A Statistical Method for the Determination of Serum Uric Acid[1]

Riichi Tawa, Yasuko Etoh, and Shingo Hirose

Kyoto College of Pharmacy[2]

A sensitive and accurate UV-assay procedure based on multiple linear regression analysis (MLR method) was developed for the measurement of uric acid with uricase. The absorbance at 290 nm of uric acid as a function of time was calculated from the color points of a mixture of uric acid, an intermediate (1-carboxy-2,4,6,8-tetraazabicyclo[3.3.0]octa-4-ene-3,7-dione) and the final product (allantoin) by the simplified complementary tristimulus colorimetry (SCTS method). The MLR method allowed the evaluation of both initial and final absorbances at the same wavelength with high accuracy. The average recovery of uric acid added to an aqueous solution was $99.9 \pm 0.02\%$, and the coefficient of variation was less than 3% ($n=4$) even at low concentration. The assay could also be performed directly on serum samples without deproteinization. An average recovery of $103.1 \pm 0.7\%$ was obtained for 0.17—1.33 mg/dl of uric acid in serum solutions, and the coefficients of variation of 0.32 mg/dl and 0.41 mg/dl were 1.6% ($n=4$) and 2.4% ($n=4$), respectively.

Keywords——serum uric acid; uricase; UV-assay; simplified complementary tristimulus colorimetry; multiple linear regression analysis

Numereous methods have been described in the literature for the assay of uric acid in serum.[3] The uricase-UV-assay is regarded as the simplest one because of the high specific activity of uricase for uric acid[4] (the apparent Michaelis constant, K_m, was calculated to be 8.06×10^{-6} M from Lineweaver–Burk plots in this study). However, an analytical difficulty arose: one of the transitory intermediates, 1-carboxy-2,4,6,8-tetraazabicyclo[3.3.0]octa-4-ene-3,7-dione (Chart 1), produced during the uricase reaction interferes with the assay of uric acid at 290 nm.[5]
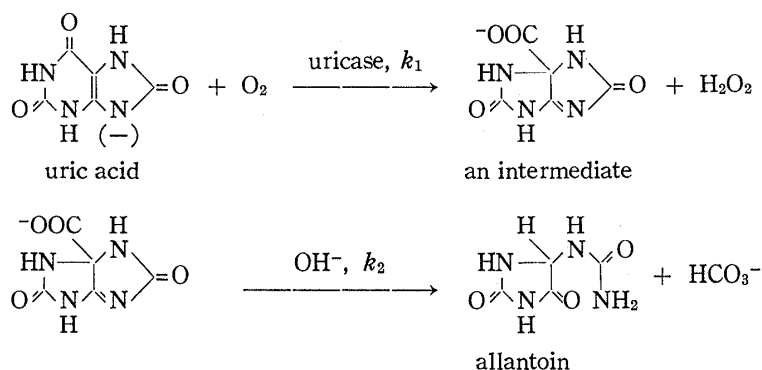

Chart 1

This paper describes a method to determine uric acid in serum without the removal of proteins, by means of two successive steps: (1) calculation of the absorbance at 290 nm of uric acid as a function of time by simplified complementary tristimulus colorimetry (SCTS

1) Presented at the 100th Annual Meeting of the Pharmaceutical Society of Japan, Tokyo, April, 1980.
2) Location: 5, *Nakauchi-cho, Yamashina-ku, Kyoto 607, Japan.*
3) P. Scheibe, E. Bernt, and H.U. Bergmeyer, "Methods of Enzymatic Analysis," Vol. 4, 2nd ed., by H.U. Bergmeyer, Verlag Chemie/Academic Press, Inc., New York, 1974, p. 1951.
4) Y. Nakagiri and T. Yamamoto, *Eisei Kensa,* 20, 751 (1971).
5) D.G. Priest and O.M. Pitts, *Anal. Biochem.,* 50, 195 (1972).

method)[6] and (2) computation of the initial and final absorbances based on a first-order reaction model by the use of multiple linear regression analysis (MLR method).[7] The advantages of the method presented are that it is accurate, selective and reproducible, does not require the use of reference blanks of serum and also allows direct comparison with known standards of uric acid.

Experimental

Materials——Uricase (grade III from *Candida utilis*, 3.1 units/mg) and uric acid were purchased from Toyobo Co. and E. Merck, respectively. A stock solution of uric acid was prepared according to the method of Henry.[8] A measuring kit using the uricase-catalase reaction (Uricolor–400®) was obtained from Ono Pharmaceutical Co. Pooled normal human sera were stored immediately at $-20°$ until use. All other reagents used were of analytical grade.

