A Spectrophotometric Method for the Determination of Aldehyde Oxidase Activity Using 3-Methyl-2-Benzothiazolinone Hydrazine: A Preliminary Study

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A B S T R A C T

Aldehyde oxidase, a molybdenum cofactor-containing cytosolic enzyme, is extensively distributed throughout the animal kingdom. The enzyme is mainly active in liver and other tissues of mammalian species and involved in the metabolism of wide range of aldehydes and nitrogen-containing molecules. A continuous spectrophotometric method for the quantitative determination of aldehyde oxidase, AO, enzyme activity is described in this article. This method is based on the coupling reaction between 3-methyl-2-benzothiazolinone hydrazone (MBTH) and the *o*-quinone. Dopamine as substrate for AO, is converted/oxidized to *o*-quinone. The latter react with MBTH to produce intensely colored products that absorb light maximally in the visible region. Then, AO activity has been kinetically characterized; the K_m (Michaelis–Menten constant) and V_{max} (maximum initial velocity) values for the oxidation of dopamine by AO were evaluated. The existence of MBTH in the reaction medium and production of stable color as well as the high ϵ values at 510 nm of MBTH-Q adduct make this direct technique more sensitive than other continuous methods for AO assay. The optimized MBTH reaction may be useful for biological staining of AO activity isolated from various biological sources in electrophoresis gels.

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Introduction

AO (aldehyde: O2 oxidoreductase EC1.2.3.1), a group of metalloflavoprotein enzymes, that is gaining attention in drug discovery and development programs. AO is broadly distributed throughout the animal kingdom and includes two identical 150-kDa subunits, each containing domains that house FAD, two iron-sulfur clusters, and molybdenum cofactor. It has been found that Flavin adenine dinucleotide and a molybdopterin cofactor are necessary for AO catalytic function [1]. Data illustrating AO enzyme structure has been obtained from the crystal structure of highly homologous enzymes such as human xanthine oxidase and murine AOX3; however, human AO has not been crystallized [2, 3]. Mounting evidence showed that AO is principally active in liver and other tissues of mammalian species and is able to terminate the metabolism of a wide range of aldehydes and nitrogen-containing molecules encompassing numerous pharmacological and/or biological activities which can lead to significant clinical impact. AO possesses broad substrate selectivity, oxidizing aldehydes and a variety of heterocyclic compounds, containing nitrogen-containing aromatic heterocycles [1]. Such heterocycles are commonly utilized as scaffolds in the development of novel chemical entities which can result in improvement of diversity of AO drug substrates [4, 5]. A large body of evidences has revealed that AOs participate in the metabolism of important drugs such as allopurinol, nicotine, famciclovir, pyridoxal, quinine, N-methylnicotinamide, carbazeran, zaleplon, brimonidine [6], methotrexate, azathioprine, and 6-mercaptopurine [1, 7-11]. Moreover, AO is associated with the metabolism of endogenous compounds like monoamine neurotransmitters, retinal, 2-phenylethylamine, as well as vitamins B3, B6, and A [1, 8-12]. Experimental studies have suggested that AO is associated with particular pathological situations such as oxidative stress and amyotrophic lateral sclerosis seizures [1, 12, 13]. Despite several investigations on AO, various aspects of physiological and biochemical properties of this enzyme have not been reviled yet.

Along with development in understanding candidates of AO substrates, a wide ranges of biochemical studies has focused on finding inhibitors of AO activity, explaining their mechanism of action, as well as revealing clinical implications, particularly probable drug-drug interactions. It has been demonstrated that various chemical compounds from different categories are able to inhibit AO activity including tricyclic antidepressants, phenothiazines, estrogens, and tricyclic atypical antipsychotic agents [1, 4]. Clinical AO-associated drug interactions have not been reported; however, *in vitro* investigations suggest an interaction risk [8, 9, 14]. Drug-drug interactions (especially those mediated by drug-metabolizing enzymes) possess a great concern due to reduce the efficacy of the drugs and enhance the risk of adverse effects. The main parts of drug interactions is attributed to interaction of components with drug metabolizing enzymes including AO which has a pivotal role in the metabolism of a wide range of substrates with different chemical structures and functionalities [5, 7, 10-12].

