however, the recovery of oligonucleotides containing purine bases from a carbon electrode should be further investigated.

Experimental

Nucleosides and Nucleotides——**1**, **5** and **6** were synthesized by a method similar to that described for *p*-*N*-tritylaminophenyl 5′-phosphorothymidine[2] from the corresponding phosphomonoesters. 4,[2] $pU(Bz)_2$,[8] $bzC(Bz)_2$,[9] $U(Bz)_2$,[9] $ibG(Bz)$[10] and $bzA(Bz)_2$[9,11] were prepared as described previously.

Apparatus——Cyclic voltammetry was carried out as reported by Sayo and Masui.[12] Controlled potential electrolysis was carried out in an open beaker using a Hokutodenko HA-101 potentiostat/galvanostat with a glassy carbon plate anode, platinium wire cathode, and saturated calomel electrode was separated from the anolyte by a unglazed cylinder and the reference electrode was connected to the anolyte through an agar bridge. When a samall volume (6 ml) of solution of compound **6** was subjected to controlled potential electrolysis, the solution was circulated by means of a peristaltic pump through a funnel bearing a glassy carbon rod anode (ϕ 3 mm × 10 cm), a carbon rod cathode (ϕ 4 mm × 4 cm) and the S.C.E. connected to the anolyte through an agar bridge.

Product Analysis——Paper chromatography was performed using ethanol-1 M ammonium acetate, pH 7.5 (7 : 3, v/v). Paper electrophoresis was performed using 0.05 M triethylammonium bicarbonate (pH 7.5) at 900 V/40 cm.

Acknowledgement This work was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture, Japan.

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[Chem. Pharm. Bull. 27(9)2245—2248(1979)]

UDC 547.857.5.08 : 543.426.061

An Ultramicro Enzymatic Method for the Fluorimetric Determination of Uric Acid in Serum

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(Received March 27, 1979)

A highly sensitive fluorimetric method is presented for the determination of uric acid in serum, based on the uricase-catalized oxidation of the acid to hydrogen peroxide, which is measured by a fluorimetric technique using the peroxidase-*p*-hydroxyphenylacetic acid system. The method is rapidly and readily performed with only 2 μl of serum without deproteinization.

Keywords——uric acid in serum; fluorimetry; enzymatic assay; uricase-peroxidase system; *p*-hydroxyphenylacetic acid; ultramicro analysis

Many colorimetric and fluorimetric methods have been reported for the determination of uric acid in serum (or plasma). Reduction methods are based on the color development

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due to uric acid reduction of phosphotungstate[2] and ferricyanide.[3] Enzymatic methods use the oxidative decomposition of uric acid catalyzed by uricase; hydrogen peroxide generated is determined by the peroxidase-catalyzed oxidation of a chromogenic (benzidine derivatives such as *o*-dianisidine[4]) or fluorigenic (homovanillic acid,[5] *p*-hydroxyphenylacetic acid[6] (HPAA) or a lutidine derivative[7]) substrate or by catalase-mediated conversion of methanol to formaldehyde, which is measured by means of Hantzsch reaction.[8] The reduction methods are not sensitive or selective for uric acid. The enzymatic methods so far described require considerable amounts of serum, and those using peroxidase require deproteinization because protein in serum shows catalase activity and act as an acceptor for hydrogen peroxide.[4,7]

We have improved the fluorimetric method using the uricase-peroxidase-HPAA system for precise determination of uric acid in only 2 μl of serum by a simple procedure, without deproteinization, based on the observation that small amounts of serum protein show no effect on hydrogen peroxide generated.

Experimental

Apparatus——Fluorescence spectra and intensities were measured with a Hitachi MPF-2A spectrofluorimeter using quartz cells of 10×10 mm optical path-length. The slit widths in the exciter and analyzer in terms of wavelengths were set at 2 and 10 nm, respectively. The fluorescence spectra are uncorrected. pH was measured with a Hitachi-Horiba M-7 pH meter at 25°.

Reagents[9]——Tris-HCl Buffer (0.1 M, pH 8.5): Prepare in the usual manner.

Uricase Solution (0.01 U[10]/ml): Dissolve 3.7 mg of uricase (2.7 U/mg, from *Candida utilis*, Toyobo) in 1000 ml of Tris-HCl buffer. The solution is usable for at least 1 month when stored in a refrigerator.

