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Simultaneous Determination of Levamisole and Triclabendazole by Multivariate Calibration Models using Spectrophotometry in Veterinary Pharmaceutical Formulation

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Abstract

The present study focuses on the development and validation of three chemometric models that are simple, accurate, and precise for the determination of levamisole and triclabendazole. These models overcome the need for prior separation techniques. The approaches under consideration are classical least squares (CLS),partial least squares (PLS-1), and principal component regression (PCR). The absorbance values of the calibration set were computed by MATLAB to construct the models. A Two-factor Five-level experimental design was employed to construct a set of 25 mixtures, each consisting of varying proportions of levamisole and triclabendazole. Thirteen mixtures were utilized as the training set, while the remaining twelve combinations were designated as the validation set. The utilization of multi-wavelengths in place of single wavelength spectrophotometry has significantly enhanced the precision and prediction capabilities of these multivariate methods. The proposed approaches have been shown to be reliable and exact, and they may be applied to the determination of the drugs in both their pure form and pharmaceutical formulations.

Keywords: Levamisole; Triclabendazole; classical least squares (CLS); principal component regression (PCR); Anthelmintic drugs; partial least squares (PLS).

1. Introduction

Intestinal nematode infections are a major source of financial loss in the sheep breeding industry [1, 2].As the prevalence of parasite resistance keeps increasingto treat animals promptly, veterinarians are combining many anthelmintic drugs together[2]. Oral suspension martibendazene medication has two active ingredients that have various effects on sheep GIT worms with distinct pharmacological actions[3, 4]. The anthelmintic medications employed in this study include levamisole HCl (LEV) and triclabendazole (TCB). The administration of LEV and TCB together has been found to yield enhanced therapeutic effects and expedited improvement of hepatic diseases in sheep infected by species of Fasciola [4]. LEV ($C_{11}H_{12}N_2S$) has been successful in significantly reducing the prevalence of ascariasis, a parasitic infection, among the people in the world.Levamisole was given FDA approval in 1990 to be used as an adjuvant therapy for colon cancer[5].Levamisole was previously used as a treatment for rheumatoid arthritis [6]. The results show the immunomodulatory properties of levamisole, which make it useful for enhancing immune response even in immunocompromised folks [7, 8].The results of the trial also revealed the therapeutic efficacy of LEV in the treatment of individuals afflicted with mild cases of coronavirus infections (COVID-19) [9].TCB ($C_{14}H_9C_{l3}N_2OS$), is an anthelmintic drug belonging to the benzimidazole class, and it has been approved for use in the treatment of worms in sheep and other bovine animals. The efficacy of this treatment in eradicating early immature and mature Fasciola species has been demonstrated in ovine and bovine animals [10, 11]. Since the 1980's, research has demonstrated that triclabendazole can be used to effectively treat Fasciola infections in livestock [12]. When

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triclabendazole is taken orally, its absorption undergoes quick hepatic clearance, making it undetectable in plasma. TCB is degraded into its metabolites, triclabendazole sulfoxide and triclabendazole sulphone, by liver metabolism[13]. Due to the novelty of LEV combined with TCB, we were required to develop and validate new methods for analyzing both drugs simultaneously in this new formulation. Several methods have been established for the quantitative analysis of LEV either on its own or in mixture with other medications. These methods involve high-performance liquid chromatography (HPLC)[13-29], thin-layer chromatographic densitometry methods (TLC) [29-31], utilization of liquid chromatography (LC) [32-36], analysis by ultra-performance liquid chromatography (UPLC) [37], gas chromatography (GC) [30, 38, 39], analysis by capillary electrophoresis [30, 40, 41], utilization of spectrophotometric methods [42, 43], utilization of potentiometric, electrochemiluminescence [44-48]. Various methods have been used to quantify the concentration of TCB alone or in a mixture, these methods involve HPLC with different application in dosage forms and biological fluids [49-57], LC-MS /MS[58, 59], spectrophotometric methods [60], andspectrofluorometric method [61].According to our knowledge, only one spectrophotometric approach among them we considered to be capable of quantitatively analyzing both drugs [62]. Our aim in this research is to create and validate rapid, simple, sensitive, and selective chemometric techniques for the simultaneous determination of triclabendazole and levamisole, these approaches including classical least squares (CLS), partial least squares (PLS-1), and principal component regression (PCR). In this work, we used chemometrics to precisely and accurately determined both drugs quantitatively using spectrophotometric analysis. The methods are sensitive and reliable for determination of both drugs in pharmaceutical dosage form.

