QUANTIFICATION OF URINARY URATE WITH ALKYLAMINE GLASS-BOUND URICASE AND ARYLAMINE GLASS-BOUND PEROXIDASE

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RUNNING TITLE: Determination of urinary urate

ABSTRACT

Uric acid in urine was analyzed using porcine liver uricase and horseradish peroxidase immobilized on alkylamine and arylamine glass beads (pore diameter 55nm) respectively. The minimum detection limit was 5.0mg/0.1 ml urine. The recovery of added uric acid was 92%. Within and between assay CVs were <1.3% and <5.3% respectively. A good correlation ($r=0.93$) was found between urinary urate values obtained by a commercial kit method and the present method. The uric acid in 24hr urine of apparently healthy adults and person suffering from various diseases was found to be 450 to 900mg/24hr, 659mg/24hr (range, mean) and 910-1400mg/24hr, 1145mg/24hr (range, mean) respectively with the present method.

KEY WORDS

Urate, uricase, peroxidase, urine, alkylamine glass, arylamine glass

INTRODUCTION

Measurement of uric acid in serum and urine is required for the diagnosis and medical management of gout, leukemia, toxemia of pregnancy, severe renal impairment and calcium urate nephrolithiasis (1). Among the various methods available for measurement of urate such as chemical (2), colorimetric (3), mass fragmentography (4), radiochemical HPLC method (5), biosensor based on chemiluminescence (6), the enzymic colorimetric method employing uricase and peroxidase is comparatively more simple, sensitive, specific and hence suitable for the routine. However, the method becomes expensive, when used for a large number of samples due to the requirement of bulk quantity of the enzymes (7). The immobilization of the enzyme onto insoluble support permits its reuse and thus reduces the cost of the procedure for large number of samples. Uricase from different sources has been immobilized on dextran, polyethylene glycol (8), glass wall (9), nylon tubing (10),

polyamide tubing (11), elastin (12), cellulose acetate membrane (6), Hornvlede (13) and alkylamine glass beads (14) for determination of uric acid in biological fluids. Among these various supports available for immobilization of enzymes, alkylamine and arylamine glass, in general, are resistant to the microbial attack, stable over a wide pH range and in various solvents such as ethanol and acetone and possess a long working life. In our laboratory, we have immobilized a commercially available porcine liver uricase onto alkylamine glass and horseradish peroxidase onto arylamine glass and employed them for determination of uric acid in serum (15). We have also examined the usefulness of alkylamine glass bound uricase in determination of uric acid in urine (14).

The present report describes a method for discrete analysis of uric acid in urine with both alkylamine glass-bound uricase and arylamine glass-bound peroxidase.

MATERIALS AND METHODS

Chemicals

Zirconia coated alkylamine glass beads (pore diameter 55nm) and arylamine glass beads (pore diameter 55nm) (Corning Glass Works, New York); porcine liver uricase, type 1, suspension in 2.0 M

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(NH₄)₂SO₄ (2.0 U/ml), peroxidase from horseradish (RZ 1.1), and 4-aminophenazone (SIGMA, USA), *p*-hydroxybenzoic acid (E. Merck) and glutaraldehyde (24%) (BDH, UK) were used.

Immobilization of uricase

The (NH₄)₂SO₄ suspension of uricase was dialyzed overnight against distilled water under continuous stirring at 4°C to remove NH₄⁺ ions. The dialyzed enzyme was immobilized onto alkylamine glass beads through glutaraldehyde coupling as described by Bhargava *et al* (15).

Immobilization of peroxidase

The lyophilized powder of horseradish peroxidase was dissolved in 0.01M sodium phosphate buffer (pH 7.0) and immobilized on arylamine glass through diazotization, as described by Pundir *et al* (16).

Preparation of standard curve for urate with immobilized uricase and peroxidase

In a 15ml conical flask wrapped with black paper, the reaction mixture containing 1.9ml 50mM glycine-NaOH buffer, pH 8.8 and 200mg alkylamine glass beads bound to uricase (4 mg/100 mg support), were preincubated at 40°C for 2 min. The reaction was started by adding 0.1ml uric acid solution containing varying concentrations of uric acid (10 to 80µg/0.1ml). After incubation at 40°C for 5 min under continuous stirring, 0.2ml reaction mixture was withdrawn from reaction flask leaving the glass beads by Eppendorff pipette carefully and transferred into another 15ml conical flask wrapped with black paper and containing 2.6ml 100mM sodium phosphate buffer, pH 7.0, 0.1ml 100mM 4-aminophenazone, 0.1ml 25mM *p*-hydroxybenzoic acid and 100mg arylamine glass beads coupled to peroxidase (28 mg/g support). After incubation at 40°C for 10 min under continuous stirring, the reaction mixture was transferred into a cuvette with the help of Eppendorff pipette. A₅₂₀ was read in Spectronic-20 (Milton & Roy, USA). A standard curve was plotted between urate concentration ranging from 10 to 80 µg/0.1ml and A₅₂₀ (Fig. 1).

