ISSN 1061-9348, Journal of Analytical Chemistry, 2022, Vol. 77, No. 3, pp. 301–307. © Pleiades Publishing, Ltd., 2022.

ARTICLES

Bienzymatic Spectrophotometric Method for Uric Acid Estimation in Human Serum and Urine

Ravani Ananda Roopa[a], Kempegowda Mantelingu[b, *], Mridula Guin[c], and Sridhar Bilgumba Thimmaiah[d]

[a] *Department of Chemistry, Pooja Bhagavat Memorial Mahajana Education Centre, Mysuru, Karnataka, 570016 India*

[b] *Synthetic laboratory, DOS in chemistry, University of Mysore, Manasagangotri, Mysuru, 570006 India*

[c] *Department of Chemistry and Biochemistry, Sharada University, Greater Noida, India*

[d] *Maharanis Science College for Women Bangalore, Karnataka, 560001 India*

**e-mail: kmlingu@gmail.com*

Received April 17, 2021; revised September 22, 2021; accepted September 22, 2021

Abstract—In this study, a novel and efficient bienzymatic method for the quantification of uric acid in serum and urine samples was developed. This method is based on the bienzymatic reaction of uricase and peroxidase in the presence of substrates pyrocatechol and 3-methyl-2-benzothiazolinone hydrazone to produce a red colored product. Under optimized conditions, a linearity of uric acid assay was obtained in the ranges of 4 to 384 μM and 2 to 256 μM by kinetic method and fixed time method, respectively. The low limit of detection and limit of quantification were found to be 0.5 and 1.6 μM, respectively. The developed assay was used to quantify uric acid in human serum and urine samples. The present method has good recovery range of 98.3–101.8% and accuracy range of 92.0–101.4%. Hence, the proposed method could be successfully adopted for the quantification of uric acid in clinical laboratories.

Keywords: uricase, peroxidase, pyrocatechol, 3-methyl-2-benzothiazolinone hydrazone, serum, urine

DOI: 10.1134/S1061934822030091

Uric acid is produced as an end product from purine derivatives during human metabolism [1]. It does not undergo further metabolism in human body and is normally excreted by kidneys and intestinal tract. The normal uric acid level in human serum is between 240 and 520 mM, and in urinary excretion it is between 1.4 and 4.4 mM. Uric acid was first associated with primary hypertension. Later, it was found to be positively associated with serum and urine glucose levels. In recent studies, it has been observed that high concentration of uric acid in blood increased the risk of diabetes and kidney disease by nearly 20 and 40%, respectively. Therefore, it is essential to know uric acid levels in blood as well as in urine [2].

The deficiency or maladjustment of uric acid levels may lead to the adverse effect on health. Uric acid is an indicator of several disorders such as renal disease [3], gout [4, 5], and Lesch–Nyhan syndrome [6]. Human blood containing abnormally large amounts of uric acid leads to hyperuricemia, and this has been found to be associated with hypertension [7, 8], metabolic syndrome [9], and cardiovascular disease [10–12]. Therefore, fast and consistent quantification of uric acid in biological samples is essentially required for diagnosis and treatment of diseases.

Several techniques are available for the quantification of uric acid such as fluorescence [13], chemiluminescence [14–16], high performance liquid chromatography (HPLC)-mass spectrometry [17], spectrophotometry [18], HPLC [19], ion chromatography [20], colorimetry [21]. These techniques have some limitations, e.g., they aretime consuming, expensive, insufficiently sensitive, lead to waste of expensive biocatalysts, etc. Hence, to overcome some of the above limitations, there is a great interest in developing simple, rapid, less interfering, and inexpensive assay for uric acid quantification as a routine analysis. Spectrophotometry is the best method to overcome some of the above limitations owing to its wide availability, facile nature, inherent simplicity, and inexpensiveness [22].

