ISSN : 0974 - 7435 Volume 9 Issue 7

BioTechnology
An Indian Journal

FULL PAPER

BTAIJ, 9(7), 2014 [267-272]

Seminal xanthine oxidase: Appropriate fluorometric assay for the examination of spermatozoa disorders

Mahmoud Hussein Hadwan[1]*, Lamia A.Almashhedy[1], Abdul Razzaq S.Alsalman[2]

[1]Chemistry Dept., College of Science, Babylon University, (IRAQ)

[2]Surgery Dept., College of Medicine, Babylon University, (IRAQ)

E-mail: mahmoudhadwan@gmail.com

ABSTRACT

A Fluorometric assay for semen xanthine oxidase activity that could be used as a back-up to current tests in the differential diagnosis of spermatozoa disorders is described. The assay is based on the H_2O_2-dependent oxidation of thiamine catalyzed by peroxidase mimetic (a mixture of hematin (HT) and hemoglobin (HB)). The method is sensitive, precise (CV below 7.9%), and linear up to 40 U/l. The analytical recovery of the present method is estimated. The comparison with the UV method gave good correlation (r = 0.994). The method is applied to the measurement of the XO activity in seminal plasma of fertile men and Asthenzoospermic patients. Reference values for seminal xanthine oxidase activities determined with the present method on 30 healthy persons are 70.37±23.1.

KEYWORDS

Xanthine oxidase;
Fluorometric assay;
Spermatozoa disorders;
Hematin;
Hemoglobin;
Thiamine.

INTRODUCTION

The key role of reactive oxygen species in the pathophysiology of sperm function has been considered comprehensively[1]. Their production in semen has been related to loss of motility, decreased capacity for sperm-oocyte fusion, loss of fertility[2] and defective sperm role[3]. Human spermatozoa are recognized to be susceptible to lipid peroxidation because of its high content of unsaturated fatty acids[4] and attack of ROS such as hydrogen peroxide on the sperm membrane phospholipids may be a reason of idiopathic subfertility. XO (XO, Xanthine:oxidoreductase, E.C.1.2.3.22) is recognized to be a potent producer of superoxide and hydrogen peroxide, which are the most injurious ROS for spermatozoa[5]. Xanthine oxidase catalyses the oxidation of hypoxanthine to xanthine and of the latter to uric acid[6], The enzyme is present in intestine, liver, milk[7] and in most mammalian tissues, including seminal fluids[2]. In view of the problem of the ROS source in seminal plasma, it is essential to examine the levels of xanthine oxidase in semen.

Previous assays for XO employ one of the following principles. In the first, XO activity is determined spectrophotometrically by measuring the formation of uric acid from xanthine[8]. This assay undergoes certain disadvantages. It is sensitive, but cannot

FULL PAPER

detect the low levels of enzyme found in human seminal fluids in normal as well as under pathological conditions.

In the second assay, XO activity is determined by using the [^{14}C]Xanthine as described by Dougherty[9]. The disadvantages of this assay involve the need for special technique, and it is reagents have a limited shelf life.

The third assay involves the high-performance liquid chromatographic techniques[10]. This type of assays has some disadvantages such as the unavailability of laboratory equipment to perform in a large number of laboratories and the need for advanced skills to complete.

Battelli *et al*., [11] described a competitive enzyme-linked immunosorbent assay (ELISA) to measure the xanthine oxidase level. This method measures both active and inactive XO protein, which makes this method unsuitable for clinical use.

Atlante *et al*., [12] and Beckman *et al*., [13] developed a method in which the conversion of pterine into isoxanthopterin by hydrogen peroxide is monitored fluorometrically to measure XO activity. This assay disregarded the interaction that results from the presence of the catalase enzyme.

In this paper, precise fluorimetric method is introduced. The present assay has used 3-aminotriazol to eliminate the interaction with catalase. The assay is uncomplicated and the reagents used are relatively stable.

