Iranian Journal of Pharmaceutical Research (2009), 8 (3): 169-177
Received: July 2008
Accepted: November 2008

Original Article

Development of a Sensitive Spectrofluorometric-Multivariate Calibration Method for Enzyme Kinetic of Aldehyde Oxidase

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Abstract

Attempts to obtain experimental values for the kinetic parameters of phenanthridine oxidation by guinea pig or rabbit liver aldehyde oxidase using common spectrophotometric methods have not been successful due to a lower limit of detection. In the present study, a new spectrofluorimetric assay in combination with a multivariate calibration method for enzymatic kinetic study of aldehyde oxidase activity, using phenanthridine as the substrate, has been developed and validated.

Phenanthridine and phenanthridinone binary mixtures were prepared in a dynamic linear range of 0.025-1 µM and the emission flourimetric spectra of the solutions recorded at the excitation and emission wavelengths of 236 and 320-450 nm, respectively. The optimized calibration model of partial least squares (PLS) method was applied for the simultaneous determination of the concentration of each chemical in the prediction set. The limits of detection for phenanthridine and phenanthridinone were found to be 2.13 ± 0.33 and 3.41 ± 0.34 nM (mean ± SD, n = 5), respectively. This method was then used for kinetic study of phenanthridine oxidation using guinea pig and rat hepatic aldehyde oxidase. The results were compared with those obtained from a univariate spectroscopic method.

Using this new spectrofluorimetric-multivariate calibration method, the K_m value for the oxidation of phenanthridine with guinea pig and rat liver aldehyde oxidase were obtained as 0.83 ± 0.08 and 2.20 ± 0.40, µM (mean ± SD, n = 3), respectively.

Keywords: Spectrofluorometry; Partial least square; Kinetic constant; Aldehyde oxidase; Phenanthridine.

Introduction

Aldehyde oxidase (AO, aldehyde: O_2 oxidoreductase EC 1.2.3.1) is a molybdenum containing cytosolic enzyme, which is widely distributed throughout the animal kingdom (1). The enzyme is predominantly active in liver and in some extent in other tissues of mammalian species and involved in the metabolism of an extensive range of aldehydes and nitrogen-containing xenobiotics and endogenous compounds including some important drugs such as famciclovir (2), methotrexate (3), 6-azathioprine (4), mercaptopurine (5), quinine and quinidine (6). Furthermore, in plants

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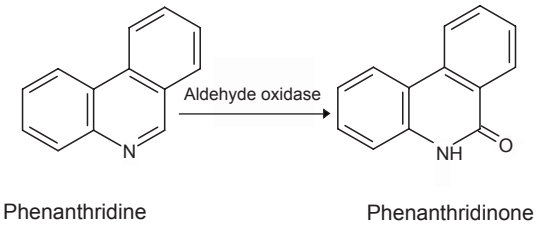

Figure 1. Oxidation of phenanthridine into phenanthridinone.

aldehyde oxidase seems to be involved in hormone biosynthesis such as those of indole-3-acetic acid and abscisic acid (7). There are also a large number of reports indicating that aldehyde oxidase is capable of catalyzing the reduction of a broad type of compounds in the presence of appropriate electron donors (8, 9). In spite of this broad range of compounds metabolized by aldehyde oxidase, only few numbers of these compounds have been used as a substrate for measuring the enzyme activity and kinetic studies (2, 10, 11). Phenanthridine is one of these compounds that its oxidation to phenanthridinone (Figure 1) has been used in many studies for monitoring aldehyde oxidase activity (2, 6, 7, 11-18).

Based on the available methods, the monitoring of the reaction is carried out by measuring phenanthridinone production at 322 nm. Although there is no spectral interference at this wavelength, the molar absorptivity of phenanthridinone at this wavelength is so low that it is difficult to measure the kinetic parameters of this reaction using common spectrophotometric methods. Accordingly, attempts to determine the K_m values for the oxidation of phenanthridine to phenanthridinone by guinea pig or rabbit liver aldehyde oxidase, using these methods, were not successful due to a lower limit of detection and only an estimated value of < 1 μM has been suggested for this reaction (1, 19).

