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A New Method for the Determination of Serum Uric Acid by measuring Allantoin produced by the Action of Uricase

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A new method for the determination of serum uric acid was developed. In this method, uric acid is converted to allantoin, hydrogen peroxide and carbon dioxide by uricase (EC 1.7.3.3) and then allantoin is measured colorimetrically at 525 nm after condensation with diacetylmonoxime-thiosemicarbazide in 6 N hydrochloric acid. In order to apply this method to serum, the coexisting urea is removed by urease (EC 3.5.1.5) treatment before the color reaction. Application of the method to patients' sera showed a good correlation with the results of a standard uricase-UV method; the regression equation was $y=0.954x+0.290$ (mg/100 ml) and the correlation coefficient was $r=0.930$.

A characteristic feature of the new method is that it is not influenced by such commonly encountered interfering substances as ascorbic acid (160 mg/100 ml), glutathione (100 mg/100 ml), glucose (500 mg/100 ml) and bilirubin (10 mg/100 ml).

Keywords——serum; uric acid; uricase; allantoin; diacetylmonoxime; determination

Introduction

The levels of serum uric acid are determined routinely in clinical laboratories these days by various methods which can be divided into two main classes. One class comprises a reductive method in which the reducing power of uric acid itself is measured colorimertically, and the other class comprises enzymatic methods in which uricase (EC 1.7.3.3) is employed to convert uric acid to allantoin, hydrogen peroxide and carbon dioxide. In the latter methods, uric acid is determined either spectrophotometrically by measuring the decrease in optical density at 293 nm (absorption maximum of uric acid) or by determining hydrogen peroxide evolved with the aid of catalase (EC 1.11.1.6) or peroxidase (EC 1.11.1.7). Although uricase is highly specific to uric acid, the measurement of hydrogen peroxide is interfered with by reducing agents commonly found in serum or urine, such as ascorbic acid and glutathione. Reducing agents in serum or urine also present difficulties in the reductive method.

Thus, we initiated studies to develop new methods to determine serum uric acid enzymatically without interference, by measuring allantoin instead of hydrogen peroxide. Since allantoin shares a ureido structure with urea, we employed the diacetylmonoxime-thiosemicarbazide (DAM–TSC) method,[2)] which is a popular method used in the determination of blood urea nitrogen, to determine allantoin. Urea, which is present in a much larger amount than uric acid (approximately 20 times on a molar basis), was to be removed completely by urease (EC 3.5.1.5) treatment prior to the color reaction with DAM–TSC.

It is the purpose of this article to demonstrate that the proposed method (uricase-DAM–TSC method) is a valid and useful one to determine serum uric acid.

Materials and Methods

Urease (Sigma, type III, 3.4 units/mg) and crystalline glutathione (reduced) were products of Sigma Chemical Co., and uricase (1.1 units/mg) was kindly provided by Seishin Seiyaku Co., Tokyo. Uric acid,

1) Location: *2-2-1 Miyama, Funabashi, Chiba, 274, Japan.*

2) *a*) C.L. Crocker, *Am. J. Med. Tech.*, 33, 361 (1967); *b*) S. Shibata and M. Sasaki, "Ultramicro Quantitative Analysis in Clinical Chemistry," Kimpo-Doh, Tokyo, 1971, pp. 239—245.

urea, lithium carbonate and diacetylmonoxime were from Koso Chemical Co., Ltd., Tokyo and thiosemicarbazide was purchased from Nakarai Chemicals Ltd., Kyoto. L-Ascorbic acid and bilirubin were products of Wako Pure Chemical Industries, Ltd., Osaka and E. Merck, Darmstadt, respectively. Allantoin was purchased from Tokyo Chemical Industry Co., Ltd., Tokyo. All other chemicals were obtained from commercial sources. All these chemicals were of reagent grade or of the highest grade available.

Nescol X (Nihon Shoji K.K., Osaka) was used as normal control serum throughout this investigation.

Spectrophotometric measurements were made with a Shimadzu UV-210A double-beam spectrophotometer (Shimadzu Seisakusho Ltd., Kyoto).

Enzyme Solutions——Urease was dissolved in ethylenediamine tetraacetic acid disodium salt solution (10 mg/ml, pH 7.0) to make a final concentration of 17 units/ml, and uricase solution (2.2 units/ml) was prepared in 0.2 M glycine–hydrochloric acid buffer (pH 9.2). These solutions were stable for at least two months when stored frozen.

Uric Acid Standard Solutions[3]——Stock solution was prepared by dissolving 100 mg of uric acid and 80 mg of lithium carbonate in about 50 ml of water by heating to 60°. The solution was cooled to room temperature, and water was added to make 100 ml; this solution was stored frozen.