Poor bioavailability of AO metabolites and failure to predict its human pharmacokinetics, due to lack of *in vitro* methods which are able to predict *in vivo* clearances and metabolism mediated by AO, has led to discontinued development of drug interaction and metabolism in medical programs [15]. Thus, exploring novel biochemical method for rapid and reliable evaluation of AO activity possesses a key contribution in the field of drug metabolism and drug interaction.

The high-throughput methods for assessing AO activity possess an important role for the desired requirement. Various high-throughput methods of AO activity are based on ultraviolet (UV) spectrophotometry assay [16] through utilizing phenanthridine, vanillin, salicylaldehyde [17], benzaldehyde, indole-3-aldehyde,Zaleplon [18], famciclovir [13], methotrexate [15, 19], zonisamide [20] as AO enzyme substrate, as well as HPLC-MS / MS methods [7]. Although above mentioned methods are sensitive ,but are not among rapid or inexpensive methods of assessment [21]. Spectrophotometric techniques have attracted much attention because they are convenient and inexpensive, compared to other methods [22].

Several spectrophotometric methods for the detection of AO activity have been described [23]. The enzyme activity can be determined using phenantridine, benzaldehyde and vanillin, as substrates, by measuring the change in the absorbance at 322, 249 and 310 nm, respectively. But their use with crude enzyme preparations is limited by the presence of other UV-absorbing material in the assay or apparent light absorbance/light scattering at ~300-400 nm range [24] (**See Fig. 1**). The 3-methyl-2-benzothiazolinone hydrazone (MBTH) is a molecule which has been introduced as a reagent in analytical chemistry for the detection and determination of different chemical compounds including aldehydes, aromatic amines, indoles, iminohetero-aromatic compounds, carbazoles, arylalkylamines, phenols [25, 26]. In addition, MBTH is applied as a reagent in the assessment of different enzyme activities, such as tyrosinases, toluene-4-monooxygenase, peroxidase [26], as well ascatecholase [27].

Here, we attempted to develop a new colorimetric high-throughput screening method for the determination of AO activity. This method is based on the coupling reaction between MBTH and the *o*-quinone product of the oxidation of dopamine mediated by AO. Finally, three well-known AO enzyme inhibitors including menadione, tamoxifen and ketoconazole (Fig. 1) [9] were employed for additional characterization of the proposed method. The aim of the current study was to develop a colorimetric assay (in visible region of light) for the quantification of AO activity and for this application, the method described here offers advantages over existing AO assays, so that this method activity causes the appearance of color against a nearly colorless blank.
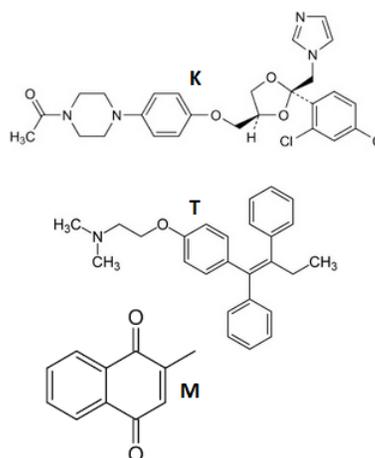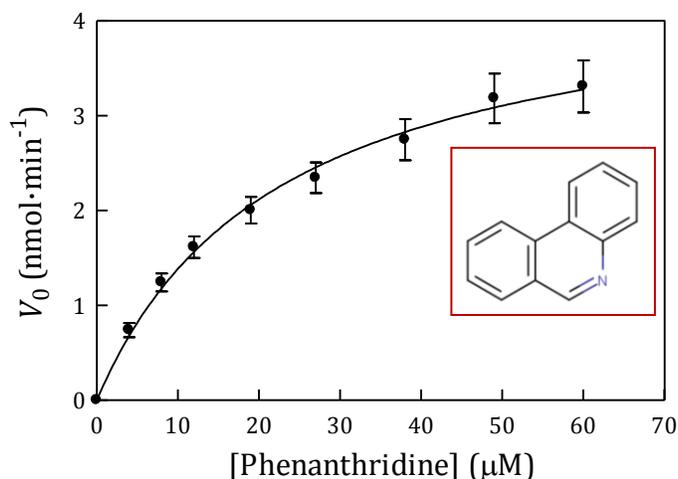
Fig. 1. (*Left*) Michaelis-Menten plot of AO activity for phenanthridine as the substrate, taken from [1] in which the enzyme activity was assayed spectrophotometrically using phenanthridine as a substrate at 322 nm (near UV region). (*Right*) Chemical structures of three known AO inhibitors; Menadione; **M**, Tamoxifen; **T**, and Ketoconazole; **K**.

