

**Original article****Simultaneous spectrophotometric determination of difloxacin and enrofloxacin in urine samples by partial least squares regression (PLS) and direct orthogonal signal correction-partial least squares regression (DOSC-PLS) methods****Kuldeep Kaur<sup>1#</sup>, Ashok Kumar Malik<sup>1\*</sup>, Baldev Singh<sup>1</sup> and M. Godarzi<sup>2</sup>**<sup>1</sup>*Department of Chemistry, Punjabi University, Patiala, 147002, Punjab, India*<sup>2</sup>*Young Researchers Club and Department of Chemistry, Faculty of Science, Azad University of Arak, Arak, Iran*<sup>#</sup>*On leave from Mata Gujri College Fatehgarh Sahib, Punjab, India**\*Corresponding author: Tel: +91-175-2359557; Fax: +91-175-2283073**E-mail address: malik\_chem2002@yahoo.co.uk***Abstract:**

Simultaneous determination of difloxacin and enrofloxacin using two chemometric methods, partial least squares regression (PLS) and direct orthogonal signal correction-partial least squares regression (DOSC-PLS) is described in this paper. The simultaneous determination of these drugs is difficult due to spectral interferences. The calibration graphs were linear in the range of 1-10  $\mu\text{g mL}^{-1}$  and 1-9  $\mu\text{g mL}^{-1}$  with detection limits of 0.52 and 0.15  $\mu\text{g mL}^{-1}$  for difloxacin and enrofloxacin, respectively. The experimental calibration matrix was designed with 25 mixtures of these drugs. The PLS and DOSC-PLS were used for multivariate calibration of spectrophotometric data. The root mean square error of prediction (RMSEP) for applying the two methods to 8 synthetic samples in the linear calibration ranges for difloxacin and enrofloxacin was 0.1499 and 0.2833 for PLS, 0.0753 and 0.0436 for DOSC-PLS respectively. The relative standard error of prediction (RSEP) for difloxacin and enrofloxacin was 3.5214 and 7.6757 with PLS, 1.7694 and 1.1809 for DOSC-PLS, respectively. The DOSC-PLS method was found to be more accurate than PLS method providing better recoveries and lower RMSEP and RSEP values. The DOSC-PLS method was successfully applied to simultaneous determination of difloxacin and enrofloxacin in urine samples.

**Keywords:** Difloxacin; Enrofloxacin; Simultaneous determination; Spectrophotometry

## Introduction

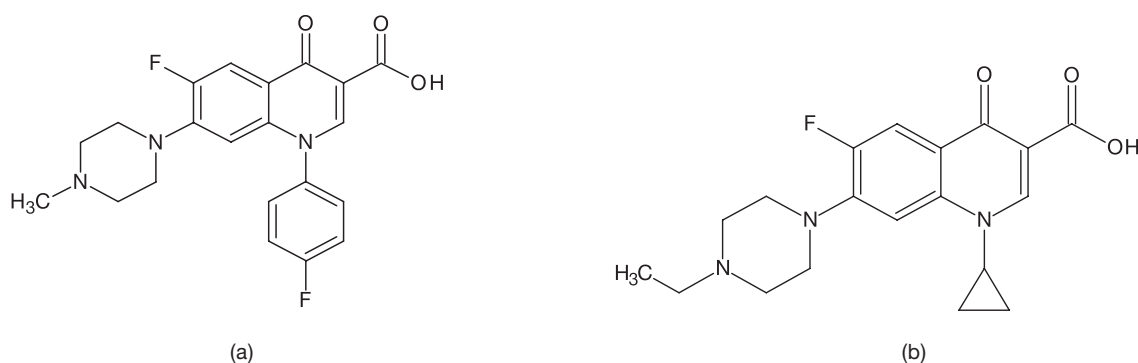
Fluoroquinolones are broad-spectrum, antibacterial drugs which are effective against a wide range of Gram-positive and Gram-negative bacteria [1]. They are extremely useful for the treatment of a variety of infections including urinary tract infections, soft tissue infections, respiratory infections, bone-joint infections and typhoid fever. Principal advantages of fluoroquinolones include good oral bioavailability, bactericidal activity at low tissue concentrations, and good penetration into phagocytic cells [2-3]. They have a large volume of distribution combined with low plasma protein binding, which allows them to reach tissue concentrations often higher than concurrent serum concentrations [4]. The fluoroquinolone drugs are structurally related to nalidixic acid.

