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# A modified xanthine oxidase activity method based on uric acid absorption

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**Abstract :** In this study, a modified xanthine oxidase activity measurement method is defined, and results obtained by the different methods were compared. In the activity measurements, a commercial milk xanthine oxidase enzyme product was used as the sample.
Significant differences were observed between the results of our modified method and those of original method (Method 1). The results of our modified method and other method (Method 2 in which no trichloroacetic acid is used) are however quite similar.
The modified method presented here eliminates faults of the previous methods and gives comparable results.


**Keywords :** Xanthine oxidase; Activity measurement; Method comparison.

## INTRODUCTION

Xanthine oxidase (XO; EC 1.2.3.2) is an enzyme catalyzing the conversion reaction of xanthine to uric acid with production of superoxide radical ($O_2^{\cdot-}$). It is the rate limiting enzyme in nucleic acid degradation, through which all purines are channelled for terminal oxidation[1]. XO activity was investigated in several disorders.

In the activity measurements, some researchers followed absorbance increases at 293 nm due to uric acid formation during the conversion reaction of xanthine to uric acid (Method-2) and others measured absorbances after the reaction was stopped by trichloroacetic acid (Method-1)[2-5]. However, there are problems in both procedures because of two reasons.

In both previous methods (Methods-1 and 2), xanthine itself (0.17 mM) gives significant absorption at this wavelength, and absence of xanthine in blank causes false increases in the activity measurements. Additionally, in the method-1, trichloroacetic acid (TCA) addition causes suppressions in absorbance values of both xanthine and uric acid, leading to measurement of lowered enzyme activity values.

Authors have given no details about their procedures in the studies. There are however two possibilities in this regard.

1. When absorbances of blank and sample tubes are obtained only after incubation period, this causes addition of absorbance value of xanthine to that of uric

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acid, which causes false increases in the activity results. By this procedure, one may determine activity in the samples having even no XO enzyme.

2. When absorbance of blank and sample tubes are obtained two times, before incubation period and after stopping the reaction by TCA, lower or sometimes no enzyme activity values are obtained due to the fact that TCA at this concentration significantly suppresses absorbance of both xanthine and uric acid. By this procedure, one may determine no enzyme activity in the samples actually having XO activity.

In order to elucidate possible sources of measurement faults in the previous methods and to eliminate them, we performed several experiments and developed a modified method to measure XO activity correctly.

## MATERIALS AND METHODS

Chemicals used in the study were of analytical grade. As enzyme source, commercial milk XO was used in the enzymatic activity measurements (Sigma, X-4500). Activity assays were performed by using 3 methods; method-1[3,4], method-2[2,5] and our modified method. The results of all the methods were compared.

### Reagents

Phosphate buffer: pH: 7.5; 46.7 mM; 0.1 mM EDTA in final concentration.

Xanthine: 0.17 mM in final xanthine concentration. TCA: 3.33% (w/v) in final concentration.

### Method-1[3,4]

Sample was preincubated at 37°C for 40 minutes.

| Reagents (mL) | Blank | Assay |
|---|---|---|
| Buffer | 2.80 | 2.70 |
| Xanthine | ---- | 0.100 |
| Sample (Enzyme solution) | 0.100 | 0.100 |
| First absorbance values were read at 293 nm ($A_1$) and incubation is made for 30 min. | | |
| TCA | 0.100 | 0.100 |
| After centrifugation at 5000 x g for 10 min., second absorbance values were read at 293 nm ($A_2$). | | |

In this method, absorbance change ($\Delta A$) can be calculated in two ways:

### Procedure 1

**$A = (A_2)_{assay} - (A_2)_{blank}$ and**

**A/min = A/30 or,**

### Procedure 2

**$A = [(A_2)_{assay} - (A_2)_{blank}] - [(A_1)_{assay} - (A_1)_{blank}]$**

**A/min = A/30**

### Method-2[2,5]

Sample was preincubated at 37°C for 40 minutes.

| Reagent (mL) | Assay |
|---|---|
| Buffer | 2.80 |
| Xanthine | 0.100 |
| Sample-(Enzyme solution) | 0.100 |
| Absorbance was read at 293 nm and absorbance change per minute was determined. | |

