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A novel spectrophotometric method for determination of kinetic constants of aldehyde oxidase using multivariate calibration method

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Abstract

Although phenanthridine has been frequently used as a specific substrate for the assessment of aldehyde oxidase activity, the use of this method is questionable due to a lower limit of detection and its validity for kinetic studies. In the present study, a novel sensitive multivariate calibration method based on partial least squares (PLS) has been developed for the measurement of aldehyde oxidase activity using phenanthridine as a substrate. Phenanthridine and phenanthridinone binary mixtures were prepared in a dynamic linear range of 0.1–30.0 μM and the absorption spectra of the solutions were recorded in the range of 210–280 nm in Sorenson's phosphate buffer (pH 7.0) containing EDTA (0.1 mM). The optimized PLS calibration model was used to calculate the concentration of each chemical in the prediction set. Hepatic rat aldehyde oxidase was partially purified and the initial oxidation rates of different concentrations of phenanthridine were calculated using the PLS method. The values were used for calculating Michaelis–Menten constants from a Lineweaver–Burk double reciprocal plot of initial velocity against the substrate concentration. The limits of detection for phenanthridine and phenanthridinone were found to be 0.04±0.01 and 0.03±0.01 μM (mean±SD, $n=5$), respectively. Using this method, the K_m value for the oxidation of phenanthridine was calculated as 1.72±0.09 μM (mean±SD, $n=3$). Thus, this study describes a novel spectrophotometric method that provides a suitable, sensitive and easily applicable means of measuring the kinetics of phenanthridine oxidation by aldehyde oxidase without the need for expensive instrumentation.

Keywords: Aldehyde oxidase; Phenanthridine; Kinetic constants; Partial least square

1. Introduction

Kinetic studies on enzymes are important tools for understanding biological interactions at the molecular level. Details of the kinetics are important because they provide essential information about how an enzyme will behave or respond in a given situation. Simple enzyme kinetics is generally described by Michaelis–Menten kinetics (Eq. (1)) [1,2].

$$v = \frac{V_{max} S}{K_m + S} \quad (1)$$

where v is the enzymatic reaction rate, S is the substrate concentration, V_{max} is the maximum or limiting reaction rate and K_m is the Michaelis–Menten constant. By rearranging Eq. (1), the following equation can be derived:

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \frac{1}{S} \quad (2)$$

When Eq. (2) is plotted as $1/v$ versus $1/S$ (known as a Lineweaver–Burk plot), we obtain an appropriate linear plot that is commonly used to determine numerical values of V_{max} and K_m.

In recent years, there have been many efforts to develop more sensitive and precise techniques for characterizing the

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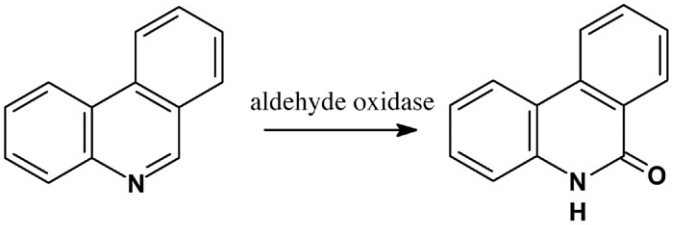
kinetics of enzyme reactions [3,4]. Aldehyde oxidase (EC 1.2.3.1), a cytosolic enzyme found in many animal species, has been shown to catalyze both oxidation and reduction of a wide range of aldehydes [5,6] and *N*-heterocyclic xenobiotics including some important drugs such as famciclovir [7], methotrexate [8], azathioprine [9], quinine and quinidine [10]. In addition, aldehyde oxidase catalyzes the metabolism of some endogenous compounds such as retinaldehyde [11], pyridoxal [12], 2-phenylethylamine [13], homovanillamine [14], 3,4-dimethoxy-2-phenylethylamine [15] and 5-HT [16]. Furthermore, aldehyde oxidase may be involved in the biosynthesis of hormones such as indole-3-acetic acid and abscisic acid in plants [17,18].