Working uric acid standard (5.0 mg/100 ml) was prepared by diluting the stock solution (100 mg/100 ml) 20-fold with water.

DAM-TSC Reagent——Diacetylmonoxime (0.6 g) and thiosemicarbazide (0.03 g) were dissolved in a final volume of 100 ml of water and the solution was kept in a brown bottle at room temperature. One part of this solution was mixed with five parts of a 7.2 N hydrochloric acid solution containing 16 mg/l of ferric chloride just prior to use. This reagent mixture should be used within an hour after preparation.

Determination of Uric Acid by the Uricase-DAM-TSC Method——One hundred μl of serum was added to each of two test tubes A and B. One hundred μl each of working uric acid standard and water were added to another two test tubes C and D. To each of the four test tubes, 100 μl of urease solution was added and the mixtures were incubated at 37°. After 10 min, 100 μl of uricase solution was added to tubes A, C and D, and 100 μl of water to tube B. Incubation was continued for a further 15 min at 37° and the reaction was terminated by adding 400 μl of 10% trichloroacetic acid (TCA). After centrifugation at 3000 rpm for 10 min, 500 μl of each supernatant was mixed well with 3.0 ml of DAM-TSC reagent and the four test tubes were immersed in a boiling water bath for 20 min. The solutions were cooled to room temperature with tap water, and the absorbances of A and C were read against B and D, respectively, at 525 nm.
Calculation:

$$\text{uric acid (mg/100 ml)} = 5.0 \times \frac{\text{absorbance of A}}{\text{absorbance of C}}$$

Determination of Uric Acid by the Uricase-UV Method[4]——The uricase-UV method was carried out using a uric acid determination set, "Urica-Ace No. 2, EIKEN" (Eiken Chemical Co., Ltd., Tokyo) which employs a borate buffer.

Results

Effects of Various Acids on the Color Reaction of Allantoin with DAM-TSC Reagent

In the routine DAM–TSC method for the determination of blood urea nitrogen, 4.9 N (28%, final concentration) phosphoric acid has been employed to avoid precipitation of serum protein.[2b] Under this condition, however, the intensity of the color developed with allantoin was not high enough for the quantitative determination of uric acid in serum. Thus, various acids were tested as possible replacements for phosphoric acid. Table I shows the relative intensities of the color reaction of allantoin in various acids at near-optimum concentrations. These results indicate that hydrochloric acid is superior to other acids. The optimum concentration of hydrochloric acid was determined to be 6 N (final concentration).

Effect of Ferric Chloride on the Color Reaction

It was found that the color intensity of allantoin after the DAM–TSC reaction was increased in the presence of ferric chloride. As shown in Fig. 1, the intensity was increased about 2-fold in the presence of 40 μg/3 ml of $FeCl_3 \cdot 6H_2O$. Therefore, this amount of ferric chloride (final concentration) was added to the DAM–TSC reagent.

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4) L. Liddle, J.E. Seegmiller, and L. Laster, *J. Lab and Clin. Med.*, **54**, 909 (1959).

Table I. Relative Intensities of the Color Reaction of Allantoin in Various Acids

Acids	Final concentration	Relative intensity %
Phosphoric acid	28.0%	12.3
Hydrochloric acid	5.0 N	112
Sulfuric acid	5.0 N	63.0
Acetic acid	83.0%	0
Trichloroacetic acid	12.5%	5.5

One hundred μl of allantoin solution (1.0 mmol/l) was reacted with 3.0 ml of DAM–TSC reagent prepared in one of the acid listed above. The DAM–TSC reagent did not contain ferric chloride.

The results are expressed as relative intensities with the color intensity of urea (1.0 mmol/l) in phosphoric acid system as 100%.

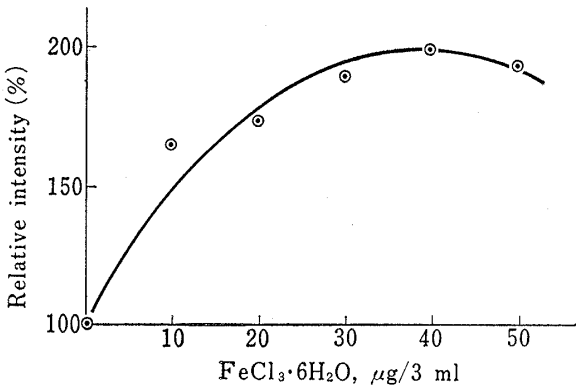

Fig. 1. Effect of Fe^{3+} on the Color Reaction with Allantion

One hundred μl of 633 μmol/l (100 mg/l) allantoin solution was mixed with 3.0 ml of DAM–TSC solution prepared in 6 mol/l hydrochloric acid. The mixture was heated for 20 min in a boiling water bath after adding 0, 5, 10, 15, 20 or 25 μl of 7.4 mmol/l (2 g/l) $FeCl_3$–$6H_2O$ solution. The results are expressed as relative color intensities.