Difloxacin {6-fluoro-1-(4-fluorophenyl)-7-(4-methyl-1-piperazinyl)-4-oxo-1,4-dihydro-3-quinolinecarboxylic acid} (Figure 1a) is a second generation fluoroquinolone drug commonly used in veterinary medicine. Like other fluoroquinolones it is broad spectrum and useful in a variety of diseases. Enrofloxacin {1-cyclopropyl-7-(4-ethyl-1-piperazinyl)-6-fluoro-1,4-dihydro-4-oxo-3-quinolinecarboxylic acid} (Figure 1b), is the first specified fluoroquinolone developed for veterinary application. Because of broad spectrum of activity and less side effects, enrofloxacin has been widely used for treatment of some infectious diseases in pets and livestock. It is mainly eliminated by renal mechanism achieving high concentrations in urine [5].

Simultaneous determination of difloxacin and enrofloxacin by ordinary spectrophotometric methods is difficult because of overlapping of absorption spectra

(Figure 2). The methods reported for the simultaneous determination of the fluoroquinolones are liquid chromatography (LC) [6], LC in combination with techniques like fluorescence detection (LC-FD) [7-9] and ultraviolet detection (LC-UV) [10-11], electrospray ionization [12], tandem mass spectrometry (LC-MS/MS) [13], mass spectrometry (MS) [14-17], capillary electrophoresis (CE) combined with UV detection [18-21], capillary zone electrophoresis (CZE) combined with tandem mass spectrometry (CZE-MS/MS) [22], high pressure liquid chromatography (HPLC) using ultraviolet [23-25] or fluorescence detection [26-28] and enzyme linked immunosorbent assay (ELISA) [29]. LC and HPLC are the most widely used techniques for separation and quantification of fluoroquinolones, while CE has been used to lesser extent. Though these techniques are sufficiently reliable and sensitive, they have some limitations such as requiring preconcentration steps, long extraction procedures, tedious work and expensive equipment. It requires expertise to handle HPLC. The spectrophotometric techniques are relatively simple and can be successfully used for analysis of fluoroquinolones. These are economical and easily available in any laboratory.

Fluoroquinolones are a widely used class of antimicrobial agents in human and veterinary medicine because of their wide spectrum of activity and their physico-chemical properties. Recently concern over the detrimental effect of these drugs on the environment and human health has increased. The extensive use of these drugs in veterinary medicine present a potential health hazard to humans because residues of these



**Figure 1** Structures of difloxacin (a) and enrofloxacin (b)

antibiotics may persist in edible tissues or foodstuffs such as milk or eggs. Often a high percentage of administered antibiotics is excreted from the dosed animals without metabolism or excreted in conjugated forms [30]. These antibiotic residues contaminate water sources including municipal wastewater and surface waters as has been indicated by some recent studies [31-34]. These residues can affect human health, develop antibiotic resistant organisms and disturb antimicrobial ecology. The main objective of the study is to develop a rapid, sensitive and economical method for the analysis of fluoroquinolone residues in urine samples. The fluoroquinolones chosen are difloxacin and enrofloxacin, which have overlapping spectra and cannot be determined by ordinary spectrophotometric methods. The method is simple and economical, does not require pre-concentration steps and therefore prevents the loss of analytes during these steps. The need of extraction with costly organic solvents is also eliminated.

Multivariate calibration methods have been successfully used in recent years for multicomponent analysis. PLS modelling is a powerful multivariate statistical tool that has been successfully applied to quantitative analysis of spectrophotometric data [35-37]. The technique is rapid so the components can be determined at a fast speed and avoids the use of prior separation that may be necessary. Generally for the evaluation of the predictive ability of a multivariate calibration model, the root mean square error of prediction (RMSEP) and relative standard error of prediction (RSEP) can be used.

$$RMSEP = \sqrt{\frac{\sum_{i=1}^n (y_{pred} - y_{obs})^2}{n}}$$

$$RSEP (\%) = 100 \times \sqrt{\frac{\sum_{i=1}^n (y_{pred} - y_{obs})^2}{\sum_{i=1}^n (y_{obs})^2}}$$

where  $y_{pred}$  is the predicted concentration in the sample,  $y_{obs}$  is the observed value of the concentration in the sample and  $n$  is the number of samples in the validation set.