In the present paper, we have proposed a simple bienzymatic spectrophotometric method for the quantification of uric acid by using uricase (**UOx**) and peroxidase (**HRP**) enzymes. This method is based on utilizing the specific enzymatic oxidation of uric acid by oxygen to produce hydrogen peroxide, allantoin, and carbon dioxide. By using this hydrogen peroxide an attempt was made to find an improved detection system for enzymatic oxidation of uric acid. We took into consideration two substrate systems, namely, pyrocatechol (**PC**) and 3-methyl-2-benzothiazolinone hydrazone (**MBTH**), which are oxidized by hydrogen peroxide in the presence of peroxidase to

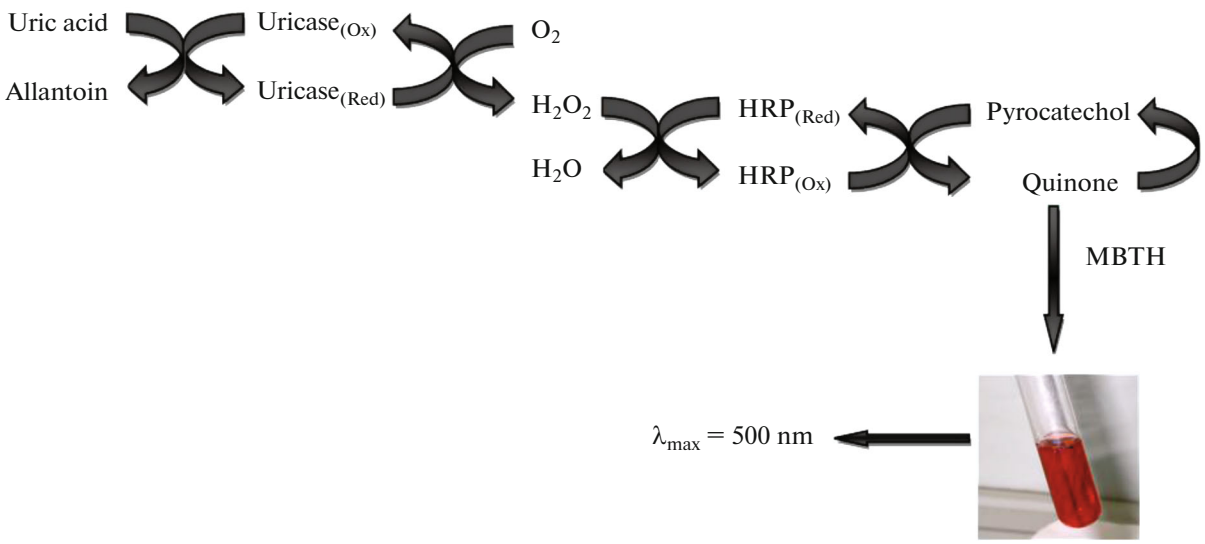


Fig. 1. The process of uric acid quantification.

form a colored product with the absorbance at the optimum wavelength of 500 nm (Fig. 1). This developed analytical assay can be adopted for the quantification of uric acid in different biological samples.

EXPERIMENTAL

Chemicals and apparatus. Uricase (94310–5MG) from *Bacillus fastidious* was purchased from Sigma-Aldrich. Uricase stock solution was prepared by dissolving 20 units/mg of enzyme in 5 mL of double distilled water and frozen at 4°C. Preparation of working solutions wasd one by the successive dilution of the stock solution. Uric acid (98%) was from Sigma-Aldrich. The stock solution of uric acid (1 mM) was prepared by dissolving the calculated amount of solid sample in a minimum volume of 0.1 M sodium hydroxide solution and diluting with water to the desired concentration [18].

Peroxidase (EC.1.11.1.7, 100 units/mg) was purchased from Himedia Laboratories (Mumbai, India). The HRP enzyme stock solution was done by dissolving 2 mg in 10 mL of 100 mM KH_2PO_4/NaOH buffer solution (pH 6.0). The stock solution was stored in refrigerator at 4°C. Working solutions were prepared by appropriate dilution of the stock solution with distilled water.

Hydrogen peroxide stock solution was prepared by proper dilution of commercially available hydrogen peroxide (30%(v/v), E. Merck, Mumbai, India), and its concentration was standardized by titration with secondary standard potassium permanganate [23]. Pyrocatechol (Merk, Germany) stock solution (27.24 mM) was prepared by dissolving 30 mg of solid sample in 10 mL of distilled water. 3-Methyl-2-benzothiazolinone hydrazone was purchased from Sigma-Aldrich. The stock solution of MBTH (37.08 mM) was done by dissolving 40 mg of solid sample in 10 mL of distilled water. All other reagents used for the uricase assay were of analytical grade.

