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The Analysis of Serum Urate Utilizing Immobilized Uricase

JANET S. COLLISS* AND JOHN M. KNOX

Department of Clinical Chemistry, Worthing Hospital, Worthing, Sussex, England

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Abstract

The evaluation of a method for the estimation of serum urate using immobilized uricase is described, the resultant hydrogen peroxide produced being measured by the oxidative coupling of 3,5-dichloro-2-hydroxybenzenesulfonate and 4-aminophenazone in the presence of peroxidase.

A continuous-flow analysis system incorporating the uricase tube was established, and the results obtained were correlated with an automated phosphotungstate method and with a manual uricase method employing an LKB 8600 Rate Reaction Analyser. The effect of ascorbic acid on the analysis of serum urate and the elimination of this interference by the use of ascorbate oxidase was also investigated.

The precision, correlation, and high specificity obtained show that this is a satisfactory method for use in routine clinical laboratory works.

Index Entries: Uricase, immobilized; serum urate analysis; urate analysis by immobilized uricase; ascorbic acid, interference in serum urate analysis.

Introduction

Methods curently available for estimating serum urate employ either chemical or enzymatic oxidation to allantoin. In chemical methods a chromogen is reduced at the same time to yield a chromophore that is measured spectrophotometrically.

The most common method is based on the reduction of an alkaline phosphotungstate complex first described in 1912 by Folin and Denis (*1*). Many variations of this basic method have been developed, but all chemical

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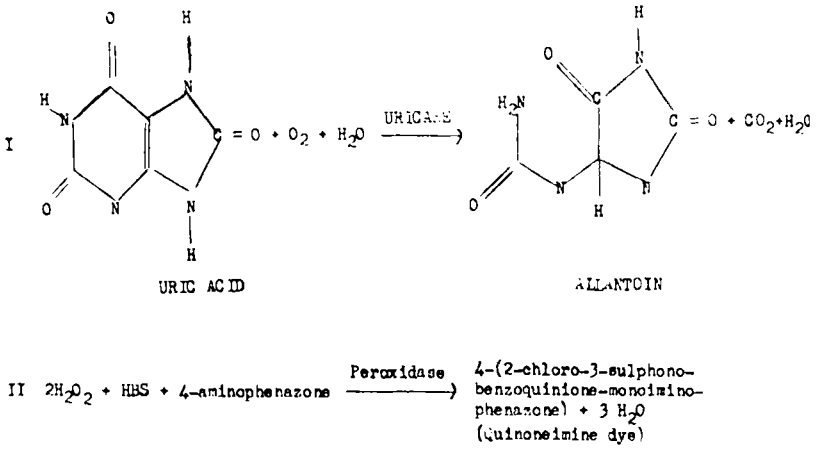

FIG. 1. Principle of serum urate analysis.

reduction methods suffer from lack of specifity, i.e., interference from other reducing agents in serum.

In the enzymatic methods, uricase (urate: oxygen oxidoreductase, EC1.7.3.3) is used to catalyze the reaction, the concentration of urate being determined by direct spectrophotometry by following the change in absorbance at 293 nm, or by measuring the amount of hydrogen peroxide produced in conjunction with a colorimetric reaction.

Measurement of the hydrogen peroxide produced following enzymatic oxidation of serum urate by uricase has been described by Lorentz and Berndt (*2*) and Domagk and Schlicke (*3*) who used peroxidase and *o*-dianisidine on uricase-treated protein filtrates. Kageyama (*4*) coupled the peroxidase formed with catalase and methanol, producing formaldehyde that was measured colorimetrically as the lutidine derivative, and Gochman and Smitz (*5*) described an automated procedure for measuring uricase-generated hydrogen peroxide by oxidative coupling of 3-methyl-2-benzothiazolinone hydrazone and *N,N*-dimethylaniline in the presence of peroxidase. Hornby et al. (*6*) in 1972 described the preparation of nylon powder-supported urate oxidase for the automated determination of uric acid. Sundaram et al. (*7*) described the use of an immobilized nylon tube reactor with high precision and low cost.

In the method described, urate is oxidized by uricase to allantoin plus hydrogen peroxide (I). The hydrogen peroxide is then reduced by peroxidase (EC1.11.1.7) coupled with another oxidative reaction involving 4-aminophenazone and 3,5-dichloro-2-hydroxybenzenesulfonate (HBS) (II). The resultant quinoneimine dye is measured spectrophotometrically at 505 nm (Fig. 1).

