Simultaneous Determination of Sulfamethoxazole and Phthalazine by HPLC and Multivariate Calibration Methods

Asadi, Sorayya*+; Gharbani, Parvin

Department of Chemistry, Ahar Branch , Islamic Azad University, Ahar, I.R. IRAN

ABSTRACT: *Two multivariate calibration methods are compared for the simultaneous chromatographic determination and separation of Sulfamethoxazole (SMX) and Phthalazine (PHZ) by High Performance Liquid Chromatography (HPLC). Multivariate calibration techniques such as Classical Least Squares (CLS) and Inverse Least Squares (ILS) were introduced into HPLC to determine the quantification by using UV detector at 235, 250, 260 and 270 nm. Sixteen binary mixtures of SMX and PHZ as calibration set and eight binary mixtures as prediction set were used. Results show that, Relative Errors of Prediction (REP) of CLS and ILS for SMX and PHZ were 0.17%, 0.63% and o.15%, 0.56%, respectively.*

KEY WORDS: *Overlapped peaks, Multivariate calibration method, Classical least squares, Inverse least squares, Sulfamethoxazole, Phthalazine.*

INTRODUCTION

Technological developments have resulted in the use of powerful analytical methods for quality control and analysis of pharmaceutical compounds [1]. HPLC as a comparison method is the common method for analysis of multicomponent pharmaceutical formulation needs a separation treatment and several injections during analysis [2]. In this paper, two pharmaceutical compounds, Sulfamethoxazole (SMX) and Phthalazine (PHZ), were chosen as model compounds. Sulfamethoxazole (SMX), a sulfonamide with well-known anti-bacterial properties, is not freely soluble in water and causes problems in its clinical applications. It is commonly used to treat urinary tract infections. In addition it can be used as an alternative to amoxicillin-based antibiotics to treat sinusitis. It can also be used to treat toxoplasmosis and it is the drug of choice

for pneumocystis pneumonia, which affects primarily patients with HIV [3].

Phthalazine, is a heterocyclic organic compound with the molecular formula $C_8H_6N_2$. It possesses basic properties and forms addition products with alkyl iodides.

A highly sensitive, selective and high-throughput Liquid Chromatography-tandem Mass Spectrometry (LC–MS/MS) method for simultaneous quantification of sulfamethoxazole (SMZ) and trimethoprim (TMP) has been developed and validated using imipramine as an internal standard [4]. Specific, accurate and precise NMR and HPLC methods were developed for determining miconazole, metronidazole and sulfamethoxazole antibiotic drugs in authentic, pharmaceutical and urine samples. F-test revealed insignificant difference in precisions between the developed

NMR methods and HPLC methods reported for analyzing miconazole, metronidazole and sulfamethoxazole [5]. The applicability of H-Point Standard Additions Method (HPSAM) to the resolving of overlapping spectra corresponding to the sulfamethoxazole and trimethoprim is verified by UV-Vis spectrophotometry. The results show that the H-point standard additions method with simultaneous addition of both analytes is suitable for the simultaneous determination of sulfamethoxazole and trimethoprim in aqueous media. The results of applying the H-point standard additions method showed that the two drugs could be determined simultaneously [6]. Gas chromatography and high-performance liquid chromatography are described for the determination of 4-N-acetylhydrazinophthalazin-1-one, 4-hydrazinophthalazin-1-one, phthalazinone and s-triazolo[3,4-a]phthalazine in human urine. Phthalazinone and s-triazolo[3,4-a]phthalazine are measured underivatised by high-performance liquid chromatography [7]. A selective high-performance liquid chromatographic assay for the separation and quantitation of the proposed hepatic microsomal metabolites of hydralazine (HP), phthalazine, phthalazinone, s-triazolo[3,4-a]phthalazine, 3-methyl-s-triazolo[3,4-a] phthalazine and 3-hydroxymethyl-s-triazolo[3,4-a] phthalazine, is described. The methods presented are accurate and reliable, permitting the baseline separation of five HP metabolites with reasonable analysis time and sensitivity [8]. An on-line UV-HPLC / APCI-MS (atmospheric pressure chemical ionization) tandem by a platinium C18 column with an isocratic solvent system after optimizing was applied to the separation and the identification of different carotenoids including astaxanthin, canthaxanthin, apocarotenoic ester, torularhodin and beta carotene.The developed method allows to distinguish torularhodin and canthaxanthin, having the same molar mass but a different chemical structure [9]. In chromatographic analysis, the main problems of this method involve the optimization conditions such as selection of column type, temperature of column, selection of one specific wavelength and composition of mobile phase [2]. So, these parameters affect the results of analysis. For this reason, use of multiwavelength detector or use of multivariate calibration methods could be eliminating the errors [1]. In recent years, chemometric calibration such as Classical Least Squares (CLS), Inverse Least Squares (ILS), Principle Component Regression (PCR) and Partial Least Squares (PLS) have been used to analysis of analytical data obtained from many instruments [10-13]. PLS, PCR, CLS and ILS are powerful mathematical methods for enhancing chemical methods and reduction of errors [14]. Considering the chromatogram of an analyte in HPLC as a spectrum, it was proposed to apply less complex multivariate calibration methods for the quantification of compounds with identical chromatograms such as CLS and ILS. These chemometric techniques have the advantage of being relatively simple, rapid and of low cost. Direct Orthogonal Signal Correction (DOSC) and Savitzky-Golay Filters (SGF) were applied as preprocessing methods on the original and first derivative absorbance data. Principle Component Regression (PCR), Partial Least Squares (PLS) and Iterative Ttarget Transformation Factor Analysis (ITTFA), were used in spectrophotometric simultaneous determination of heavy divalent metal ions, lead, zinc, mercury and cadmium, using 4-(2-pyridylazo) resorcinol (PAR) as metallochromic indicator [15]. Mathematical modeling and simulation of microbial Polyhydroxybutyrate (PHB) production process was used to simulate the process in MATLAB environment. It was revealed that the kinetic model parameters were estimated off the optimal or at a local optimal point [16]. The aim of this paper is use of chemometric approach to simultaneous chromatographic determination and separation of sulfamethoxazole and Phthalazine and so CLS and ILS calibration techniques were applied to HPLC data set at multiwavelengths for binary mixture analysis.