Peroxidase Solution (7 purpurogallin units[11]/ml): Dissolve 2.3 mg of peroxidase (300 purpurogallin units/mg, from horseradish, Toyobo) in 100 ml of Tris-HCl buffer. The solution remains usable for at least 1 month when stored in a refrigerator.

Peroxidase-HPAA Solution (0.7 purpurogallin units/ml and 1 mg/ml, respectively): Dissolve 100 mg of HPAA in 10 ml of peroxidase solution and dilute with Tris-HCl buffer to 100 ml, store in a refrigerator and use within a week.

Uric Acid Standard Solutions (2—20 mg/dl): Dissolve 100 mg of uric acid in 15 ml of 0.4% Li_2CO_3 solution and dilute with H_2O to 100 ml, store in a refrigerator and use within 50 days. By diluting this solution with H_2O, prepare 2, 5, 10, 15 and 20 mg/dl solutions. Use within 2 days.

Procedure——Place 2.5 ml of Tris-HCl buffer in a test tube, add 2 μl of serum and 0.5 ml of uricase solution and allow to stand at room temperature for approximately 15 min. Then add 0.5 ml of peroxidase-HPAA solution and allow to stand at room temperature for approximately 5 min. Prepare a blank by treating 2 μl of H_2O. Measure the fluorescence intensities of the test and blank at 404 nm with irradiation at 321 nm and calculate the net intensity. Read the concentration of uric acid from a calibration graph prepared with uric acid standard solutions.

Results and Discussion

The excitation and emission spectra of the final mixture have their maxima at 321 and 404 nm, respectively. They are identical to those of the final mixtures obtained through

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the procedure with uric acid standard solution and uric acid-free serum[12] spiked with the acid. This indicates that a calibration graph can be constructed in the absence of serum. The calibration graph is linear up to at least 20 mg/dl uric acid.

The optimal pHs for the uricase and peroxidase reactions are at 8.5 and approximately 7, respectively, and the product from HPAA fluoresces most intensely at pH 10—11. At pH 8.5 the fluorescence intensity increases with increasing concentrations of uricase and peroxidase in the ranges tested (0.1—0.001 U/ml and 0.3—1.0 purpurogallin units/ml, respectively) (Figs. 1 and 2). The peroxidase reaction proceeds more rapidly than the uricase reaction and is completed within 10 min even with 0.01 U/ml uricase when examined with serum containing a very high concentration of uric acid. Thus, pH 8.5 and concentrations of 0.01 U/ml (uricase) and 0.7 purpurogallin units/ml (peroxidase) were selected as optima for the procedure.

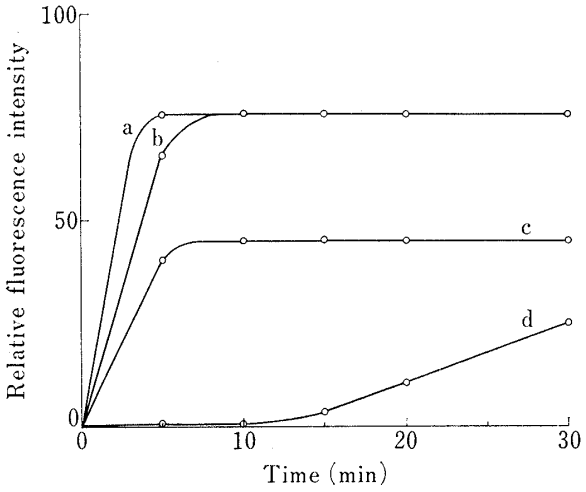

Fig. 1. Effect of Uricase Concentration and Incubation Time on the Fluorescence Development

Uricase concentration: a, 0.1; b and c, 0.01; d, 0.001 U/ml. Uric acid concentration in serum: a, b and d, 16.5; c, 9.8 mg/dl.

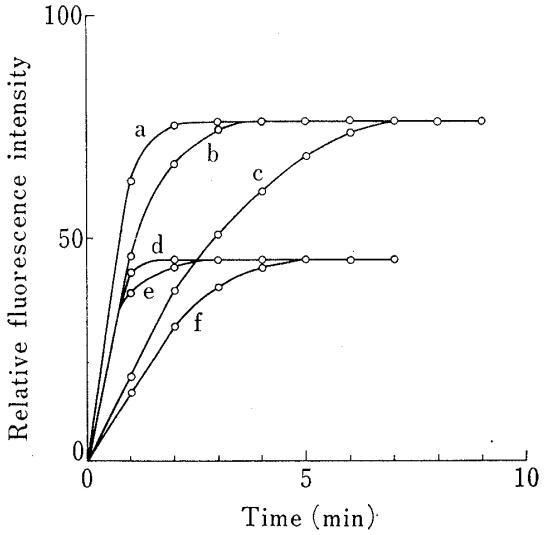

Fig. 2. Effect of Peroxidase Concentration and Incubation Time on the Fluorescence Development

Peroxidase concentration: a and d, 1.0; b and e, 0.7; c and f, 0.3 purpurogallin units/ml. Uric acid concentration in serum: a, b and c, 16.5; d, e and f, 9.8 mg/dl.

