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Simultaneous spectrophotometric determination of phenanthridine, phenanthridinone and phenanthridine N-oxide using multivariate calibration methods

Research Article

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Abstract: The multivariate calibration methods, partial least squares (PLS) and principle component regression (PCR) have been used to determine phenanthridine, phenanthridinone and phenanthridine N-oxide in spiked human plasma samples. Resolution of binary and ternary mixtures of analytes with minimum sample pre-treatment and without analyte separation has been successfully achieved analyzing the UV spectral data. The net analyte signal (NAS) concept was also used to calculate multivariate analytical figures of merit such as limit of detection, selectivity and sensitivity. The simultaneous determination of three analytes was possible by PLS and PCR processing of sample absorbance in the 210-355 nm region. Good recoveries were obtained for both synthetic mixtures and spiked human plasma samples.

Keywords: *Multivariate calibration • Spectrophotometry • Phenanthridine • Phenanthridinone • Phenanthridine N-oxide*

1. Introduction

Aza-aernes are a group of N-heterocyclic aromatic compounds that have been detected as components of urban airborne particulates, crude petroleum distillates, shale oil, effluents produced during coal combustion and conversion and cigarette smoke. Certain aza-arenes, including phenanthridine are carcinogenic. Phenanthridine (Fig. 1) is a fused azaheterocyclic compound which could be found in a number of plants [1]. Some of its analogues have been used as DNA binding fluorescent dyes in molecular biology and genetics laboratories [2,3]. Phenanthridine can be oxidized by some enzymes including CYP450 and aldehyde oxidase [1,4]. CYP450 can oxidize phenanthridine into several metabolites with phenanthridinone and phenanthridine N-oxide as the major metabolites [1]. By aldehyde oxidaze (EC 1.2.3.1: aldehyde: oxygen oxidoreductase), phenanthridine is oxidized only to phenanthridinone [4], although according to some authors the production of phenanthridinone may pass through phenanthridine N-oxide [5-7]. Its oxidation by aldehyde oxidase has been used widely to monitor the activity of this enzyme [4]. Therefore, it would be useful and important to have available an efficient and simple method for quantitative analysis of phenanthridine and its metabolites. Several

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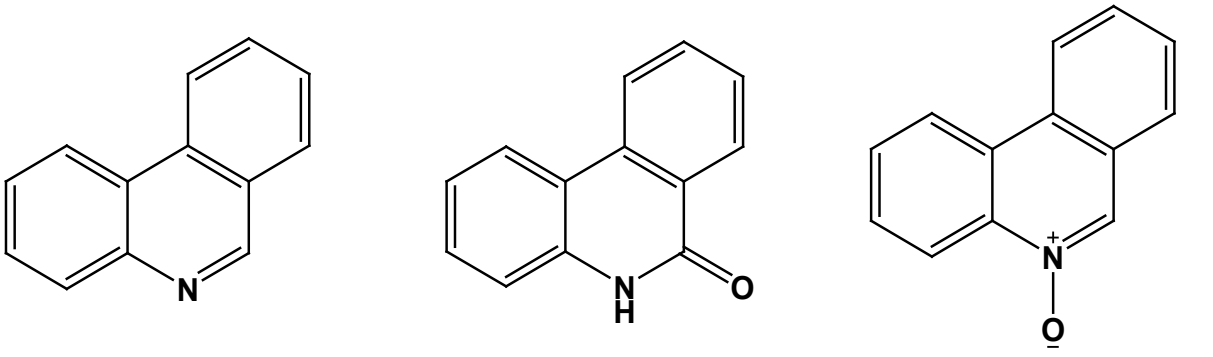

