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Pure component contribution (PCCA) and synergy interval partial least squares (siPLS) algorithms for efficient resolution and quantification of overlapped signals; an application to novel antiviral tablets of daclatasvir, sofosbuvir and ribavirin

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ABSTRACT

Daclatasvir (DAC), sofosbuvir (SOF) and ribavirin (RIB) have been recently co-formulated in tablet dosage form for the treatment of Hepatitis C virus infections. In this work, the resolution and quantitation of overlapped spectral signals was achieved by both univariate and multivariate algorithms. Pure component contribution algorithm (PCCA) as a novel approach was applied along with factor based partial least squares (PLS) algorithms using both full range and synergistic intervals (siPLS). Each drug could be determined at its λ_{max} using PCCA, while PLS and siPLS were used for multivariate determination of the three components. Good linear relationships were obtained in the ranges of 5.45-16.35, 4.40-44.00 and 5.50-35.00 µg/mL for DAC, SOF and RIB, respectively, by PCCA. The PLS and siPLS models were built for the three compounds each in the concentration range of 2.00-10.00, 10.00-20.00 and 10.00-26.00 µg/mL for DAC, SOF and RIB, respectively. Validation of the proposed methods was ascertained according to ICH guidelines for PCCA and through the use of internal and external validation sets for PLS and SiPLS models. The three methods were successfully applied for determination of DAC, SOF and RIB in pure form and in tablets.

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1. Introduction

Chronic hepatitis C virus (HCV) has infected about 130 million people. HCV infection has been thought to be widespread in many populations for many years [1]. Direct acting antivirals (DAAs) have newly arisen as a promising therapy for HCV. It is now established that it is essential to use DAA combination therapy to cure HCV totally [2]. In this work, we focus on combinations of three different active species.

Daclatasvir dihydrochloride, (Figure 1(a)), methyl [(2S)-1-{(2S)-2-[4-(4'-{2-[(2S)-1-{(2S)-2-[(methoxycarbonyl) amino]-3-methylbutanoyl}-2-pyrrolidinyl]-1*H*-imidazol-4-yl}-4-biphenylyl)-1*H*-imidazol-2-yl]-1-pyrrolidinyl}-3-methyl-1-oxo-2-butanyl] carbamate dihydrochloride is a non-structural 5A protein inhibitor. It has been used concomitantly with several drugs for several HCV genotypes under different conditions [3-5]. Sofosbuvir (Figure 1 (b)) is propan-2-yl(2S)-2-[[[(2R, 3R, 4R, 5R)-5-(2, 4-dioxopyrimidin-1-yl)-4-fluoro-3-hydroxy-4-methyloxolan-2-yl]methoxy phenoxyphosphoryl] amino]propanoate. Sofosbuvir is an orally administered HCV nucleotide polymerase NS5B inhibitor [6]. Ribavirin (Figure 1 (c)) is 1-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-1,2,4-triazole-3-carboxamide. RIB is a synthetic nucleoside analogue related to guanine. It inhibits many RNA and DNA viruses replication [7].

SOF in combination with DAC is used for patients infected with HCV genotype 1 or 4. The DAC/SOF combination with RIB is used for the treatment of cirrhosis and treatment experienced patients from 12 to 24 weeks [8,9].

Co-formulating these three drugs in one tablet is preferable regarding patient compliance as it is a major point in HCV therapy. Also, co-formulation will reduce the cost of the