Recently, we have developed a spectrophotometric–multivariate calibration method to determine K_m value of this reaction, using rat liver fraction through simultaneous determination of phenanthridine and phenanthridinone (20). Using this method, the corresponding value was found to be 1.72 μM, which is higher than the estimated value for the guinea pig and rabbit hepatic enzyme. Hepatic aldehyde oxidase shows a marked species variation, which makes it difficult to extrapolate the results obtained for the assay of enzyme activity from one animal species to another (1, 21).

Fluorescence spectroscopy is a powerful method, widely used in biomedical analyses due to its extremely high sensitivity and selectivity. Both phenanthridine and phenanthridinone possess a significant native fluorescence in solution, making it possible to analyze their concentration by using fluorescence spectroscopy. However, the main problem encountered is the spectral overlapping of phenanthridine and phenanthridinone. Based on our previous study (20), it is possible to perform aldehyde oxidase kinetic analysis using chemometric methods. Chemometric methods, including multivariate calibration and multivariate curve resolution, have found increasing applications for determination of enzymatic activity in those cases that spectra of the substrates and products are overlapping (22, 23).

Multivariate calibration methods applied to emissive spectral data as well as to absorptive data are increasingly being used for the analysis of complex biological mixtures. They have the advantage of using full spectral information and allow for a rapid determination of mixture components, often with no need of prior separation or sample pretreatment (24, 25). In the present study, it has been tried to take the benefits of both fluorescence spectroscopy and the partial least squares (PLS), as multivariate calibration methods, to develop and validate a sensitive method for determining enzyme kinetic parameters with hepatic guinea pig aldehyde oxidase using phenanthridine as a substrate.

Experimental

Chemicals

All experiments were performed with analytical-reagent grade chemicals. Phenanthridine and phenanthridinone were purchased from Alderich Chemical Company (Gillingham, Dorset,UK).

Stock standard solutions, 1 mM, of

Table 1. Analytical figures of merit for phenanthridine and phenanthridinone in Sorenson's phosphate buffer pH 7.0 ($\lambda_{(ex)}$ 236 nm).

Parameters	Phenanthridine	Phenanthridinone
$\lambda_{max(em)}$ (nm)	369	364
Dynamic linear range (nM)	10-1800	10-1700
Correlation coefficient	0.9995	0.9996
Limit of detection (nM) (n = 5)	2.13 ± 0.33	3.41 ± 0.34
RSD (%)	0.15	0.10
Equation of calibration curve (emission flouresence intensity versus nM of analyte)	y = 0.52x + 15.59	y = 0.35x + 13.33

phenanthridine and phenanthridinone were prepared in ethanol. Solutions of lower concentrations were prepared by appropriate dilution of the stock solutions with double distilled water.

Apparatuses and software

All fluorescence measurements were porformed on a Shimadzu RF-5301 spectroflourometer, equipped with a xenon lamp pulsed at 80 Hz. The measurements were carried out using 1.00 cm quartz cell, whith slit widths of 3 and 5 nm for excitation and emission monochromators, respectively. The instrument was connected to a Shimadzu cell temperature control unit. The cuvette used had a path length of 1 cm and the total volume was constant at 3.0 mL.

All spectra were saved in ASCII format and transferred to a PC computer for subsequent manipulation by PLS. The data were handled using MATLAB software (6.5) and PLS-Toolbox (26).

Calibration curves

Individual calibration curves were constructed with several points as emission fluorescence intensity versus phenanthridine and phenanthridinone concentration in the range of 10-1800 nM and 10-1700 nM for phenanthridine and phenanthridinone, respectively, and evaluated by linear regression. In order to obtain the calibration curves of compounds, emission fluorescence intensity was measured at 369 and 364 nm for phenanthridine and phenanthridinone, respectively, using 236 nm as the excitation wavelength. The characteristics of calibration graph and the statistical parameters for determination of phenanthridine and phenanthridinone under optimum conditions have been summarized in Table1.