PRINCIPLE

XO oxidizes xanthine to hydrogen peroxide (H_2O_2):

$$\text{Xanthine} + H_2O + O_2 \xrightarrow{\text{Xanthine Oxidase}} \text{Uric Acid} + H_2O_2$$

Hydrogen peroxide (H_2O_2) reacts stoichiometrically with thiamine to generate fluorescence thiochrome (at Ex/Em = 370/425 nm) in the presence of a mixture of hematine and hemoglobin, which acts as peroxidase mimics. Aminotriazole (AT) is included to inhibit catalase enzyme and prevents the interference with measurements, as shown in Figure 1. Since the fluorescence, intensity is proportional to XO level; the XO activity can be accurately measured.

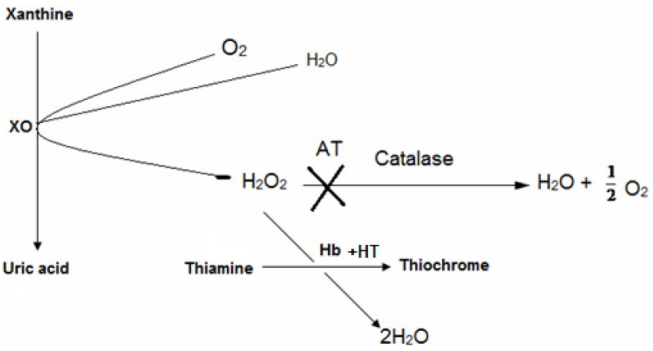

Figure 1 : The Fluorometric Measurement of Xanthine Oxidase (XO).

REAGENTS

1. Hemoglobin (5x 10^{-7} M).
2. Hematin stock solution is prepared by dissolving 0.05 g hematin in 25 ml 0.2 N sodium hydroxide. This solution remains stable for 3 months when refrigerated.
3. Hematin working solution is prepared by diluting 0.5-ml of hematin stock solution to 50 ml of 25 mM sodium phosphate buffer (pH 7.4).
4. Peroxidase mimics solution is prepared by mixing equal volumes of Hemoglobin and Hematin working solution. (It is prepared immediately before using).
5. Xanthine Solution (0.15 mM) is prepared by dissolving 0.228 gm in a minimal volume of NaOH 100 ml. Add approximately 90 ml of phosphate buffer (pH 7.4). Adjust to pH 7.4 at 25°C with either 1 M NaOH or 1 M HCl. Dilute to a final volume of 100 ml. prepared fresh)
6. Thiamin stock solution (10 mM) is prepared by dissolving 337 mg of thiamine hydrochloride in 100 ml of water. Refrigerated at 4 °C, this solution stable for one month. A working solution (2x10^{-3}M) is prepared daily by diluting one volume of stock solution to five volumes with distilled water.
7. Hydrogen peroxide (100µM) is freshly diluted and standardized daily using a molar extinction coefficient of 43.6 M^{-1} cm^{-1} at 240 nm.
8. Sodium phosphate buffer pH 7.4 (50 mM) is prepared by dissolving 1.1 g of Na_2HPO_4 and 0.27 g of KH_2PO_4 in 100 ml distilled water.
9. 3-aminotriazole (50mM): is prepared by dissolv-

ing 0.42 gm of 3-aminotriazole in 100 ml of phosphate buffer (pH 7.4).

10. An K_2HPO_4 –NaOH buffer solution (pH-12) is prepared by adjusting 100 mM K_2HPO_4 (1.36 g of KH_2PO_4 dissolve in 100 ml D.W) to pH-12 with 2 M NaOH (8 g of NaOH in 100 ml dissolve in D.W).