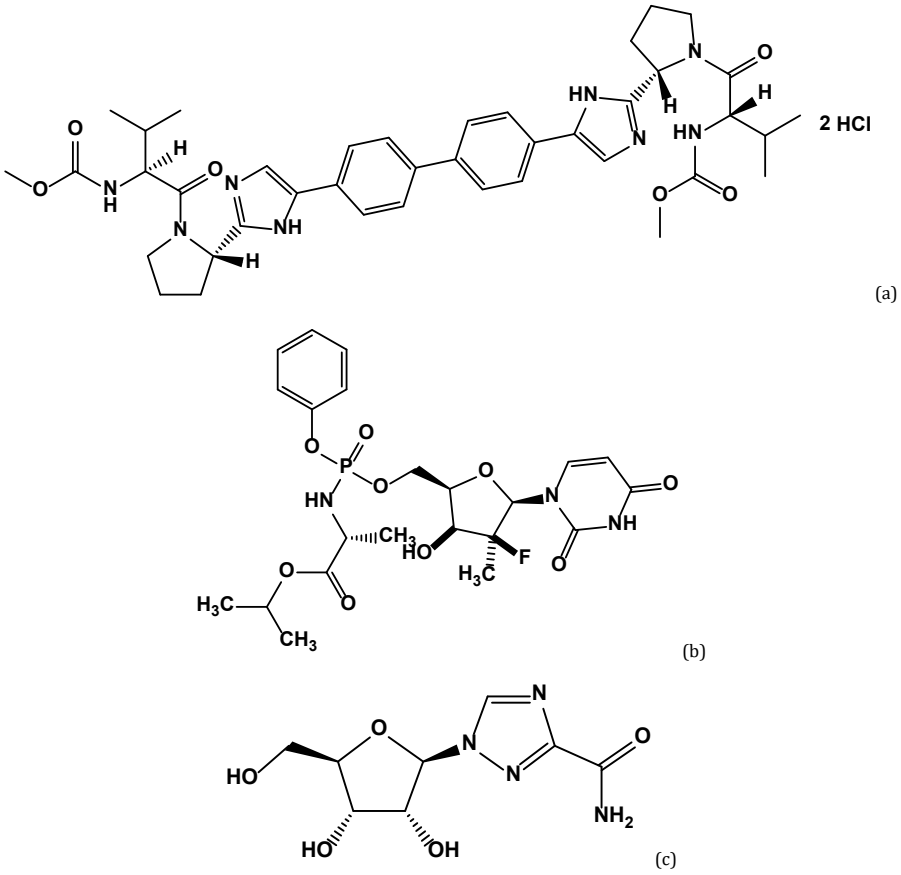

Figure 1. Chemical structure of (a) daclatasvir dihydrochloride, (b) sofosbuvir, and (c) ribavirin.

treatment. To date, only two reported methods have been used for the simultaneous determination of DAC, SOF and RIB, which are an HPLC method [10] and a spectrophotometric method [11]. In this work, the efficiency of the pure component contribution algorithm and full and synergy interval partial least squares (PLS and siPLS) were studied and evaluated for spectral resolution and quantitation of the studied drugs in their pure forms and in two different brands of tablet dosage forms.

2. Experimental

2.1. Instruments and software

All absorbance measurements were carried out using a JASCO (V-630) double beam UV-Visible spectrophotometer (Japan), with 1 cm matched quartz cells. JASCO UV-Probe (VWS-580 Spectra Manager software) was used to obtain all the spectra automatically and the scan was performed from 200-350 nm using a 0.1 nm interval. Matlab® for Windows TM version 6.5 was used to perform all the calculations [12]. The PLS procedure was taken from PLS-Toolbox 2.0 for use with Matlab® version 2.1, 2000 [13].

2.2. Chemicals and solvents

2.2.1. Pure samples

Daclatasvir and sofosbuvir with a certified purity of 99.2 and 99.6%, respectively, were kindly supplied by the National Organization for Drug & Control Research (NODCAR) (Giza, Egypt). Ribavirin with a certified purity of 99.5% was supplied by the Pharmaceutical Egyptian Association.

2.2.2. Market samples

Pharmaceutical preparations containing the drugs were purchased from the local market. Daklanork® tablets (MSD, Egypt), Batch no. M1010417 were labelled as containing 65.92 mg Daclatasvir dihydrochloride equivalent to 60 mg DAC per one tablet, Gratisovir® tablets (Pharco, Egypt), Batch no. 7107040 was labelled as containing 400 mg Sofosbuvir and Ribavirin® tablets (Memphis, Egypt), Batch no. 317059 contained 200 mg Ribavirin and all were purchased from the local market.

2.2.3. Solvents

Methanol of HPLC grade was purchased from Sigma-Aldrich (Germany) and highly purified double distilled water were used.

2.3. Solutions

Standard solutions containing 110.00 µg/mL of SOF and RIB, and 109.00 µg/mL of DAC were prepared by dissolving 11.00 mg of SOF and RIB, and 10.90 mg of DAC in 100.0 mL methanol.

