J.Serb.Chem.Soc. 68(8–9)691–698(2003)
JSCS – 3078

UDC 612.461.25+543-4:543.645:574.152
Original scientific paper

Determination of uric acid in human serum by an enzymatic method using *N*-methyl-*N*-(4-aminophenyl)-3-methoxyaniline reagent

MILENA JELIKIĆ-STANKOV[1, *, #], PREDRAG DJURDJEVIĆ[2] and DEJAN STANKOV[3]

[1]*Faculty of Pharmacy, Department of Analytical Chemistry, Vojvode Stepe 450, P. O. Box. 146, 11224 Belgrade,* [2]*Faculty of Science, P. O. Box 60, 34000 Kragujevac and* [3]*Hospital "Dr Dragiša Mišović", Biochemistry Department, 11000 Belgrade, Serbia and Montenegro*

(Received 8 March 2003)

Abstract: In this work a new enzymatic method for the determination of uric acid in human serum has been developed. The method is based on the oxidative coupling reaction between the *N*-methyl-*N*-(4-aminophenyl)-3-methoxyaniline (NCP) reagent and the hydrogen – donor reagent, *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-3-methylaniline (TOOS), in the system involving three enzymes: uricase, peroxidase and ascorbate oxidase. Using this method uric acid could be determined in concentrations up to 1.428 mmol/L, with a relative standard deviation of up to 1.8 %. The effect of the medium pH and the NCP concentration on the linearity of the chromogen absorbance *versus* the uric acid concentration curve was investigated. The influence of the uricase activity on the maximum rate of uric acid oxidation was also examined. The use of the NCP reagent demonstrated a more precise and more sensitive determination of the uric acid compared to the determination with 4-aminoantipyrine (4-AA) as the coupling regent. The sensitivity of the method determined from the calibration curve was 0.71 absorbance units per mmol/L of uric acid; the limit of detection was LOD = 0.0035 mmol/L and the limit of quantification was LOQ = 0.015 mmol/L of uric acid.

Keywords: uric acid, human serum, enzymatic determination, NCP reagent.

INTRODUCTION

Uric acid is a metabolic product of exogenous (brought in with food) or endogenous purine bases. Since uric acid is found in human serum in relatively low concentrations (reference range is 0.21 to 0.42 mmol/L in men and 0.16 to 0.36 mmol/L in women) it is necessary to use specific and sensitive methods for its determination. The existing methods could be conveniently devided into two groups: reductive and enzymatic. The reductive methods are non-specific and involve the oxidation of uric acid with phosphotungstate reagent to allantoin with resultant blue coloring of tungstate solution. The enxymatic methods are specific. They involve the catalytic oxidation of uric acid with the enzyme uricase

* Author for correspondence.
\# Serbian Chemical Socitey active member.

to allantoin with the formation of hydrogen peroxide.[1] The peroxide, the concentration of which is directly proportional to the concentration of uric acid, could then be determined by a number of methods.[2–10]

Owing to their specificity, enzymatic methods have found widespread use. The reagents mixture used in this method contains the enzymes: uricase, peroxidase, hydrogen – donor reagent and coupling reagent. As hydrogen – donor reagent various derivatives of phenol or aniline have been used.[11] Formerly, as the coupling reagent, 4-AA was used. In the present work the use of the NCP reagent instead of 4-AA is proposed. This reagent forms a colored compound (chromogen) with TOOS reagent in the presence of H_2O_2 (oxidative coupling). The concentration of the chromogen is directly proportional to the concentration of uric acid. The chromogen shows an absorption maximum at 750 nm. The chromogen formed with 4-AA possesses an absorption maximum at 556 nm. This batochromic shift of 194 nm is especially convenient in the analysis of hemolyzed or lipemic serum where undesirable products are colored with an absorption near 500 nm and, thus, may strongly interfere with the uric acid determination. The sensitivity of the uric acid determination with the NCP reagent is considerably higher than that with 4-AA, as well.

EXPERIMENTAL

Apparatus

The absorbance measurements were made with a double beam UV-VIS spectrophotometer Cintra model 40 (GBC, Australia). The cells were thermostated with a temperature precision to ± 0.01 ºC. The absorbance – concentration curves were obtained by using a single beam UV spectrophotometer Ultrospec model 2000 (LKB-Pharmacia, UK). The pH measurements were made with a Corning model 250 pH-meter equipped with an Orion sure – flow combined electrode (USA).