## Theory

### PLS modeling

PLS is a method for building regression models on the latent variable decomposition relating two blocks, matrices **X** and **Y**, which contain the independent,  $x$ , and dependent,  $y$ , variables, respectively. These matrices can be simultaneously decomposed into a sum of  $f$  latent variables, as follows:

$$X = TP^T + E = \sum t_f p_f' + E \tag{1}$$

$$Y = UQ^T + F = \sum u_f q_f' + F \tag{2}$$

in which  $T$  and  $U$  are the score matrices for  $X$  and  $Y$ , respectively;  $P$  and  $Q$  are the loadings matrices for  $X$  and  $Y$ , respectively,  $E$  and  $F$  are the residual matrices. The two matrices are correlated by the scores  $T$  and  $U$ , for each latent variable, as follows:

$$u_f = b_f t_f \tag{3}$$

in which  $b_f$  is the regression coefficient for the  $f$  latent variable. The matrix  $Y$  can be calculated from  $u_f$ , as Eq. (4), and new samples can be estimated from the new scores  $T^*$ , which are substituted in Eq. (4), leading to Eq. (5)

$$Y = TBQ^T + F \tag{4}$$

$$Y_{new} = T^* BQ^T \tag{5}$$

In this procedure, it is necessary to find the best number of latent variables, which normally is performed by using cross-validation, based on determination of minimum prediction error. Applications of PLS have been discussed by several workers [38-41].

### Selection of the optimum number of factors

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 cross-validated models using a high number of factors (half the number of total standard +1), which is defined as follows:

$$PRESS = \sum (y_i - \hat{y}_i)^2 \quad (6)$$

where  $y_i$  is the reference concentration for the  $i^{\text{th}}$  sample and  $\hat{y}_i$  represents the estimated concentration. The cross-validation method employed was to eliminate only one sample at a time and then PLS calibrate the remaining standard spectra. By using this calibration the concentration of the sample, left out was predicted. This process was repeated until each standard had been left out once.

One reasonable choice for the optimum number of factors would be that number which yielded the minimum PRESS. Since there are a finite number of samples in the training set, in many cases the minimum PRESS value causes overfitting for unknown samples that were not included in the model. A solution to this problem has been suggested by Haaland *et al.* [42-43] in which the PRESS values for all previous factors are compared to the PRESS value at the minimum. The F-statistical test can be used to determine the significance of PRESS values greater than the minimum.

### **DOSC-PLS modeling**

In 1998, Wold *et al.* published the original paper on orthogonal signal correction [44]. The objective was to construct a filter that removes from the spectral matrix  $\mathbf{X}$  only the part that definitely is uncorrelated to  $\mathbf{Y}$ . It was followed by a number of different OSC filters [41,45-47]. Since all of these methods had some problems, Westerhuis *et al.* [48] introduced direct orthogonal signal correction.

The DOSC algorithm calculates directions in  $\mathbf{X}$  that are orthogonal to  $\mathbf{Y}$  and account for the largest variance of  $\mathbf{X}$ . These directions are obtained by only using least squares steps. The first step of DOSC is to decompose  $\mathbf{Y}$  into two orthogonal parts, the projection of  $\mathbf{Y}$  onto  $\mathbf{X}$ ,  $\hat{\mathbf{Y}}$ , and the residual part,  $\mathbf{F}$  that is orthogonal to  $\mathbf{X}$ .

$$\mathbf{Y} = \mathbf{P}_X \mathbf{Y} + \mathbf{A}_X \mathbf{Y} = \hat{\mathbf{Y}} + \mathbf{F}$$

$$\text{(where, } \mathbf{P}_D = \mathbf{D}\mathbf{D}^+ \text{ and } \mathbf{A}_D = \mathbf{I} - \mathbf{P}_D = \mathbf{I} - \mathbf{D}\mathbf{D}^+ \text{)} \quad (7)$$

Next,  $\mathbf{X}$  is decomposed into two orthogonal parts, one part that has the same range as  $\hat{\mathbf{Y}}$  and another part that is orthogonal to it.

$$\mathbf{X} = \mathbf{P}_{\hat{\mathbf{Y}}} \mathbf{X} + \mathbf{A}_{\hat{\mathbf{Y}}} \mathbf{X} \quad (8)$$

$$\mathbf{A}_{\hat{\mathbf{Y}}} \mathbf{X} = \mathbf{X} - \mathbf{P}_{\hat{\mathbf{Y}}} \mathbf{X} \quad (9)$$

Therefore, the columns of  $\mathbf{A}_{\hat{\mathbf{Y}}} \mathbf{X}$  span a subspace of  $\mathbf{X}$  that is orthogonal both to  $\hat{\mathbf{Y}}$  and to  $\mathbf{Y} = \hat{\mathbf{Y}} + \mathbf{F}$ , since  $\mathbf{F}$  is also orthogonal to  $\mathbf{X}$ . Having found this orthogonal subspace  $\mathbf{A}_{\hat{\mathbf{Y}}} \mathbf{X}$ , PCA is now applied to find the principal component  $\mathbf{t}$  corresponding to the largest singular value. If more DOSC components are necessary, more principal components can be obtained in this step. The direction  $\mathbf{t}$  finally is expressed as linear combinations of  $\mathbf{X}$ .