A Jasco model UVIDEC-610 UV-Vis spectrophotometer was used for all absorption measurements. All pH measurements and adjustments of pH were done by a digital pH meter (model EQ-614, Equip-tronics, Mumbai, India). A temperature controlled thermostat (model 206-88950-93, Shimadzu, Japan) was used for maintaining the reaction temperature.

Blood sample collection. The human blood samples were collected from the local clinical laboratories in heparinized tubes. Serum was separated just after sample collection by centrifugation at 4000 rpm for 15 min. The serum samples were stored at –20°C. To analyze biological samples, the permission was taken from Institutional Human Ethical Committee (IHEC-UOM no. 22/Ph.D/2008–09) of University of Mysore [24].

Urine sample collection. Random urine samples from apparently healthy individuals were collected and stored with sodium borate (~0.5 g/L $Na_2B_4O_7{\cdot}10H_2O$) at 0–8°C. These samples were stable and could be used for the analysis up to one month.

Experimental procedure. The uric acid assay was carried out in a 3 mL reaction mixture containing 4.63 mM MBTH, 4.02 mM PC, 9.46 nM HRP and 0.32 units/mg of UOx in 50 mM KH_2PO_4/K_2HPO_4 buffer (pH 7.5) with varying concentrations of uric acid (1–400 μM). The reaction started at 28°C, the progress of the reaction was assessed by measuring the absorbance of the colored solution for about 5 min against the corresponding control at 500 nm. The calibration graph was constructed by plotting the reaction

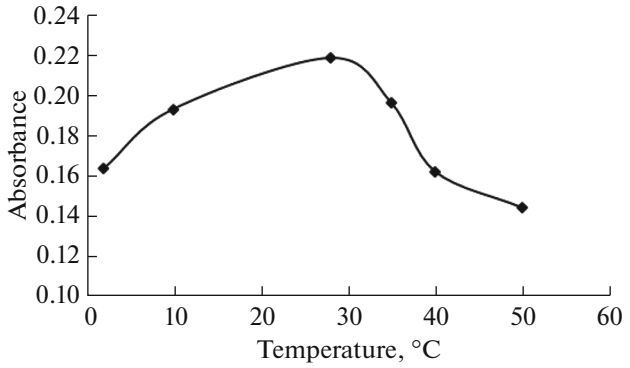


Fig. 2. Effect of temperature on enzyme activity.

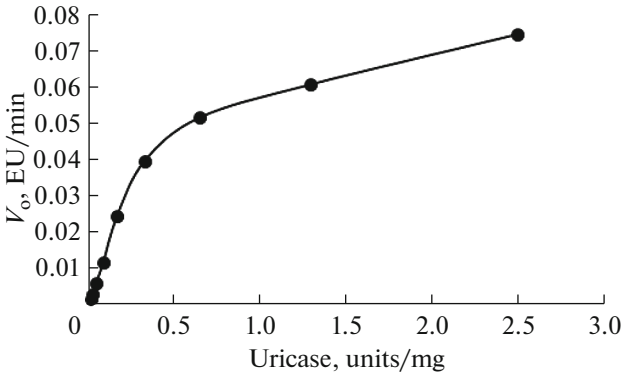


Fig. 3. Effect of uricase concentration on the reaction rate.

rate against uric acid concentrations. In fixed time assay, the linearity of uric acid was measured by incubating the reaction mixture for 5 min at 28°C, and the absorbance of the colored solutions was measured at 500 nm.

Reference method procedure. Uric acid assay in serum and urine samples was employed by using a commercially available kit. This method is based on the reaction of 3,5-dichloro-2-hydroxybenzene sulfonic acid and 4-aminophenazone chromogenic system in the presence of horseradish peroxidase and uricase enzymes. The sample was added to 2 mL of working reagent prepared from 20 mL of 4 mM 3,5-dichloro-2-hydroxybenzene sulfonic acid, 1.0 mL of 2 mM 4-aminophenazone in phosphate buffer (pH 7) containing ≥0.2 kU/L of uricase and ≥0.2 kU/L of peroxidase enzyme. The mixture was left for 15 min at room temperature, and the absorbance was measured at 520 nm against reagent blank [25].

RESULTS AND DISCUSSION

Optimization of experimental variables. Different analytical parameters influencing the catalytic activity of uricase enzyme and uric acid were studied which included pH of the solution, reaction mixture temperature, and substrates concentration.