Materials and methods

Chemicals and Reagents

Menadione, Tamoxifen, and Ketoconazole were generous gifts from Dr. Bahrami, Dr. Fatahi and Dr. Shirazi (Kermanshah/Shaheed Beheshti Universities of Medical Sciences). Dopamine and 3-Methyle-2-benzothiazolinone Hydrazone (MBTH) were obtained from Merck (Darmstadt, Germany). All other compounds utilized were of analytical grade.

Enzyme Preparation

Partially purified molybdenum hydroxylases were prepared from mature male Dunkin-Hartley guinea pig liver (400—600 g, Medical Biology Research Center, Kermanshah University of Medical Sciences) according to the method of Johnson *et al.* [19]. Animals were handled with humane care in accordance with the National Institute of Health guidelines, and the study was approved by the local ethic committee. Briefly, the animal was killed between 9:00—10:00 a.m. by cervical dislocation, and the liver was immediately excised and placed in ice-cold isotonic potassium chloride solution (1.15% KCl w/v) containing 0.1mM EDTA. Then, the liver was homogenized at 3000—4000 rpm for 1 min at 4 °C using Potters homogenizer. The homogenate was heated on a steam bath at 55—57 °C for 10 min, cooled to 4 °C and centrifuged at 15000 *g* for 45 min at 4 °C and the supernatant was treated by 50% saturated solution of ammonium sulphate (35.3 g/100 ml) at 4 °C. The resulting suspension was re-centrifuged at 6000 *g* for 20 min at 4 °C. The precipitate was dissolved in a minimum volume of 0.1 mM EDTA solution and then was kept at -50 °C until use [28].

AO activity assay in the presence and absence of inhibitors

All spectrophotometric determinations were carried out at 25 °C using a PerkinElmer spectrometer. Guinea pig AO activity was assayed spectrophotometrically using phenanthridine as a substrate at 322 nm according to [1]. The enzyme activity was also determined using different concentrations of dopamine as substrate, in the presence of MBTH, by measuring the change in the absorbance 510 nm. Substrate was separately incubated with the enzyme fraction in Sorenson's phosphate buffer pH 7.0 containing 0.1mM EDTA and MBTH (5 mM), and the initial oxidation rates were measured as a function of time (0-12 h), by registering A_{510} at different time intervals. The initial velocities of enzymatic reactions were calculated and used for determination of K_m and V_{max} values (for the oxidation of phenanthridine/dopamine by guinea pig liver fraction) from Lineweaver–Burk double reciprocal plot of 1/V against 1/[S]. The line of the best fit through the points on the plot was calculated using linear regression by the least square method [1].

The enzyme was also assayed in the presence of different concentrations of the drug inhibitors; menadione, tamoxifen, and ketoconazole. All drugs were dissolved in DMSO as 10 mM stock and diluted to proper concentrations with the assay buffer. Likewise, we utilized the same volumes of DMSO for the reference samples (AO solutions with no inhibitor), as vehicle control and found no significant alteration in the enzyme initial velocity under the effect of changes in DMSO concentration. DMSO final concentration was ≤3% during this study. Before enzymatic reaction, all of the solutions were equilibrated at 25°C, except the enzyme fraction which was kept on ice bath prior to addition to the incubation solution. In addition, possible enzymatic and non-enzymatic interactions of the drugs in the absence of the substrates with the constituents of the incubation mixture, including MBTH, were tested before measurement of enzymatic reaction. After incubation at room temperature for at 12 h, absorption spectra of different reaction mixtures were then recorded over a range (of 450-600 nm) that included the wavelength of 510 nm at the end of each experiment.