Reagents and solutions

Bidistilled water and analytical grade reagents were used for the preparation of the solutions. Uric acid standard, uricase, peroxidase (POD) and ascorbate oxidase were the products of SERVA (FRG). The NCP and TOOS reagents were the products of Dojindo Lab (Japan). The phosphate buffer and 4-aminoantipyrine (4-AA) were the products of Merck (FRG). Sodium tetraborate, EDTA and Triton X-100 detergent were the products of Serva (FRG); hydrogen peroxide (30 %) was from Zorka – Šabac (Serbia nad Montenegro). The control serum with a declared amount of uric acid was obtained from Boehringer Mannheim – Precinorm (Austria).

Reagent for the determination of the uricase activity

Prior to the uric acid determination, the optimal uricase activity needed for the determination had to be established. The composition of the solution used for uricase activity determination was: phosphate buffer (pH 7.40), 0.1 mol/L; sodium tetraborate, 20 mmol/L; EDTA, 1 mmol/L; Triton X-1000, 0.01 %. The activity of uricase were 100, 200, 300 and 400 U/L. The primary standard solution of uric acid had a concentration of 1.428 mmol/L and was used as the analyt. An amount of 25 μL of the sample was added into 1.0 mL of the reagent and the absorbance was measured at 750 nm using the reagent solution as the blank at a temperature of 37 ºC.

Reagent for the determination of uric acid

The composition of the reagent solution used for uric acid determination in serum was: phosphate buffer (pH 7.40), 0.1 mol/L; NCP reagent, 0.075 mmol/L; TOOS reagent, 0.2 mmol/L; sodium tetraborate, 20 mmol/L; EDTA, 1 mmol/L; peroxidase (POD), 1000 U/L; ascorbate oxidase, 1000 U/L; uricase, 200 U/L and Triton X-100, 0.01 %. Ascorbate oxidase was added in order to prevent the reaction between hydrogen peroxide and vitamin C present in serum.

Analytical procedure

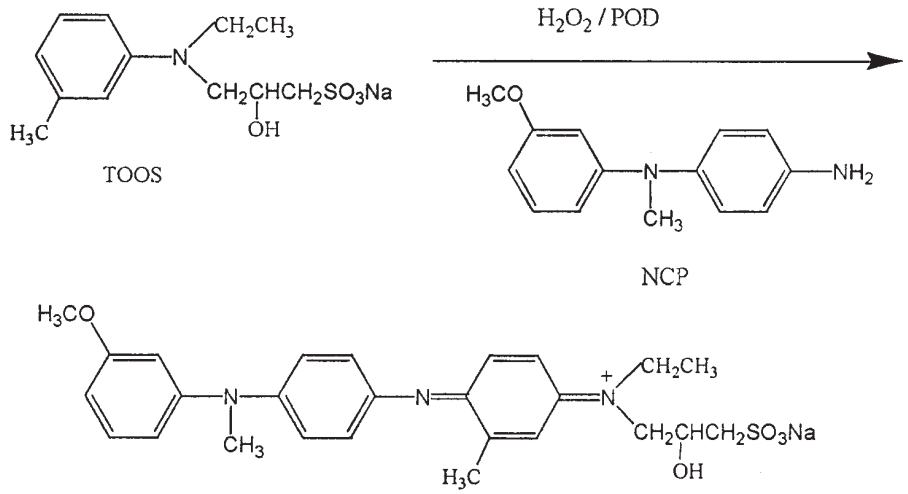
A serum sample of 25 μL volume was added into 1.0 mL of the reagent solution (2.4). The absorbance of the solution was measured at 750 nm after five minutes incubation at 37.0 ºC using the reagent solution as the blank.

RESULTS AND DISCUSSION

The procedure of uric acid determination is based on the reactions:

$$\text{Uric acid} \xrightarrow{uricase} \text{allantoine} + H_2O_2 \qquad (1)$$

The concentration of chromogen (green colored dye stable fifteen minutes) which is formed by oxidative coupling of NCP and TOOS reagents is directly proportional to the concentration of the uric acid.