2.4. Procedure

2.4.1. Construction of calibration curves for PCCA method

Aliquots equivalent to 0.5-1.5 mL of DAC, 0.4-4.0 mL of SOF and 0.5-3.0 mL of RIB were accurately and separately transferred from their corresponding standard solutions (109.00 µg/mL for DAC and 110.00 µg/mL for SOF and RIB)

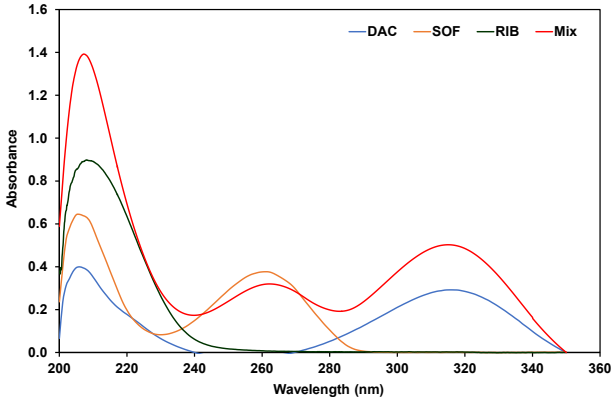

Figure 2. Zero order absorption spectra of 5.45 µg/mL DAC (λ_{max} = 315.0 nm), 17.6 µg/mL SOF (λ_{max} = 261.0 nm) and 24.2 µg/mL RIB (λ_{max} = 209.0 nm) using methanol as a blank.

using calibrated micro pipettes to a series of 10 mL volumetric flasks. The volume was completed with methanol to reach a final concentration range of 5.45-16.35, 4.40-44.00 and 5.50-35.00 µg/mL for DAC, SOF and RIB, respectively. The spectra of the prepared standard solutions were scanned from 200-350 nm with 0.1 nm intervals. The absorbance values obtained from the scanned spectra at 315.0, 261.0 and 209.0 nm for DAC, SOF and RIB, respectively, were recorded and plotted against the corresponding concentrations and the regression parameters were calculated.

2.4.2. Construction of calibration PLS and siPLS models

Five levels three factor experimental design [14] was applied to prepare mixtures of DAC, SOF and RIB. Twenty-five mixtures were prepared, the calibration model was built by 20 of them, while five mixtures were randomly chosen and used as an independent validation set. The prepared mixtures contained the three drugs with different ratios and within the concentration range of 2.00-10.00, 10.00-20.00 and 10.00-26.00 µg/mL for DAC, SOF and RIB, respectively.

The prepared mixtures absorption spectra were recorded in the range 200-350 nm at 0.1 nm intervals using methanol as a blank. For construction of the calibration models (PLS) and siPLS, the absorbance and concentration matrices for the training set were used. The validity of the developed models was evaluated via an external validation set.

A siPLs algorithm was applied to find the optimal informative regions, improve prediction ability of active ingredients, reduce interference and reduce the number of latent variables compared to PLS. Combinations of the previously mentioned equidistant subintervals under PLS were examined by the algorithm, each combination of 4 sub-intervals was used for building a PLS model. Different numbers of combinations were selected and tried with a combination of 4 sub-intervals used to obtain the best results. Good performance for the resulted models was indicated by the different combination of sub-intervals using siPLS.

2.4.3. Assay of pharmaceutical formulations

Ten tablets of each of Daklanork®, Gratisovir® and Ribavirin® were accurately weighed and finely powdered. An accurate weight of the powdered tablets equivalent to 10.9 mg DAC for Daklanork® tablets, 11 mg SOF for Gratisovir® tablets and 11 mg RIB for Ribavirin® tablets were sonicated with 70.0 mL methanol for 30 min, then the solutions were completed to the volume with methanol, mixed well and filtered using a disposable syringe filter (0.45 µm). Aliquots of the filtrates were transferred into 10 mL volumetric flasks, diluted to volume with methanol and mixed well. The prepared solutions were further diluted to have concentrations within the calibration ranges of each of the applied methods.

3. Results and discussion

Spectral overlap of two or more analytes was usually experienced when analyzing complex mixtures by spectrophotometry which cannot be resolved by traditional spectrophotometric methods. The overlapped spectral signals of the three studied compounds are shown in Figure 2. In such cases, mathematical manipulation using some algorithms could be successfully applied for resolution of such analyte signals and consequently their quantification. In this work, simultaneous determination of DAC, SOF and RIB was achieved by the application of one univariate and two multivariate spectrophotometric methods which are the novel PCCA and PLS and siPLS chemometric methods, respectively.