Data were analyzed using GraphPad Prism version 5.00 for Windows, (GraphPad Software, San Diego California USA, www.graphpad.com). Some experimental data are representative mean of three independent experiments, and the other values are presented as mean± standard deviations (SD). P value <0.05 was considered statistically significant.

Results

A known amount of substrate was incubated with AO and MBTH. A dark color formed in the reaction medium. The wavelength at which the *o*-quinone–MBTH adducts absorb maximally was determined (as ~510 nm) by scanning the reaction mixture at wavelengths between 450 and 600 nm, so this wavelength was selected for quantitative

measurement of AO activity. It is indicated in Fig. 2 that activity (*dA/dt*) changes in a concentration-dependent manner related to AO amount. The extent of dopamine oxidation linearly increases with increase of AO concentration, indicating that the enzyme velocity, in term of MBTH-quinone formation, is first order with respect to AO concentration, with the correlation coefficient equal to 0.976. The AO-mediated dopamine oxidation in the presence of MBTH resulted in formation of MBTH-quinone adduct which possesses an intensive red-pink color and an absorbance maximum at ~510 nm (Fig. 2). This is indicative of the transformation of dopamine by AO activity resulting in the formation of colored *o*-quinone–MBTH adducts. The kinetic behavior of guinea pig liver AO was then studied during the oxidation of dopamine. Fig. 3, shows the oxidation reaction of dopamine by guinea pig liver AO under the effect of substrate concentration. A plot of the reaction velocity as a function of the dopamine concentration that obeys Michaelis-Menten kinetics was also documented.
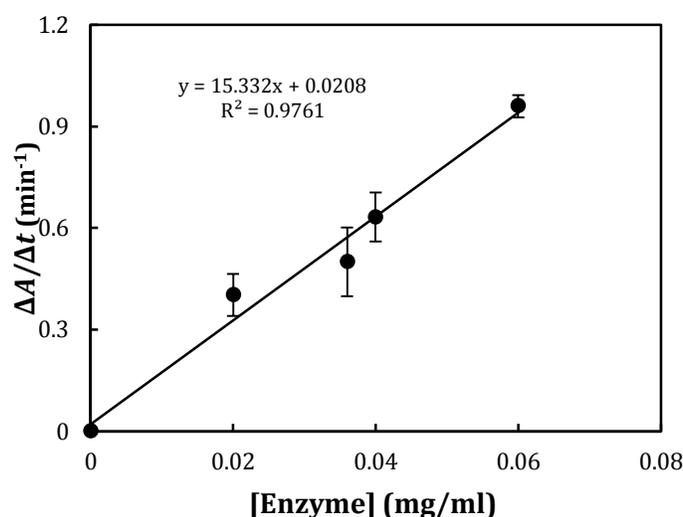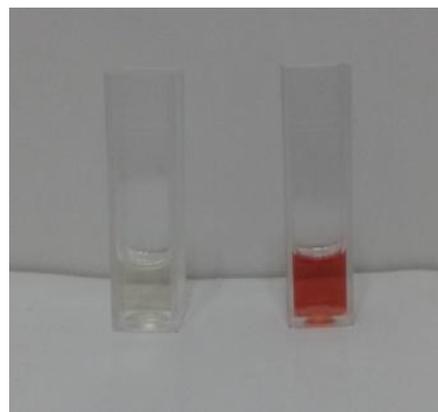
Fig. 2. (*Left*) Absorbance vs amount of AO in the one-step colorimetric method. Linear relationship between AO concentration and absorbance at λ_{max} of the *o*-quinone–MBTH product is documented. Assays were performed under the standard assay conditions as described in Materials and Methods. (***Right***) The AO-mediated dopamine oxidation in the presence of MBTH results in formation of MBTH-quinone adduct which possesses an intensive color and an absorbance maximum at ~510 nm.