EXPERIMENTAL SECTION
Chemicals, Reagents and Solutions
Sulfamethoxazole (SMX) and phthalazine (PHZ) were purchased by Aldrich (Dowel, UK). HPLC-grade acetonitrilie, methanol and all other chemicals were supplied from Merck (Germany). Water was obtained by double distillation and additionally purified with a Milli-Q system.

Stock solutions of 633 µg/mL of SMX and 325 µg/mL of PHZ were prepared by dissolving 15.82 mg of SMX and 8.12 mg of PHZ, in the mobile phase. The mobile phase consisted of 38% of acetonitrile, 4% of methanol and 58% of potassium dihydrogen phosphate (10 mmol^{-1}, pH =6) in 25 mL

Table 1: Linear regression analysis, limit of determination (LOD), and limit of quantification (LOQ)
for determination Sulfamethoxazole (SMX) and phthalazine (PHZ) by HPLC.

Drug	λ (nm)	Regression equation	r	LOD (µg/mL)	LOQ (µg/mL)
SMX	235	$A = 7.80 \times 10^3 \, C_{SMX} - 84$	0.9998	0.009	0.033
	250	$A = 9.93 \times 10^3 \, C_{SMX} - 88$	0.9997	0.008	0.026
	260	$A = 1.91 \times 10^4 \, C_{SMX} - 61$	0.9999	0.005	0.017
	270	$A = 2.26 \times 10^4 \, C_{SMX} - 82$	0.9999	0.002	0.007
PHZ	235	$A = 4.34 \times 10^3 \, C_{PHZ} + 344$	0.9997	0.015	0.050
	250	$A = 5.80 \times 10^3 \, C_{PHZ} + 638$	0.9998	0.012	0.041
	260	$A = 8.06 \times 10^3 \, C_{PHZ} + 394$	0.9999	0.010	0.036
	270	$A = 6.40 \times 10^3 \, C_{PHZ} + 612$	0.9998	0.011	0.038

* HPLC: high performance liquid chromatography

Equipment, Instrumentation and Software

Experiments were performed using an Agilent-1100 liquid chromatographic system (Agilent Technologies, USA) equipped with a binary HPLC pump (Waters Model 515), a thermostatted auto sampler, and a column heater. Sykam S3240 Programmable 4-channel UV-Vis Detector (Laserchrom, UK) was used as a detector. So, it is possible to obtain the chromatogram at the four wavelengths in a single injection.

The absorption spectra were recorded using a Beckman DU 640 UV-Vis spectrophotometer. All data were saved in ASCII format and transferred to a PC computer for subsequent manipulation. Data were handled using Microsoft excel and MATLAB software (7.1 versions).