Figure 1. The structure of phenanthridine, phenanthridinone and phenanthridine N-oxide

methods have been reported for the determination of phenanthridine and its metabolites [4,8,9]. The separation and determination of phenanthridine and its metabolites are achieved by chromatographic methods which are usually inconvenient, expensive and time-consuming [8,9]. The simultaneous determination of several components in a mixture, in particular in the biological matrices, can be a difficult task, especially when their analytical characteristics are not very different, which is the case with phenanthridine and its metabolites. Recently, multicomponent systems based on chemometrics methods have become an important tool in resolution of mixtures into their components in many different fields including biomedical, clinical, environmental and drug analysis [10]. Among different regression methods exist for multivariate calibration, the factor analysis-based methods including partial least squares (PLS) regression and principal component regression (PCR) have received considerable attention in the chemometrics for multicomponent analysis [11]. These techniques are powerful multivariate statistical tools that have been successfully and widely applied to the quantitative analysis of spectroscopic data because of their ability to overcome problems common to this data such as co-linearity, band overlaps and interactions and ease of their implementation due to the availability of software [11–15]. To overcome the problems encountered with phenanthridine and its metabolites measurement, in the present study, PLS and PCR methods have been developed and validated as simple methods for simultaneous spectrophotometric determination of phenanthridine, phenanthridinone and phenanthridine N-oxide in human plasma. Furthermore, NAS calculations were performed with both of these methods in order to obtain multivariate limit of detection, selectivity and sensitivity values.

2. Experimental procedure

2.1. Chemicals

All experiments were performed with analytical-reagent grade chemicals. Phenanthridine and phenanthridinone were purchased from Aldrich Chemical Company (Gillingham, Dorset, UK). Trichloroacetic acid (TCA) was obtained from Merck (Darmstatd, Germany). Phenanthridine N-oxide was prepared by reaction of phenanthridine with 40% peracetic acid as described previously [16]. Human plasma was obtained from the Iranian Blood Transfusion Organization. Stock standard solutions of phenanthridine, phenanthridinone and phenanthridine N-oxide (1 mmol L^{-1}) were prepared in ethanol. Solutions of lower concentrations were prepared by appropriate dilution of the stock solutions with Sorenson's phosphate buffer (pH 7.0) containing 0.1 mmol L^{-1} EDTA.

2.2. Apparatus and software

Spectrophotometric measurements were carried out at 25°C using a Shimadzu 2550 UV/VIS spectrophotometer that was controlled by the Shimadzu UV Probe personal software package. The instrument was connected to a Shimadzu cell temperature control unit. Path length of used cuvette was 1 cm. The total volume used for the spectrophotometric measurements was constant at 3.0 mL. All spectra were saved in the ASCII format. The spectrophotometric data were transferred to a PC for subsequent manipulation by PLS-1 and PCR. The data were handled using MATLAB software (7.0) and PLS-1 and PCR were applied with the program MULTIVAR, written in Visual Basic 5.0 [17] and available online at ftp://www.fbioyf.unr.edu.ar/cientifico/multivar.exe.

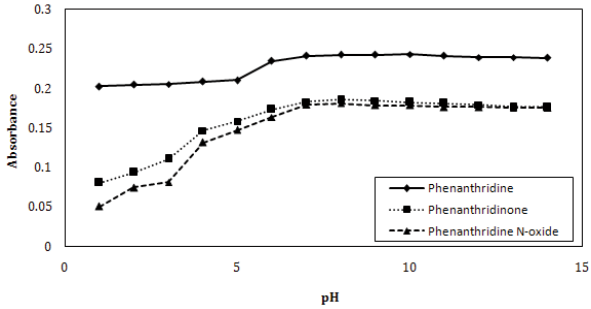

Figure 2. Variation of absorbance of 5 μmol L^{-1} phenanthridine, phenanthridinone and phenanthridine N-oxide measured at 248, 236 and 237 nm, respectively, *versus* pH