Determination of urinary urate

24 hr urine from the apparently healthy male adults were collected in 2L plastic bottle containing 15 ml conc. HCl. The acidified urine was diluted with distilled water in 1:4 ratio and its pH was adjusted to 7.0 by adding NaOH. The assay of urinary urate

was carried out as described for the preparation of standard curve, except that uric acid solution was replaced by 0.1 ml pre-treated urine. The value of urinary urate was extrapolated from the standard curve of uric acid vs. A₅₂₀ (Fig 1).

Reusability and storage of immobilized enzymes

To reuse the alkylamine glass-bound uricase and arylamine glass-bound peroxidase, the glass beads were washed off with their respective reaction buffers 3-4 times prior to the use in next assay. During each washing, the buffer was withdrawn by Eppendorff pipette to avoid the loss of glass beads. The glass beads were stored in distilled water at 4°C, when not in use.

RESULTS AND DISCUSSION

Commercially available uricase from porcine liver and peroxidase from horseradish were immobilized covalently on alkylamine and arylamine glass beads with respective conjugation yields of 16.5mg/g and 15.4mg/g with 83.3% and 72% retention of initial specific activity (Table 1). Alkylamine conjugated uricase showed maximum activity at pH 8.8 in 0.05M glycine-NaOH buffer, when incubated at 40°C for 25 min; while arylamine conjugated peroxidase had optimum activity at pH 7.5 in 0.05M sodium phosphate buffer, when incubated at 40°C for 25 min.

An enzymic colorimetric method for discrete analysis of oxalate in urine has been developed employing alkylamine glass bound uricase and arylamine glass bound peroxidase under their optimal assay conditions. The method is based on measurement of H₂O₂, generated from urinary urate by immobilized uricase, with a colour reaction consisting of 4-aminophenazone, *p*-hydroxybenzoic acid and immobilized peroxidase as chromogenic system. The following analytic parameters were determined to evaluate the method:

Linearity

A linear relation was obtained between the uric acid concentration (ranging from 10-80 µg/2.0 ml reaction mixture) and colour intensity at 520nm upto an absorbance of 0.16 (Fig 1). The lower detection limit of the method is 5mg uric acid/ 0.1ml urine, which is lower than earlier methods such as centrifugal fast analyzer using soluble (50µg/0.1 ml) (17) but higher than by auto-analyzer employing immobilized uricase (0.22mg/0.1 ml) (13).

Recovery

The mean analytical recoveries of added solid urate in urine (200 mg/dL and 400 mg/dL) in urine samples were $93.1 \pm 8.3\%$ (Mean $\pm$ S.D.; n=6) and $91.58 \pm 10.1\%$ (Mean $\pm$ S.D.; n=6) (Table 2).

Precision

To assess the reproducibility of the method, uric acid contents in the same urine sample in one run (within batch) and after one week of storage at -20°C (between the batch) were determined. The results given in Table 3 show that uric acid concentrations agreed with each other and within and between day coefficients of variation (CV) were $<1.3\%$ and $<5.3\%$ respectively, indicating the reproducibility of the method.

Uric acid values in 24 hr urine

The uric acid concentration in 24hr urine of apparently healthy adults, as measured by the present method, was found in the range 450 to 900 mg/24 hr with a mean of 659 mg/24 hr (Table 4) which is in good agreement with those reported by other methods such as centrifugal fast analyzer (695 mg/24 hr) and continuous flow analyzer (500 mg/24 hr), employing free and immobilized uricase respectively (17 & 18).

The uric acid in 24 hr urine of the patients of gout, leukemia, toxemia of pregnancy and renal impairment, as determined by the present method, was found in the range 910 – 1400 mg/24 hr with a mean of 1145 mg/24 hr (Table 2).

Accuracy

To test the accuracy of the method, we determined the urinary urate values of 20 urine samples of both healthy and diseased persons by the present method (y) and correlated with those obtained by the commercial enzymic colourimetric method supplied by M/s Miles India Ltd. Baroda (x). The uric acid values determined by the present method (y) showed good correlation ($r = 0.993$) with the commercial method (x), the regression equation being ' $y = 0.842x + 86.3$ ' (Fig 2).