Chromatographic Conditions

The original mobile phase optimization procedure was carried out based on the selectivity triangle approach using three solvents and taking into consideration the molar fraction of the solvents [17-18]. The mobile phase consisted acetonitrile, methanol, potassium dihydrogen phosphate buffer (10 mM, pH =6) (38:4:58 v/v/v).

The separations were performed on a Symmetry C18 HPLC column (250 mm×4.6 mm, 5 µm particle size) from Waters (USA). The flow rate was maintained at 0.9 mL/min and the injection volume was 25 µL. The mobile phase was prepared daily, filtered through a 0.45 µm membrane filter, and degassed before use.

Individual Calibrations

Individual calibration curves were constructed using several points as peak height versus SMX and PHZ concentration in the range of 0.12-2.53 µg/mL and

0.06-1.30 µg/mL for SMX and PHZ, respectively and the results evaluated by linear regression. The peak height for sets was obtained at four different wavelengths for both compounds. The HPLC data characteristic of calibration graph and the validation parameters for determination of SMX and PHZ are given in Table 1. As shown lower LOD and LOQ for SMX and PHZ was obtained in 270 and 260 nm, respectively. So, separation of mixture in low concentration is possible.

Calibration Procedure for the Simultaneous Determination

SMX and PHZ as binary mixtures were prepared as follows: appropriate volumes of the standard solutions (in the dynamic linear range) were transferred into a 10 mL volumetric flask and made up to the mark. The chromatograms of the analyte solutions were obtained at four wavelengths 235, 250, 260 and 270 nm by HPLC/ UV detector. These wavelengths were selected based on the spectra of the analytes (Fig. 1). The optimized calibration models were applied to calculate the concentration of each analyte in the prediction

THEORITICAL SECTION

These approaches are based on the application of Multi Linear Regression (MLR) to the ratio of the peak height of each analyte [19]. The matrix equation describing the system is given as follows:

$$R = CK \tag{1}$$

Where, R is M×N matrix and represents the peak height responses of M samples at N wavelengths, C denotes the concentrations of L for the investigated compounds in M samples, and K is L×N matrix
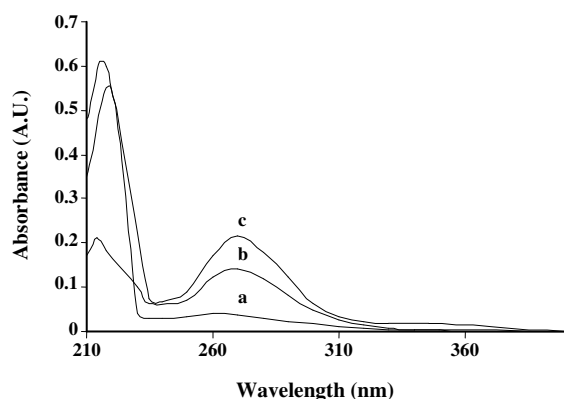
Fig. 1: The UV spectra of 1.3 µg/mL phthalazine (PHZ) (a), 2.51µg/mL Sulfamethoxazole (SMX) (b), and the mixture of these two solutions (c).

Fig. 2: The chromatogram obtained from a standard solution containing 1.3 µg/mL PHZ (a), 2.51 µg/mL SMX (b) and their mixture (c).

of the calibration coefficients. In calibration step, it is assumed that a series of experiments are performed in which C is known (e.g. a set of mixtures of compounds with known concentrations are recorded). C^T is the transpose of the matrix C. An estimate of K can then be obtained by:

$$K = (C^T C)^{-1} C^T R \qquad (2)$$

And can be used in the prediction step to predict the concentrations in any unknown samples (C_{un}):

$$C_{un} = R_{un} K(K^T K) \qquad (3)$$

Where R_{un} is the unknown peak height response and K^T represents the transpose of the matrix K.

The approach described above is a form of classical calibration, and it is also possible to envisage an inverse calibration model (Inverse Least Square, ILS) [19].

$$C = RB \qquad (4)$$

The matrix B is given by

$$B = (R^T R) R^T C \qquad (5)$$

Where R^T is the transpose of the matrix R.

This can be extended to estimate the concentrations in any unknown sample:

$$C_{un} = R_{un} B \qquad (6)$$

This use of the inverse model is only practicable if: 1) the number of experiments and wavelengths is at least equal to the number of components in the mixture, and 2) the number of experiments is at least equal to the number of wavelengths [19].