Calibration procedure for simultaneous spectrofluorometric determination

Phenanthridine and phenanthridinone binary mixtures were prepared as follows: appropriate volumes of the standard solutions (in dynamic linear range) were transferred into a 10 mL volumetric flask and made up to the mark with Sorenson's phosphate buffer pH 7.0 containing 0.1 mM EDTA. The emission fluorescence spectra of solutions were recorded in the range of 320-460 nm, using 236 nm as the excitation wavelength ($\lambda_{(ex)}$). An optimized calibration model of PLS method was applied to calculate the concentration of each chemical in the prediction set.

Enzyme preparation

Mature male Sprague-Dawley rats and guinea pigs (150-250 g, Tabriz University of Medical Sciences), previously maintained on a standard laboratory diet, were sacrificed between 9.00 and 10.00 am by cervical dislocation. Livers were immediately excised, placed in ice-cold isotonic potassium chloride solution (1.15 % KCl w/v) containing 0.1 mM EDTA and the gall bladder and excess fat was removed. Partially purified aldehyde oxidase was prepared from liver homogenates by heat treatment and ammonium sulfate precipitation, as described elsewhere (27).

Aldehyde oxidase activity assay

Aldehyde oxidase activity was determined spectrofluorometrically in Sorenson's phosphate

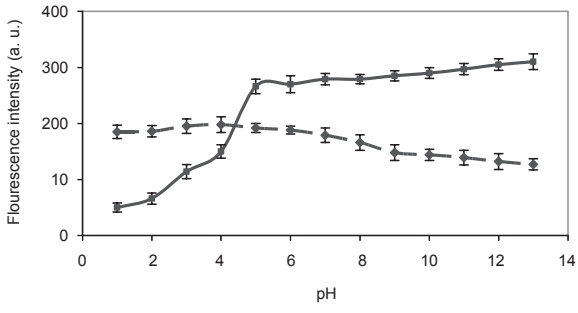

Figure 2. The variation of fluorescence intensity of 0.5 µM phenanthridine (-) and 0.5 µM phenanthridinone (--) versus pH variation ($\lambda_{(ex)}$= 236 nm, $\lambda_{(em)}$ = 364 nm).

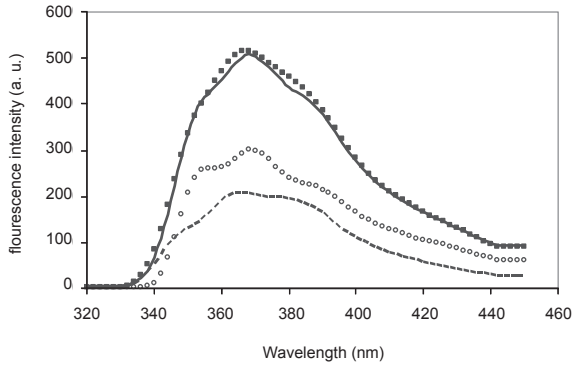

Figure 3. The emission spectra of 0.5 µM of phenanthridine (○), phenanthridinone (**--**) and their theoretical (-) and experimental mixture (■) in 30 mM Sorenson's phosphate buffer pH 7.0 containing 0.1 mM EDTA ($\lambda_{(ex)}$= 236 nm).

buffer pH 7.0 containing 0.1 mM EDTA at 37 °C, using phenanthridine as the substrate. Eight concentrations of phenanthridine, ranging from 0.2 to 0.9 µM, were prepared in 5.8 mL phosphate buffer and the reaction started by adding 200 µL of the enzyme fraction. Then, 1 mL of the reaction solution was taken at 10 sec intervals up to 50 sec and added into the test tubes containing 0.5 mL of HCl (10 M) to stop the reaction. The pH was adjusted to 7.0 with NaOH (10 M) and the emission fluorescence spectrum of each test tube was recorded in the range of 320-450 nm, using 236 nm as the excitation wavelength. The emission spectrum of enzyme solution at the same conditions (pH 7.0 containing 0.1 mM EDTA at 37°C, 236 nm as the excitation wavelength) was subtracted from the emission spectrum of each reaction sample and then concentrations of phenanthridine and phenanthridinone in each solution were calculated using PLS. These values were used for calculating initial velocities and then K_m and V_{max} values from a Lineweaver-Burk double reciprocal plot for the phenanthridine oxidation catalyzed by hepatic guinea pig and rat aldehyde oxidase. The line of the best fit through the points on the plot was calculated using linear regression by the least squares method.