HPAA was dissolved in the Tris-hydrochloric acid buffer with peroxidase for convenience. Reagent solution containing more than 0.5 mg/ml HPAA gives a maximum and constant fluorescence with sera containing uric acid in the range of 1.5—16.5 mg/dl. Thus, a 1.0 mg/ml solution of HPAA was used in the procedure. The values of uric acid determined did not change over the temperature range examined, 15—37°.

The values of uric acid obtained by this method were identical to those obtained using sera deproteinized with ethanol[4,5] or acetic acid,[6,7] up to serum sample size of 5 μl. Bilirubin in serum does not affect the value of uric acid at concentrations less than 10 mg/dl, but higher concentrations cause an apparent decrease of about 0.05 mg/dl per 1 mg/dl bilirubin. Ascorbic acid at concentrations less than approximately 20 mg/dl in serum does not interfere with the method, but at higher concentrations, the measured value is dependent on the serum and the conditions of its strage. Cysteine and glutathione do not interfere when added to serum at 5 and 10 mg/dl, respectively, though their aqueous solutions act as inhibitors of peroxidase.[13] Glucose and hemoglobin added to serum do not affect the value of uric acid at

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concentrations of 400 and 100 mg/dl, respectively. Heparin and EDTA used as anticoagulants for blood have no effect at the concentrations usually employed.[14]

Recovery of uric acid was checked by adding known amounts of the acid (3.0 and 5.0 mg/dl) to sera with 5.5 and 9.8 mg/dl uric acid. Recoveries of 96±3% were obtained. The lower limit of determination for uric acid is 1 ng, which gives a fluorescence intensity of twice the blank. This sensitivity may permit the determination of uric acid in only 0.5 μl of serum. The within-day precision was examined using serum with a mean uric acid value of 3.20 mg/dl (n=30). The standard deviation was 0.05 mg/dl. The day-to-day precision was obtained by repeating the determination for 7 days on serum stored frozen at −20° with a mean uric acid value of 3.39 mg/dl, freshly prepared reagents being used each day (n=30). The standard deviation was 0.08 mg/dl.

Comparisons with the phosphotungstate method[2] and a fluorimetric method based on the same principle as the present method but requiring 25 μl of deproteinized serum[6a] showed correlation coefficients of 0.984 (n=18) and 0.991 (n=33), respectively, and the regression equations for the present method (x) against these methods were y=0.94x+0.77 and y=0.98x+0.07, respectively. This suggests that the phosphotungstate method gives a higher value, perhaps due to the presence of some reducing substances other than uric acid.

The proposed method for the determination of uric acid in serum is simple, precise and rapid, and should be useful in pediatric research and in cases where only an extremely small amount of serum is obtainable.

Acknowledgement The supply of pathological sera from the Pediatric and Central Clinical Laboratories of Kyushu University Hospital is acknowledged.

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[Chem. Pharm. Bull. 27(9)2248—2251(1979)]

UDC 547.597.02.05 : 581.192

Studies on the Constituents of *Marsdenia formosana* Masamune. V. Isolation and Structure of a New Triterpenoid, Marsformosanone

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(Received March 20, 1979)

Further examination of the petroleum ether extracts of *Marsdenia formosana* Masamune (Asclepiadaceae) led to the isolation and characterization of a new triterpenoid, marsformosanone (III) together with a known steroidal compound, stigmast-5-en-3β, 7α-diol (I).

Keywords——*Marsdenia formosana* Masamune; Asclepiadaceae; marsformoxide A; marsformosanone; stigmast-5-en-3β,7α-diol; urs-9(11),12-dien-3β-yl acetate; D-friedours-11,14-dien-3β-yl acetate

In preceding papers, we have reported the isolation and characterization of thirteen triterpenoids including five new compounds (α-amyrin formate, lupenyl cinnamate, mars-

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