Chromogen

The spectra of the NCP reagent (Fig. 1, curve 1), TOOS reagent (Fig. 1, curve 2) and the chromogen compound (Fig. 1, curve 3) were taken at pH 7.40. The absorption maximum of the chromogen is at 750 nm. Thus, its absorption maximum is batochromically shifted compared to its components.

The adventage of the application of NCP reagent lays in the fact that the formed chromogen (Fig. 2, curve 2) has a batochromic shift of the absorption maximum at 750 nm compared to the method that uses 4-AA instead of NCP, as a coupling reagent, when the formed chromogen has an absorption maximum at 556 nm (Fig. 2, curve 1).

The effect of pH on the chromogen spectra was investigated in the pH interval from 6.5 to 7.5. The reason for choosing the pH interval was in the fact that all the enzymes used show optimal activity under such conditions. The obtained spectra (Fig. 3) show that variation of pH has no significant effect on the intensity and position of the adsorption maximum of the chromogen. Thus, the working pH was set at 7.40.

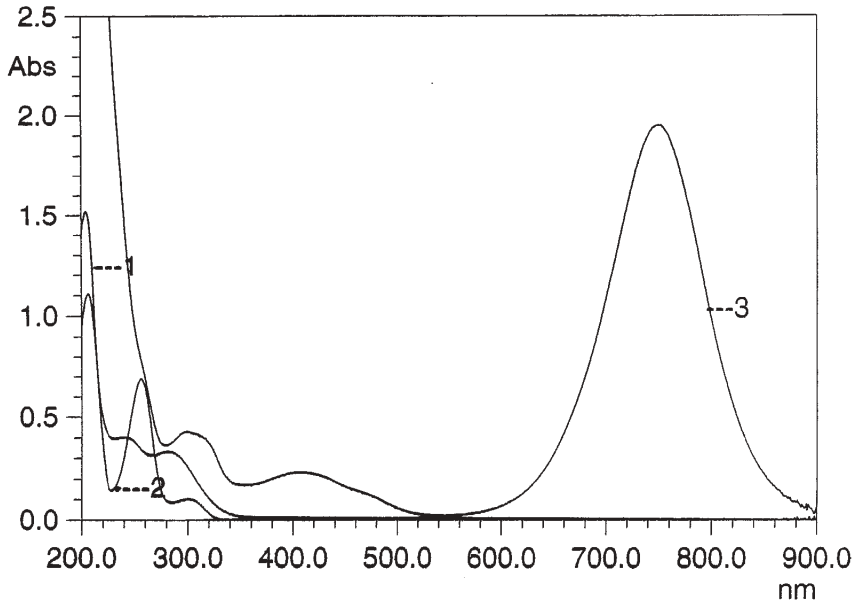


Fig. 1. Absorption spectra of NCP (curve 1, 0.05 mmol/L; pH 7.40), TOOS (curve 2, 0.05 mmol/L; pH 7.40) and the chromogen (curve 3, NCP – 0.05 mmol/L, TOOS – 0.05 mmol/L; pH 7.40).