For the purpose of comparison, the initial oxidation rates for obtaining the K_m and V_{max} values for oxidation of phenanthridine by guinea pig and rat liver aldehyde oxidase were also determined, using a common spectrophotometric method as described before (17, 19).

Protein determination

Protein concentrations of partially purified enzyme fractions were determined using a Pierce BCA Protein assay kit with bovine serum albumin as the protein standard (28).

Results and Discussion

Spectra characterization of phenanthridine and phenanthridinone: optimization

The excitation spectrum of phenanthridine and phenanthridinone shows maxima located at 248 nm and 236 nm respectively. All the fluorescence spectra were recorded at the maximum excitation wavelength of phenanthridinone (236 nm) to favor the fluorescence enhancement of the weaker emitter.

Influence of pH on the fluorescence range and its intensity for each compound was studied by adding a small volume of dilute solution of HCl and NaOH to adjust the pH (Figure 2). The maximum emission wavelength of phenanthridinone ($\lambda_{(em)}$ 364 nm) was constant over pH 1-13. Also, there was no change in the fluorescence intensity of phenanthridinone in the pH range of 1-8. On the other hand, phenanthridine has two different emission spectra versus pH range. Sequentially two different maximum emission wavelengths were observed for phenanthridine over the pH range of 1-13. The maximum emission wavelengths were 401 and 369 nm in the pH range of

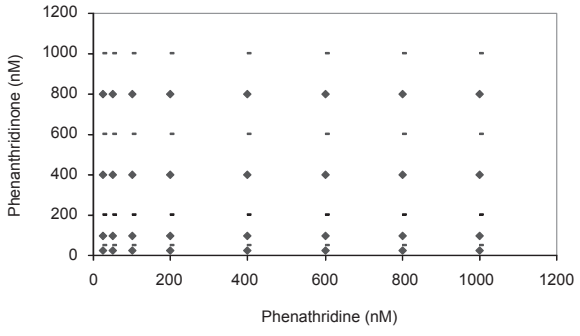

Figure 4. The composition of calibration (♦) and prediction (-) sets.

1-4 and 5-13, respectively. The fluorescence intensity of phenanthridine in acidic media (pH range 1-4) is lower than the neutral and basic media. Neutral pH (pH 7) was selected as the optimum pH.

Emission spectra of phenanthridine (0.5 μM) and phenanthridinone (0.5 μM) in pH 7 are shown in Figure 3. The extensive overlapping makes it difficult to distinguish between the two compounds in their mixture. Trials involving the use of either acidic or basic media in order to resolve the fluorescence emission spectra of these compounds, by either direct or synchronous fluorescence, were not successful.

The univariate analysis method cannot be applied for resolving this mixture. Hence, full-spectrum multivariate calibration methods should be used. The fluorescence intensity of these two compounds has good additive properties (Figure 3) Thus, linear models, of multivariate calibration, such as PLS, can be used for simultaneous determination of these components.

Multivariate analysis

Multivariate calibrations, such as the PLS method, involve the decomposition of the experimental data, such as spectrofluorometric data in this case, into systematic variations (principal components or factors) that explain the observed variance in data. The purpose of both methods is to build a calibration model between the concentration of the analyte under study and the factors of the data matrix (29).

The first step in the simultaneous determination by PLS method, involves constructing the calibration matrix for the binary mixture. PLS regression as a full spectrum multivariate calibration method only requires the concentration of the analyte of interest to be known in the calibration samples and knowing the concentration of other analytes are not necessary. In this study, calibration sets were optimized with the aid of the orthogonal design method (30). Figure 4 shows the composition of the calibration and prediction samples. The calibration and prediction sets were designed using the orthogonal design method, with eight concentration levels in the range of 0.025-1 μM for both phenanthridine and phenanthridinone. Each solution was prepared to contain combinations of two compounds. Thirty two mixtures of these solutions were used as the calibration set for PLS model development. Another 32 mixtures, not included in the previous set, were employed as an independent test set called the prediction set. The PLS-1 algorithm that performed the PLS analysis one component at a time, has been selected to perform the determination (29). In the calibration procedure for this method, the first step is to select the optimum number of factors, which depends on the number of independent chemical variables, and other sources of systematic signal variation, such as any interaction between the chemical components, change of shape of the component peak and detector noise. Selection of the optimum number of factors in the PLS algorithm was estimated by the cross- validation method, leaving out one sample at a time. The prediction error was calculated for each component for the prediction set, which are the samples not participating in the construction of the model. The optimum number of factors (latent variables) to be included in the calibration model was determined by computing the prediction error sum of squares (PRESS) for the first variable, which helps to build the PLS modeling in the calibration step. Then, another latent variable was added for the model building and the PRESS was re-calculated. This process was repeated for one to 10 latent variables, which were used in the PLS modeling.