Effect of PC concentration on the reaction rate was studied in the range of 0.25–24 mM. The result indicates that the reaction rate increases with the increase in the concentration of PC up to 4.02 mM, above this concentration the reaction rate does not depend on the concentration of PC. Therefore, 4.02 mM PC was selected for further analysis. Similarly, the reaction rate is maximum at MBTH concentration of 4.63 mM. Above this concentration, the reaction rate was found to be independent of MBTH concentration.

The influence of pH on spectrophotometric response for uric acid assay was studied by using different buffer solutions such as citric acid/potassium citrate buffer in the pH range from 3.6 to 5.6, acetate/acetic acid buffer in the pH range from 3.6 to 5.6, potassium dihydrogen phosphate/sodium hydroxide buffer in the pH range from 6.0 to 8.0, potassium dihydrogen orthophosphate/dipotassium hydrogen phosphate buffer from pH 6.0 to 8.0, and tris buffer with pH value of 9.8. Among these buffer solutions, KH_2PO_4/K_2HPO_4 (pH 7.5) shows the highest enzyme activity. Thus, to get the highest activity, KH_2PO_4/K_2HPO_4 buffer solution (pH 7.5) was selected for further assay.

The temperature effect was studied by pre-incubating optimized reaction mixture at different temperatures ranging from 5 to 80°C for 5 min. The results show that the enzyme activity increases with the increase in the temperature up to 28°C, while at higher temperature the enzyme activity decreased. This is because of heat inactivation of HRP enzyme. Therefore, the temperature of 28°C was chosen for further assays. The data are shown in Fig. 2.

Analytical figures of merit for uricase assay. H_2O_2 was quantified under optimized conditions. The linear range obtained for H_2O_2 assay by both kinetic method and fixed time method was 4–125 μM. The apparent molar absorptivity of H_2O_2 assay was 0.48×10^4 L/mol/cm. The limit of detection was found to be 1.2 μM, and the limit of quantification was found to be 4 μM. The linearity ranges for HRP assay were 0.15–9 nM by kinetic method and 0.04–2.4 nM by fixed time method. The limits of detection and quantification for HRP assay were found to be 0.013 nM (3δ) and 0.05 nM (10δ), respectively. The linear equations for H_2O_2 and HRP assay are shown in Table 1. Figure 3 for UOx assay shows linearity from 0.01 to 0.32 units/mg, above this concentration the reaction rate was independent of the concentration of UOx enzyme.

The calibration graph for the quantification of uric acid was measured in the range of 1–400 μM. The calibration graph shows the linearity for uric acid between 4 and 384 μM by kinetic method and 2–256 μM by fixed time method with good regression coefficients (Table 1). The apparent molar absorptivity of uric acid

Table 1. List of linearity equations and R^2 values for H_2O_2, peroxidase (HRP), uric acid assay and comparison plots (a straight line passes through an origin in all assays)

Assay	Linear equation	R^2 value
H_2O_2 assay by rate method	$y = 0.0040x$	0.9958
H_2O_2 assay by fixed time method	$y = 0.0031x$	0.9984
HRP assay by rate method	$y = 0.0073x$	0.9973
HRP assay by fixed time method	$y = 0.1331x$	0.9971
Uric acid assay by rate method	$y = 0.0012x$	0.9985
Uric acid assay by fixed time method	$y = 0.0006x$	0.9982
Comparison plot for serum samples	$y = 1.0014x - 0.1841$	0.9999
Comparison plot for urine samples	$y = 1.0023x + 0.1160$	0.9997

Table 2. Comparison of different enzymatic uric acid methods

Analytical method	Instrument	Linear range, μM	Limit of detection, μM	Real sample	Reference
Uricase/MIL-53(Fe)	Colorimetry	4.5–60	1.30	Urine and serum	[26]
Uricase/MOF-Th	Colorimetry	4.0–70	1.15	Urine and serum	[27]
TMB + H_2O_2 and PtNPs	Colorimetry	0–8 mM	4.20	Urine	[28]
TCPO/H_2O_2/rubren	Chemiluminescence	10–1000	5.00	Serum	[29]
Uricase/AuNPs/MWCNT	Amperometry	10–800	10.00	Serum	[30]
Uricase/PC-MBTH	Colorimetry	4–384	0.5	Urine and serum	This work