3.1. Theoretical background

3.1.1. Pure component contribution algorithm

The novel PCCA algorithm applied here was developed and validated by Hegazy *et al.* [15,16] which efficiently extracts the pure contribution of each component in binary and ternary mixtures. Coded function was involved through the algorithm after which the following equations were run automatically upon the use of the code [16].

The following theory was reported for the developed algorithm

$$A_m = \alpha_X C_X + \alpha_Y C_Y + \alpha_Z C_Z \quad (1)$$

$$B = A_m/\alpha_Z = \alpha_X C_X / \alpha_Z + \alpha_Y C_Y / \alpha_Z + C_Z \quad (2)$$

$$C = MC\ (B) = MC\ (\alpha_X C_X / \alpha_Z) + MC\ (\alpha_Y C_Y / \alpha_Z) \quad (3)$$

$$D = C / MC\ (\alpha_Y / \alpha_Z) = MC\ (\alpha_X C_X / \alpha_Z) / MC\ (\alpha_Y / \alpha_Z) + C_Y \quad (4)$$

$$E = MC\ (D) = MC\ [MC\ (\alpha_X C_X / \alpha_Z) / MC\ (\alpha_Y / \alpha_Z)] \quad (5)$$

$$F = MC\ [MC\ (\alpha_X C_X / \alpha_Z) / MC\ (\alpha_Y / \alpha_Z)] / MC\ [MC\ (\alpha_X / \alpha_Z) / MC\ (\alpha_Y / \alpha_Z)] = C_X \quad (6)$$

$$G = C_X * \alpha_X = \alpha_X C_X \quad (7)$$

Table 1. Regression and validation parameters of PCCA method for the determination of the studied drugs in their pure form by the proposed method *.

Parameters	DAC	SOF	RIB
Concentration range (µg/mL)	5.45-16.35	4.40-44.00	5.50-35.00
Slope	0.049197	0.021502	0.046149
Intercept	0.022538	-0.00176	0.066779
% mean	100.85	99.97	98.37
SD	2.47	1.47	8.28
Correlation coefficient	0.999	0.9998	0.9962
Limit of detection (µg/mL)	0.11	0.06	0.18
Limit of quantification (µg/mL)	0.33	0.18	0.56
S.E. of intercept (S_a)	0.012196	0.005981	0.040184
S.E. of slope (S_b)	0.001124	0.000234	0.001617
Standard deviation of residuals	0.001614	0.000389	0.002591
Repeatability (% RSD)	1.13	1.34	0.95
Intermediate precision (% RSD)	1.45	1.57	1.31

* RSD is the relative standard deviation; SD is the Standard Deviation; S.E. is the Standard Error.

where, A_m is the vector of the absorbance of the mixture, α_X, α_Y and α_Z are the molar absorptivity vectors of X, Y and Z and C_X, C_Y and C_Z are the concentrations of X, Y and Z, respectively, and MC is the mean centering process. The detailed description of the equations have been reported elsewhere [15,16]. Equation (7) shows that the obtained spectra permit the determination of component X by direct measurement of the estimated absorbance value at its λ_{max} using the corresponding regression equation obtained by plotting the absorbance of the pure spectra of X at its λ_{max} versus its corresponding concentration. After obtaining pure component contribution for X, then the Y and Z pure component contributions could also be acquired.

3.1.2 Partial least squares

PLS is the predictive chemometric algorithm used for the separation and resolution of complex mixture being well recognized depending on factor analysis [14]. PLS deals with the full raw spectral data for building the model where the optimum number of latent variables was chosen according to Haaland and Thomas criteria [17,18]. Cross validation and an external validation set were used to test the developed model.

3.1.3. Variables selection algorithms

As the full spectral data using simple univariate spectrophotometry failed for efficient determination and explanation of the complex systems, variables (wavelengths) selection could improve such resolution in the points of collinearity and can improve prediction ability through finding out the most informative regions in spectra [18]. Thus, more efficient determination with lower number of LVs could be obtained. iPLS and siPLS are considered among the developed algorithms of variable selection, where one informative region and synergistic regions are selected for modeling, respectively [19].

3.1.3.1. Synergy interval partial least squares

A number of equidistant intervals which is variable wise are formed by siPLS [18,20] via dividing the data sets, and then calculating all possible PLS models from a combination of two, three or four intervals. Many models were processed depending on the number of intervals and the selected number of intervals to be combined. The results are represented automatically as number of PLS components, intervals combinations, and root mean squares error of cross-validation (RMSECV) for best models according intervals original number [14].