PROCEDURE

Pipette the following reagents into suitable tubes:

Mix by vortex, leave test tubes for 5 min at room temperature, transfer the solutions to suitable cuvettes and read fluorescent intensity. Fluorescent intensity was linearly related to hydrogen peroxide concentration with

Pipette the following reagents into suitable tubes:

	Test	Test Blank	STD1	STD2	STD3	STD4	STD5	STD Blank
Sample	30 µL	-------	-------	-------	-------	-------	-------	-------
H_2O_2 STD	-------	-------	30 µL	50 µL	100 µL	200 µL	300 µL	-------
AT	500 µL	500 µL	500 µL	480 µL	430 µL	330 µL	230 µL	530 µL
Mix by inversion and equilibrate to 37°C for 5 min. Then add:								
xanthine solution	400 µL	400 µL	400 µL	400 µL	400 µL	400 µL	400 µL	400 µL
Mix by inversion and incubate at 37C for exactly 30 minutes. Then add:								
*Phosphate buffer (pH 12).	1500 µL	1500 µL	1500 µL	1500 µL	1500 µL	1500 µL	1500 µL	1500 µL
Thiamin	250 µL	250 µL	250 µL	250 µL	250 µL	250 µL	250 µL	250 µL
Peroxidase- mimics	250 µL	250 µL	250 µL	250 µL	250 µL	250 µL	250 µL	250 µL
** Sample	-------	30 µL	-------	-------	-------	-------	-------	-------

*** There is no need to use concentrated acids or high temperature to stop the reaction that catalyzed by a xanthine oxidase because this enzyme inhibited completely when the pH equal to 12. (ref. 14); ** Serum is added to test blank to insure the prevention of overlapping of peroxides in sample (seminal plasma) with hydrogen peroxide that formed from the reaction, which catalyzed by xanthine oxidase.**

the fluorophotometer (excitation, 370 nm; emission, 425 nm).

CALCULATIONS

Standard curve

ΔI Standard = I Standard - I Standard blank

Plot the ΔI of the Standards vs. µmoles of H_2O_2

Sample determination

I Sample = I Test - I Test Blank (I: fluorcence intensity)

Determine the µmoles of H_2O_2 liberated using the Standard Curve.

$$\text{Units/L enzyme} = \frac{B}{(30)(0.03)} * df = (\mu mol/min)/L = (U/L) = mU/ml$$

B = µmoles of H_2O_2 liberated according to standard curve; df = Dilution factor; 30 = Time of assay in minutes; 0.03 = Volume (in milliliter) of enzyme used.

INSTRUMENT

Spectrofluorometer shimadzu RF- 5301.

Statistical analysis

Statistical parameters such as: mean value (X), standard deviation (SD), and coefficient of variations (CV) are calculated with EXCEL.

RESULTS AND DISCUSSION

Oxidation of non-fluorescent thiamine to fluorescent thiochrome with peroxidase or peroxidase mimics as a catalyst by hydrogen peroxide is often used to estimate thymine[15,16] or to estimate hydrogen peroxide[17]. Previous methods, which focused on measuring hydrogen peroxide, have used hemin, hematin[18], metal-porphyrin complex [19] or hemoglobin [17] as peroxidase mimics. In the present assay, a new fluorometry for assay xanthine oxidase via hydrogen peroxide determination is presented, in which a mixture of hematin (HT) and hemoglobin (HB) is used as mimetic enzyme of peroxidase for fluorogenic reaction between thiamin and hydrogen peroxide. The obtained results demonstrated that the mixture of HB and HT is a promising peroxidase mimic. The emission spectra of thiamine is shown

FULL PAPER

in Figure 2.

The concentration of 3-aminotriazole required to prevent interference with catalase that is founded in the sample, is investigated by assaying catalase activity in the presence of different concentrations of this inhibitor. Figure 3 indicates that the degree of catalase inactivation depends on the concentration and duration of pre-incubation with aminotriazole.

Aminotriazole (50mM) is included in all subsequent assays of XO; this concentration is likely to be in excess of that necessary to inactivate the catalase present in seminal fluids. The increment of xanthine oxidase activity after adding 50mM-aminotriazole reaches up to 40% and stays constant with higher concentration of this inhibitor. However, five min incubation with 50mM-aminotriazole allowed adding 100 k-unit of catalase(obtained from Himedia (Product Code: TC037)) to reaction mixture without any effect on the measured oxidase activity.