Interference study

To test the interference of various urinary ions and metabolites in our method, following compounds were added in the reaction mixture of uricase individually, each at a final concentration of 1mM: NaCl, KCl, KI, MnCl_2 , KNO_3 , FeSO_4 , NaHCO_3 , ZnSO_4 , FeCl_3 , CuSO_4 , pyruvate, glutamate, glucose, fructose, citrate, cysteine, glycine, oxalate, ascorbate, NAD, NADH, bilirubin, creatinine, albumin, Hb, urea, cholesterol and xanthine. Among these compounds, only ZnSO_4 , bilirubin, ascorbate, glutamate and xanthine caused 70%, 65%, 60%, 52% and 40% inhibition of the immobilized uricase respectively. The interference by ascorbate and bilirubin was removed by adding sodium nitrate (35 mg/100ml) and potassium ferrocyanide (40 mg/100ml) respectively in the sample, prior to measurement of urate as suggested (7).

Reusability and storage

Alkylamine glass bound uricase lost 41% of its initial activity after its 200 uses over a period of 6 months when stored in distilled water at 4°C while arylamine glass bound peroxidase lost only 19% of its initial activity for the similar uses under the similar storage conditions (14 & 15). The net cost (including the losses of enzyme activity due to repeated uses) of 200 urinary urate determinations by the present method was calculated and found to be US\$ 48.28, which is 53.4% less than that of Sigma kit method for single reagent system which cost US\$ 90.40 for 200 estimations. Further the present method avoids the use of costly equipment and likely to be adopted by developing countries where clinical laboratories are not well equipped.

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Fig. 1 Standard curve for uric acid plotted between urate concentrations in reaction mixture of uricase vs. A_{520} .

Fig. 2 : Correlation between results by the present method and assigned values for proficiency test samples

Table 1. Immobilization of porcine liver uricase and horseradish peroxidase onto alkylamine glass and arylamine glass beads (Pore diameter 55nm)

Enzyme	Enzyme added to 200mg glass beads (mg)	Enzyme coupled to 200mg glass beads (%)	Total activity bound (Units)	Conjugation yield (mg/g)	% Retention of initial activity
Uricase	5.0	66	0.46	16.5	83.3
Peroxidase	4.0	77	2.25	15.4	72.0

One unit of enzyme is defined as the amount of enzyme protein required to produce one mmole H_2O_2 /min for uricase utilized and one mmole H_2O_2 /min for peroxidase under the standard assay conditions.

Table 2. Analytical recovery of added uric acid in urine as measured by alkylamine uricase and arylamine peroxidase

Uric acid added (mg/24 hr)	Uric acid found (mg/ 24hr) (mean)	% Recovery (mean $\pm$ SD) (n = 6)
Nil	670	—
200	856	93.1 $\pm$ 8.3
400	1036	91.58 $\pm$ 10.1

Table 3. Within and between assay coefficients of variation of uric acid determination in urine, as determined by alkylamine glass bound uricase and arylamine glass bound peroxidase.

N	Uric acid (mg/24 hr) (mean)	CV%
Within assay ^a		
6	539.4	<1.3
Between assay ^b		
6	564.6	<5.3

^a Samples assayed on the same day.

^b Same samples assayed after one week storage at -20°C.

Standard assay conditions were used in each assay, the data shown are the mean of six observations.

Table 4. Urinary uric acid values in healthy individuals and diseased persons as determined by immobilized uricase and peroxidase.

Age Group (years)	Sex	Uric acid (mg/ 24 hr)			
		Healthy individuals		Patients*	
		Range	Mean	Range	Mean
10 – 20	M	450 – 800	625	910 – 1340 ^a	1125
	F	460 – 880	670	900 – 1300 ^a	1100
21 – 30	M	460 – 900	680	950 – 1370 ^b	1160
	F	480 – 895	687	960 – 1375 ^d	1167
31 – 40	M	480 – 900	690	930 – 1200 ^a	1065
	F	497 – 900	698	930 – 1190 ^c	1060
41 – 50	M	500 – 900	700	940 – 1400 ^d	1170
	F	500 – 900	700	935 – 1400 ^d	1167
51 and above	M	550 – 900	725	940 – 1390 ^d	1165
	F	560 – 890	725	950 – 1400 ^d	1175

10 samples in each group were collected from local Medical Institute.

*Patients suffering from gout^a, leukemia^b, toxemia of pregnancy^c and renal impairment^d

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