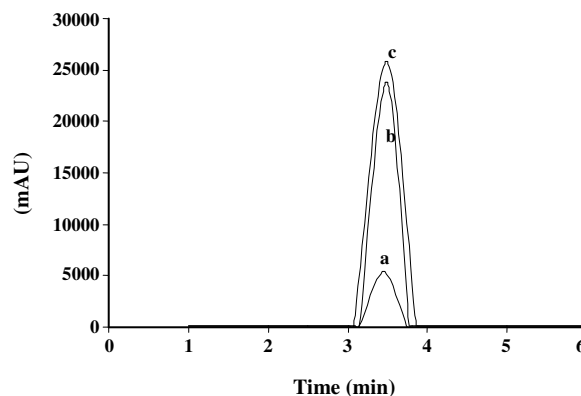The condition 2 requires a large number of extra experiments to be performed. There have been a number of algorithms developed for wavelength selection, enabling inverse models to be produced, but it has been proposed that there is no real advantage over classical least squares in these situations [19].

Such equations make assumptions that the concentrations of the significant analytes are all known and they work well only if this is true. In those cases that there is an application to mixtures with unknown components, it can result in serious estimation errors.

In this paper, the signals in data matrix are heights of the peak in the chromatograms at four wavelengths. So there is a vector, r, with the size 1×4 for each sample. R is column augmented matrix, each row is the vector that was obtained for each sample. Since the obtained data are first order, the mentioned simple first order methods, CLS and ILS, can be used to analyze them. The proposed method can be used not only to analyze components with overlapped chromatograms, but also to resolve the components with identical chromatograms. It is necessary to emphasis that the proposed method can be used when there is no linear dependency between UV spectra of two components at selected wavelengths.

RESULTS AND DISCUSSION

The chromatograms of the SMX and PHZ standard solutions and the mixed solutions are shown in Fig. 2. As shown, the chromatograms of SMX and PHZ are completely overlapped and separation of them is impossible by single variation. So, it is necessary to use of multivariate calibration methods. So, chemometric methods, discussed above, can be used to resolve the

Table 2: The composition of the calibration samples with four different concentration levels in the dynamic linear range for Sulfamethoxazole (SMX) and phthalazine (PHZ) obtained from the orthogonal design method.

Experiment	SMX	PHZ
1	2.53	0.65
2	2.53	1.30
3	2.53	0.13
4	2.53	0.065
5	1.26	0.65
6	1.26	1.30
7	1.26	0.13
8	1.26	0.065
9	0.25	0.65
10	0.25	1.30
11	0.25	0.13
12	0.25	0.065
13	0.12	0.65
14	0.12	1.30
15	0.12	0.13
16	0.12	0.065
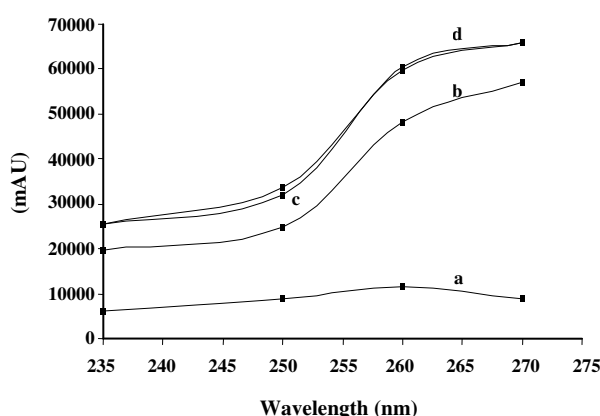
Fig. 3: The UV spectra of PHZ (a), SMX (b), a mixture solution of PHZ and SMX (c), and the spectrum obtained from the sum of PHZ and SMX signals at 235-270 nm (d).

components in the mixtures. Thus it can be suggested that the multivariate calibration methods coupled with HPLC could be determined the analytes even when there is an observed overlapping of the chromatographic peaks.

The first step in linear models of multivariate calibration methods such as CLS and ILS is checking additive property. This step is necessary in order to check that the other sources of signal variation, such as any interactions between the chemical components, changes of shape of the component peak and detector noise, exist or not. Additive property is a requirement for successful application of these quantification methods. As shown, the peak heights, UV signals, of two compounds showed good additive properties at selected wavelengths and thus the linear models of multivariate calibration of CLS and ILS can be used for the simultaneous determination of these components. Four wavelengths in the UV region were used as detector sensors in recording chromatograms. Fig. 3. Indicates the signal of peak height of compounds for SMX, PHZ and binary mixture of them, as theatrical and experimental results.

The second step in the simultaneous determination using the multivariate calibration methods involves constructing of the calibration matrix for the binary mixture. In the study, calibration sets were optimized with the aid of the orthogonal design method. Table 2 shows the composition of the calibration and prediction samples with four concentration levels in the dynamic linear range for SMX and PHZ. Therefore, the applied limits for SMX and PHZ were in the range of 0.12-2.53 and 0.65- 1.3 µg/mL respectively.