A crude xanthine oxidase has been purified from bovine milk according to method described by Shivraj *et al.*,[20]. It is used to compare present method with others that are used for assessment of xanthine oxidase. Results obtained by the present method are compared with those of UV-method[8]. Identical sample, buffer, and substrate were used in both methods. Results have been shown in TABLE 1.

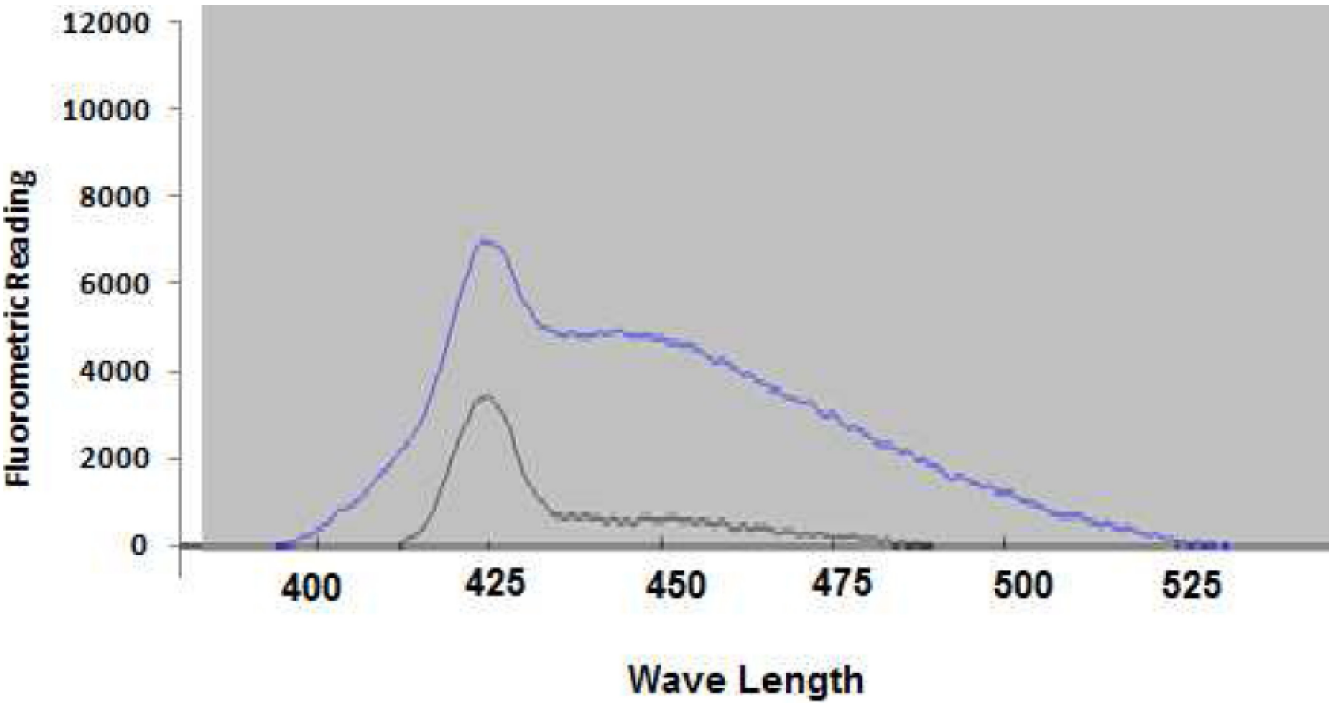

Figure 2 :Emission Spectra of Reagent Thiochrome. Excitation Spectra were Monitored at 370 nm, Emission Spectra were Monitored at 425 nm.