3.2. Methods consideration

3.2.1. Pure component contribution algorithm

PCCA was established, validated and effectively used for the spectral resolution of severely overlapped bands [19]. In this work, the algorithm was applied for the simultaneous determination of DAC, SOF and RIB in laboratory prepared mixtures and tablets. It relies on the elimination of the interfering components and ultimately extracting the pure contribution of each component in a mixture. Moreover, the method is beneficial as it allows the quantitative determination of each component at a single wavelength (λ_{max}) giving the highest sensitivity, accuracy and precision results. Linearity was constructed by plotting the absorbance of DAC, SOF and RIB at 315.0, 261.0 and 209.0 nm, respectively, against their corresponding concentrations, all regression and validation parameters are represented in Table 1.

Two fit values were calculated by the algorithm and compared between different divisors to accomplish maximum accuracy and precision. One fit value is the absorptivity at $\lambda_{max}(\alpha)$ and the value will be accepted when it falls within the confidence limits of the regression slope of the pure standard. The second fit value is the correlation coefficient (r) between the extracted pure spectrum and the standard spectrum of the component. As the r value reaches unity, it indicates high fitness. Different divisors were attempted, and the use of normalized divisors gave the best fit values and thus were selected. The extracted pure components from one of the tertiary mixtures are shown in Figure 3. Precision of the proposed PCCA method was evaluated using three different concentrations of 2.00, 4.00, 6.00 µg/mL, 12.50, 17.50, 20.00 µg/mL, and 14.00, 18.00 and 22.00 µg/mL for DAC, SOF and RIB, respectively.

3.2.2. Full spectrum PLS Model

The full and raw spectral data without any preprocessing was used for building a full spectrum PLS model and a five levels-three factors calibration design was used for construction of the regression models. Table 2 shows the selected concentration levels along with the samples used as training and validation sets. During model construction, Leave one out (Loo) was used as a cross validation tool. The optimum number of latent variables (LVs) contributing to the variance among the data was chosen according to Haaland and Thomas criteria [17] and was found to be five. The developed model was tested for prediction by an external validation set where the three components were determined and the performance characteristics of the developed model was evaluated, with details in Table 3.

3.2.3. siPLS Model

Effective determination of the three components could not be accomplished by the full spectral data. Thus, variables (wavelengths) selection could amend such resolution.

Table 2. Determination of DAC, SOF and RIB in laboratory prepared mixtures by the proposed PCCA method.

Mixture no	DAC	SOF	RIB
1	100.25	100.03	99.90
2	100.30	100.28	100.18
3	99.08	99.66	99.99
4	99.88	100.90	100.33
5	100.02	100.15	100.38
6	98.32	100.62	100.43
Mean±SD	99.64±0.78	100.27±0.44	100.20±0.22

Table 3. Concentration of mixtures of DAC, SOF and RIB in the calibration and validation sets using PLS and siPLS.

Sample	DAC	SOF	RIB
1	6	15	18
2	6	10	10
3	2	10	26
4	2	20	14
5	10	12.5	26
6	4	20	18
7	10	15	14
8	6	12.5	14
9	4	12.5	22
10	4	17.5	26
11	8	20	22
12	10	17.5	18
13	8	15	26
14	6	20	26
15	10	20	10
16	10	10	22
17	2	17.5	10
18	8	10	18
19	2	15	22
20	6	17.5	22
21	8	17.5	14
22	8	12.5	10
23	4	10	14
24	2	12.5	18
25	4	15	10

* The bold samples are those used as an external validation set.

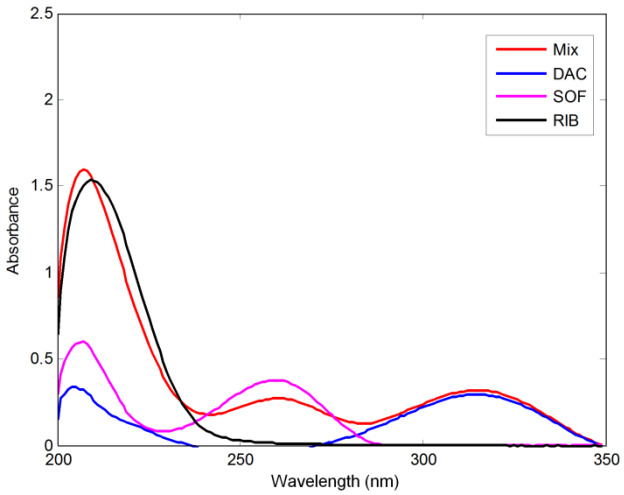

Figure 3. The extracted pure component contribution of each of mixture of 6.00 µg/mL of DAC, 17.50 µg/mL of SOF and 22.00 µg/mL of RIB, and the extracted pure spectrum of each component by the PCCA method.