Materials and Methods

Instrumentation

1. A continuous flow automated analysis system was used for the immobilized uricase method with the following modules:

Chemlab CS40 sampler (Chemlab Instruments Ltd., Hornminster House, Hornchurch, Essex, UK)
CPP 15 proportioning pump
24-in dry block dialyzer
Chemlab colorimeter
Servoscribe recorder

2. SMA PLUS system (Technicon Instruments Co. Ltd., Hamilton Close, Houndmills, Basingstoke, UK) incorporating Technicon method number SF-0013FJ5 for the estimation of serum urate.
3. LKB 8600 reaction rate analyser (LKB Clinicon Systems Ltd., Bell Lane, Lewes, East Sussex, UK) utilizing the Uricaquant kit method (Cat. No. 124761) for the enzymatic determination of serum urate (Boehringer Corporation Ltd., Bell Lane, Lewes, East Sussex, UK).
4. Nylon tube-immobilized uricase. The enzyme tube (Miles Laboratories Ltd., Stoke Court, Stoke Poges, Slough, UK) is a 1-mm internal diameter nylon tube with uricase covalently bound to the inner surface; 50-cm lengths of tube were used and were stored at + 4°C filled with EDTA/tris buffer, pH 9.2, when not in use.

Reagents

All chemicals were analytical grade (unless otherwise stated) (British Drug Houses Ltd., Poole, Dorset, UK).

Saline. (150 mmol/L). 9 g of sodium chloride are dissolved in 800 cm^3 of deionized water. 2 cm^3 of Brij-35 surfactant are added (300 g/L solution) and the solution is diluted to 1 L with deionized water.

HBS buffer. (25.0 mmol/L glycine, 0.20 mmol/L EDTA, 6.5 mmol/L HBS). 1.875 g glycine, 0.744 g EDTA disodium salt, and 2.1 g HBS disodium salt (supplied by Miles Laboratories Ltd.) are dissolved in 800 cm^3 of deionized water. The pH is adjusted to 9.2 with solid sodium carbonate, and 2.0 cm^3 of Brij-35 surfactant are added. The solution is made up to 1 L with deionized water. The reagent is stable at room temperature for at least 3 weeks and at 4° C for at least 6 weeks.

Peroxidase/4-aminophenazone reagent. (potassium phosphate, pH 6.5, 0.2 mmol/L, EDTA disodium salt 12.5 mmol/L, 4-aminophenazone 1.2 mmol/L, peroxidase > 3000 U/L). Supplied as a solid reagent by Miles Laboratories Ltd. Dissolved in 1 L of deionized water for use, and 1 cm^3 of Brij-35 surfactant is added.

Urate standards. Aqueous standards (Preciset) supplied by the Boehringer Corporation Ltd. were used with urate concentrations of 0.12, 0.24, 0.36, 0.48, 0.60, and 0.72 mmol/L. An aqueous drift control (Precimat) supplied by the Boehringer Corporation Ltd. was used.

Ascorbate oxidase (EC1.10.3.3). Specific activity = *c* 1000 U/mg obtained from the Boehringer Corporation Ltd.

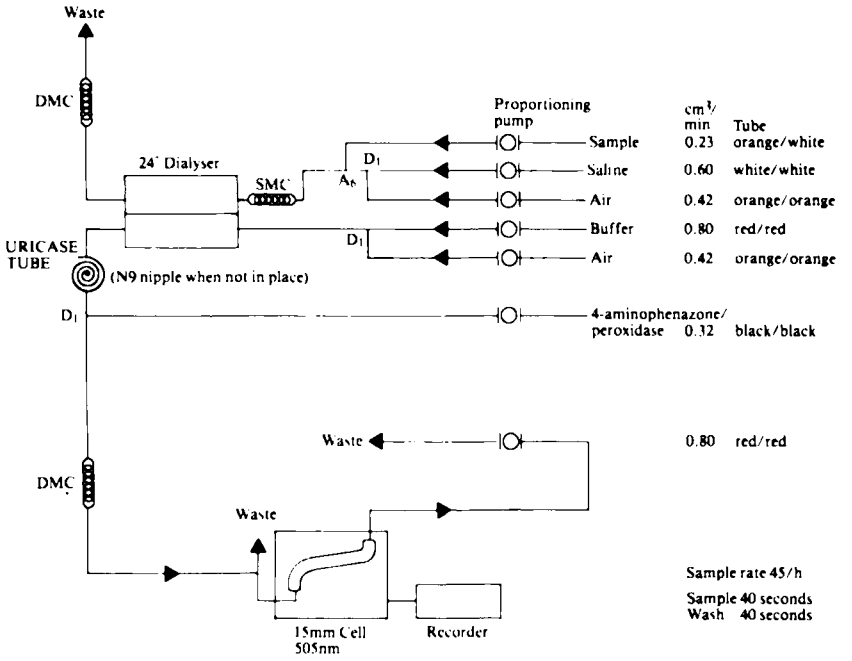

FIG. 2. Flow diagram for determination of serum urate using immobilized uricase.