MIL—materials of Institute Lavoisier, MOF—metal organic frameworks, TMB—3,3,5,5-tetramethylbenzidine, AuNPs—gold nanoparticles, TCPO—bis(2,4,6-trichlorophenyl) oxalate, MWCNTs—multi-walled carbon nanotubes.

was found to be 0.8×10^4 L/mol/cm. The high apparent molar absorptivity value indicates greater stability of red colored product, which in turn indicates that the proposed method is highly stable. The limits of detection and quantification of uric acid were 0.5 and 1.6 μM, respectively. The proposed method analytical parameters were compared with several previously reported methods, the comparison is summarized in Table 2 [26–30]. The linear range and detection limit of uric acid in the proposed method are comparable or even better than those of the reported methods. In addition, low values of detection limits indicate that the proposed method is sensitive compared to the reported methods. The coefficient of variation was found to be 2.2% for 64 μM uric acid in 10 successive measurements.

Determination of catalytic parameters. Enzymatic kinetic parameters for hydrogen peroxide were calculated by using Lineweaver–Burk plot in the presence of peroxidase. A linear regression equation is $y = 1467.2x + 10.48$ with R^2 value of 0.9933. The Lineweaver–Burk plot for uric acid shows a linear regression equation of $y = 968.43x - 2.44$ with R^2 value of 0.9988.

The Michaelis–Menten parameters for peroxidase reaction are as follows: Michaelis–Menten constant (K_m) is 140 μM, maximum velocity (V_{max}) is 0.0954 μM^{-1} min^{-1}. The catalytic power (V_{max}/K_m) and catalytic constant (K_{cat}) were found to be 7×10^{-4} and 0.0202 min^{-1}, respectively. The specificity constant value was 0.001 μM^{-1} min^{-1}, and catalytic efficiency was 0.001×10^6 M^{-1} min^{-1}.

The Michaelis–Menten parameters for uricase reaction are as follows: Michaelis–Menten constant (K_m) is 400 μM, maximum velocity (V_{max}) is 0.4086 μM^{-1} min^{-1}. Low value of Michaelis–Menten constant for MBTH and PC system implies that stronger interaction takes place between active sites of enzyme and substrates. The catalytic power (V_{max}/K_m) and catalytic constant (K_{cat}) were found to be 0.001 min^{-1} and 1.276 μM/min EU, respectively. The specificity constant value was 0.0032 $(EU)^{-1}$ min^{-1}, and catalytic efficiency value was 0.0032 EU/μM min.

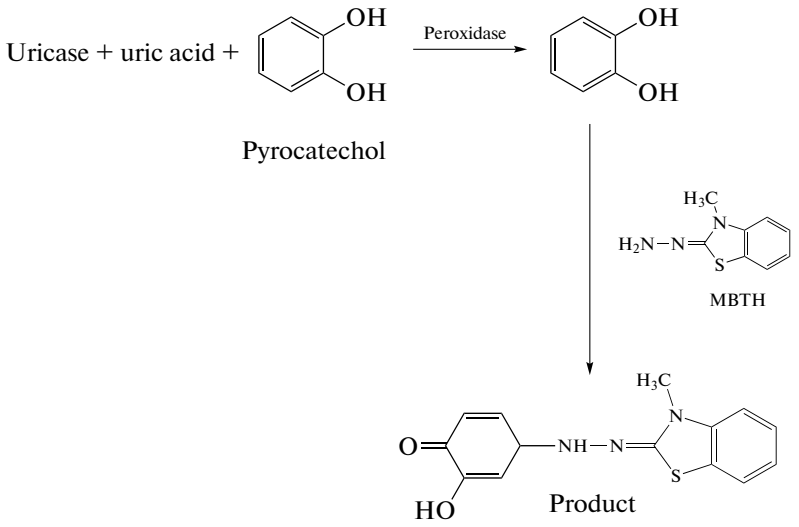
Proposed reaction pathway for uricase catalyzed reaction. Probable mechanism for uricase catalyzed reaction of MBTH and PC is similar to Napolitano et al. [31], Karon et al. [32] and Reinkensmeier et al. [33] reports. Uric acid oxidation takes place by uricase enzyme in the presence of water, and dissolved oxygen produces allointon, CO_2, and H_2O_2 [34, 35]. Then, oxidation of H_2O_2 takes place in the presence of peroxidase enzyme, and hydroxyl radical is produced [36]. Under optimized reaction conditions, pyrocatechol