K_m (Michaelis–Menten constant) and V_{max} (maximum initial velocity) values for the oxidation of dopamine by guinea pig liver fraction in the presence of MBTH were determined spectrophotometrically from a Lineweaver–Burke double reciprocal plot (Figure 3). The values obtained for the K_m and V_{max} were 14.13±1.51 mM and 0.127±0.006 µM.min^{-1}, respectively. Although these kinetic values for dopamine oxidation are much different from K_m/V_{max} values of the phenantridine (higher K_m and lower V_{max}, see [1], but, is yet worthwhile because it is generally accepted that colometric enzyme assay in visible region has an undeniable advantage over methods that use shorter wavelengths in UV region (<400 nm).

So, as described in Materials and methods, for quantification of AO activity the final absorbance at 510 nm (λ_{max}) was suggested, as a continuous enzyme assay.
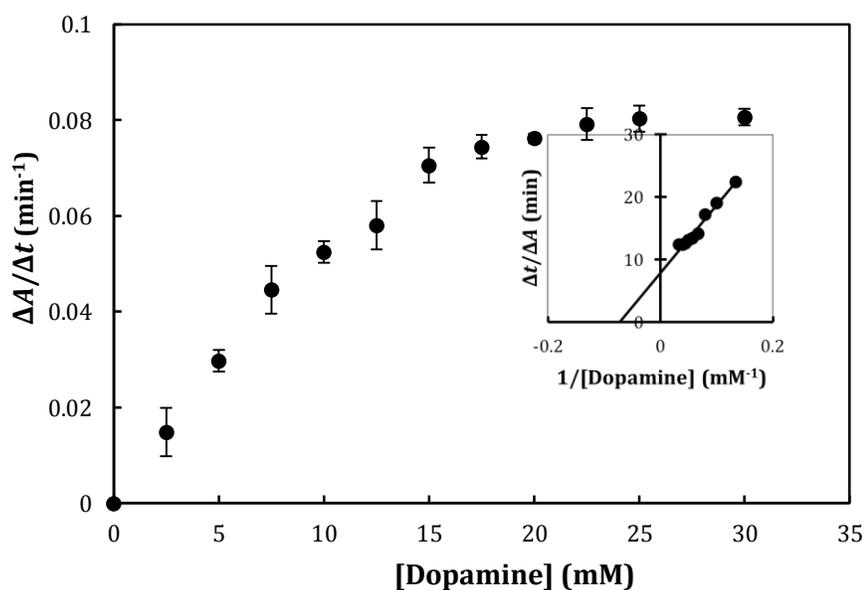
Fig. 3. Substrate titration curve and Lineweaver-burk plot (as inset) of AO (initial) activity for dopamine (as the substrate).

AO-mediated formation of "MBTH-quinone" adduct

It appears likely that cells expressing the tyrosinase gene are present in a wide range of human tissues [29]. Tyrosinase is an oxidase that is the rate-limiting enzyme for controlling the production of melanin. The enzyme is mainly involved in two distinct reactions of melanin synthesis; firstly, the hydroxylation of a monophenol and secondly, the conversion of an o-diphenol to the corresponding *o*-quinone [30]. It is likely that the observed conversion of diphenol-to-quinone is related to tyrosinase activity. As reported earlier [29], liver was negative for tyrosinase gene expression. So, it can be assumed that AO is responsible for dopamine-to-quinone conversion. Additional characterization was performed to determine whether AO catalyzes dopamine oxidation or not, using known AO inhibitors.