Procedure

The manifold was prepared according to the flow diagram shown in Fig. 2. The uricase tube was inserted prior to operation; the reagents were then pumped and the baseline adjusted to zero on the recorder chart. Aqueous urate standards were sampled in ascending order, followed by serum samples and controls. After the run, the system was washed through with deionized water for 10 min, the uricase tube removed, and the N9 nipple replaced before lifting the platen. The uricase tube was filled with EDTA/tris buffer, pH 9.2, and stored at + 4°C.

Results

A typical recorder tracing obtained using the immobilized uricase method is illustrated in Fig. 3 showing aqueous standard peaks and serum sample peaks.

Linearity

The linearity of the immobilized uricase method is shown in Fig. 4, in which absorbance (peak height) is plotted against urate concentration in mmol/L. Linearity exists from 0 to at least 0.72 mmol/L; samples with higher urate levels should be diluted with physiological saline (150 mmol/L) into this range.

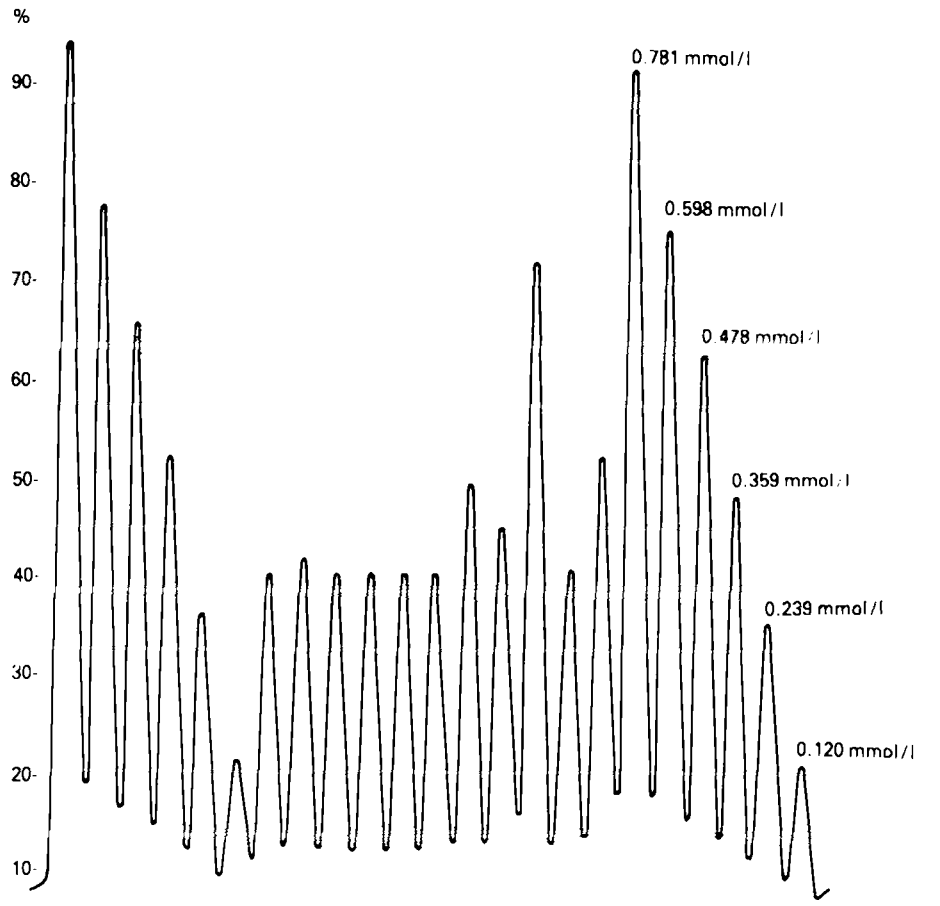

FIG. 3. Recorder tracing obtained using the immobilized uricase method for serum urate.

Recovery

The analytical recovery of the immobilized uricase method was determined by adding accurately measured quantities of urate standard solutions to a previously assayed pooled human serum. These samples were then analyzed and the recoveries obtained ranged from 87 to 93%, apparently being greater at higher levels. The results are shown in Table 1.