The data could be used to extract points of collinearity improving prediction ability via finding out the most informative regions in spectra. Hence, more efficient determination with lower number of LVs could be acquired. The siPLS model was established in which synergistic regions are chosen for modeling [19]. The prediction ability of active ingredients, interference minimization and number of latent variables reduction compared to PLS could be improved for finding the optimal informative regions through applying a siPLs algorithm. Different numbers of combinations were chosen and tried. The best results were obtained using combination of 4 sub-intervals. The different combination of subintervals by siPLS showed better performance for the acquired models. A better combination of subintervals number 5, 11, 13 and 16 with four number of latent variables, showed the lowest RMSECV value of 1.992 (Table 4).

3.3. Methods validation

3.3.1 PCCA

Method validation was performed according to ICH guidelines [21] for PCCA.

3.3.1.1 Linearity

Table 4. Statistical results of siPLS model for DAC, SOF and RIB *.

PLS component	Intervals	RMSE
4	5 11 13 16	1.992
4	6 10 18 19	1.995
4	6 9 18 19	1.997
7	5 12 13 16	2.005
4	6 10 19 20	2.007
4	6 8 18 19	2.009
5	5 13 15 16	2.009
4	6 9 19 20	2.009
4	6 9 10 20	2.010
4	6 9 12 19	2.016

* Original number of intervals is 20.

Table 5. Summary of results obtained by applying the diagnostic tools for model validation of PLS and siPLS chemometric methods.

Validation parameters	PLS			siPLS		
	DAC	SOF	RIB	DAC	SOF	RIB
Predicted *vs* known concentration plot						
Slope	0.9906	0.9852	0.9976	1.0071	0.9836	1.0034
Intercept	0.0868	0.3517	0.166	-0.0728	0.2627	-0.049
Correlation coefficient	0.9964	0.9940	0.9991	0.9993	0.99995	0.9995
RMSEP - Recovery of validation set (Mean±SD)	101.21±2.69	100.55±1.78	100.63±0.98	99.05±1.44	99.86±0.57	100.08±0.36

The linearity of the proposed PCCA method was evaluated by analyzing six different concentrations of each of DAC, SOF and RIB ranging between 5.45-16.35, 4.40-44.00 and 5.50-35.00 µg/mL for DAC, SOF and RIB, respectively. Each concentration was repeated three times and the results are represented in Table 1. Good linearity of the calibration graphs was revealed through the high values of the correlation coefficient (r) and small values of residuals standard deviation ($S_{y/x}$).

3.3.1.2 Accuracy

To study the accuracy of the proposed methods, construction of calibration curves for DAC, SOF and RIB were repeated three times for the determination of five different concentrations of each drug. The accuracy was expressed as percentage recoveries as shown in Table 1. The results obtained show good accuracy of the developed method.

3.3.1.3. Precision

Replicate analysis of three different concentrations for DAC, SOF and RIB were used to evaluate the repeatability and intermediate precision. Each concentration was measured three successive times for intra and inter-daily, respectively. The percentage relative standard deviation was computed and the results are displayed in Table 1. The low value of %RSD indicates the high precision of the proposed method. Detection and quantitation limits were determined using the SD of the response and they are listed in Table 1.

3.3.1.4 Selectivity

Different laboratory prepared mixtures of DAC, SOF and RIB within the linearity range were prepared to show the selectivity of the proposed method. Acceptable results are shown in Table 2.

3.3.1.5. Solution stability

No absorbance changes were noticed for the prepared solutions of the studied drugs for one day when kept at room temperature and for about one week when stored in the refrigerator at 4 °C.