The absorption spectra of reaction mixtures in the presence and absence of menadione are summarized in Fig. 4. However, as depicted in this figure, there was a relative decrease in the MBTH-quinone absorption intensity at maximum wavelength, probably confirming that AO activity was strongly inhibited by menadione, as the standard inhibitor of the enzyme. Additionally, different concentrations of the other nonspecific AO inhibitors (tamoxifen and ketoconazole) showed decrease in production of pink color (Fig. 4). The results also showed that menadione was stronger AO inhibitor, as judged by less color production at lower concentrations of the drug. Taking data into account, it can be concluded that AO is responsible for dopamine oxidation, so it can be established, as a simple/reliable colorimetric method for the AO enzyme assay.
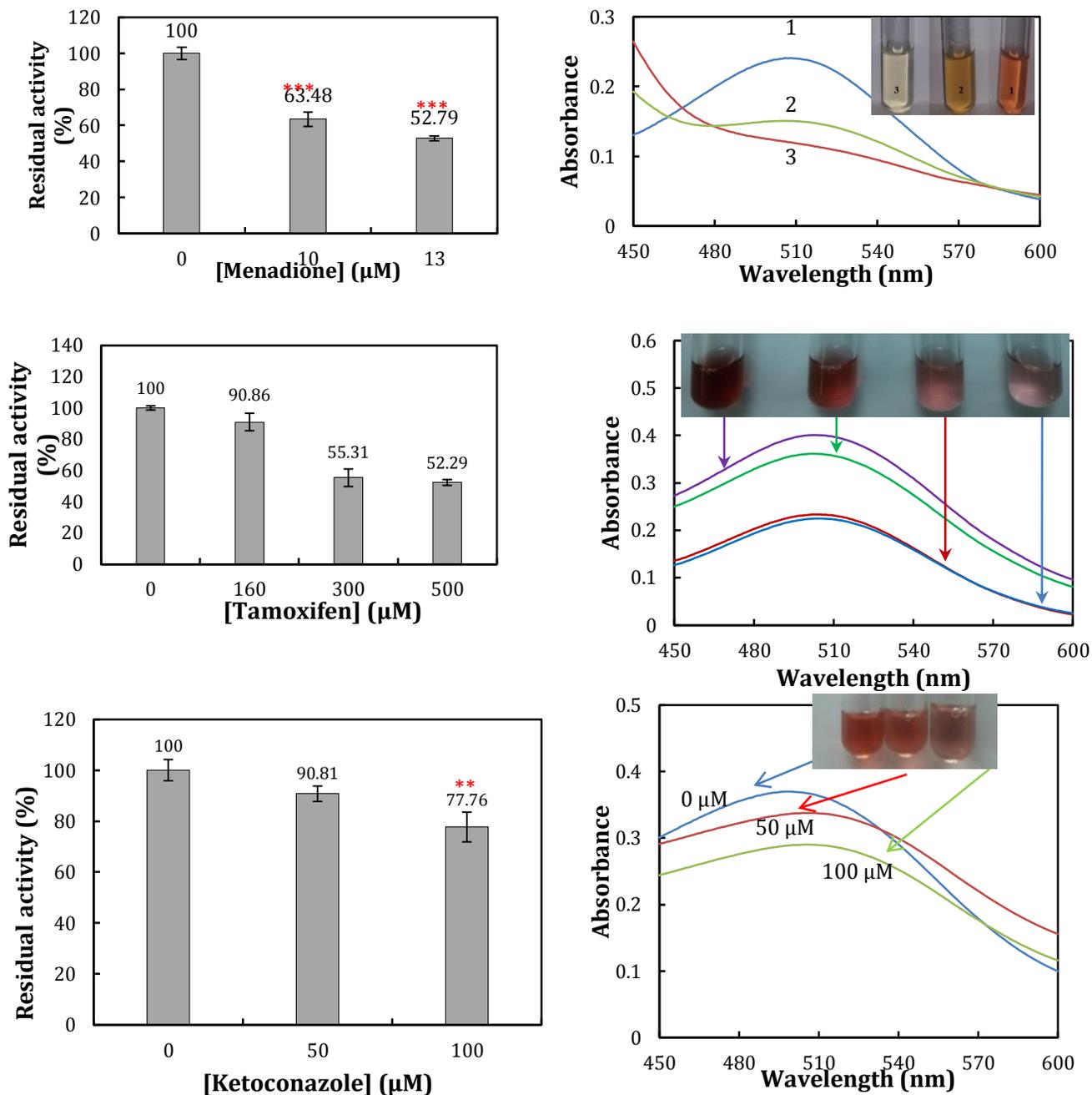
Fig. 4. (*Top*) Effect of menadione on AO-mediated dopamine oxidation, as measured by monitoring changes in MBTH-quinone absorbance spectra. AO assay mixture (AO+dopamine+MBTH), in the presence (10 μM, curve 2; 30 μM, curve 3) or absence (curve 1) of the drug was incubated at 25 °C. Difference spectra are shown, which have been corrected for the contribution from appropriate blank. Further details are given in experimental procedures. (*Middle and Bottom*) Inhibitory effects of different concentrations of tamoxifen (0, 160, 300, 500 μM) and ketoconazole (0, 50, 100 μM) on the oxidation of dopamine by guinea pig liver AO. (Residual activity: the activity of the enzyme in the presence of inhibitor).