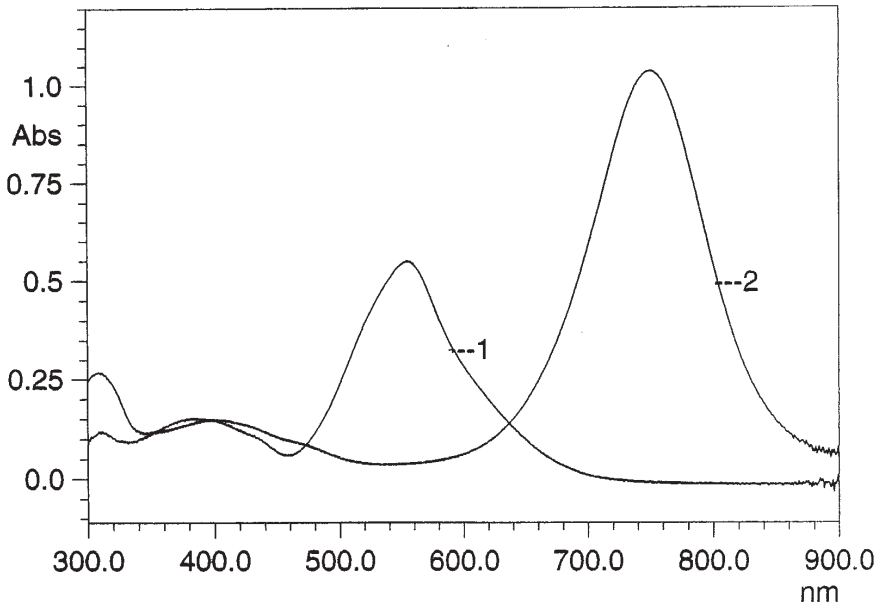


Fig. 2. Absorption spectra of the chromogen with 4-AA (curve 1, uric acid – 1.428 mmol/L, pH 7.40) and the chromogen with NCP (curve 2, uric acid – 1.428 mmol/L, pH 7.40).

The effect of the NCP concentration on the absorbance of the chromogen (Fig. 4) was investigated using NCP reagent in the concentration range from 0.025 to 0.075 mmol/L at

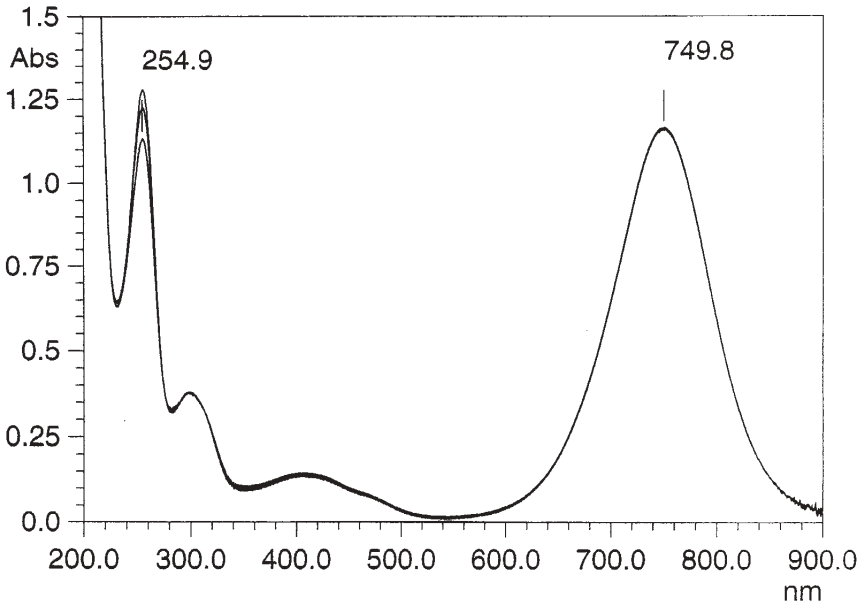


Fig. 3. Absorption spectra of the chromogen (0.1 M TOOS and 0.025 M NCP) in the pH region 6.50 – 7.50.

constant concentration of TOOS, 0.2 mmol/L. The maximal absorbance of formed chromogen, at 750 nm, was 3 units which is enough for the determination of uric acid up to a concentration of 1.428 mmol/L.

During the investigations of the chromogen formation (Fig. 1, curve 3), the effect of pH (Fig. 3) and NCP concentration on the absorbance of chromogene (Fig. 4), a hydrogen peroxide concentration of 5.0 mmol/L was used.

Kinetic studies

The activity of uricase was followed kinetically as the rate of uric acid oxidation to peroxide and allantoine (Eq. (1)). It was assumed that the reaction was finished when $dA/dt = 0$. At a uricase activity of 200 U/L, complete oxidation of the uric acid (1.428 mmol/L), was achieved in 5 min. Thus, the uricase was used at this level of activity in the determination of uric acid (Fig. 5).

For the construction of a calibration curve for the determination of uric acid, standard solutions of uric acid in the concentration range from 0 to 1.428 mmol/L were used. The linear equation was $y = 0.709\,x + 0.00061$, $n = 5$ with a correlation coefficient $r = 0.9998$, where y denotes the absorbance and x is the concentration of the uric acid in mmol/L.