Table 3. Inter-day and intra-day precision and accuracy

Inter-day precision[a]			Accuracy range, %	Intra-day precision[a]			Accuracy range, %
uric acid, μM	SD[b] ($n^c = 5$)	RSD[d], %	–	uric acid, μM	SD ($n = 5$)	RSD, %	–
16	0.0028	2.2	92.3–95.8	16	0.0019	3.0	92.0–94.9
128	0.0044	1.7	94.2–98.5	128	0.0052	2.3	94.6–97.9
256	0.0100	2.5	93.2–99.5	256	0.0168	3.3	98.7–101.4

[a]Triplicate measurements, [b]SD is standard deviation, [c]*n* is number of measurements, [d]RSD is relative standard deviation.

forms quinone which interacts with MBTH to form a red colored product showing a maximum absorbance at 500 nm. Uricase and peroxidase oxidation of MBTH and PC is shown in Scheme1.



Scheme 1. Uricase and peroxidase oxidation of 3-methyl-2-benzothiazolinone hydrazone (MBTH) and pyrocatechol (PC).

Method validation. *Accuracy and precision.* Accuracy of the method was studied by analyzing solutions containing known amounts of uric acid within the Beer's Law range [37]. Three different concentrations of uric acid were selected for the accuracy study. The results are given in Table 3. The accuracy range for uric acid obtained from the proposed method indicates that the developed assay for the quantification of uric acid is interesting, convenient, and more accurate with respect to the methods and parameters.

Also, precision of the proposed method was studied. The inter-day and intra-day precision was calculated for 5 successive measurements. These two measurements had very low values of standard deviation and coefficient of variation, which indicates that the proposed method is highly precise and reproducible (Table 3).

Interference study. The interference of various ions and compounds commonly present in urine and serum samples was studied by using optimized reaction conditions and 150 μM uric acid concentration. The tolerance ratios are shown in Table 4. The tolerance ratios correspond to the concentrations of interfering species that cause an interference of ±3% in its quantification [38]. The results show that except ascorbic acid, bilirubin, iron(II), nitrite, and L-cysteine most of the species did not interfere with the determination of uric acid in urine and serum samples. Many authors investigated how to minimize some of these interfering species [25, 39–41]. Ascorbic acid interference was minimized by using Triton X-100, and bilirubin interference was minimized by using potassium hexacyanoferrate. Except these five species, all other species did not interfere in the proposed assay protocol, which indicates that it is highly specific and sensitive.

Comparison of proposed method with reference method. Comparison study was carried out for serum and urine samples by using the proposed method and reference method [25]. The comparison plots were

Table 4. Influence of potential interfering species on the quantification of uric acid

Interfering species	Tolerance ratio[a]
Ascorbic acid, bilirubin	0.1
Iron(II), nitrite, L-cysteine	0.5
Iron(III), L-tyrosine, L-tryptophan, F^-	4.8
L-Histidine, lactose, L-cystine, molybdenum, fructose, galactose	25.4
Isoleucine, D-asparagine, copper (II), EDTA magnesium(II), cobalt(II), urea, chloride	31.0
Sucrose, maltose, magnesium(II), potassium	45.3
Citric acid, DL-methionine, oxalic acid	71.0
Ammonium, sulfate, allantoin	99.2
Carbonate, L-serine, creatinine	135.0
Fructose, D-galactose, zinc(II), mannose	275.0
Glycine, sodium	450.0
Acetone	756.0

[a]Tolerance ratio corresponds to the ratio of limit of interfering species concentration to that of concentration of uric acid used.

constructed by using results, and comparison plots for serum and urine samples show the correlation coefficients of 0.9999 and 0.9997, respectively (Table 1). The results show that the proposed assay protocol is comparable with the standard reference assay.

Application of uricase assay. The feasibility and effectiveness of the proposed assay were measured by the quantification of uric acid in human serum and urine samples. The samples were analyzed by the proposed method, and the results were compared with the reference method [25]; more modern methods [42, 43] were also available for the determination of uric acid. In these resent works, the same reference method was also used for comparison. The results obtained are summarized in Table 5. The recovery test was also performed for 3 serum and 3 urine samples spiked with known concentrations of uric acid. The proposed method provides good recovery, which indicates minimum interference from the reducing substance and good reproducibility of the proposed assay. The proposed method is highly simple, sensitive, stable, fast (5 min), requires inexpensive instrumentation, has good recovery and reproducibility – these are the advantages of the proposed method compared to the reference method.