Comparison Studies

A number of patients' samples were analyzed for serum urate by the immobilized uricase method, the SMA PLUS method, and the Uricaquant-LKB method. The correlation and regression lines are shown in Figs. 5 and 6.

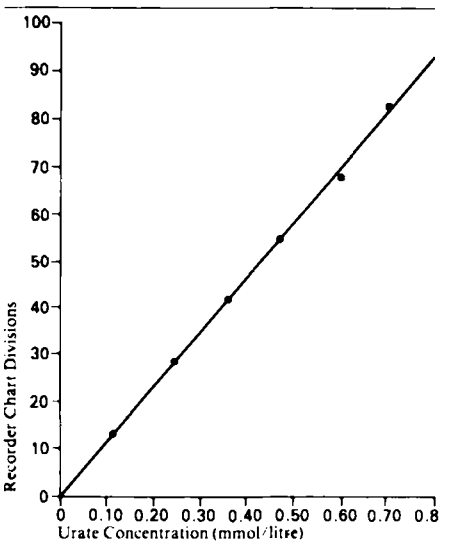

FIG. 4. Absorbance vs concentration plot of aqueous urate standards using the immobilized uricase method.

TABLE 1
Analytical Recovery of Added Urate from Serum Using the Immobilized Uricase Method

Added urate concentration, mmol	Recovery, %
0.12	87
0.24	89
0.36	91
0.48	92
0.60	91
0.72	93

Sample Interaction

The carry-over coefficient was calculated according to the method of Broughton et al. (*8*). When using three samples with a urate concentration of 0.72 mmol/L followed by three samples with a urate concentration of 0.12 mmol/L, this coefficient was found to be 1.69%.

Precision

Within-run precision was determined at three levels using pooled samples with low, intermediate, and high urate concentrations. The results are shown in Table 2.

Between-batch precision was determined using a commercially prepared

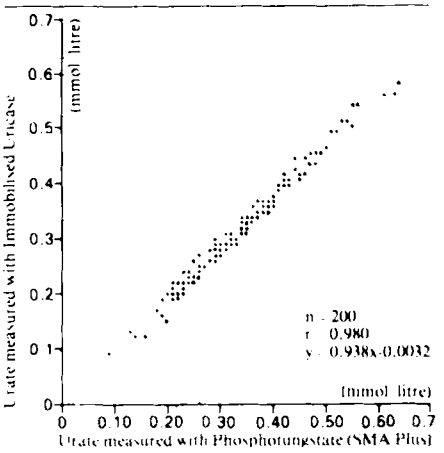

FIG. 5. Correlation and regression line for serum urate by immobilized uricase against serum urate by phosphotungstate (SMA Plus).

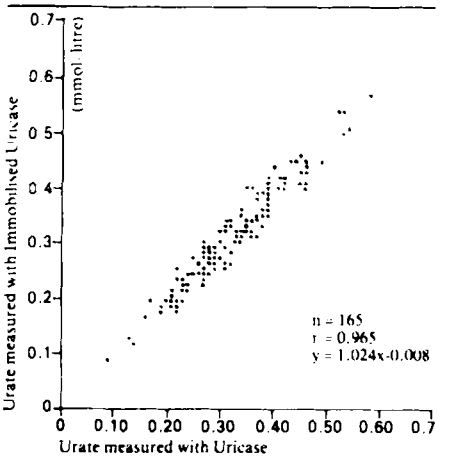

FIG. 6. Correlation and regression line for serum urate by immobilized uricase against serum urate by uricase (Uricaquant-LKB).

TABLE 2
Within-Run Precision of the Immobilized Uricase Method, Performed by Analysis of Serum Samples with Low, Intermediate, and High Urate Concentrations

	Low	Intermediate	High
Mean (mmol/L)	0.290	0.390	0.576
SD	1.90×10^{-3}	1.03×10^{-3}	5.70×10^{-3}
CV	0.65	0.26	0.99
n	20	20	20

TABLE 3
Day-to-day Precision of the Immobilized Uricase Method Performed by Daily Analysis of an Assayed Quality Control Serum

Mean (mmol/L)	0.402
SD	6.88×10^{-3}
CV	1.72
n	25

lyophilized control serum. Table 3 summarizes the results, the between-batch coefficient of variation being 1.72%.

Interference Studies

Interference of ascorbic acid with serum urate determinations using immobilized uricase was investigated by adding increasing amounts of ascorbic acid to two serum pools with high and low urate concentrations. Because of the short half life of ascorbic acid, the effect of its interference was investigated over a period of three days, the two pools of control sera containing varying amounts of ascorbic acid being assayed for urate on three consecutive days. In addition, on day 1, the experiment was repeated replacing the donor stream by a 50 mmol/L phosphate buffer containing varying concentrations of ascorbate oxidase. The percentage inhibition by ascorbic acid was calculated in each case, and the results are shown in Table 4.