On the basis of the obtained calibration curve, uric acid was determined in samples from 50 patients. Analysis was performed as described in the section Analytical Procedure.

In order to check the obtained results, uric acid was determined in the same samples of human serum by the standard enzymatic method based on the use 4-AA as the coupling reagent instead of NCP. The obtained results indicated a high level of correlation ($a = 0.006$, $b = 0.998$ and $r = 0.9993$).

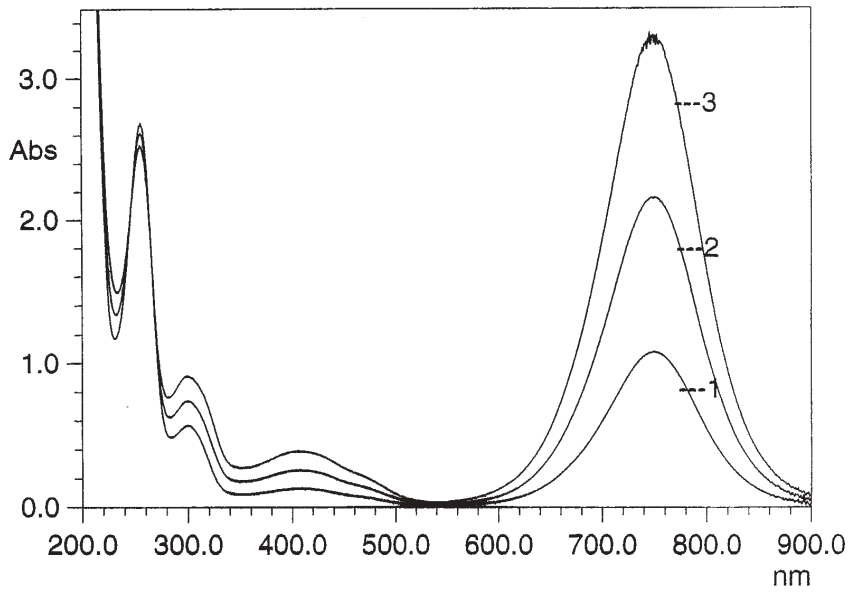


Fig. 4. The effect of the NCP concentration on the formation of the chromogen (curve 1, NCP – 0.025 mmol/L, curve 2, NCP – 0.05 mmol/L, curve 3, NCP – 0.075 mmol/L; TOOS – 0.2 mmol/L, pH 7.40).

Accuracy of the method was examined by the recovery test. To hyman pool serum known amounts of uric acid were added and the concentration of the uric acid was determined from the calibration curve. The recovery was calculated from the expression:

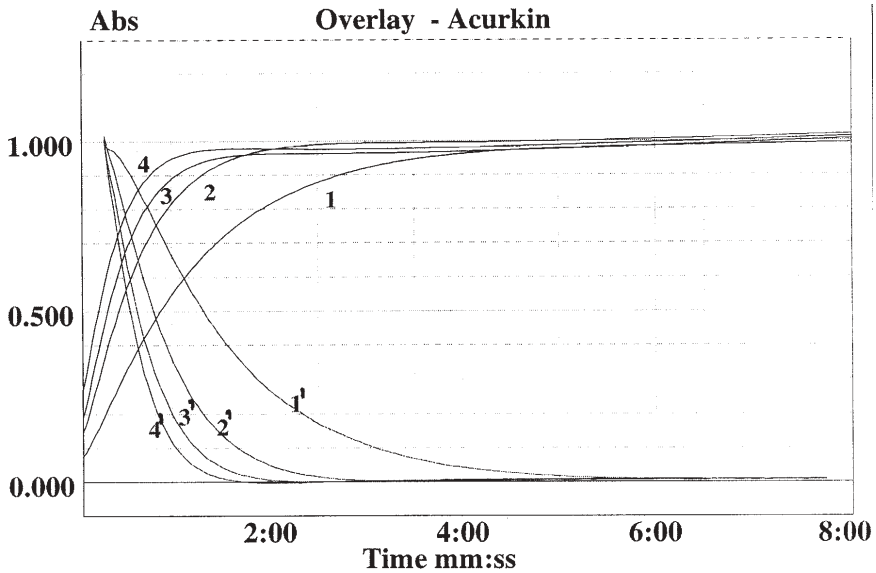


Fig. 5. The influence of the uricase activity on the rate of uric acid oxidation (uricase activity: curve 1 – 100 U/L, curve 2 – 200 U/L, curve 3 – 300 U/L, curve 4 – 400 U/L: uricase activity shown as d*A*/d*t* *vs.* time (curve 1' – 4').