2. Experimental

2.1. Apparatus

Spectrophotometer: SHIMADZU UV-1900i (SHIMADZU, Japan), two identical 1 cm quartz cells in a dual beam UV-visible spectrophotometer, and electronic balance (Vibra, Japan).

2.2. Software

- Software for multiple data processing (Lab solutions UV-Vis.software) was applied to all of the measurements.
- The chemometric techniques were applied in MATLAB 8.5.0.197613 (R2015a).
- PLS Toolbox software version 2.1 was used to run the CLS, PLS, and PCR models.
- Microsoft® Excel® 365 was used to run the t-test and F-test.

2.3. Materials

2.3.1. Pure samples

LEV and TCB standards were kindly gifted by (Pharma Swede, 10th of Ramadan City, Egypt) with purity of(99.7%) and (99.6%), respectively.

2.3.2. Pharmaceutical dosage form

Martiros for pharmaceutical industrial Co., was provided by the Martibendazene® oral suspension (Batch No. 23120), each (1 mL) consist of(7.5 gm) of LEV and 12 gm of TCB.

2.3.3. Chemicals and reagents

Methanol of the HPLC grade was purchased from (Sigma-Aldrich, Germany).

2.4. Standard solutions

2.4.1. Stock standard solutions (1000µg/mL)

(100 mg) of each of LEV and TCB were accurately weighed and placed into two separate (100-mL) volumetric flasks, each flask was filled with (50 mL) of methanol and shaken to dissolve the powder. Then the flask was made up to the mark with methanol.

2.4.2. Working standard solutions (100µg/mL)

Two separate (100-mL) volumetric flasks were used to precisely transfer (10 mL)of both LEV and TCB stock standard solutions. The remaining volume in each flask was then filled to the mark using methanol.

3. Procedure

3.1. Experimental design

To construct the chemometric models, a series of 25 combinations containing the two drugs within a concentration range of (1-8µg/mL), were prepared in accordance with Beer's law. This design consisted of thirteen mixtures that represented a calibration set and twelve mixtures that represented a validation set, within the spectral region spanning from 200 to 320 nm, with an interval of 1 nm, the central concentration level of the experimental design was determined to be (3 µg/mL) for TCB and (6 µg/mL) for LEV. The second derivative of the absorbance

values of the calibration set were computed by the MATLAB program to build up the models. The leave one out cross validation method was assigned for the CLS, PLS, and PCR models. With PLS toolbox software version 2.1, CLS, PCR, and PLS models were developed. The resulting spectral data matrix consists of 25 rows, each of which describes a sample and 101 columns, each of which describes a wavelength (25 × 101).

3.2. Application to laboratory prepared mixtures

A series of (10-mL) volumetric flasks were filled with aliquots of working solutions having varying concentrations of TCB 1-5 µg/mL and LEV (4-8 µg/mL), and then mixed to produce a variety of TCB: LEV combinations. Then volumes were completed up to the mark with methanol. After scanning and saving the constructed series' spectra in the 200-400 nm region, the models were generated as mentioned in section 3.1Then, they were validated by making predictions about TCB and LEV concentrations in the validation set.

3.3. Application to pharmaceutical formulation

(1 mL) of Martibendazene® oral suspension consist of (7.5 gm) of levamisole and (12 gm) of triclabendazole, transfer precisely into a (100-mL) volumetric flask and sonicate it for 15 minutes in (50-mL) of methanol. Subsequently, the solution underwent filtration, followed by the addition of methanol to achieve the desired volume, resulting in a solution with a concentration of (120 mg/mL) of TCB and(75 mg/mL) of LEV.

3.4. Degradation studies

The degradation studies were conducted using drug solutions with a concentration of (100 µg/mL) for each. As reported [29]triclabendazole and levamisole were subjected to stress testing under the following conditions: a solution of hydrochloric acid (HCl) with a concentration of (0.5 M), a solution of sodium hydroxide (NaOH) with a concentration of (0.5 M), and a hydrogen peroxide (H_2O_2) solution with a concentration of 30% are all present at a temperature of 100°C. Through the application of forced degradation on both drugs TCB underwent total degradation when exposed to H_2O_2, while undergoing only partial degradation when subjected to HCl, NaOH, and temperature. However, when the identical degradation techniques were applied to LEV, the drug remained unaffected and did not undergo degradation. Consequently, when a combination of TCB and LEV was subjected to the aforementioned stress conditions, no interaction between them was seen.