Correlation studies were also carried out with and without ascorbate oxidase incorporated in the donor stream. The results are shown in Table 5.

Discussion

This paper describes a simple, specific automated method for the determination of serum urate. Although the recoveries obtained were not as high as might optimally have been expected, both within-run and day-to-day precision were good. The method may also be used for urine samples if the urine is first prediluted with physiological saline.

Continuous running of the system indicated that the uricase tube could be used for approximately 3500 analyses, after which the limiting factor seemed to be the lack of sensitivity of the equipment rather than loss of activity of the enzyme tube. There is, however, a problem with the storage of the tube when used for relatively short routine runs, an appreciable loss of sensitivity occurring after 1000–2000 assays. The cause of this has yet to be determined.

The main advantage of a uricase method over a phosphotungstic acid method is its specificity. Many drugs and reducing substances are known to produce falsely elevated values for serum urate measured with phosphotungstic acid, but uricase methods are less affected by these interfering substances. However, Gochman and Schmitz (*5*) observed that some reducing

TABLE 4
Percentage Inhibition of Serum Urate Measured with Immobilized Uricase by Ascorbic Acid over Two Days and the Effect of Including Ascorbate Oxidase in the Donor Stream[a]

Ascorbic acid concentration, mmol/L	% Inhibition with no added ascorbate oxidase					
	0 h		24 h		48 h	
	Low	High	Low	High	Low	High
0.5	83	72	47	51	19	36
0.2	43	33	10	14	7	6
0.1	22	17	2	4	2	2

Effect of addition of ascorbate oxidase, % inhibition at 0 h

Ascorbic acid concentration, mmol/L	Ascorbate oxidase, U/mL					
	1.0 U		0.5 U		0.3 U	
	Low	High	Low	High	Low	High
0.5	0	2	2	3	7	6
0.2	0	0	2	0	3	0
0.1	0	1	0	1	6	0

[a]Pooled serum assayed values: low 0.31 mmol/L; high 0.50 mmol/L.

TABLE 5
Correlation between the Immobilized Uricase Method and the Phosphotungstate Method

No ascorbate oxidase:	$y = 0.938x - 0.0032$; $r = 0.980$
With ascorbate oxidase:	$y = 1.088x - 0.0698$; $r = 0.972$

substances produced an apparent depression of serum urate values when measured with uricase, notably ascorbic acid. Klose et al. (9) examined the interference of 32 drugs and some anticoagulants on a uricase method and found a minor interference by α-methyldopa; there was no interference by hemoglobin, bilirubin, or lipemia and 0.5 U/cm^3 of ascorbate oxidase included in the donor stream was used to eliminate any ascorbic acid present in the sample.

From the interference studies carried out, allowing for within and between-batch variation, it appears that 1.0 U/cm^3 of ascorbate oxidase would be the ideal concentration required to be included in the donor stream for sera containing high ascorbic acid levels, but in routine conditions 0.5 U/cm^3 would be acceptable. We consider that in most cases it is not necessary to include

ascorbate oxidase in the donor stream, especially in the routine laboratory where serum is frequently left for a day or more before urate analysis. In addition, the correlation between the immobilized uricase method without ascorbate oxidase in the donor stream and the phosphotungstate method was comparable with the immobilized uricase method with ascorbate oxidase in the donor stream and the phosphotungstate method.

Ascorbic acid (0.1 mmol/L) is probably the highest physiological concentration one would expect in serum; Klose et al. (*9*) stated in their work that 193 μmol/L would be the expected level after oral doses of 2 g of ascorbic acid daily for nine days. In our studies, the interference effect of serum ascorbic acid levels of up to 0.5 mmol/L were studied, and it was found that where the level of ascorbic acid was over 0.2 mmol/L, there was a distortion in the peak shape.

With the regression lines produced and the correlation coefficients of $r = 0.980$ between the immobilized uricase and phosphotungstate methods and $r = 0.965$ between the immobilized uricase and Uricaquant methods, it was felt that the immobilized uricase method could readily be adopted for routine use in the laboratory. The Uricaquant method was not adopted owing to its apparent lack of precision. This is shown by the wide scatter of points in Fig. 6 and a between-batch CV of 4.0%. The sensitivity of the equipment, which is the limiting factor on the life of the enzyme tube, would need to be considered when evaluating the running costs of the immobilized uricase method.

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