$$\% \text{ recovery} = (FS - US) / ADD \times 100$$

where *FS* is concentration of the uric acid in the fortified serum sample. *US* is the concentration of uric acid in the unfortified (blank) serum and *ADD* is concentration of uric acid in the added aliquot. The obtained results are given in Table I.

TABLE I. Application of the proposed method to the analysis of uric acid

Added/(mmol/L)	Found[a]/(mmol/L)	Recovery ± RSD/%
0.286	0.282	98.6 ± 1.80
0.571	0.568	99.5 ± 1.52
0.857	0.863	100.7 ± 1.29
1.142	1.152	100.9 ± 1.35
1.428	1.417	99.2 ± 1.40

[a]Mean value of ten determinations

The sensitivity of the method as determined from the calibration curve was 0.71 absorbance units per mmol/L of uric acid. This is significantly higher than that obtained using 4-AA (0.35 absorbance units per mmol/L of uric acid). The detection limit (LOD) was 0.0035 mmol/L and the quantification limit (LOQ) for uric acid was 0.015 mmol/L.

CONCLUSIONS

Owing to the significantly higher absorbance of the chromogen and the increased sensitivity of the method as a result of the batochromic shift of the absorption maximum of the chromogen, the interference from the serum components on the determination of uric acid was diminished. This enabled precise and accurate determination of uric acid in human serum. The NCP reagent possesses better analytical properties than the so far used 4-AA reagent.

Acknowledgement: The authors are grateful to the Ministry of Science, Technology and Development for financial support. (project 1941/2002).

ИЗВОД

ОДРЕЂИВАЊЕ МОКРАЋНЕ КИСЕЛИНЕ У СЕРУМУ ЕНЗИМСКОМ МЕТОДОМ ПРИМЕНОМ *N*-МЕТИЛ-*N*-(4-АМИНОФЕНИЛ)-3-МЕТОКСИАНИЛИН РЕАГЕНСА

МИЛЕНА ЈЕЛИКИЋ-СТАНКОВ[1], ПРЕДРАГ ЂУРЂЕВИЋ[2] И ДЕЈАН СТАНКОВ[3]

[1]Фармацеутски факултет, Одсек за аналитичку хемију, Војводе Степе 450, п. пр. 146, 11224 Београд, [2]Природно-математички факултет, п. пр. 60, 34000 Крагујевац и [3]Болница "Др Драгиша Мишовић", Одсек за биохемију, Универзитет у Београду, 11000 Београд

У раду је предложена нова ензимска метода за одређивање мокраћне киселине у хуманом серуму. Метода је заснована на купловању *N*-метил-*N*-(4-аминофенил)-3-метоксианилин (NCP), реагенса са *N*-етил-*N*-(2-хидрокси-3-сулфопропил)-3-метиланилином (TOOS), хидроген–донор реагенсом, у ензимском систему три ензима: уриказа, пероксидаза и аскорбат оксидаза. Мокраћна киселина се овом методом може одређивати у концентрацијама до 1.428

mmol/L са релативном стандардном девијацијом до 1.8 %. Испитан је утицај pH медијума и концентрације NCP реагенса на линеарност зависности апсорбанције хромогена од концентрације мокраћне киселине. Такође је испитан утицај активности уриказе на максималну брзину оксидације мокраћне киселине. Примена NCP реагенса омогућава прецизније и осетљивије одређивање мокраћне киселине у поређењу са одређивањем уз 4-аминоантипирин (4-AA) као реагенса за кукпловање. Осетљивост методе, одређена из калибрационе криве, износила је 0.71 апсорпциону јединицу по mmol/L мокраћне киселине; граница детекције је износила 0.0035 mmol/L а граница квантификације 0.015 mmol/L.

(Примљено 8. марта 2003)

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