**A/min = A/1**

### Modified Method

Sample was preincubated at 37°C for 40 minutes.

| Reagents (mL) | Blank | Assay |
|---|---|---|
| Buffer | 2.70 | 2.70 |
| Xanthine | 0.100 | 0.100 |
| Sample-(Enzyme solution) | 0.100 | 0.100 |
| TCA | 0.100 | ----- |
| Incubation was made at 37°C for 30 minutes | | |
| TCA | ----- | 0.100 |
| After centrifugation at 5000xg for 10 min., absorbance values were read at 293 nm. | | |

**$A = (A)_{assay} - (A)_{blank}$**

**A/min = A/30**

XO activities were calculated by using extinction coefficients (ε) established in each assay condition. For the establishment of extinction coefficients, defined concentrations of the xanthine and uric acid were prepared in the buffer above with and without TCA and absorbance values at 293 nm were recorded. Then, ε values were calculated by using the formula of A= (ε) L C.

Here A=Absorbance; L= Light path (1 cm); C= Concentration.

| Extinction Coefficients (ε) as $cm^{-1}$. $\mu mol^{-1}$.L | | |
|---|---|---|
| | Before TCA addition | After TCA addition |
| Uric acid | $10.0 \times 10^{-3}$ | $7.0 \times 10^{-3}$ |
| Xanthine | $1.0 \times 10^{-3}$ | $0.2 \times 10^{-3}$ |

**XO Activity (IU/mL) = (A/min) x $(1/\varepsilon_{uric\ acid})$ x $V_T / V_S$ x (1/1000 mL)**

$V_T$, $V_S$: Total and Sample volumes (mL)

Here,

A/min is calculated dividing absorbance change by incubation period (For the method-1 and modified method, it is 30 min. See tables)

In fact, we suppose that it would be better to make a further correction analysis for all of the methods given here in order to obtain correct activity values.

The problem is that xanthine is consumed during the conversion reaction to the same amount of uric acid, and its absorbance is decreased in final mixture. This leads to lowered readings of uric acid absorption and thereby, lowered XO activity. It is possible to correct this fault by using a correction analysis as described below.

**X= Observed absorbance (A2)**

**Y= Real absorbance**

$$Y = X + X\left(\varepsilon_{xanthine} / \varepsilon_{uric\ acid}\right)$$

After this correction, (Y/min) becomes the real absorbance change per minute and used instead of (A/min).

## RESULTS

Results obtained by the methods as described above are given in TABLE 1. As seen from the results, there are great differences between the results of original procedures (Method-1) and, those of both method-2 and modified method. However, the results of method-1 and modified method are quite similar.

### Evaluation of the methods

The results obtained by the methods mentioned are seen in TABLE 1.

### (a) Linearity

Following equations were obtained by linearity analysis:

$$\mathcal{Y} = 0.444\chi - 0.115$$ **(for reference method)**

$$\mathcal{Y} = 0.428\chi + 1.200$$ **(for modified method)**

Where, $\mathcal{Y}$= enzyme activity (IU/ml); $\chi$= sample amount (enzyme solution) (l)

### (b) Correlation analysis

Correlation coefficient value between enzyme activity and enzyme amount was r=0.99 for reference and modified methods. Recovery of added enzyme activity was 98±3.1 % for the reference method and 99±2.9 % for the modified method. The recovery was established by measuring enzyme activity after adding certain amounts of enzyme (sample) solutions into the reaction mixture. As seen from the results above, there are significant differences between the results of method-1 (procedure 1) and, those of method-2 and our modified methods. In this regard, our modified method gives precise and comparable results.

### (c) Precision analyses

Results of precision analyses are given in TABLES 2 and 3. Intra run analysis was performed by measuring activity ten times a day in the same sample, and inter run analysis was performed by measuring activity one time a day for consecutive 10 days. Then, coefficients of variation (CV) values were established by using the formula of (Standard deviation/mean) x 100 for each analysis.