4. Results and discussion

The suggested models' main benefit was that they used green analytical techniques, which minimized waste, sample and solvent consumption, analysis time, and cost. The absorption spectra of TCB and LEV overlapped significantly as shown in Fig. 1; consequently, the proposed drugs were not indicated quantitively by direct spectrophotometric analysis. The chemometric models (CLS, PLS, and PCR) were made by following the general procedure, The MATLAB® program was used to process the second derivative of the absorbance values ranged from 200 to 400 nm, with 1 nm interval and center design level was (3µg/mL) for TCB and (6µg/mL) for LEV as shown in Fig 2-3.

The proposed models have several advantages. Firstly, the PCR method requires only a small number of principal components, which simplifies the analysis. Secondly, the PLS method minimizes errors by evaluating the second derivative of the absorbance values across a wide range of wavelengths, specifically in the region of 200-310 nm. CLS has the benefit of being the first chemometric method established and has strong qualitative capabilities.

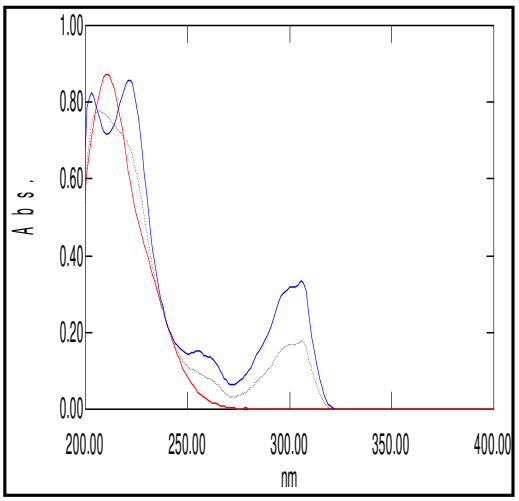


Fig. 1: Overlain absorption spectra of levamisole (10µg/mL), triclabendazole (6µg/mL) and a mixture containing (4µg/mL) of LEV and (3µg/mL) of TCB using methanol as a solvent.

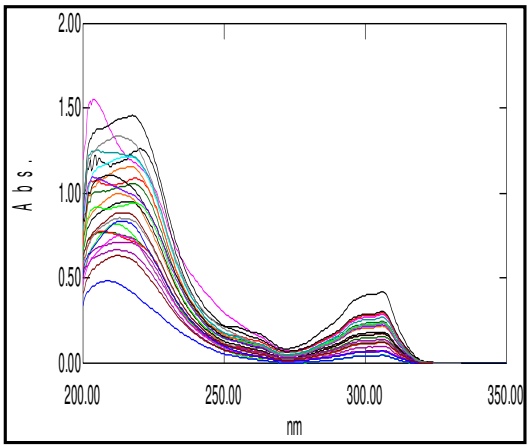


Fig. 2: Absorption spectra at zero-order for different concentrations.

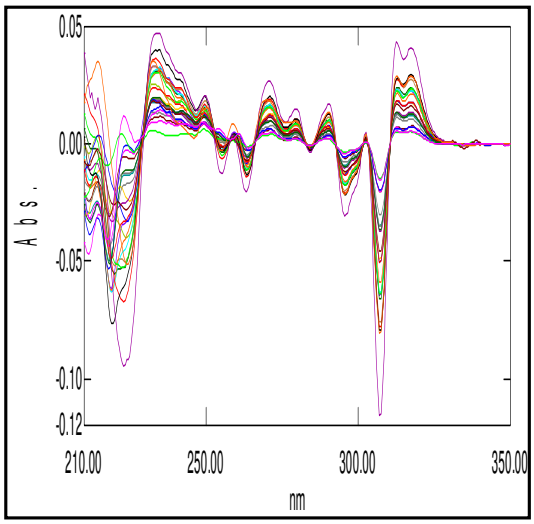


Fig. 3: The second derivative of the absorbance values used in the experimental design.

5. Methods validation

Predicted TCB and LEV concentrationsin the validation set were used to test the validity of the suggested models. The concentrations used in experimental design matrix are illustrated in Table 1.The calibration process involved the calculation of the root mean squares error of calibration (RMSEC), whereas the prediction process involved the calculation of the root mean square errors of prediction (RMSEP) for each method as shown in Table 2.