Although aldehyde oxidase can metabolize an extensive range of aldehydes and *N*-heterocycles, few of these compounds have been used as a substrate for measuring its enzyme activity and kinetic studies [5,7,19]. Phenanthridine has been shown to be an excellent specific substrate for monitoring aldehyde oxidase activity in many studies [7,14–23]. Phenanthridine is a *N*-heterocyclic compound that is oxidized to phenanthridinone by aldehyde oxidase using molecular oxygen as an electron acceptor [2].



Phenanthridine Phenanthridinone

The K_m value for the oxidation of phenanthridine has previously been estimated to be <1 μM [24]. To obtain a valid K_m value, it is necessary to obtain some of the initial rates at phenanthridine concentrations around the K_m value. Aldehyde oxidase activity can be determined spectrophotometrically from the oxidation of phenanthridine to phenanthridinone by monitoring the production of the latter compound at 322 nm. Although there is no spectral interference at this wavelength, the molar absorptivity of phenanthridinone at 322 nm is not very high. Thus, there may be some difficulties in measuring the initial oxidation rates of phenanthridine at the concentrations required to measure the kinetic parameters (Fig. 1). The use of phenanthridine as a substrate for aldehyde oxidase in kinetic studies measured by common spectrophotometric methods may therefore be questionable due to a lower limit of detection and validity of the method. Although phenanthridinone has higher molar absorptivity values in other spectral regions, it is not possible to perform kinetic studies at these wavelengths by univariate calibration methods due to extensive spectral interference between the phenanthridine and phenanthridinone spectra (Fig. 1). A univariate calibration refers to the construction of a relationship between two variables, x and c, such that x can be used to predict c. Determination of the concentration of a single compound using a single wavelength is one of the simplest problems that a univariate calibration model can be used to solve. A univariate calibration model can only provide accurate results when the measured signal does not have contributions from other sources. In other words, full selectivity for the analyte of interest is required.

Chemometrics, is the art of extracting chemically relevant information from data produced in chemical experiments, and enables the chemist to determine the constituents of a complex system without the need of prior separation steps [25,26]. Chemometric methods, including multivariate calibration and multivariate curve resolution, have been shown to have increasing applications for the determination of enzymatic activity [27].

In recent years, multicomponent analysis has become an important tool in the resolution of mixtures into their components in many different fields including biomedical, clinical, and environmental and drug analysis [28]. Increasing attention has been paid to multivariate calibration methods, such as multiple linear regression and principal component regression, and in particular to those that use the partial least squares (PLS) method with decomposition into latent variables. The basic concept of PLS regression was originally developed by Wold [29] and its first chemical application was carried out by Wold [30]. PLS has been used in combination with various spectroscopic techniques such as UV–VIS (including UV optosensing devices) and fluorescence spectroscopy. In order to perform a PLS regression as a full-spectrum multivariate calibration method, only the concentration of the analyte of interest in the calibration samples is required. Unlike univariate calibration, multivariate calibration such as PLS, allows a mixture of analytes with overlapping signals to be analyzed. Furthermore, full selectivity is not required in multivariate calibration.

In the present study, the PLS multivariate calibration method was used for simultaneous determination of phenanthridine and phenanthridinone in synthetic samples. In addition, we measured the Michaelis–Menten constants for the oxidation of phenanthridine by aldehyde oxidase. This was possible due to the good additive property of the phenanthridine and phenanthridinone spectra, as well as the ability of PLS to analyze data with strongly collinear (correlated), noisy and numerous response variables.

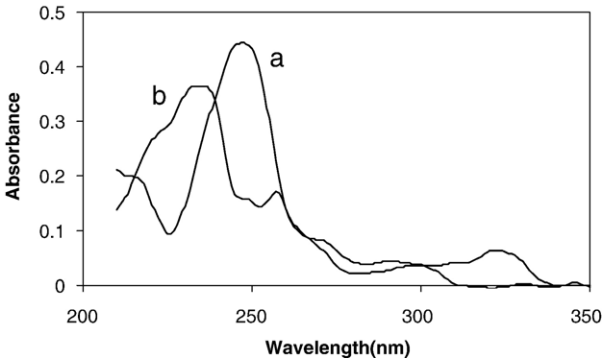


Fig. 1. The UV-spectra of 6 μM of phenanthridine (a) and phenanthridinone (b) in 30 mM Sorenson's phosphate buffer pH 7.0 containing 0.1 mM EDTA.