MBTH is a potent nucleophile through its amino group, which is in different degrees of protonation-deprotonation, depending on the pH. The suggested mechanism for the oxidation of dopamine catalyzed by AO and the proposed reaction scheme of dopaquinone and MBTH, inspired by [31] is indicated in Fig. 5. In current study dopamine is used as the substrate of AO enzyme and its oxidation resulted in producing *o*-quinone. As *o*-quinone is unstable, the MBTH was used as a nucleophile to react with this compound. Deprotonation of MBTH ammonium group, nuclophilic attack of the NH2 group on the *o*-quinone yields MBTH-quinone adduct with a pink color.
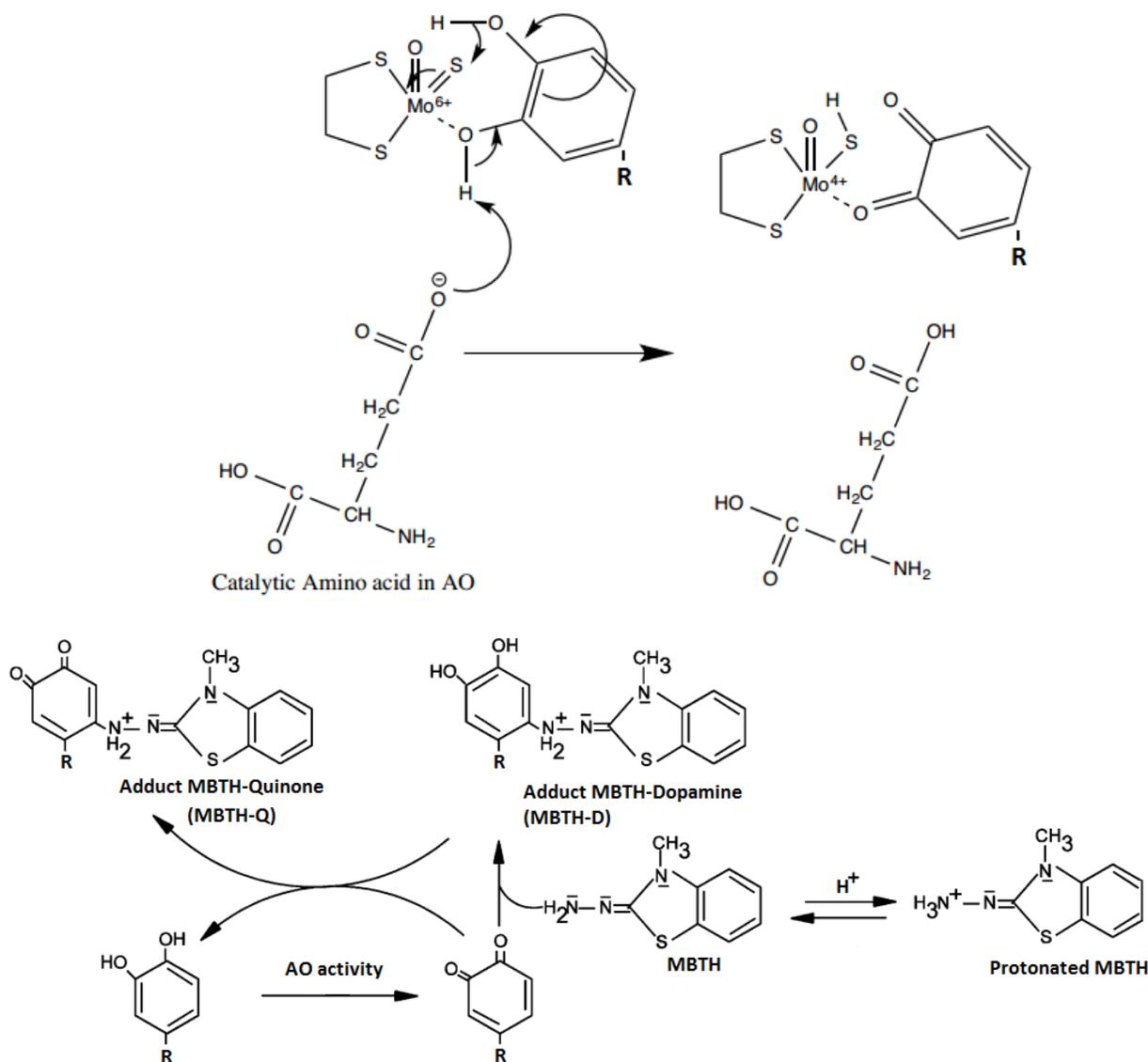
Fig. 5. (Top) possible AO-mediated oxidation of dopamine. (Bottom) Proposed reaction scheme of dopaquinone and MBTH. Adapted from [31].