Figure 5 shows a plot of PRESS against the number of latent variable (principal component)

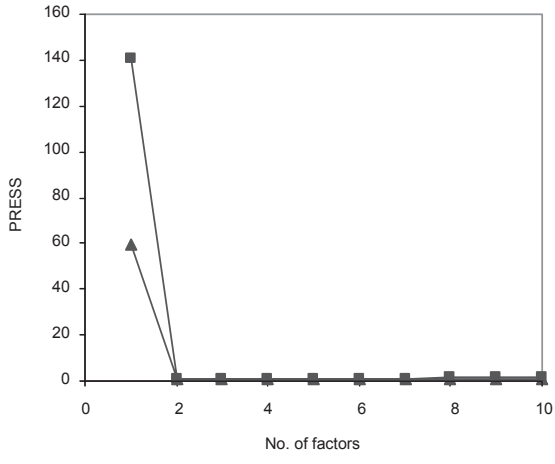

Figure 5. Cross-validation plot for phenanthridine (▲) and phenanthridinone (■).

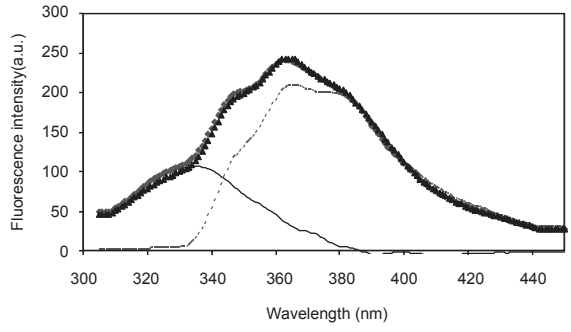

Figure 6. The emission spectra of enzyme (-), phenanthridinone (--) and their theoretical (▲) and experimental mixture (■) in 30 mM Sorenson's phosphate buffer pH 7.0 containing 0.1 mM EDTA ($\lambda_{(ex)}$ = 236 nm).

for each individual component. The F-statistical test can be used to determine the significance of PRESS values greater than the minimum. Two factors were selected as the optimum number of factors for both compounds. Also, when a plot of the second principal component (PC) versus the first principal component (score plot) is constructed, the score matrix is certainly rotated and not very much disturbed with respect to the calibration matrix. In fact, the distribution of scores in the plane formed by these first two PCs, reproduces the experimental data. This confirms that two factors are enough to construct a PLS model.

Simultaneous determination of phenanthridine and phenanthridinone in synthetic samples

The obtained model in the calibration step was validated with 10 synthetic mixtures, containing the considered compounds in different proportions that were randomly selected. The results obtained from spectrofluorometric simultaneous analysis of phenanthridine and phenanthridinone by PLS method have been presented in Table 2. The values of root mean square difference (RMSD), the square of the correlation coefficient obtained when plotting actual versus predicted concentration (R^2), and the relative error of prediction (REP) for each component in ten synthetic samples are included in order to give an indication of both the average error in analysis and the quality of fit of all data to a straight line.

Determination of kinetic constants for the oxidation of phenanthridine by aldehyde oxidase

The proposed PLS model was applied for determination of phenanthridine and phenanthridinone in the samples obtained from reactions with different initial concentrations of the substrate. In the present study, a phenanthridinone detection assay was developed to estimate the initial oxidation rates of phenanthridine.

Figure 6 shows the emission spectra of enzyme, using 236 nm as the excitation wavelength. As can be seen in this figure, fluorescence intensities of enzyme and phenanthridinone have good additive properties. In order to remove the enzyme contribution, spectrum of enzyme was subtracted from the spectrum of the reaction samples.