3.3.2. Chemometric method

The factor analysis based methods including partial least squares regression is extensively known to draw considerable attention in the chemometrics literature among the different regression methods found for multivariate calibration [22-25]. The studied drugs have overlapping spectra, as shown in Figure 2, thus the PLS method was used for simultaneous deter-mination of the three drugs. Five level three factor design was utilized for the preparation of the calibration and validation sets. The concentrations of the prepared mixtures are shown in Table 3. The choice of the number of principal components (PCs) or factors is essential for PLS calibrations. The number of factors should account as much as possible for the experimental data without over fitting. Several principles have been developed to select the optimum number [26]. Cross-validation methods leaving out one sample at a time was performed [27]. The predicted concentrations were compared with the known concentrations of the compounds in each calibration sample. The root mean squares error of cross-validation (RMSECV) was calculated for each method to analyze the errors in the predicted concentrations. The optimum number of factors was selected by following the principle of Haaland and Thomas [17].

The chosen model was the one with the smallest number of factors in which RMSECV for that model was not greater than the RMSECV from the model with additional factors. Many factors were found to be five which is questionable for the mixture of DAC, SOF and RIB using PLS, Figure 4.

The applied siPLS model has been effectively applied to the validation set. The developed model was described by four LVs which was the best fit for the three component mixture. The values of RMSECV plotted against the number of LVs of the siPLS model is shown in Figure 5.

Figure 6 displays the selected spectral regions to build the models and results represented by the predicted concent-rations of the three components (µg/mL) when compared to the average concentration of the three drugs. To evaluate the validity of the developed models, an external validation set was utilized. The root mean squared error of prediction (RMSEP) values were computed [27,28] Tables 4 and 5 show the percentage recoveries of the validation samples.

The suggested PCCA, PLS and siPLS methods were valid and applicable for the analysis of DAC, SOF and RIB in Daklanork®, Gratisovir® and Ribavirin® tablets, respectively. The validity of the proposed method is further proven by applying the standard addition technique. The results obtained are shown in Table 6.

Table 6. Summary of results obtained by applying the proposed methods for determination of the three drugs in pharmaceutical formulations and application of standard addition technique.

Parameter	PCCA			PLS			siPLS		
	DAC	SOF	RIB	DAC	SOF	RIB	DAC	SOF	RIB
% Found	99.41	101.55	101.35	98.07	101.63	101.72	99.92	98.92	99.70
Standard addition Mean±SD	99.14±1.28	99.82±0.19	100.03±0.64	101.13±1.17	101.02±0.12	100.02±1.07	100.14±0.55	99.99±0.12	99.3±1.05

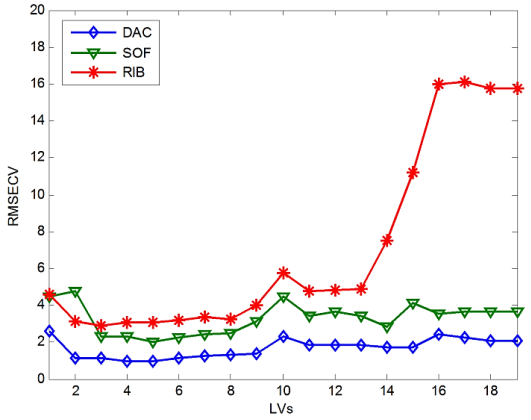

Figure 4. The number of latent variables (LVs) of the developed full spectrum PLS model for DAC, SOF and RIB.

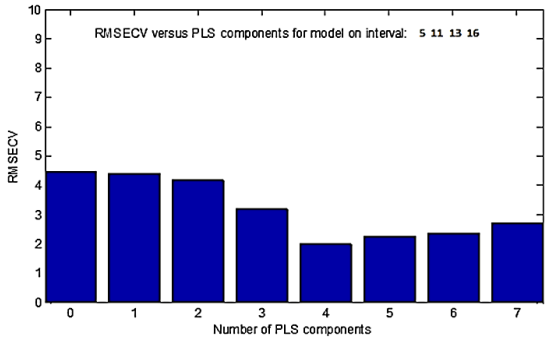

Figure 5. RMSECV *vs* PLS components for siPLS model on interval of interval of 5:11:13:16.