$$\mathbf{t} = \mathbf{X}\mathbf{r} \quad (10)$$

$\mathbf{r} = \mathbf{X}^+ \mathbf{t}$ , where  $\mathbf{X}^+$  is the Moore-Penrose inverse of  $\mathbf{X}$

$$\quad (11)$$

$$\mathbf{X}^{\text{DOSC}} = \mathbf{X} - \mathbf{P}_{\hat{\mathbf{Y}}} \mathbf{X} = \mathbf{X} - \mathbf{t}(\mathbf{t}^T \mathbf{t})^{-1} \mathbf{t}^T \mathbf{X} = \mathbf{X} - \mathbf{t}\mathbf{p}^T =$$

$$\mathbf{X} - \mathbf{X}\mathbf{r}\mathbf{p}^T \quad (12)$$

$$\mathbf{P} = \mathbf{X}^T \mathbf{t}(\mathbf{t}^T \mathbf{t})^{-1} \quad (13)$$

For spectra of new samples  $\mathbf{X}_{\text{new}}$ , the correction can be performed as follows:

$$\mathbf{X}_{\text{new}}^{\text{DOSC}} = \mathbf{X}_{\text{new}} - \mathbf{r}^T \mathbf{X}_{\text{new}} \mathbf{P} \quad (14)$$

Now  $\mathbf{X}_{\text{new}}^{\text{DOSC}}$  can be used in calibration model instead of  $\mathbf{X}_{\text{new}}$  to predict  $\mathbf{Y}_{\text{new}}$  [48].

## **Experimental**

### **Apparatus and software**

A UV-1800 Pharmaspec UV-Visible spectrophotometer (Shimadzu, Japan) was used to record all absorption spectra. All spectral measurements were obtained against blank using 1-cm path length quartz cuvettes. An ATC pH meter model 132-E (Electronics, India) was used for all pH measurements. All the programs were written in MATLAB (Mathworks, version 6.5) [48-49]. PLS analysis

was performed using PLS toolbox in the MATLAB program.

### Reagents

All reagents were of analytical reagent grade. Triply-distilled and deionized water was used in all the studies. Stock solutions of difloxacin and enrofloxacin ( $100 \mu\text{g mL}^{-1}$ ) were prepared by direct weighing of the requisite amount of commercially available reagent (Sigma Aldrich, Steinheim, Germany) and then dissolving in dilute acetic acid. These solutions were stored in the dark and were found stable for at least 4 weeks spectrophotometrically. Working solutions of each drug were prepared by the appropriate dilution to the stock solutions and were stored at room temperature.

### Procedures

#### Individual calibration

For individual calibration, known amounts of standard solutions were placed in 10-ml volumetric flasks and completed to the final volume with triply-distilled water. The final concentrations of drugs in these solutions varied between  $1\text{-}10 \mu\text{g mL}^{-1}$  for difloxacin and  $1\text{-}9 \mu\text{g mL}^{-1}$  for enrofloxacin. The absorbances were then measured at 280 nm and 274.5 nm for difloxacin and enrofloxacin respectively against a reagent blank. The absorbance of each solution was noted and plotted

against concentration to obtain the calibration curves.

#### Multivariate calibration

Each calibration, prediction and validation set was prepared as follows: appropriate amounts of both the drugs were taken into volumetric flasks and the final volume was made upto 10 mL with triply-distilled water. The concentration ranges in these mixtures were selected so that the final concentration was in the range of  $1\text{-}10 \mu\text{g mL}^{-1}$  for difloxacin and  $1\text{-}9 \mu\text{g mL}^{-1}$  for enrofloxacin. The concentration ranges were chosen so that absorbances obtained for all standard samples were  $< 1.5$ . In order to obtain maximum information on each drug from calibration procedure, the compositions of the samples were randomly designed.