Table 1: Experimental design of concentrations of TCB and LEV mixtures used in chemometric methods

Mixture No.	Triclabendazole	Levamisole
1	3	6
2	3	4
3	1	4
4	1	8
5	5	5
6	2	8
7	5	6
8	3	5
9	2	5
10	2	7
11	4	8
12	5	7
13	4	6
14	3	8
15	5	8
16	5	4
17	1	7
18	4	4
19	1	6
20	3	7
21	4	7
22	4	5
23	2	4
24	1	5
25	2	6

5.1. Principal component regression (PCR) and Partial least squares (PLS)

The PCR-model is designed to minimize the number of predictor variables while requiring a high degree of correlation between the predictor variables. One of the advantages of this approach is that it requires just a small number of principal components. Additionally, it proves to be particularly beneficial in cases where the predictor variables exhibit a high degree of correlation. The PLS-model is closely associated with (PCR). However, it has the advantage of error minimization by the measurement of thesecond derivative of absorbance values at many sites throughout the wavelength range of 200-310 nm.

The distinction between PCR and PLS models lies in their respective approaches to decomposition. In the PCR model, decomposition is performed only based on spectrum variations, without considering the concentrations, while in the PLS-model had a correlation between the spectrum decomposition and the concentrationas shown in Fig 4-5.

Table 2. Determination of LEV and TCB in Validation set by theproposed chemometric methods.

Validation mixture	CLS		PCR		PLS	
	TCB	LEV	TCB	LEV	TCB	LEV
2	99.41	98.67	99.32	99.75	99.37	99.37
4	101.06	98.51	98.64	101.40	101.58	100.24
6	101.10	99.39	99.05	99.02	101.64	100.41
8	99.53	99.08	99.40	100.86	98.99	98.40
10	98.96	98.54	97.97	99.19	100.49	100.14
12	99.75	102.18	100.48	99.26	99.33	99.52
14	95.61	99.69	99.16	99.55	99.12	99.67
16	99.25	101.33	99.78	100.90	99.91	99.77
18	98.99	100.96	100.82	101.24	98.74	98.74
20	102.85	99.55	101.42	101.84	100.52	100.22
22	100.45	100.40	102.10	100.42	99.30	99.44
24	102.14	99.56	100.39	100.53	101.98	100.40
Mean±%RSD	99.92±1.85	99.82±1.17	99.70±0.90	99.63±0.98	100.08±1.14	99.69±0.65
RMSEP*	0.050	0.072	0.024	0.064	0.027	0.035

*Root mean square error of prediction.

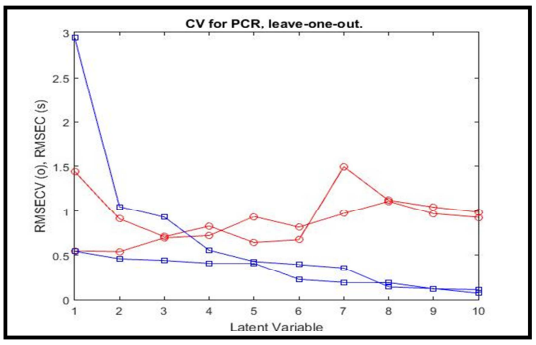


Fig. 4: RMSECV plot showing the cross-validation obtained from the calibration set as a function of the number of latent variables (LVs) used to build the PCR-model

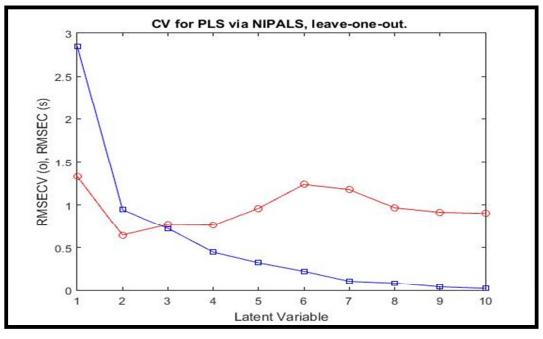


Fig. 5: RMSECV plot illustrating the cross-validation obtained from the calibration set as a function of the number of latent variables (LVs) used to build the PLS- model.