Apparatus——A Shimadzu UV-350 spectrophotometer equipped with a temperature-controlled cell holder, and a Hitachi-Horiba pH meter (type F-7) were used. Calculations of the complementary color points were made by the method described previously.[9] MLR method was carried out with a Hewlett-Packard HP9835A desktop computer (memory size 64 KB).

Determination of the Rate Constants——Since uric acid decays to allantoin by consecutive irreversible first-order kinetics, as depicted in Chart 1,[10] the rate constants, k_1 and k_2, can be determined by the use of Eqs. (1), (2) and (3).

$$[1]_t/[1]_0 = e^{-k_1 t} \tag{1}$$

$$[2]_t/[1]_0 = k_1(e^{-k_2 t} - e^{-k_1 t})/(k_1 - k_2) \tag{2}$$

$$[3]_t/[1]_0 = 1 + [(k_2 e^{-k_1 t} - k_1 e^{-k_2 t})/(k_1 - k_2)] \tag{3}$$

where $[1]_t$, $[2]_t$, $[3]_t$ and $[1]_0$ are the concentrations of uric acid, the intermediate and allantoin at time t and time zero, respectively.[11] Since $[1]_0 = [1]_t + [2]_t + [3]_t$, the mole fractions of uric acid, the intermediate and allantoin at time t can be calculated by the SCTS method easily and with good accuracy,[9] and k_1 and k_2 were determined by means of Eqs. (1), (2) and (3) with the use of these values.[6b] In the present study, k_1 and k_2 were calculated to be 0.134 ± 0.001 min^{-1} and 0.0497 ± 0.004 min^{-1} under the conditions used, respectively.

MLR Method——For the process of a first-order decomposition of uric acid, the absorbance at 290 nm is given by

$$A_t = A_\infty(1 - e^{-k_1 t}) + A_0 e^{-k_1 t} \tag{4}$$

where A_t, A_0 and A_∞ are the absorbances of uric acid at 290 nm at time t, time zero and infinite time, respectively. Since Eq. (4) is non-linear, the Taylor's series expansion is taken, with the usual nomenclature for the partial derivatives:[12]

$$\hat{A}_t = A_t^0 + (\partial A_t^0/\partial A_0)dA_0 + (\partial A_t^0/\partial A_\infty)dA_\infty + (\partial A_t^0/\partial k_1)dk_1 \tag{5}$$

$$= \beta_0 + \beta_1 P_1 + \beta_2 P_2 + \beta_3 P_3 \tag{5'}$$

where $\hat{A}_t$ is the predicted value with respect to the regression coefficients β_j's ($J = 0, 1, 2$ and 3), A_t^0 is A_t evaluated by Eq. (4) using the initial estimates of A_0, A_∞ and k_1, and P_j's are the independent variables which can be derived from the following equations, respectively.

$$P_1 = (\partial A_t^0/\partial A_0) = e^{-k_1 t} \tag{6}$$

$$P_2 = (\partial A_t^0/\partial A_\infty) = 1 - e^{-k_1 t} \tag{7}$$

$$P_3 = (\partial A_t^0/\partial k_1) = t(A_\infty - A_0)e^{-k_1 t} \tag{8}$$

6) *a*) R. Tawa and S. Hirose, *Talanta*, **26**, 237 (1979); *b*) Idem, *ibid.*, **27**, 759 (1980).

7) L.A. Currie, J.J. Filliben, and J.R. DeVoe, *Anal. Chem.*, **44**, 497R (1972); G.E. Mieling and H.L. Pardue, *ibid.*, **50**, 1611 (1978).

8) R.J. Henry, C. Sobel, and J. Kim, *Am. J. Clin. Path.*, **28**, 152 (1957).

9) R. Tawa and S. Hirose, *Chem. Pharm. Bull.*, **28**, 2136 (1980).

10) H.R. Mahler, H.M. Baum, and G. Hübscher, *Science*, **124**, 705 (1956); idem, *Biochim. Biophys. Acta*, **22**, 514 (1956).

11) A.A. Frost and R.G. Pearson, "Kinetics and Mechanism," 2nd ed., John Wiley and Sons, Inc., New York, 1961, p. 166.

12) D.M. Himmelblau, "Process Analysis by Statistical Methods," John Wiley and Sons, Inc., New York, 1968, p. 143.