2.3. Individual calibration

Individual curves plotting absorbance against concentration of phenanthridine, phenanthridinone and phenanthridine N-oxide in the range of 0.1-50.0 μmol L^{-1} were evaluated by linear regression. In order to obtain calibration curves of the compounds, the absorbance of phenanthridine, phenanthridinone and phenanthridine N-oxide was measured at 248, 236 and 237 nm, respectively. The characteristics of the calibration graph and the statistical parameters for determination of phenanthridine, phenanthridinone and phenanthridine N-oxide under optimum conditions are summarized in Table 1. The limit of detection (LOD) and the limit of quantification (LOQ) were computed using the following expressions: LOD=$3S_b$/m, and LOQ=$10S_b$/m, where S_b is the standard deviation of the blank and m is the slope of the calibration curve [18]. As the selected wavelengths used for the individual calibration of each of the compounds were not selective for their measurements in mixtures, the results shown in Table 1 could not be used for determination of these three compounds in mixed solutions.

2.4. Calibration procedure for the simultaneous spectrophotometric determination

An orthogonal designed calibration mixture of phenanthridine, phenanthridinone and phenanthridine N-oxide using a 25 sample set and a prediction set, was prepared as follows: appropriate volumes of the standard solutions in the dynamic linear range of 0.1-50.0 μmol L^{-1} were prepared in Sorenson's phosphate buffer (pH 7.0) containing 0.1 mmol L^{-1} EDTA. The absorption spectra of these solutions were recorded in the range of 210-355 nm. The optimized PLS-1 and PCR calibration models were used to calculate the concentration of each chemical in the prediction set.

2.5. Analysis of plasma samples

Plasma spiked with phenanthridine, phenanthridinone and phenanthridine N-oxide was obtained by diluting aliquots of the stock standard solutions of these compounds with the human plasma in a ratio of 1:1. To precipitate proteins, 0.5 mL of TCA (1.5 mol L^{-1}) was added to 1 mL of the spiked plasma and the precipitated proteins were separated by centrifugation for 10 min at 8500 rpm. The pH of the clear supernatant layer was adjusted to 7 with NaOH (1.5 mol L^{-1}) and the spectrum of the solution was recorded in the range of 210-355 nm. The concentrations of phenanthridine, phenanthridinone and phenanthridine N-oxide in each solution were predicted.

3. Results and Discussion

3.1. Spectral characterization of phenanthridine, phenanthridinone and phenanthridine N-oxide: optimization

The absorbance intensity of phenanthridine (λ_{max}=248), phenanthridinone (λ_{max}=236) and phenanthridine N-oxide (λ_{max}=237) varied with pH (Fig. 2). The optimum pH range for simultaneous determination of these components was found to be 7-14. In this pH range, the highest absorbance intensity of the compounds with no significant change can be achieved. Considering the physiological pH range, pH 7.0 was selected as

Table 1. Analytical figures of merit for phenanthridine, phenanthridinone and phenanthridine N-oxide

Parameters	Phenanthridine	Phenanthridinone	Phenanthridine N-oxide
λ_{max}	248	236	237
Dynamic linear range (μmol L^{-1})	0.1-50	0.1-50	0.1-50
Correlation coefficient	0.9997	0.9993	0.9999
Limit of detection (μmol L^{-1}) (n=5)	0.05±0.01	0.04±0.01	0.03±0.01
Limit of quantification (μmol L^{-1}) (n=5)	0.16±0.01	0.13±0.01	0.10±0.01
RSD (%)	0.3	0.4	0.4
Equation of calibration curve (absorbance versus μmol L^{-1} of analyte)	A=0.045C + 0.009	A=0.037C + 0.007	A=0.023C + 0.007

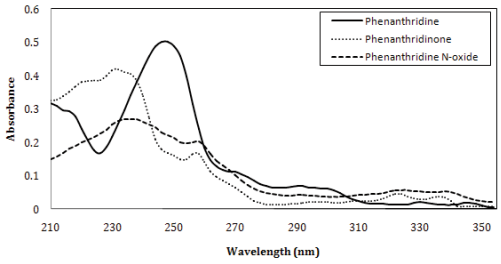

Figure 3. Absorption spectra of 10 μmol L^{-1} phenanthridine, phenanthridinone and phenanthridine N-oxide in 67 mmol L^{-1} Sorenson's phosphate buffer, pH 7.0 containing 0.1 mmol L^{-1} EDTA

the optimum. Fig. 3 shows that the absorption spectra of these three compounds overlap extensively at pH 7. Since the univariate analysis method cannot be applied for resolving this mixture, full-spectrum multivariate calibration methods of PLS and PCR were used to determine these compounds in the sample analysis.