In order to check that the enzyme fraction dose not interfere with the determination of phenanthridinone by the proposed method, four samples with different concentrations of phenanthridinone in the presence of enzyme fraction were prepared and the concentration of each sample was predicted by the proposed method. The recovery was > 99%, indicating that there was no interference from the enzyme fraction on the determination of phenanthridinone

Table 2. Statistical parameters for phenanthridine and phenanthridinone in validation set of the PLS model.

Parameters	Phenanthridine	Phenanthridinone
Recovery (%)	96.64	101.70
RMSD [a]	0.04	0.03
REP (%) [b]	1.96	2.23
R^2 [c]	0.9972	0.9870

[a] RMSD calculated according to: $RMSD = [(sum (C_{real} - C_{found})^2) / sum (C_{found})^2]^{1/2}$

[b] REP calculated according to: $REP = 100*[(sum (C_{real} - C_{found})^2) / n]^{1/2}$

[c] Correlation coefficient for plotting C_{real} versus C_{found}

in the samples.

The K_m and V_{max} values for the oxidation of phenanthridine by guinea pig and rat liver aldehyde oxidase obtained by different methods used in the present study have been summarized in Table 3. With the guinea pig enzyme, the spectrofluorometric-multivariate calibration method developed in the present study gave 0.83 ± 0.08 (mean ± SD, n = 3) µM for the K_m value, which is lower than the 1 µM estimated by others (1, 19). In this study, it was also found that the initial oxidation rate of phenanthridine by guinea pig liver fraction did not vary between 5-100 µM, which indicates that the K_m value for phenanthridine oxidation by guinea pig liver aldehyde oxidase could be < 1 µM this is in good agreement with previous reports (1, 19). The K_m value for the phenanthridine oxidation by rat liver aldehyde oxidase, using the spectrofluorometric-multivariate calibration and common UV-spectroscopic methods, were obtained as 2.20 ± 0.40 and 5.79 ± 0.84 µM, respectively. The former value is consistent with the 1.72 µM value, which has been obtained from the UV-spectrophotometric-multivariate calibration method reported before (20).

Conclusions

Phenanthridine, as a specific substrate of aldehyde oxidase, has been used in many studies for the measurement of the enzyme activity (2, 6, 7, 11-18). however due to some limitations in the methods of assay, there is no experimental value for the enzyme kinetic parameters of its oxidation by rabbit and guinea pig hepatic enzyme. It is particularly important. for the guinea pig enzyme, which has a closer resemblance to the human liver aldehyde oxidase (1, 19). Resolution of the binary mixture has been accomplished by partial least squares. These methods have the advantage of using full spectra information and allow for a rapid determination of mixture component with no need of prior separation or sample pre-treatment.

The proposed method has been shown to be useful for simultaneous determination of phenanthridine and phenanthridinone. This spectroflourimetric method used along whit PLS as a suitable, sensitive and easy method, can be applied for these N-heterocyclic compounds without the need for an expensive instrumentation. This method is very sensitive and efficient for accurate determination of K_m and V_{max} values of phenanthridine oxidation to phenanthridinone, as one of the best and common reactions for aldehyde oxidase assay. Since phenanthridine has mutagenic and carcinogenic affects and its biotransformation has been the subject of some studies (31, 32), the method developed in the present study also has the potential for analysis of this compound

Table 3. The enzyme kinetic parameters for the oxidation of phenanthridine by guinea pig and rat liver aldehyde oxidase, obtained by spectroflourometric-multivariate calibration and spectrophotometric-univariate calibration methods* (n = 3, data expressed as mean ± SD).

	Spectroflourometric-multivariate calibration method		Spectrophotometric-univariate calibration method	
	K_m (µM)	V_{max} (nmol/min/mg protein)	K_m (µM)	V_{max} (nmol/min/mg protein)
Guinea pig	0.83 ± 0.08	98.84 ± 7.36	>1*	-
rat	2.20 ± 0.40	36.81 ± 2.43	5.79 ± 0.84	46.8 ± 3.93

** An estimated value.

and its metabolite, phenanthridinone.

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