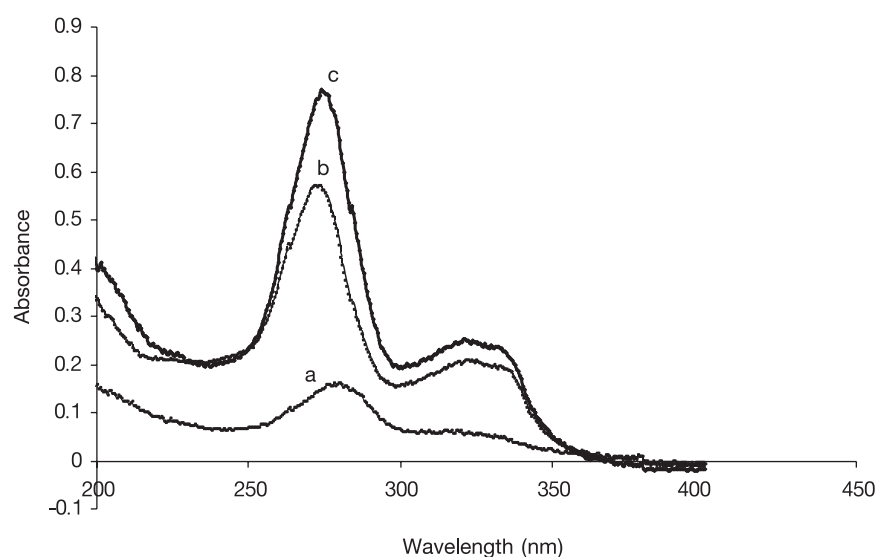
#### Sample preparation

For analysis of drug in urine, the urine samples were collected from dogs. The samples were then centrifuged at 3000 rpm for 10 min. A suitable aliquot of the supernatant was taken, spiked with standard drug solution and diluted to obtain drug concentrations in the linear calibration range and absorption spectra were recorded.

## Results and Discussion

### Absorption spectra

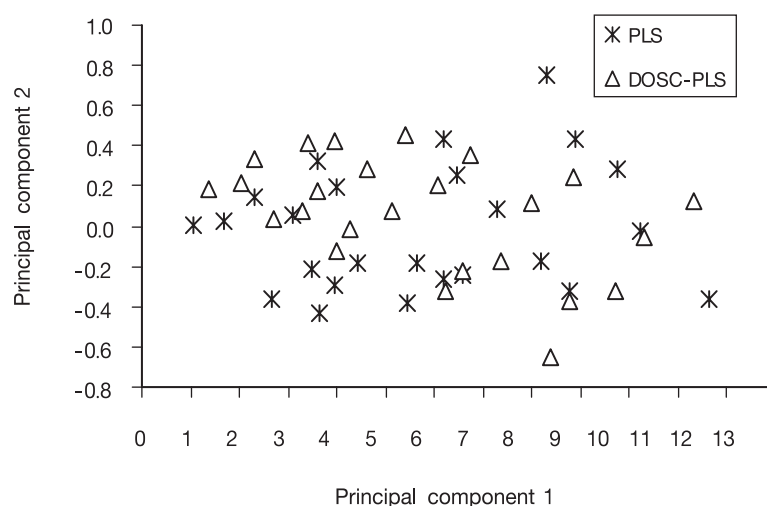
Difloxacin and enrofloxacin show absorption maxima at 280 nm and 274.5 nm respectively as shown in Figure 2. The determination of these drugs by ordinary



**Figure 2** Absorption spectra of difloxacin (a), enrofloxacin (b) and their mixture (c) (concentrations of difloxacin and enrofloxacin are  $5 \mu\text{g mL}^{-1}$ )

**Table 1** Statistical analysis of the determination of difloxacin and enrofloxacin in mixtures by zero derivative spectrophotometry

Parameters	Zero derivative	
	Difloxacin	Enrofloxacin
	$\lambda_{\max} = 280 \text{ nm}$	$\lambda_{\max} = 274.5 \text{ nm}$
Molar absorptivity, $\epsilon$ ( $\text{L mol}^{-1} \text{ cm}^{-1}$ )	$1.26 \times 10^4$	$3.98 \times 10^4$
Specific absorptivity, $a$ ( $\text{L g}^{-1} \text{ cm}^{-1}$ )	31.6	110.3
Sandell's sensitivity, $S$ ( $\mu\text{g cm}^{-2}$ )	$31.50 \times 10^{-3}$	$9.06 \times 10^{-3}$
Beer's law ( $\mu\text{g mL}^{-1}$ )	1-10	1-9
Limit of detection ( $\mu\text{g mL}^{-1}$ )	0.52	0.15

**Figure 3** Plots of first principal component against second principal component for difloxacin and enrofloxacin determination by PLS model and DOSC-PLS model

spectrophotometric methods is difficult because the spectral bands of the two drugs overlap. Various parameters including molar absorptivity, Sandell's sensitivity, specific absorptivity, limit of detection and linear range were calculated for both the drugs as shown in Table 1.