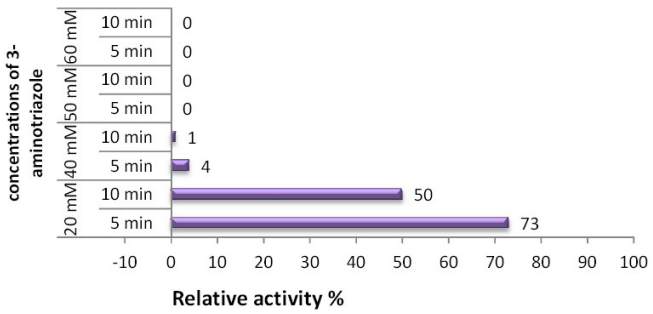

Figure 3 : The dependence of catalase activity (50 k-unit/L) on time of incubation with different concentrations of 3-aminotriazole.

The method is applied to the measurement of the XO activity in seminal plasma of 30 fertile men (age 31.6±3.3 year) and 30 Asthenzoospermic patients (age 32.5±3.23 year). Results have been shown in TABLE 2.

It is evident from the above that the results of this method can be used to assess oxidative stress of seminal fluids. The obtained results are consistent with results of previous studies[21].

Accuracy of the entire assay protocol is measured by recovery of hydrogen peroxide added to sample detailed in TABLE 3.

TABLE 1: Statistical Analysis of The Values Obtained for Xanthine Oxidase by UV- Method and Present Method.

No. of Samples	20
Mean of UV- method (U/L)	9.84
Mean of test method (U/L)	9.94
Mean of both methods (U/L)	9.89
Regression coefficient B	0.9863
Regression coefficient A	0.0137
Correlation coefficient	0.9942

TABLE 2 : Levels of Xanthine Oxidase in seminal plasma of fertile and subfertile men.

	Healthy controls	Asthenzoospermic patients
Xanthine oxidase (mU/L) (mean± SD)	70.37±23.1	132.37±30.15

The precision of the assay is measured in-run on a single sample specimen and between run over a period

TABLE 3 : Analytical Recovery of Hydrogen Peroxide Added to Sample

Present in assay	Equivalents of Hydrogen Peroxide	Calculated activity U/liter	Observed activity[a] U/liter	Recovery %
Pooled sample	-----	-----	10.62	-----
Pooled sample + peroxide [b]	10	20.62	20.69	99.99
Pooled sample + peroxide	20	30.62	29.85	97.48
Pooled sample + peroxide	30	40.62	38.345	95.00
Pooled sample + peroxide	40	50.62	48.576	96.00
Pooled sample + peroxide	100	100.62	93.19	82.75

[a] mean of triplicate determinations; [b] present as H_2O_2

TABLE 4 : The precision of the present assay.

	N	Mean (±SD)U/liter	CV%
Within-run	20	10.6 ± 0.272	2.5
Between-run	20	9.26 ± 0.819	7.9

of three weeks with aliquots from the same sample pool but different reagent's preparations. The results are shown in TABLE 4.

The assay presents a number of advantages more than the existing methodologies. These advantages include; less quantity of sample is required (0.03 ml); the reagents are relatively stable; instrumentals, and apparatus are not complicated and available in most research laboratories, and the assay is free from interference.

REFERENCES

[1] M.Hadwan, L.A.Al-mashhedy, A.Al salman; BMC Urology, http://www.biomedcentral.com/1471-2490/12/32, **12**, 32 **(2012)**.

[2] A.Agarwal, S.R.Shyam; Middle East Fertility Society Journal, http://www.bioline.org.br/request?mf04034, **9**, 187 **(2004)**.

[3] E.de Lamirande, H.Jiang, A.Zini, H.Kodama, C.Gagnon; Rev.Reprod, http://ror.reproduction-online.org/cgi/reprint/2/1/48, **2**, 48 **(1997)**.

[4] M.Marmunti, A.M.Gutiérrez, M.Gavazza, S.Williams, A.Palacios; Rev.vet., http://www.cabdirect.org/abstracts/20123291771.html;jsessionid=D1F5B1401F309B546E6E7F565E32EDD7, **23**, 8 **(2012)**.

[5] E.Ee Lamirande, C.Gagnon; J Androl., http://onlinelibrary.wiley.com/doi/10.1002/j.1939-4640.1992.tb03327.x/abstract;jsessionid=80FE8992684BE374314A24A66A80951B.d01t0, **13**, 368 **(1992)**.