## DISCUSSION

XO is an important enzyme participating in purine catabolism[1], in iron absorption and mobilization[6] and in oxidation reactions of a wide variety of substrates. It also serves as an ubiquitous source of oxidizing agents and is responsible for the production of oxygen free

**TABLE 1 : Mean±SD values (mIU/mL) established by the XO activity methods (n=10).**

| Original Method | I | | II | | III | | IV | |
|---|---|---|---|---|---|---|---|---|
| Procedure1 | 71.4±8.2 | | 34.2±6.1 | | 17.9±2.1 | | 7.9±0.9 | |
| Procedure2 | 19.3±2.8 | | n.a | | n.a | | n.a | |
| | $A_o$ | $A_c$ | $A_o$ | $A_c$ | $A_o$ | $A_c$ | $A_o$ | $A_c$ |
| Reference Method | 40.0±3.2 | 44.4±3.6 | 19.5±2.0 | 21.7±2.1 | 9.0±0.6 | 9.9±0.7 | 4.0±0.3 | 4.4±0.3 |
| Modified Method | 42.9±4.0 | 44.1±4.6 | 21.1±1.8 | 21.8±2.1 | 11.4±1.2 | 11.8±1.3 | 4.0±0.3 | 4.1±0.3 |

**I: Activities of undiluted enzyme (sample) solution; II, III and IV: Activities of 2.0, 5.0 and 10.0 fold diluted enzyme solution; $A_o$: Observed activity; $A_c$: Corrected activity; n.a: No activity**

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**TABLE 2 : Intra-run and inter-run coefficient of variation values (n=10 for each) established by reference and modified methods.**

| CV % | I | | II | | III | | IV | |
|---|---|---|---|---|---|---|---|---|
| | M2. | M.M. | M2. | M.M. | M2. | M.M. | M2. | M.M. |
| Intra – run | 2.5 | 2.4 | 2.8 | 2.4 | 3.2 | 2.9 | 2.5 | 3.3 |
| Inter – run | 5.4 | 6.0 | 6.2 | 5.7 | 3.9 | 5.1 | 4.2 | 6.6 |

**M2: Method-2; M.M: Modified Method; I: undiluted enzyme solution; II, III and IV: 2.0, 5.0 and 10.0 fold diluted enzyme solution.**

**TABLE 3 : Intra-run coefficient of variation values on different days (n=10) established by reference and modified methods.**

| CV % | 1st day | | 3rd day | | 7th day | | 10th day | |
|---|---|---|---|---|---|---|---|---|
| | M2 | M.M | M2 | M.M. | M2 | M.M. | M2 | M.M. |
| Intra – run | 2.5 | 2.4 | 3.3 | 2.5 | 2.8 | 3.3 | 2.6 | 2.8 |

**M2: Method-2; M.M: Modified Method**

radicals in the living cells[7]. The physiological importance of xanthine oxidase is exemplified by the disorders of gout and xanthinuria. In several diseases, XO activities were found to be altered in the tissues, cells and biological fluids and many studies were carried out to establish XO activities in some pathological conditions[8-10]. For this reason, establishment of a precise and accurate method seems to be essential.

Indeed, several methods have been used to measure XO activity in the biological materials. Spectrophotometric methods based on manometric measurement of oxygen uptake, measurement of methylene blue or cytochrome c reduction[11], several colorimetric methods[12], radioisotopic assays[13], fluorometric procedures[14] and radioimmunoassay techniques[15] have been described by several researchers. Each method has both advantages and disadvantages in laboratory applications. In any case, the method to be used should be correct and give accurate and precise results.

In this respect, XO activity method used by Prajda *et al*[3] and Kökoglu *et al*[4] presents an important problem due to methodological problems. As seen from our results, there are great differences between the results of the methods. These differences mainly arise from two reasons. First, as described in the methods blank contains no xanthine even it gives significant absorbance at this wavelength. For this reason, even though there is no XO activity in a sample, one can measure false activity value by using procedure 1 in the method-1. This is mainly due to presence of xanthine in the assay mixture. Second, TCA suppresses absorbances of both xanthine and uric acid significantly, thereby causing measurement of lowered enzyme activity. It is also possible to find no activity with the method-1 (procedure 2), particularly while working with samples having low XO activity (TABLE 1). Infact, when a sample with low XO activity is being studied, it is better to use lower xanthine concentrations (such as 0.033 mM) because high xanthine concentration causes high absorbance values, which decreases assay sensitivity. Our modified method eliminates all these faults. As seen, the results obtained by modified method and method-2 are quite similar and, precision and accuracy values of the methods are comparable. There are however great differences between the results of procedure1 and 2 of the method-1 and other methods.