For each concentration of uric acid, the absorbances at 290 nm were calculated by the SCTS method at regular intervals ($t = 1.0$ min) over the time range 0.5 to 33.5 min. The iterative procedure was then carried out based on Eqs. (5'), (6), (7) and (8). The values of dA_0, dA_∞ and dk_1 were calculated as the estimated shifts from the initial values of A_0, A_∞ and k_1, and the calculations were repeated for new corrections [e.g., $k_1(\text{improved}) = k_1(\text{previous}) + dk_1$]. We thus attempted to find the minimum of $\sum(\hat{A}_t - A_t)^2$.[13]

Assay Procedure——1.0 ml of uric acid solution (1.0—8.0 mg/dl) and 4.8 ml of 50 mM phosphate buffer (pH 8.5) were pipetted into a cuvette. The mixture was placed in a water bath at 37° for 10 min and then 100 μl of uricase solution (0.1 mg/ml) was added. The absorbances at three fixed wavelengths (u, v and w) were measured as a function of time against a blank solution. The serum samples were run in exactly the same manner after a preliminary 1:10 dilution of serum with distilled water.

Results and Discussion

The first-derivative curves of UV spectra were used for measurement of the appearance of the short-lived intermediate, because the curves were greatly preferable for identifying features.[14] As shown in Fig. 1, the curves indicate that the intermediate absorbs strongly at the 220—230 and 300—340 nm regions, and for the aqueous solutions, 315, 290 and 220 nm were chosen in the SCTS method. For serum samples, 315, 290 and 240 nm were chosen, as the serum protein made the absorbance at 220 nm difficult to measure. The color point, $Q_{m,tr}$ ($r = u$, v and w), of a binary mixture consisting of the intermediate and allantoin at any time (m_t) was calculated by using the color points of a reaction mixture of uric acid, the intermediate and allantoin at the same time (M_t) and of uric acid under the conditions described
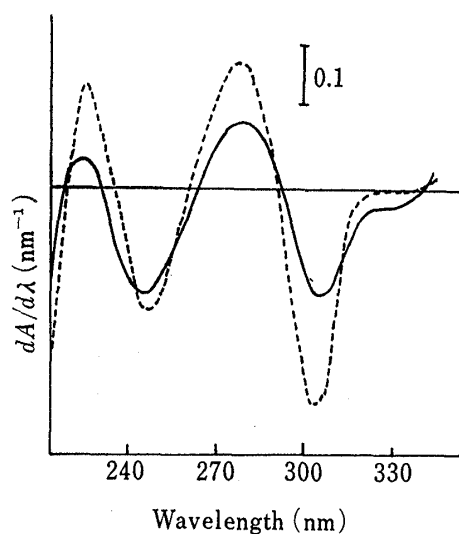

Fig. 1. First–Derivative Spectral Changes during the Oxidation of Uric Acid to Allantoin

————: initial; ————: after 10 min.
The reaction solution consisted of 8.7×10^{-5} M uric acid and 0.009 unit of uricase in a final volume of 3.4 ml. The experiment was carried out at pH 8.5 (50 mM phosphate buffer) and 25°.
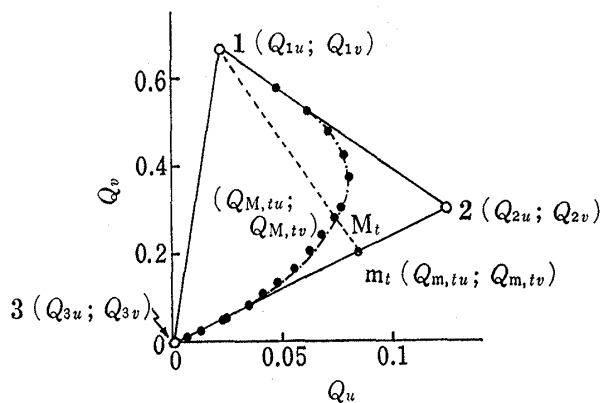

Fig. 2. Q_u—Q_v Plot for the Oxidation of Uric Acid to Allantoin by Uricase

Complementary color point: 1: uric acid; 2: the intermediate; 3: allantoin; M_t: reaction mixture of 1, 2 and 3 at time t; m_t: reaction mixture of 2 and 3 at the same time. The procedures are described in the legend to Fig. 1. The following wavelengths were chosen: u: 315 nm; v: 290 nm; w: 220 nm.

previously, $Q_{M,tr}$ and Q_{1r}, (Fig. 2).[9] On the other hand, $Q_{m,tr}$ is given by the following relationship.