3.2. PLS analysis

Simultaneous determination of the analytes were performed through application of the PLS regression on the spectral data. There are two general classes of PLS algorithm usually designed as PLS-1 and PLS-2 [19]. Unlike PLS-2 which calculates all the scores and

Table 2. Statistical parameters for phenanthridine, phenanthridinone and phenanthridine N-oxide in validation sets of PLS-1 and PCR methods

Calibration method	Statistical parameters	Component		
		Phenanthridine	Phenanthridinone	Phenanthridine N-oxide
PLS-1	Spectral rage (nm)	210-355	210-355	210-355
	Factors	4	4	4
	PRESS*	12.4	8.9	3.9
	REP** (%)	5.83	8.52	8.21
PCR	Spectral rage (nm)	210-355	210-355	210-355
	Factors	4	4	4

**PRESS = prediction residual error sum of squares*
*** REP (%) = relative error of prediction*

Table 3. Multivariate analytical figures of merit for phenanthridine, phenanthridinone and phenanthridine N-oxide

Figures of merit	Phenanthridine		Phenanthridinone		Phenanthridine N-oxide	
	PLS-1	PCR	PLS-1	PCR	PLS-1	PCR
Limit of detection(μmol L^{-1})	0.351	0.315	0.722	0.796	0. 631	0.714
Selectivity	0.219	0.237	0.596	0.561	0.500	0.591
Sensitivity (L μmol^{-1})	0.236	0.292	0.533	0.523	0.441	0.685

Table 4. Recoveries (%) of phenanthridine, phenanthridinone and phenanthridine N-oxide in the resolution of synthetic mixtures of these compounds by using PLS-1 and PCR*

Concentration (μmol L^{-1})	Phenanthridine		Phenanthridinone		Phenanthridine N-oxide	
	PLS-1	PCR	PLS-1	PCR	PLS-1	PCR
1	102.12±0.33	96.42±0.34	96.33±0.22	101.27±0.12	101.05±0.11	93.65±0.10
	(0.88-1.12)	(0.87-1.13)	(0.89-1.11)	(0.90-1.10)	(0.92-1.08)	(0.89-1.11)
10	98.86±1.73	103.22±3.00	94.27±1.99	96.71±2.31	100.67±1.52	99.84±1.85
	(9.37-10.63)	(9.34-10.66)	(9.82-10.18)	(9.79-10.21)	(9.62-10.38)	(9.59-10.41)
20	101.41±1.54	101.02±2.38	95.10±0.74	98.56±1.34	99.24±1.84	105.02±2.24
	(19.28-20.72)	(19.24-20.76)	(19. 76-20.24)	(19.67-20.33)	(19.57-20.43)	(19.42-20.58)

** The data are expressed as mean ± standard deviation. Values in parentheses represent 95% confidence intervals.*

Table 5. Recoveries (%) for the target compounds in human plasma samples using PLS-1 and PCR models*

Concentration (μmol L^{-1})	Phenanthridine		Phenanthridinone		Phenanthridine N-oxide	
	PLS-1	PCR	PLS-1	PCR	PLS-1	PCR
1	99.51±0.47	92.23±0.39	102.27±0.15	99.36±0.15	106.41±0.11	93.78±0.14
	(0.87-1.13)	(0.86-1.14)	(0.88-1.12)	(0.87-1.13)	(0.93-1.07)	(0.91-1.09)
10	93.62±2.47	104.02±3.00	99.51±2.79	96.70±2.38	101.72±1.96	96.90±2.04
	(9.34-10.66)	(9.32-10.68)	(9.77-10.23)	(9.81-10.19)	(9.59-10.41)	(9.53-10.47)
20	99.11±1.79	103.85±2.39	99.93±0.99	97.41±1.09	99.50±2.21	102.63±2.59
	(19.22-20.78)	(19.20-20.80)	(19.69-20.31)	(19.65-20.35)	(19.23-20.77)	(19.22-20.78)