### PLS and DOSC-PLS methods

Individual calibration graphs were constructed for both the drugs using linearity equation  $y = mx + c$  where  $x$  is the concentration of the drug in  $\mu\text{g mL}^{-1}$  (Figure 3). The first step in the simultaneous determination of the drugs by PLS methodology involved constructing the calibration matrix for the binary mixture, difloxacin-enrofloxacin. A set of 33 binary mixtures was prepared. Each solution was prepared to contain combination of concentration levels from 1-10  $\mu\text{g mL}^{-1}$  for difloxacin and 1-9  $\mu\text{g mL}^{-1}$  for enrofloxacin. Twenty-five binary

mixtures were selected as the calibration set. The composition of the calibration set is shown in Table 2. PLS method was developed by recording the absorption spectra for calibration set of 25 mixtures. A linear predictive model was constructed for the two drugs based on their spectra. The calibration model was validated using a set of 8 synthetic mixtures and considered as prediction set as shown in Table 3. To ensure that the prediction and real samples are in the subspace of the training set, the score plot of the first principal component versus the second was sketched and it was concluded that all the samples are spanned with the training set scores (Figure 4) The number of factors in the PLS algorithm were selected by using cross validation. According to this method, one sample is eliminated at a time. For the mentioned set of 25 calibration spectra, PLS1 calibration on 24 calibration spectra was performed, and using this calibration, the

**Table 2** Concentration data of the different mixtures used in the calibration set for the determination of difloxacin and enrofloxacin ( $\mu\text{g mL}^{-1}$ )

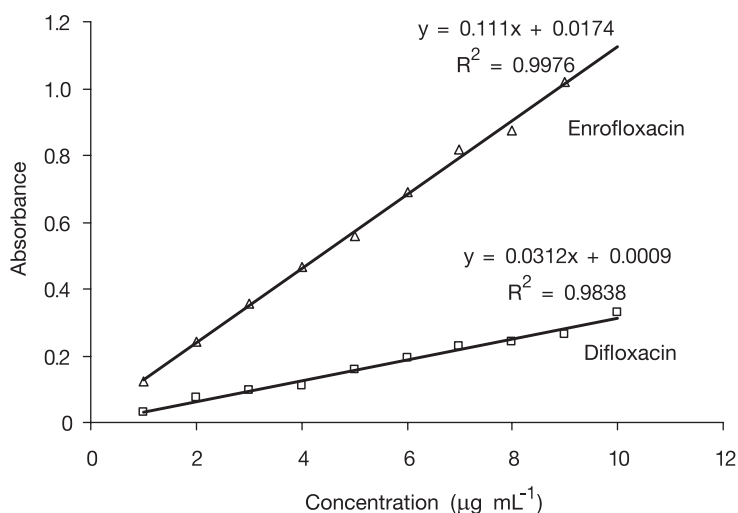
Mixture	Difloxacin ( $\mu\text{g mL}^{-1}$ )	Enrofloxacin ( $\mu\text{g mL}^{-1}$ )	Mixture	Difloxacin ( $\mu\text{g mL}^{-1}$ )	Enrofloxacin ( $\mu\text{g mL}^{-1}$ )
M1	1.00	1.00	M14	4.50	6.50
M2	1.00	2.50	M15	4.50	9.00
M3	1.00	4.00	M16	7.25	1.00
M4	1.00	6.50	M17	7.25	2.50
M5	1.00	9.00	M18	7.25	4.00
M6	2.75	1.00	M19	7.25	6.50
M7	2.75	2.50	M20	7.25	9.00
M8	2.75	4.00	M21	10.0	1.00
M9	2.75	6.50	M22	10.0	2.50
M10	2.75	9.00	M23	10.0	4.00
M11	4.50	1.00	M24	10.0	6.50
M12	4.50	2.50	M25	10.0	9.00
M13	4.50	4.00			

**Table 3** Composition of synthetic samples of difloxacin and enrofloxacin ( $\mu\text{g mL}^{-1}$ ), their prediction by PLS and DOSC-PLS model; result with PLS methods

Synthetic, $\mu\text{g mL}^{-1}$		Prediction by PLS, $\mu\text{g mL}^{-1}$ (recovery, %)		Prediction by DOSC-PLS, $\mu\text{g mL}^{-1}$ (recovery, %)	
Difloxacin	Enrofloxacin	Difloxacin	Enrofloxacin	Difloxacin	Enrofloxacin
3.00	3.00	3.13 (104.3)	2.84 (94.7)	3.03 (101.0)	2.90 (96.6)
3.00	4.00	3.11 (103.6)	3.70 (92.5)	3.12 (104.0)	4.01 (100.2)
3.00	5.00	3.05 (101.6)	4.80 (96.0)	2.96 (98.7)	5.00 (100.0)
4.00	2.00	3.90 (97.5)	2.00 (100.0)	3.96 (99.0)	2.00 (100.0)
4.00	3.00	4.26 (106.5)	2.89 (96.3)	4.10 (102.5)	3.00 (100.0)
5.00	3.00	4.87 (97.4)	2.60 (86.7)	4.87 (97.4)	3.05 (101.6)
5.00	6.00	4.97 (99.4)	5.48 (91.3)	5.00 (100.0)	5.95 (99.2)
6.00	1.00	5.77 (96.2)	1.21 (121.0)	6.00 (100.0)	0.99 (99.0)