CONCLUSIONS

A new bienzymatic method for the quantification of serum and urine uric acid was developed based on coupling of MBTH and PC. This method offers a new and economical protocol for the investigation of uricase enzyme. The proposed method requires less expensive reagents, small quantity of reagents, less reaction time (5 min), inexpensive instrumentation and provides high sensitivity. This method has a broad linear range of 4 to 384 μM, high apparent molar absorptivity of 0.8×10^4 L/mol/cm, lower detection limit. Low Michaelis–Menten constant (400 μM) and relative standard deviation values indicate a stronger affinity between active sites of enzyme and substrates. The proposed method has excellent correlation with the reference method, good recovery between 98.3–101.8% and high accuracy between 92.0–101.4%, which indicates that the proposed assay is highly precise and reproducible. In the literature, few techniques show greater sensitivity compared to the proposed assay, but they need multiple steps for the preparation of a biosensor, expensive instrument, skilled operator, and are time consuming. Hence, the proposed method is a feasible choice for the quantification of uric acid in clinical laboratories.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

Table 5. Quantification of uric acid in human serum and urine samples

Sample	Proposed method ($n = 5$)		Reference method ($n = 5$)	Added uric acid, μM	Obtained uric acid by proposed method[b], μM	Recovery, %
	found, μM	RSD[a], %	found, μM			
Serum A	230	3.2	214	22	254	100.8
Serum B	340	3.1	310	30	368	99.5
Serum C	145	1.6	156	15	163	101.8
Urine A	380	1.5	390	18	400	100.4
Urine B	330	1.9	313	40	368	99.4
Urine C	270	2.0	246	35	300	98.3

[a]RSD—relative standard deviation, [b]mean of triplicate measurements.