** The data are expressed as mean ± standard deviation. Values in parentheses represent 95% confidence intervals.*

loadings at one time, with just one set of loadings for all the components, PLS-1 calculates these parameters separately [19]. In the present study, PLS-1 has been employed. The optimum number of PLS-latent variables for each analyte was obtained by leave-one out cross-validation procedure. The number of factors that produced the least prediction residual error sum of squares (PRESS) was selected as the optimum value. The number of PLS latent variables (LV) used to model absorbance–concentration is higher than the number of analytes, which can be attributed to the interaction between the organic compounds in the mixtures.

3.3. PCR analysis

To compare the effect of the type of regression method on the analysis of phenanthridine, phenanthridinone and phenanthridine N-oxide by spectrophotometric technique, in addition to PLS, PCR method was employed. The values obtained for the PRESS and relative error of prediction (REP %), as well as the optimum numbers of factors following the application of PCR on the spectral region absorbance data are given in Table 2. These results indicate that there is no significant difference between the results obtained by PLS-1 and PCR models, which indicates that both models have a good promise for field use.

PCR and PLS techniques have many properties in common. Although PCR technique was introduced before the later one [20–24], PLS appears to be the method of choice among chemists [25–27]. According to the literature survey made by Wentzell and Montono, PLS produces better results than PCR, however, most studies have indicated no real difference in performance between these two techniques [28]. Therefore, according to the results obtained in the present study, PLS and PCR methods have no advantages over each other and both can be used as a chemometric model to analyze the spectral data collected on synthetic and human plasma samples to predict the concentrations of phenanthridine, phenanthridinone and phenanthridine N-oxide. One of the recent advances in the field of multivariate analysis is the estimation of analytical figures of merit which makes it possible to compare the employed methods [29]. In this study, net analyte signal (NAS) method was employed to calculate the analytical figures of merit as described elsewhere [30] for phenanthridine, phenanthridinone and phenanthridin N-oxide. The results for analytical figures of merit obtained by PLS and PCR are shown in Table 3.

3.4. Analysis of phenanthridine, phenanthridinone and phenanthridine N-oxide in spiked human plasma samples

Table 4 indicates the results obtained in the resolution of the three components in binary and ternary synthetic mixtures expressed as recovery (%) values, the standard deviation of three replicate analyses, and the 95% confidence interval. To examine the applicability of the proposed multivariate calibration methods in the analysis of the real samples, the quantification of phenanthridine, phenanthridinone and phenanthridine N-oxide were analyzed in spiked human plasma samples by the PLS and PCR regression methods. The absorbance spectrum of the solution was recorded and transferred to the PLS and PCR calibration models to predict the concentrations of three analytes (Table 5). The recoveries (relative to the added amounts of components in the human plasma) varied between 96–103%, 94–101% and 93-105% for phenanthridine, phenanthridinone and phenanthridine N-oxide, respectively. Therefore, it is possible to analyze accurately and precisely phenanthridine, phenanthridinone and phenanthridine N-oxide simultaneously in a complex mixture.

4. Conclusions

PLS and PCR as two powerful and the most widely used multivariate calibration methods were employed for simultaneous spectrophotometric determination of phenanthridine and its two major metabolites - phenanthridinone and phenanthridine N-oxide. The application of PLS and PCR was found to be of comparable quality to analyze phenanthridine, phenanthridinone and phenanthridine N-oxide in human plasma and obtain their analytical figures of merit. Both models provided excellent results. Simplicity of pretreatment and measurements could make the proposed method an effective analytical tool to analyze accurately and precisely aza-hetrocyclic compounds simultaneously in a complex mixture by using simple instrumentation and low costs of materials.

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