concentration of the sample left out during the calibration process was evaluated. This process was repeated 25 times and each sample was left out once. The concentration of each sample was then predicted and compared with the known concentration of this reference sample. The plots of predicted concentration versus actual concentration for difloxacin and enrofloxacin by both the methods are given in Figure 5. The prediction residual sum of squares (PRESS) was calculated. The PRESS value is a direct measure of how well a calibration predicts the concentration left out during a cross validation. The maximum number of factors used to calculate the optimum PRESS was selected as 13. The PRESS values and the optimum number of factors obtained by the application of PLS and DOSC-PLS

models are summarized in Table 4 along with other statistical parameters including mean recovery, RMSEP and RSEP (%) values for both the methods. In all instances, the number of factors for the first PRESS values whose F-ratio probability drops below 0.75 was selected as the optimum. In Figure 6, the PRESS obtained by optimizing the calibration matrix of the absorbance data with PLS and DOSC-PLS models is shown. As is clear from Table 4, better recoveries and lower RMSEP and RSEP values were obtained with DOSC-PLS method as compared to PLS method for both the drugs. The calculated values certainly show the superiority of DOSC-PLS method over PLS method. Therefore DOSC-PLS was used to determine drug concentrations in real sample.



**Figure 4** Individual calibration graphs for difloxacin ( $\lambda_{\max} = 280 \text{ nm}$ ) and enrofloxacin ( $\lambda_{\max} = 274.5 \text{ nm}$ )

**Table 4** Statistical parameters for difloxacin and enrofloxacin calculated by PLS and DOSC-PLS models

Parameters	By PLS method		By DOSC-PLS method	
	Difloxacin	Enrofloxacin	Difloxacin	Enrofloxacin
Mean recovery	100.81	97.31	100.32	99.59
PRESS	0.4357	1.5912	0.3369	0.9048
Number of factors	2	3	4	4
RMSEP	0.1499	0.2833	0.0753	0.0436
RSEP (%)	3.5214	7.6757	1.7694	1.1809

PRESS, prediction residual sum of squares; RMSEP, root mean square error of prediction; RSEP, relative standard error of prediction.

**Table 5** DOSC-PLS results applied on dog urine samples.

Samples	Difloxacin ( $\mu\text{g mL}^{-1}$ )			Enrofloxacin ( $\mu\text{g mL}^{-1}$ )		
	Added	Found	R.S.D*	Added	Found	R.S.D*
Sample 1	1.00	1.02	0.09	1.00	1.01	0.08
Sample 2	1.00	0.98	0.12	2.50	2.48	0.15

\*Relative standard deviation (n=5)

### Application

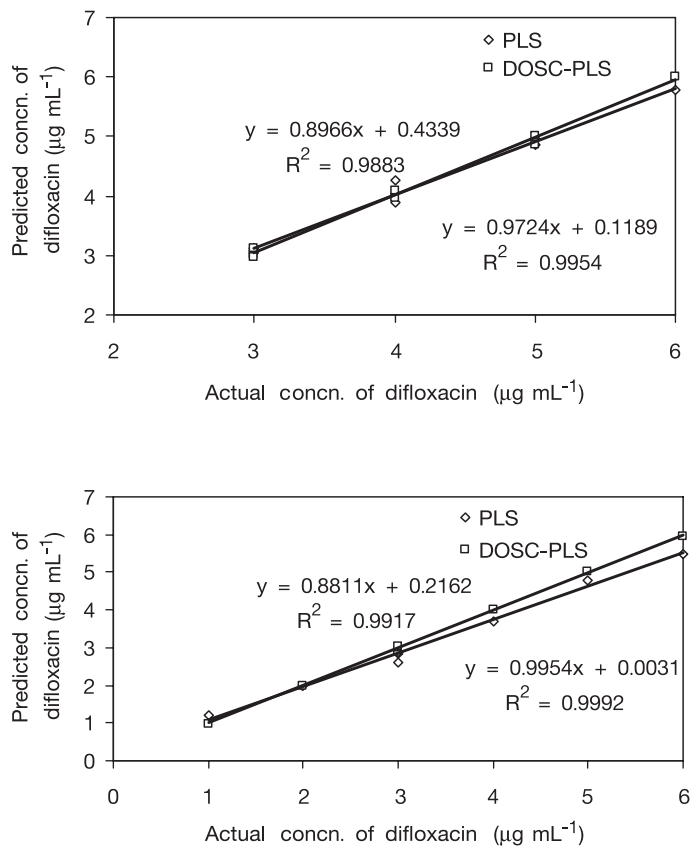
DOSC-PLS method was applied for simultaneous determination of difloxacin and enrofloxacin in urine samples. For this, urine samples were prepared according to the sample preparation procedure mentioned above and then analysed by the proposed method. The results for the simultaneous determination of difloxacin and enrofloxacin in urine samples by DOSC-PLS method are shown in Table 5. Table 5 shows that good recovery values were obtained for the samples assayed.