5.2. Classical least square (CLS)

Unlike the PCR and PLS models, where knowledge of all overlapping spectrum components is required, the CLS-model just requires knowledge of the components in the calibration mixtures. The K-matrix produced from the CLS model construction at 110 various wavelengths was then utilized to forecast the unknown concentration of each component, based on the training set. To assess the prediction capabilities of the models, the known concentrations were graphed versus the predicted concentrations [63]as shown in Fig 6, values for the mean, standard deviation, root mean square error of prediction, slope and correlation coefficient were presented in Table 3, that demonstrating strong prediction capabilities of the models.

Table 3. Assay validation for LEV and TCB by the proposed chemometric models

Parameters	**PLS**		**PCR**		**CLS**	
	LEV	TCB	LEV		LEV	TCB
Mean	99.69	100.1	99.63	99.70	99.82	99.92
SD [a]	0.65	1.14	0.98	0.90	1.17	1.85
RMSEP [b]	0.035	0.027	0.064	0.024	0.072	0.050
Slope [c]	1.01	0.987	0.976	0.992	0.987	0.991
Intercept [c]	- 0.07	0.03	0.11	0.009	0.064	0.018
R^2 [c]	0.999	0.999	0.999	0.999	0.998	0.999

[a] Stander deviation.
[b] Root mean square error of prediction.
[c] Data of the straight line plotted between predicted concentration versus actual concentration.

6. Application to pharmaceutical preparation

Levamisole and triclabendazole concentrations in Martibendazene® oral suspension were determined using the suggested methodologies, and a statistical comparison was made between the data obtained using the suggested methods and the results obtained using the reported methods [43, 60], as shown in Table 4-5, The proposed methods for analyzing the investigated drugs in its Veterinary pharmaceutical formulation were accurate and precise, since no significant discrepancies were discovered when using t-test and F-test at 95% confidence level [64].

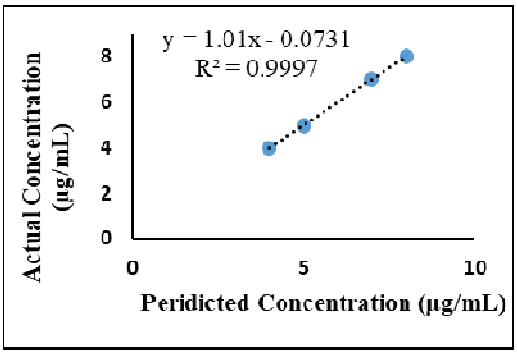
Table 4. Statistical comparison for the results obtained the proposed methods and reported methods for the analysis of LEVand TCB in Martibendazene® oral suspension.

Parameters	LEV			Method [43]	TCB			Method [60]
	CLS	PCR	PLS		CLS	PCR	PLS	
N*	5	5	5	5	5	5	5	5
$\bar{X}$**	99.57	99.15	99.30	99.77	100.30	99.72	100.10	100.66
SD	1.112	0.965	0.802	0.938	0.782	0.697	1.044	0.856
Variance	1.237	0.930	0.642	0.879	0.611	0.485	1.091	0.733
Student's-t-test*** (2.306)	0.301	1.017	0.846	------	0.709	1.910	0.939	-----
F-value *** (6.388)	1.407	1.059	1.367	------	1.199	1.509	1.488	-----

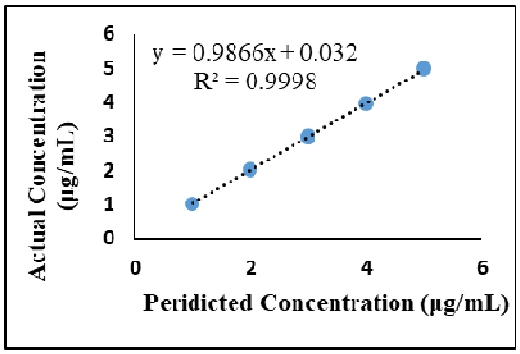
* Number of experiments.
** The mean of percent recovery of pharmaceutical preparation.
*** The values in parenthesis are tabulated values of "t "and "F" at (P = 0.05).

