperature was varied from 25° to 70°C in the procedure (Fig. 3). The highest constant fluorescence intensity was obtained after standing for 5 to 60 min at 30° to 40°C. Therefore, standing for 20 min at 30°C was selected for the procedure.

Effect of DMPD concentration

The highest constant fluorescence intensity was obtained in the range of 0.008–0.1 g dl^{-1} DMPD.

Fig. 2 Effects of reaction time and temperature of the NBS oxidation reaction on the fluorescence intensity. Uric acid at $1.0 \mu\text{g ml}^{-1}$ was used; a, 0°–2°C; b, 15°C; c, 25°C; d, 30°C.

Fig. 3 Effects of reaction time and temperature of the DMPD reaction on the fluorescence intensity. Uric acid at $1.0 \mu\text{g ml}^{-1}$ was used; a, 25°C; b, 30°C; c, 40°C; d, 70°C.

Therefore, 0.01 g dl⁻¹ DMPD solution was used.

Excitation and emission spectra

The excitation and emission spectra of fluorescent product derived from uric acid are shown in Fig. 4.

The excitation spectra showed maxima at 350 and 400 nm and the emission spectra showed a maximum at 500 nm. In the procedure, excitation and emission wavelengths at 350 and 500 nm were selected.

Calibration curve and stability of the fluorescence

The calibration curve for uric acid was linear in the range of 0.01 to 5.0 µg ml⁻¹. The relative standard deviations were 0.51 and 0.57% (*n*=10) at 1.0 and 0.1 µg ml⁻¹ of uric acid aqueous solution. The fluorescence developed was stable at least for 2 d at room temperature.

Fig. 4 Excitation and emission spectra of the fluorescent product formed from uric acid. Uric acid at 1.0 µg ml⁻¹ was used.

Effects of diverse substances

The effects of various substances were examined in the presence of 1.0 µg ml⁻¹ of uric acid. The diverse substances which interfere with the proposed method are shown in Table 1. As a result, xanthine (1.0 µg ml⁻¹) gave a positive error of about 40 percent. Co-existence of other substances did not much affect the determination of uric acid.

Determination of uric acid in serum

Uric acid concentration in a male human serum and four samples of controlled sera was measured with the proposed method. Controlled sera used were Moni-Trol® I, Moni-Trol® II and Q-PAK®-Chemistry I, Q-PAK®-Chemistry II. A human and controlled sera were diluted 1000 times with water, respectively. The diluted sample solutions were used for the determination of uric acid. The measurements showed that the male human serum contained 3.24 mg dl⁻¹ of uric acid and the values for moni-Trol® I, Moni-Trol® II, Q-PAK®-Chemistry I and Q-PAK®-Chemistry II were 4.11, 7.03, 4.60 and 8.03 mg dl⁻¹, respectively. The values of uric acid from four controlled sera were identical with the amounts stated in the experimental part. The relative standard deviations were 1.52% for the male serum, 1.48, 1.18, 1.63 and 1.10% for four controlled sera, respectively (each *n*=6). The recoveries of uric acid (5.0 µg ml⁻¹) added to the above five sera were 99.1±0.60, 99.8±0.59, 99.2±0.55, 101.2±0.61 and 99.3±0.67% (mean±standard deviation, each *n*=6).

Comparison with uricase-peroxidase method

The present method was compared with uricase-peroxidase system kit (UA Shinotest®); uric acid

Table 1 Effects of diverse substances

Substance	µg ml ⁻¹	Found, %	Substance	µg ml ⁻¹	Found, %
Fe ²⁺ (FeSO ₄ ·7H ₂ O)	10	92.3	Urea	10	86.8
Fe ³⁺ (FeCl ₃ ·6H ₂ O)	1	96.0		1	100.0
Cu ²⁺ (CuSO ₄ ·5H ₂ O)	10	98.5	Allantoin	100	93.6
Zn ²⁺ (ZnSO ₄ ·7H ₂ O)	10	99.8		10	100.0
NO ₂ ⁻ (NaNO ₂)	10	94.0	Albumin	100	30.3
NO ₃ ⁻ (NaNO ₃)	10	99.7		10	100.0
Cl ⁻ (KCl)	100	99.6	Bilirubin	10	97.6
Br ⁻ (KBr)	100	100.0		1	100.0
L-Ascorbic acid	1	103.1	L-Pyruvic acid	1	101.2
Thiamine·HCl	10	97.9	Xanthine	1	140.8
	1	100.0	Theophylline	100	73.3
Riboflavine	1	168.3	Theobromine	10	105.7
D-Glucose	100	100.0	Caffeine	1	100.1
Lactose	10	97.7	Hypoxanthine	100	100.0
Citric acid	10	100.8	Adenine	1	95.8
Glutathione	10	76.6	Guanine	1	94.0
	1	100.0	Uracil	10	92.3
Creatine	100	100.0	Cytosine	1	99.1
	1	100.0	Thymine	1	100.0
Creatinine	10	96.0			

Uric acid taken; 1 µg ml⁻¹.

concentration in 20 samples of sera was measured by both methods. The coefficient of correlation was 0.992 ($n=20$) and the regression equation was $y=0.96x-0.26$.

The fluorescent product formed from uric acid in the proposed method is unknown. However, uric acid is oxidized to alloxan with oxidizing agents such as bromine^{11,12}, and the alloxan gives alloxazine and quinoxaline derivatives by the reaction with *o*-phenylenediamine.^{14,15} Therefore, the fluorescent product may be alloxazine or quinoxaline derivative.

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