$$(A_{M,tr} - A_{1,tr})/(J - X) = Q_{m,tr} \tag{9}$$

13) H.O. Hartley, Technometrics, **3**, 269 (1961).
14) L.J. Saidel, Arch. Biochem. Biophys., **54**, 185 (1955).

where $A_{M,tr}$ and J are the absorbance of M_t corresponding to wavelength r and the sum of $A_{M,tr}$ ($r=u$, v and w), respectively, and $A_{1,tr}$ and X are the absorbance of uric acid at the same wavelength at time t and the sum of $A_{1,tr}$ ($r=u$, v and w). Since Q_{1r} is a known constant, being independent of the concentration of uric acid under the conditions used, and can be calculated at any given concentration by means of the following equation,

$$A_{1,\ tr}/X = Q_{1r} \tag{10}$$

the values of $A_{1,tr}$ as a function of time could be determined by means of Eqs. (9) and (10).

TABLE I.　Comparison of Observed and Predicted
Absorbances at 290 nm, A_t and $\hat{A}_t$, calculated
by the MLR Method[a]

Time (min)	A_t	$\hat{A}_t$	Δ[b]
0.5	1.244	1.254	−0.010
1.5	1.154	1.185	−0.031
2.5	1.055	1.059	−0.004
3.5	0.863	0.846	0.017
4.5	0.773	0.775	0.018
5.5	0.693	0.674	0.019
6.5	0.616	0.601	0.015
7.5	0.550	0.550	0.000
8.5	0.486	0.477	0.009
9.5	0.429	0.424	0.005

a) The reaction solution consisted of 1.19×10^{-4} M uric acid and 0.0062 unit of uricase in a final volume of 5.0 ml. The experiment was carried out at pH 8.5 (50 mM phosphate buffer) and 37 $\pm 0.1°$.
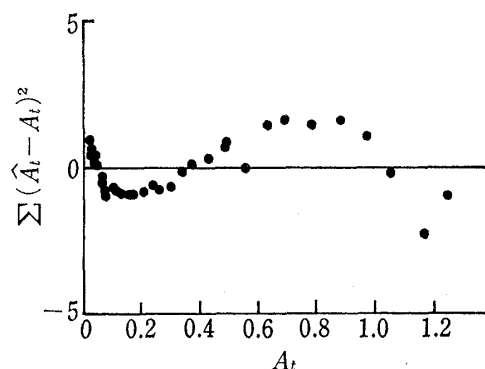b) $\Delta = A_t - \hat{A}_t$.

Fig. 3.　Standardized Residuals Plots for A_t

The values of $\sum (A_t - \hat{A}_t)^2$ were calculated by using the results in Table I.

The best fit of the absorbances at 290 nm *versus* time as calculated by the MLR method based on Eq. (4) was obtained for each concentration of uric acid. A typical result is shown in Table I and Fig. 3. The standard errors, $\sigma\hat{A}_0$, $\sigma\hat{A}_\infty$ and $\sigma\hat{k}_1$, of the regression values, $\hat{A}_0$, $\hat{A}_\infty$ and $\hat{k}_1$, were less than 0.034, 0.227 and 0.079, respectively, for all concentrations of uric acid studied. The standard errors of $\hat{A}_t$ were less than 0.002. With an appropriate standard curve, the analyte concentration of uric acid was evaluated as follows:

$$\delta\hat{A} = \hat{A}_0 - \hat{A}_\infty \tag{11}$$

The average recovery of uric acid added to an aqueous solution was $99.9 \pm 0.02\%$ in the concentration range tested (0.17—1.33 mg/dl). The coefficients of variation determined at two different concentrations, 0.40 mg/dl and 1.33 mg/dl, were 1.3% ($n=4$) and 2.4% ($n=4$), respectively. For serum samples, the average recovery of uric acid was $103.1 \pm 0.70\%$, and the coefficients of variation determined at levels of 0.32 mg/dl and 0.41 mg/dl were 1.6% ($n=4$) and 2.4% ($n=4$), respectively, under the assay conditions given.

Statistical analysis for 50 sera samples involving concentrations of uric acid from 3.0 mg/dl to 12 mg/dl showed that the present method performed well compared to the uricase-catalase method.[15] The regression equation was $y=1.07\ x-0.02$, where y is the results of the present method and x is the results of the uricase-catalase method; the coefficient of correlation was 0.998.

15) N. Kageyama, *Clin. Chim. Acta*, **31**, 421 (1971).