Enzymatic Reactions

The levels of urease and uricase to be used in the present system were determined so that 60 mg urea nitrogen/100 ml of urea and 20 mg/100 ml of uric acid were consumed completely within 5—10 min at 37°. It was observed with normal sera that the urease reaction was completed within 5 min and the uricase reaction within 6—10 min. Thus, reaction times of 10 and 15 min were selected for the urease and uricase steps, respectively. These two enzyme reactions could be performed simultaneously if the uricase preparation was free from inhibitors of the urease reaction, such as the borate ion. In this case, the enzymatic reactions were carried out for 15 min at 37°.

Deproteinization

Since the color reaction was carried out under strongly acidic conditions (6 N HCl), a heavy precipitate of serum protein was formed after treatment of the reaction mixture with DAM–TSC reagent. Therefore, it was decided to remove proteins from the reaction mixture prior to the color reaction. In view of the fact that the color reaction was performed under strongly acidic conditions, TCA was applied after the enzymatic steps. The supernatant of the TCA-treated reaction mixture was then used for the DAM–TSC reaction.

Calibration Graphs

Calibration graphs were prepared by using various concentrations of standard solutions of allantion, uric acid, and uric acid in the presence of a constant amount of urea (15 mg urea nitrogen/100 ml) according to the method described above. As can be seen in Fig. 2, the plots were linear up to 20 mg/100 ml, which covers the possible range of uric acid levels in serum.

Comparison with the Uricase-UV Method

The serum uric acid levels obtained by the proposed method were compared with those obtained by a standard uricase-UV method using sera from 38 patients (Fig. 3). The relationship between the two methods is expressed by the following linear regression equation: $y = 0.954x + 0.290$, where y is the uricase-UV result and x is the result of the proposed method, expressed in mg/100 ml. The correlation coefficient (r) was 0.930.

Recovery

As shown in Table II, recovery tests were carried out with sera to which 1 to 15 mg/100 ml uric acid had been added. Mean recovery was 93.8% of the calculated theoretical values.

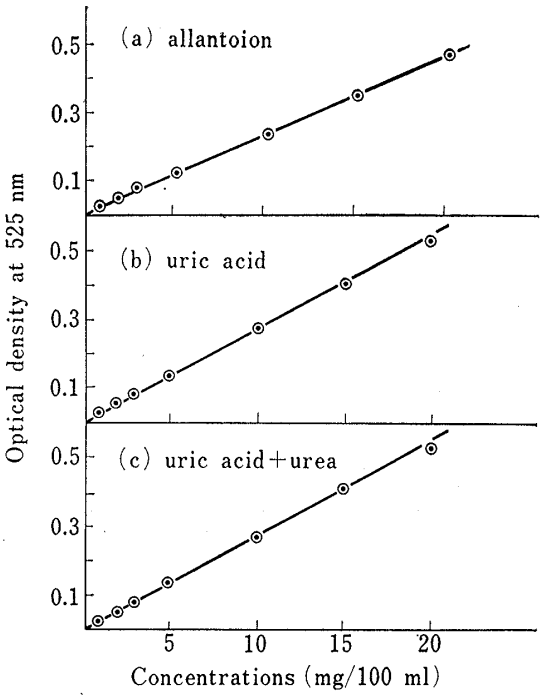

Fig. 2. Calibration Graphs of Allantion and Uric Acid

One hundred μl of standard solutions of allantoin (a) or uric acid (b, c) was mixed with 100 μl of water (a, b) or urea solution (150 mg urea nitrogen/l, c) and the mixtures were incubated with 100 μl of urease solution at 37° for 10 min. Subsequent procedures were carried out as described in "Materials and Methods."