Discussion

Aldehyde oxidase (AO) is a cytosolic molybdenum-containing hydroxylases that is recently gaining attention in drug design and development. AO catalyzed oxidation of hetero-aromatic rings which commonly incorporated into new drug entities and form the backbone of several marketed drugs and drug candidates [13]. Assessing the potential effect of chemical as well as natural compounds on drug metabolizing enzymes can be used to predict metabolism based dietary substance-drug as well as drug-drug interactions. Various investigations have focused on cytochrome P450-associated interactions; however, other phase-I drug metabolizing enzymes like AO have been systematically evaluated by some rare experimental studies. A wide range of biochemical studies have suggested heterocycles as substrates of AO rather than aldehydes and several investigations have been conducted by using guinea pig liver AO because of high degree of overall amino acid sequence homology between the human and guinea pig AO [1].

One of the routine methods for determination of AO activity was based on utilizing phthalazine as a heterocyclic substrate and potassium ferricyanide (1 mM) as the electron acceptor and measuring the change in the absorbance at 420 nm [32]. Another method for assessing AO activity was based on utilizing phenanthridine as a substrate, following spectrophotometrically the decrease in absorbance at 322 nm.

In the current study, AO enzyme activity converts dopamine to *o*-quinone that reacts with MBTH to produce intensely colored product where the intensity of color (optical density) correlates with the concentration of AO/substrate used. The mentioned property, together with the high **ε** value for *o*-quinone–MBTH adduct [31], makes this a versatile and sensitive method for the quantification of enzyme activities. The sensitivity of the method described here may be comparable favorably with previously reported MBTH-utilizing methods and for detection limit. Furthermore, assessing enzyme activity in visible wavelength, increase in the speed of reaction and no need to expensive laboratory equipment are the main advantages of this method in comparison with previous biochemical method of AO analysis.

Conclusion

The current study was conducted to evaluate a continuous spectrophotometric method for the simple determination of AO activity. This method is based on the coupling reaction between MBTH and the *o*-quinone product of the oxidation of dopamine. Formation of MBTH-quinone adduct has been kinetically characterized, the K_m and V_{max} values for the oxidation of dopamine by AO were evaluated. The existence of MBTH and the high ϵ values at 510 nm of the adducts make this technique more sensitive than other continuous methods. The optimized MBTH method may be suitable to stain AO activity isolated from various biological sources in electrophoresis gels.

Acknowledgment

Conflict of Interests

Authors certify that no actual or potential conflict of interest in relation to this article exists.

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