Table 5. Comparative study with previously reported methods

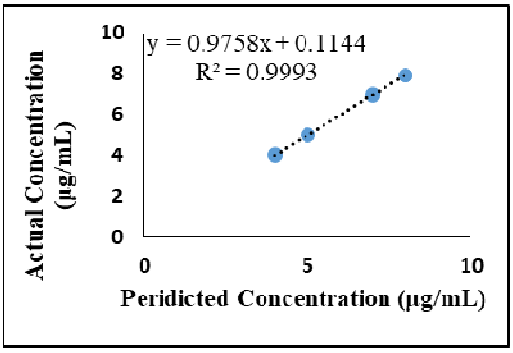
Parameters	**Method 1 [65]**		**Method 2 [66]**	**Method 3 [67]**		**Method 4[29]**				**Proposed Method**	
Techniques	Spectrophotometry		LC-MS/MS	Chemometric		HPLC		TLC		Multivariate calibration models using second derivative spectrophotometry	
Drugs	TCB	LEV	Mix of ten drugs	TCB	LEV	TCB	LEV	TCB	LEV	TCB	LEV
Linearity	2-20 µg/mL	2-14 µg/mL	0-500 µg/L Ten drugs mix linearity	1-9 µg/mL	5-25 µg/mL	6-60 µg/mL	3.75 - 37.5 µg/ mL	2–14 µg/spot	2–14 µg/spot	1-9 µg /mL	2-14 µg /mL
LOD	0.08 µg/mL	0.19 µg/mL	Less than 1 µg/L	-----	-----	1.09 µg/mL	0.71 µg/mL	0.44 µg/mL	0.53 µg/mL	-----	-----
LOQ	0.23 µg/mL	0.58 µg/mL	------	-----	-----	3.33 µg/mL	2.15 µg/mL	1.35 µg/mL	1.63 µg/mL	-----	-----
Application	Veterinary formulation		Milk	Veterinary formulation		Pharmaceutical dosage form				Veterinary pharmaceutical for-mulation	



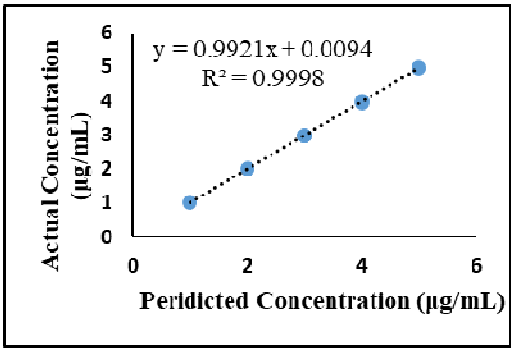
The actual known concentration versus predicted concentration of LEV by PLS Model



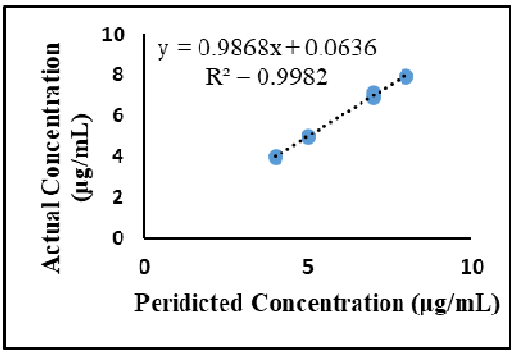
The actual known concentration versus predicted concentration of TCB by PLS Model



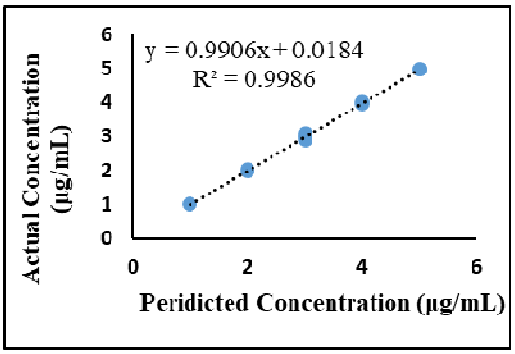
The actual known concentration versus predicted concentration of LEV by PCR Model



The actual known concentration versus predicted concentration of TCB by PCR Model



The actual known concentration versus predicted concentration of LEV by CLS Model



The actual known concentration versus predicted concentration of TCB by CLS Model

Fig. 6. The actual known concentration versus predicted concentration (expressed µg/mL) of LEV and TCB by PCR, PLS and CLS models

6. Conclusion

We could conclude from the results we computed from CLS, PLS and PCR chemometric models that simultaneous analysis of LEV and TCB binary mixtures is applicable in an effective and accurate way. In this study, various mathematical approaches and various sets of data to manipulate the second-derivative spectra were applied. The proposed models are simple, sensitive, selective, accurate, quick and easy, and cost-effective, making them potent tools for separating LEV and TCB in their pure or oral suspension form.

Conflicts of interest

There are no conflicts to declare.

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