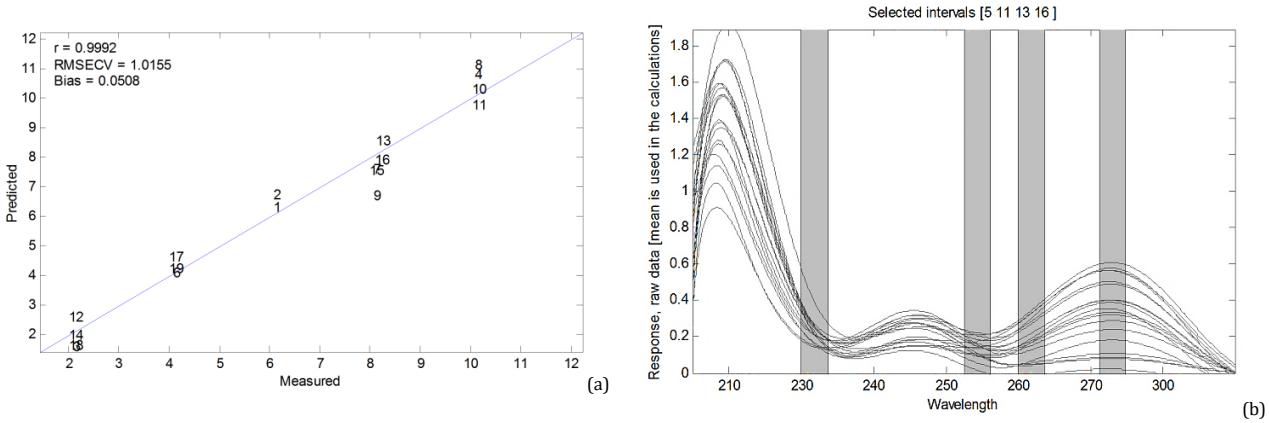

Figure 6. Spectral regions selected to build the models and results: (a) siPLS model by combination of subintervals 5, 11, 13 and 16 for quantification; (b) average content of the three components (µg/mL) *vs* the predicted values by cross-validation for the siPLS model with 4 LVs.

3.4. Statistical comparison

Comparing the results obtained by applying the PCCA and siPLS methods for analysis of pure DAC, SOF and RIB to those obtained by applying the reported method [10] revealed that there is no significant difference regarding accuracy and precision represented by Student's t-test and Variance ratio F-test, respectively [29]. The results are shown in Table 7.

4. Conclusion

The improved data analysis PCCA, PLS and siPLS methods are found to enable better use of spectrophotometric methods as a viable option for rapid mixture analysis and circumventing the cost and time associated with additional separation steps.

Table 7. Statistical comparison of the results obtained by the PCCA, PLA and siPLS spectrophotometric methods and the reported method [10] for DAC, SOF and RIB *.

Method	PCCA			PLS			siPLS			Reference method [10]		
	DAC	SOF	RIB	DAC	SOF	RIB	DAC	SOF	RIB	DAC	SOF	RIB
% Mean **	99.64	100.27	100.2	101.21	100.55	100.63	99.38	99.86	100.08	100.81	100.16	99.97
SD	0.78	0.44	0.22	2.68	1.78	0.982	1.44	0.57	0.357	1.37	1.49	1.46
N	6	6	6	6	6	6	6	6	6	7	8	5
Variance	0.61	0.19	0.05	7.22	3.17	0.96	2.84	0.33	0.127	1.88	2.22	2.14
F-test	0.33 (4.39)	0.09 (3.97)	0.02 (6.26)	3.84 (4.39)	1.42 (3.97)	0.45 (6.26)	1.11 (4.39)	0.15 (3.97)	0.06 (6.26)			
t-test	1.84 (2.20)	0.18 (2.18)	0.39 (2.26)	0.35 (2.20)	0.45 (2.18)	0.89 (2.26)	1.69 (2.20)	0.46 (2.18)	0.18 (2.26)			

* N.B. Figures between parentheses are the tabulated F and t values, respectively, at p = 0.05 [29].
** The mean of three determinations.

They are sensitive, selective, accurate and have a significant role in solving the problem of highly overlapped spectrum with no significant difference of the precision compared with the reference HPLC method [10]. They are applicable for routine analysis of pure DAC, SOF and RIB or in their pharmaceutical formulation without interference from the excipients and could also be easily used in quality control laboratory for their routine analysis. They do not need any sophisticated instruments, critical reactions or any prior separation steps. The methods are also applicable in laboratories with no liquid chromatographic instruments.

Disclosure statement

Conflict of interests: The authors declare that they have no conflict of interest.
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Sample availability: Samples of the compounds are available from the author.

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