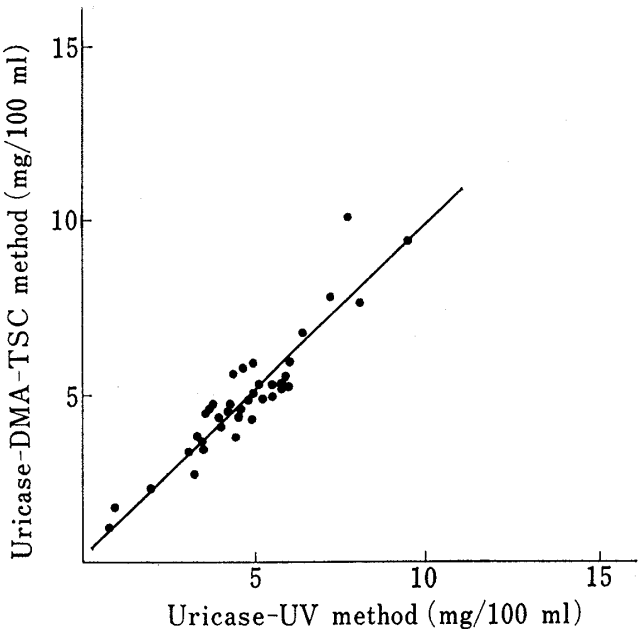

Fig. 3. Comparison with the Uricase-UV Method

Thirty-eight patients' sera were analyzed for uric acid by the method described in "Materials and Methods." The uricase-UV method was also carried out (uric acid determination set "Urica-Ace No. 2 EIKEN").
$n=38$, $r=0.930$, $y=0.954x+0.290$.

Table II. Recoveries of Uric Acid Added to Serum

Uric acid		Recovery (%)
Added mg/100 ml	Found mg/100 ml	
0	3.27	
1.0	4.23	96.0
2.0	5.19	96.0
5.0	7.92	93.0
10.0	12.7	94.3
15.0	16.7	89.5

Precision

Within-day precision was determined by testing normal pooled serum 10 times during 6 hours.

Day-to-day precision was evaluated by testing the same serum in triplicate each day for 10 days.

The results shown in Table III demonstrate that the proposed method has fairly good precision.

Effects of Ascorbic Acid, Glutathione, Glucose and Bilirubin

It was found that the proposed method was not interfered with at all by up to 160 mg/100 ml of ascorbic acid and 100 mg/100 ml of glutathione. Bilirubin (10 mg/100 ml) and

Table III. Precision of Uric Acid Determination by the Uricase–DAM–TSC Method

	Within-day	Day-to-day
Mean (mg/100 ml)	3.84	3.63
SD (mg/100 ml)	0.134	0.130
CV (%)	3.49	3.59

glucose (500 mg/100 ml) were also found to have no interfering effect in the determination of uric acid.

Discussion

One of the intrinsic problems associated with uric acid determination by a reductive method or enzymatic methods in which hydrogen peroxide is measured in some way is the interference by reducing substances in body fluids. To avoid this problem, alkaline conditions have been employed to destroy ascorbic acid in the reductive method[5] and ascorbic acid oxidase in the uricase method.[6] However, the influence of reducing substances other than ascorbic acid is still unavoidable. On the other hand, we have successfully avoided the problem of reducing substances by determining allantoin instead of hydrogen peroxide in the method proposed here. It was also found that neither bilirubin nor glucose interfered with our new method.

Various substances are known to interfer in the DAM–TSC reaction. The possible serum constituents which could interfere with the present method are arginine and citrulline, the blood levels of which are increased considerably in patients with rare metabolic diseases, hyperarginemia and citrullinemia, respectively. In these patients, the levels of both amino acids were found to be as high as 2.5 mmol/l,[7] high enough to give a strongly positive DAM–TSC reaction.[8] However, the levels of these amino acids in otherwise normal sera were found to be too low to give a false-positive reaction.[8]

According to a current hypothesis on the mechanism of uricase action, in the presence of borate, uricase produces urea, alloxanate and hydrogen peroxide instead of allantoin, carbon dioxide and hydrogen peroxide.[9] This implies that allantoin might be a poor choice as a target for determination of uric acid, because allantoin may not be produced quantitatively in the body fluids, which contain various ions. However, as far as we have tested, uric acid can be determined quantitatively with sera from various patients. This favorable result is supported by a good correlation coefficient and regression line obtained with patients' sera in comparison with the results of a standard uricase-UV method.

Notwithstanding the advantage mentioned above, the proposed method has some shortcomings that remain to be solved. The procedure is composed of many steps, such as enzymatic reactions, deproteinization, and heating for the color reaction. Of these steps, however, deproteinization could perhaps be deleted by using non-ionic detergents. Unfortunately, however, the serum blank in the presence of the detergents tested (Brij-35 and Triton X-100) was found to be higher than that observed in the normal procedure, so we are investigating the use of other detergents.

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6) S. Klose, M. Stoltz, E. Munz, and R. Portnhauser, *Clin. Chem.*, **24**, 250 (1978).
7) T. Gerritsen and A. Niederwieser, "Clinical Biochemistry: Principles and Methods," Vol. II, ed. by H.C. Curtius and M. Roth, Walter de Gruyter, Berlin and New York, 1974, p. 1064.
8) unpublished results.
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Although we have been trying to simplify the whole procedure so that it is acceptable as a routine manual method, we consider that the proposed method may be suitable for use in an automatic analyzer equipped with a dialyzer as well as a heating device. We are also pursuing this possibility.

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