The precision of the proposed methods was investigated by the analysis of the real samples five times each. The result showed that the relative standard deviation (RSD) obtained was acceptable. Therefore, DOSC-PLS method can successfully predict the concentrations of each drug, difloxacin and enrofloxacin in urine samples.

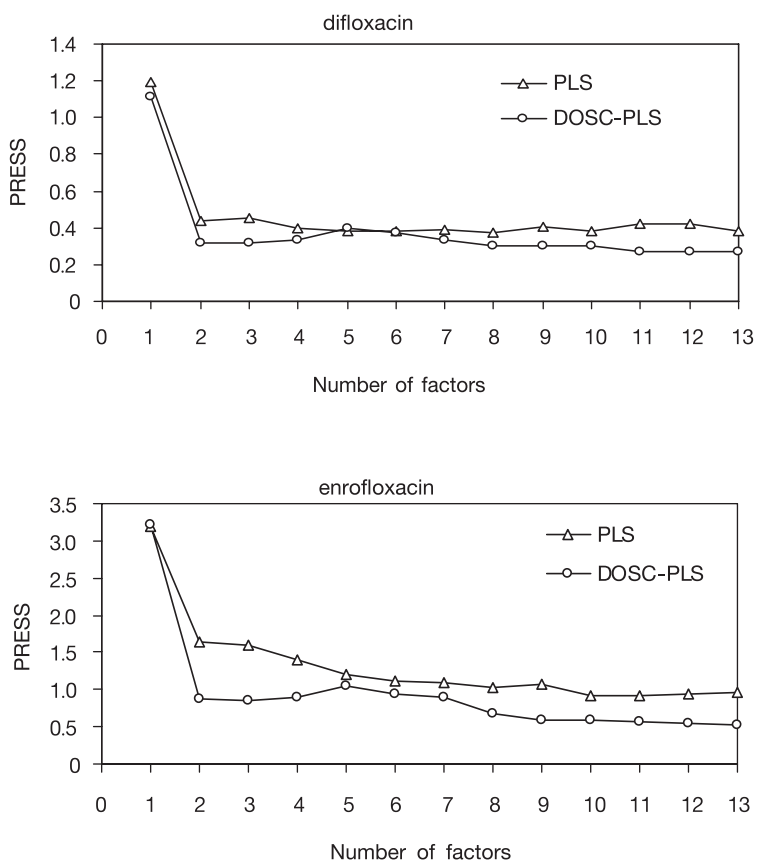
### Conclusion

Simultaneous determination of difloxacin and enrofloxacin using spectrophotometric data and





**Figure 5** Plots of predicted concentration ( $\mu\text{g mL}^{-1}$ ) versus actual concentration ( $\mu\text{g mL}^{-1}$ ) for difloxacin and enrofloxacin by PLS and DOSC-PLS



**Figure 6** Plots of prediction residual sum of squares (PRESS) versus number of factors by PLS and DOSC-PLS models

chemometric tools, PLS and DOSC-PLS is proposed in this paper. Difloxacin and enrofloxacin show spectral overlapping which makes the resolution of these drugs difficult. The results obtained by applying PLS and DOSC-PLS methods can be used for overcoming these spectral interferences. However DOSC-PLS method gives better results than PLS and can be successfully used for rapid, simple and accurate resolution of enrofloxacin and difloxacin in their mixtures. An important characteristic of this work is that no extraction step is required and hence the use of organic solvents is avoided making the proposed methods non-polluting. It also prevents the loss of analytes during multisteps involved in extraction procedures. However the proposed methods are less sensitive as compared to commonly used chromatographic methods for determination of these drugs and cannot be used for trace determination of these drugs in environmental samples. But the proposed methods are simple, fast and cost effective, as they do not require expensive equipment and can be successfully used at ppm levels. The methods are selective and sensitive as compared to ordinary spectrophotometric methods.

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