[6] A.Baghiani, M.Djarmouni, S.Boumerfeg, H.Trabsa, N.Charef, S.Khennouf, L.Arrar; European Journal of Medicinal Plants, http://www.sciencedomain.org/download.php?f=132880013 1-Baghianietal_2011EJMP898.pdf&aid=356, **2**, 42 **(2012)**.

[7] K.Vettenranta, K.O.Raivio; Pediatr Res, http://www.nature.com/pr/journal/v27/n3/abs/pr199066a.html, **27**,286 **(1990)**.

[8] Bergmeyer, H.U., Gawehn, K.and Grassl, M., 1974, in Methods of Enzymatic Analysis (Bergmeyer, H.U.ed.) Second Edition, Academic Press Inc., New York, **1**, 521-522.

[9] H.M.Dougherty; Anal.Biochem., http://wyndmoor.arserrc.gov/Page/1976/4135.pdf, **74**, 604 **(1976)**.

[10] T.Sasaoka, N.Kaneda, T.Nagatsu; Journal of Chromatography, http://www.ncbi.nlm.nih.gov/pubmed/3372632, **424**, 392 **(1988)**.

[11] M.G.Battelli, A.Abbondanza, S.Musiani,

L.Buonamici, P.Strocchi, P.L.Tazzari, D.L.Gramantieri, F.Stirpe; Clinica Chimica Acta, http://www.ncbi.nlm.nih.gov/pubmed/10217635, **281**, 147 **(1999)**.

[12] A.Atlante, D.Valenti, S.Gagliardi, S.Passarella; Brain Research Protocols, http://www.ncbi.nlm.nih.gov/pubmed/11086257, **6**, 1 **(2000)**.

[13] J.S.Beckman, D.A.Parks, J.D.Pearson, P.A.Marshall, B.A.Freeman; Free Radical Biology & Medicine, http://www.ncbi.nlm.nih.gov/pubmed/2753392, **6**, 607 **(1989)**.

[14] http://www.toyobo-global.com/seihin/xr/enzyme/pdf_files/277_280XTO_212.pdf

[15] W.Horwitz, (Ed); Official Methods of Analysis of AOAC International, 17th Edition, Thiamin Fluorometric Methods 942.23 Ch. 45, 957.17 Ch. 45, **2**, 6-7, 8-9 **(2000)**.

[16] W.C.Ellefson; Thiamin, In : Augustin J., B.P.Klein, D.A.Beker, P.B.Enugopal, (Eds); Methods of Vitamin Assay, 4th Editon, John Wiley & Sons, Inc., New York, U.S.A., 349-363 **(1985)**.

[17] Xu.Chunli, Zhujun Zhang; Analytical Sciences, https://www.jstage.jst.go.jp/article/analsci/17/12/17_12_1449/_pdf, **17**, 1449 **(2001)**.

[18] J.Li, P.K.Dasgupta; Anal.Chem., http://www.lib.ctgu.edu.cn:8080/wxcd/qw/1468.pdf; **72**, 5338 **(2000)**.

[19] C.Q.Zhu, D.H.Li, H.Zheng, Q.Z.Zhu, Q.-Y.Chen, J.G.Xu; Anal.Sci., https://www.jstage.jst.go.jp/article/analsci/16/3/16_3_253/_pdf, **16**, 253 **(2000)**.

[20] S.H.Nile, C.Khobragade; Indian Journal of Natural Products and Resources, http://nopr.niscair.res.in/bitstream/123456789/11540/1/IJNPR%202%281%29%2052-58.pdf, **2**, 52 **(2011)**.

[21] D.Sanocka, R.Miesel, P.Jedrzejczak, M.K.Kurpisz; Journal of Andrology, http://onlinelibrary.wiley.com/doi/10.1002/j.1939-4640.1996.tb01813.x/abstract, **17**, 449 **(1996)**.