In our opinion, another important point in the XO activity assay, which has not been evaluated in the previous methods, is that, absorbance of xanthine decreases after incubation period due to the conversion reaction of xanthine to uric acid. For this reason, background absorption of xanthine is lowered in sample tube after incubation. This difference causes lowered estimation of uric acid absorption and XO activity. This can be corrected by the analysis as described in the materials and methods section. With this correction analysis, we found that real XO activity was higher by approximately 5% than that of observed activity.

Modified XO activity method presented here gives comparable results eliminating fault sources in the previous methods. This method can be easily and accurately used for the XO activity measurement in the biological samples.

## Simplified description of the method and its future application

The method presented here is based on determination of absorption increases at 293 nm due to increase in uric acid production by the effect of xanthine oxidase. First, TCA addition to stop the reaction causes suppression of absorbances of both xanthine and uric acid and second, xanthine itself gives significant absorption at 293nm, previous method causes false increases in the activity measurements. Our modified method eliminates these faults and gives more correct and precise results. Therefore it is important to measure XO

activity in the biological samples by using the method given in the present study.

## REFERENCES

**[1]** D.A.Parks, D.N.Granger; Xanthine oxidase: Biochemistry, distribution and physiology, Acta Physiologica Scandinavica, Supplementum, **548**, 87-99 **(1986)**.

**[2]** S.Hashimoto; A new spectrophotometric assay method of xanthine oxidase in crude tissue homogenate, Analytical Biochemistry, **62**, 426-435 **(1974)**.

**[3]** N.Prajda, *et al.*; Imbalance of purine metabolism in hepatomas of different growth rates as expressed in behavior of xanthine oxidase (EC 1.2.3.2), Cancer Res., **36**, 4639-4646 **(1976)**.

**[4]** E.Kokoglu, *et al.*; Xanthine oxidase levels in human brain tumors, Cancer Letters, **50**, 179-181 **(1990)**.

**[5]** H.U.Bergmeyer; In Methods of Enzymatic Analysis: Enzymes as Biochemical Reagent, Academic Press, 521-522 **(1974)**.

**[6]** R.W.Topham, *et al.*; Evidence for the participation of intestinal xanthine oxidase in the mucosal processing of iron, Biochemistry, **21**, 4529-4535 **(1982)**.

**[7]** E.Tubaro, *et al.*; Xanthine oxidase: An enzyme playing a role in the killing mechanism of polymorphonuclear leucocytes, Biochemical Pharmacology, **29**, 3018-3020 **(1980)**.

**[8]** O.Akyol, *et al.*; Activites of free-radical and DNA turn-over enzymes in cancerous and non-cancerous human brain-tissues, Redox Report, **1**, 255-259 **(1995)**.

**[9]** I.Durak, *et al.*; Activities of DNA turn-over and free radical-metabolising enzymes and levels of peroxidation indices in human hepatic cancer tissues, Cancer Research Therapy & Control, **5**, 195-201 **(1998)**.

**[10]** D.J.Hearse, *et al.*; Xanthine oxidase: A critical mediator of myocardial injury during ischemia and reperfusion? Acta Physiologica Scandinavica, Supplementum, **548**, 65-78 **(1986)**.

**[11]** E.Della Corte, *et al.*; Properties of the xanthine oxidase from human liver, Biochimica et Biophysica Acta, **191**, 164-166 **(1969)**.

**[12]** M.Sugiura, *et al.*; A new method for the assay of xanthine oxidase activity, Chemical & Pharmaceutical Bulletin, **29**, 430-432 **(1981)**.

**[13]** C.R.Ramboer; A sensitive and nonradioactive assay for serum and tissue xanthine oxidase, The Journal of Laboratory and Clinical Medicine, **74**, 828-835 **(1969)**.

**[14]** J.L.Haining, J.S.Legan; Fluorometric assay for xanthine oxidase, Analytical Biochemistry, **21**, 337-343 **(1967)**.

**[15]** G.Bruder, *et al.*; Immunological identification and determination of xanthine oxidase in cells and tissues, Differentiation: Research in Biological Diversity, **23**, 218-225 **(1983)**.