REFERENCES

1. Grabowska, I., Chudy, M., Dybko, A., and Brzozka, Z., *Sens. Actuators, B*, 2008, vol. 130, p. 508.
2. Retna Raj, C. and Ohsaka, T., *J. Electroanal. Chem.*, 2003, vol. 540, p. 69.
3. Nakagawa, T., Kang, D.H., Feig, D., Sanchez-Lozada, L.G., Srinivas, T.R., Sautin, Y., Ejaz, A.A., Segal, M., and Johnson, R.J., *Kidney Int.*, 2006, vol. 69, p. 1722.
4. Falasca, G.F., *Clin. Dermatol.*, 2006, vol. 24, p. 498.
5. Merriman, T.R. and Dalbeth, N., *Joint Bone Spine*, 2011, vol. 78, p. 35.
6. Nyhan, W.L., *J. Inherited Metab. Dis.*, 1997, vol. 20, p. 171.
7. Cannon, P.J., Stason, W.B., Demartini, F.E., Sommers, S.C., and Laragh, J.H., *N. Engl. J. Med.*, 1966, vol. 275, p. 457.
8. Jossa, F., Farinaro, E., Panico, S., Krogh, V., Celentano, E., Galasso, R., Mancini, M., and Trevisan, M., *J. Hum. Hypertens.*, 1994, vol. 8, p. 677.
9. Lohsoonthorn, V., Dhanamun, B., and Williams, M.A., *Arch. Med. Res.*, 2006, vol. 37, p. 883.
10. Gagliardi, A.C.M., Miname, M.H., and Santos, R.D., *Atherosclerosis,* 2009, vol. 202, p. 11.
11. Fang, J. and Alderman, M.H., *JAMA*, 2000, vol. 283, p. 2404.
12. Lippi, G., Montagnana, M., Franchini, M., Favaloro, E.J., and Targher, G., *Clin. Chim. Acta*, 2008, vol. 392, p. 1.
13. Galban, J., Andreu, Y., Almenara, M.J., Marcos, S., and Castillo, J.R., *Talanta*, 2001, vol. 54, p. 847.
14. Wu, F., Huang, Y., and Li, Q., *Anal. Chim. Acta*, 2005, vol. 536, p. 107.
15. Hong, H.C. and Huang, H.J., *Anal. Chim. Acta*, 2003, vol. 499, p. 41.
16. He, D., Zhang, Z., Huang, Y., Hu, Y., Zhou, H., and Chen, D., *Luminescence*, 2005, vol. 20, p. 271.
17. Perello, J., Sanchis, P., and Grases, F., *J. Chromatogr. B*, 2005, vol. 824, p. 175.
18. Moghadam, M.R., Dadfarnia, S., Shabani, A.M.H., and Shahbazikhah, P., *Anal. Biochem.*, 2011, vol. 410, p. 289.
19. Rocha, D.L. and Rocha, F.R.P., *Microchem. J.*, 2010, vol. 94, p. 53.
20. Dai, X., Fang, X., Zhang, C., Xu, R., and Xu, B., *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.*, 2007, vol. 857, p. 287.
21. Zhao, F.Y., Wang, Z.H., Wang, H., Zhao, R., and Ding, M.Y., *Chin. Chem. Lett.*, 2011, vol. 22, p. 342.
22. Bhargava, A.K., Lal, H., and Pundir, C.S., *J. Biochem. Biophys. Methods*, 1999, vol. 39, p. 125.
23. Vogel A.I., *Vogel's Text Book of Quantitative Chemical Analysis*, New Delhi: Pearson Education, 2006, 6th ed.
24. Roopa, R.A., Mantelingu, K., Rangappa, K.S., and Kamara, M.N., *Asian J. Pharm.*, 2016, vol. 10, no. 2, p. S170.
25. Fossati, P., Prencipe, L., and Berti, G., *Clin. Chem.*, 1980, vol. 26, p. 227.
26. Lu, J., Xiong, Y., Liao, C., and Ye, F., *Anal. Methods*, 2015, vol. 7, p. 9894.
27. Badoei-dalfard, A., Sohrabi N., Zahra Karami, and Sargazi, G., *Biosens. Bioelectron.*, 2009, vol. 141, 111420.
28. Muhsin Ali., Asad Ullah Khalid, M., Imran Shah, Wan Kim, S., Su Kim, Y., Lim, J.H., and Choi, K.H., *New J. Chem.*, 2019, vol. 43, p. 7636.
29. Yao, D., Vlessidis, A.G., and Evmiridis, N.P., *Anal. Chim. Acta*, 2003, vol, 478, p. 23.
30. Chauhan, N. and Pundir, C.S., *Anal. Biochem.*, 2011, vol. 413, p. 97.
31. Napolitano, A., Crescenzi, O., Pezzella, A., and Prota, G., *J. Med. Chem.*, 1995, vol. 38, p. 917.
32. Karon, B.S., Daly, T.M., and Scott, M.G., *Clin. Chem.*, 1998, vol. 44, p. 155.
33. Annika, R., Katrin, S., Thomas, H., Bußler, S., Sascha, R., and Hashadrai, M.R., *Food Chem.*, 2016, vol. 194, p. 76.
34. Zhao, Y., Yang, X., Lu, W., Liao, H., and Liao, F., *Microchim. Acta*, 2009, vol. 164, p. 1.
35. Nevenka Modrić Poje, M., Watkin Alison, D.J., and Edwards, J., *Tetrahedron Lett.*, 1993, vol. 34, p. 4679.
36. Regalado, C., García-Almendárez, B.E., and Duarte-Vázquez, M.A., *Phytochem. Rev.*, 2004, vol. 3, p. 243.
37. Taverniers, I., De Loose, M., and van Bockstaele, E., *TrAC, Trends Anal. Chem.*, 2004, vol. 23, p. 535.
38. Roopa, R.A., Mantelingu, K., and Mridula Guin, *Anal. Chem. Lett.*, 2017, vol. 7, no. 6, p. 779.
39. Kabasakalian, P., Kalliney, S., and Westcott, A., *Clin. Chem.*, 1973, vol. 19, p. 522.
40. Fossati, P. and Prencipe, L., *Clin. Chem.*, 1982, vol. 28, p. 2077.
41. Klose, S., Stoltz, M., Munz, E., and Portenhauser, R., *Clin. Chem.*, 1978, vol. 24, p. 250.
42. Duplancic, D., Kukoc-Modun, L., Modun, D., and Radic, N., *Molecules*, 2011, vol. 16, p. 7058.
43. Diogo, L.R. and Fábio, R.P.R., *Microchem. J.*, 2010, vol. 94, p. 53.