The recovery and Relative Error of Prediction (REP) and data obtained by application of the mixtures for SMX and PHZ by CLS and ILS have been summarized in Table 3. The statistical parameters of recovery percentage were used to evaluate the prediction ability for the proposed models. The formula for the REP parameter was calculated by the following equations:

$$REP(\%) = 100 \times \left[\frac{\sum_{i=1}^{m}(C_{pre} - C_{act})^2}{\sum_{i=1}^{m}C_{act}^2} \right]^{1/2} \tag{7}$$

where, C_{act} indicates the actual concentration in the sample, C_{pre} is the predicted concentration and m is the number of samples in the prediction set. As shown in Table 3, amounts of REP and recovery for both compounds by ILS and CLS

Table 3: The recovery and relative error of prediction (REP) for SMX and PHZ by CLS and ILS methods.

	HPLC-CLS		HPLC-ILS	
Drug	SMX	PHZ	SMX	PHZ
Recovery (%)	107.2	109.1	102.3	110.0
REP* (%)	0.17	0.63	0.15	0.56

* REP: relative error of prediction

Table 4: Statistical results for SMX and PHZ in binary mixture.

Drug	SMX	PHZ
LOD (µg/mL)	0.004	0.014
LOQ (µg/mL)	0.013	0.047

Table 5: The predicted concentration of each compound (µg ml^{-1}) by two methods multivariatie calibration methods (ILS and CLS) in prediction sets. In the paranthesis, low standard deviation shows the accuracy and repeatability of methods.

Drug	SMX (µg/mL)			PHZ (µg/mL)		
NO.	HPLC	CLS	ILS	HPLC	CLS	ILS
1	2.53	2.52 (0.00)	2.52 (0.00)	1.30	1.31 (0.02)	1031 (0.02)
2	1.89	1.90 (0.02)	1.88 (0.01)	0.97	0.95 (0.00)	0-96 (0.01)
3	1.26	1.25 (0.00)	1.24 (0.01)	0.65	0.64 (0.00)	0.64 (0.00)
4	0.63	0.60 (0.00)	0.60 (0.00)	0.32	0.31 (0.00)	0.31 (0.00)
5	0.50	0.48 (0.00)	0.45 (0.07)	0.26	0.28 (0.02)	0.27 (0.00)
6	0.25	0.23 (0.00)	0.24 (0.00)	0.13	0.14 (0.02)	0.14 (0.02)
7	0.18	0.16 (0.01)	0.17 (0.00)	0.097	0.098 (0.02)	0.098 (0.02)
8	0.12	0.11 (0.01)	0.11 (0.01)	0.067	0.066 (0.00)	0.065 (0.01)

methods indicate the proposed methods were successfully to simultaneous determination and separation of overlapped peaks in HPLC.

Some wavelengths contain colinear absorbance data (i.e. the same peak height for analytes) and some have uninformative data. To investigate the prediction ability of the resultant CLS and ILS models and to compare the effect of wavelength, the calibrated models were used to determine the analytes in a separate prediction set that did not have contribution in the model building steps. Four wavelengths and different ternary and binary sets of wavelengths were used to construct the models and predict the concentration of analytes in prediction set. The advantage of the constructed model is likely to be its simplicity, if it can be used with low numbers of wavelengths. As can be seen in Table 4, the results for the binary sets of wavelengths are as good as the result of four wavelengths and lower LOD and LOQ was obtained for SMX in binary mixture. Table 5, show the results and statically parameters for SMX and PHZ by two multivariate calibration methods (ILS and CLS) in prediction sets. The predicted concentration of each compound (µg/mL) by two methods is summarized in Table 5. As shown, agreement between predicted and actual concentration indicates the successfully of both methods Also, low standard deviation (in the parentheses) shows the accuracy and repeatability of methods. While, comparison of HPLC with Photo Diode Array (PDA) detection for the simultaneous determination of some drugs shows best results [1,2, 14].

CONCLUSIONS

HPLC as a powerful separation and analysis method is widely used for simultaneous determination of analytes. But there can be problems when two compounds have the identical chromatograms. In this work, CLS and ILS are two simple, but powerful chemometric methods which can be used for simultaneous determination of compounds with the overlapped and identical chromatograms. When it is not possible to resolve of two components by changing or modification of mobile phase, the proposed method is a good alternative. In comparison with second order algorithms the proposed method is simple, rapid and easy to understand and apply.

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