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Simultaneous diffuse reflectance infrared determination of clavulanic acid and amoxicillin using multivariate calibration techniques

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A method for simultaneous determination of clavulanic acid (CA) and amoxicillin (AMO) in commercial tablets was developed using diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) and multivariate calibration. Twenty-five samples (10 commercial and 15 synthetic) were used as a calibration set and 15 samples (10 commercial and 5 synthetic) were used for a prediction set. Calibration models were developed using partial least squares (PLS), interval PLS (iPLS), and synergy interval PLS (siPLS) algorithms. The best algorithm for CA determination was siPLS model with spectra divided in 30 intervals and combinations of 2 intervals. This model showed a root mean square error of prediction (RMSEP) of 5.1 mg g^{-1}. For AMO determination, the best siPLS model was obtained with spectra divided in 10 intervals and combinations of 4 intervals. This model showed a RMSEP of 22.3 mg g^{-1}. The proposed method was considered as a suitable for the simultaneous determination of CA and AMO in commercial pharmaceuticals products.

Keywords: amoxicillin; clavulanic acid; DRIFTS; interval PLS; synergy interval PLS.

Introduction

Amoxicillin (AMO) has been used for antibacterial treatment for many years, but the occurrence of resistant bacterial strains has resulted in limited use of this antibiotic. Clavulanic acid (CA) can improve the antibacterial therapy and overcome the bacterial resistance when administered in combination with amoxicillin.[1] Nowadays, pharmaceutical formulations containing both antibacterial agents is currently used. A number of analytical methods have been reported for the determination of these substances in pharmaceutical preparation and biological fluids, which include high performance liquid chromatography (HPLC),[2–5] capillary electrophoresis,[6–9] and luminescence spectroscopy.[10] Methods described in current pharmacopoeias use HPLC as an official assay for quality control of CA and AMO in pharmaceutical preparations.[11] However, this technique could present some drawbacks related to the throughput, consumption, and generation of chemical residues, and excessive time for analysis.

On the other hand, alternative techniques for drug quality control using measurements in real time and with minimum sample preparation have been proposed in recent years.[12–17] Diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) is a fast and non-destructive method and, combined with chemometrics algorithms, it has become a powerful tool in analysis of pharmaceutical products.[17,18]

Infrared spectroscopy combined with multivariate analysis allows improving the quality of results obtained for complex mixtures by overcoming problems related to overlapped signals. Partial least-squares (PLS) regression is the most popular multivariate calibration method for quantitative analysis because it can minimize problems such as the loss of resolution of analytical signal.[19,20] Generally, PLS performs the calibration using information from the full spectrum to build a regression model in order to determine the property of interest, called a full-spectrum method. However, recent applications have proposed the use of methods for spectral region selection with suitable algorithms to improve the performance of PLS regression.[21–25] In practice, these methods are based on the identification of a interval of spectrum that will produce the lowest prediction error.[21]

Interval PLS model (iPLS) is based on the division of the spectrum into smaller intervals followed by the construction of a PLS regression model for each interval. Root mean square error of cross-validation (RMSECV) is calculated for each model and compared to the obtained value for the full spectrum model.[26] One of the

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main advantages of this method is the possibility of obtaining a regression model for each interval in a graphical display. This graphical display allows the selection of better intervals and compares the RMSECV values of the different intervals models and the full spectrum model.[21]

The Synergy iPLS algorithm also splits the spectrum into a number of intervals (variable-wise) and allows the application of PLS regression models for all possible combinations of two, three, or more intervals. However, despite some advantages over conventional procedures, the use of the variables selection methods has been restricted because few works deal with pharmaceutical quantification using infrared spectroscopy data.[25,27–29]

In this study, the feasibility of DRIFTS associated with different PLS regression models was evaluated for the simultaneous determination of CA and AMO in pharmaceutical formulations as an alternative to the method recommended by pharmacopoeias using HPLC. PLS algorithm was employed for data modelling using full spectra information, while iPLS and siPLS models were used to select variable intervals. Results obtained by the proposed method were compared with those obtained using the recommended procedure described in official pharmacopoeias.

Experimental

Samples

Twenty commercial samples of 10 different pharmaceutical industries were acquired in local drugstores with a nominal value in tablets of 500 and 125 mg, or 875 mg and 125 mg of amoxicillin and clavulanic acid, respectively. The medium weight of these samples was carried out as recommended by the United States Pharmacopoeia (USP 31).[11] Potassium clavulanate and amoxicillin trihydrate were purchased in manipulation drugstore and used for synthetic sample preparation. Twenty synthetic samples were prepared in laboratory through the mixture of the correspondent active ingredients and excipients mixture (78% microcrystalline cellulose, 20% talc, 1% colloidal silicon dioxide, and 1% magnesium stearate). The concentration of CA and AMO in synthetic samples ranged from 20% below and 20% above the respective lower and higher nominal concentration for both substances in commercial products. Commercial and synthetic samples were ground and mixed in a cryogenic mill Spex Certiprep (model 6750 Freezer Mill, Metuchen, EUA). Samples were frozen in liquid argon for 2 min and also ground for 2 min. Samples with particle size less than 80 μm were obtained and used for the subsequent tests.

Reference method by HPLC

HPLC assay recommended by the United States Pharmacopoeia (USP 31) was used as reference method for AMO and CA determination in commercial and synthetic samples.[11] This procedure was performed using a model Agilent 1100 Series system equipped with pump (model G1311A), UV-VIS detector (model G133A ALS) and a sampling loop of 20 μl. Detector was set at 220 nm and peak areas were manually integrated using a Chemstation® software program (Agilent Technologies Inc., Santa Clara City, California State – USA). Separation was carried out at ambient temperature using a C18 column-Zorbax® SBC-18 (250 mm × 4.5 mm i.d., 5 μm particle size). A Zorbax® SBC-18 column (12.5 mm × 4.5 mm i.d., 5 μm particle size) guard cartridge system was used to safeguard the analytical column. The mobile phase was a mixture of methanol : sodium phosphate buffer 5:95 *v/v* (pH 4.4 sodium phosphate buffer adjusted with 10 mol L^{-1} sodium hydroxide). Ten commercial tablets and synthetic samples were dissolved in water using a mechanical stirrer and transferred to a suitable volumetric flask. The determination of AMO and CA was performed in triplicate for synthetic and commercial samples.

Recording spectra

All spectra were collected in the range of 4000 to 400 cm^{-1} using an infrared spectrometer (PerkinElmer, Spectrum One FTIR spectrometer, EUA) with 16 scans and resolution of 4 cm^{-1}. This instrument was equipped with a diffuse reflectance sampling accessory (Pike Technologies, EUA, Madison City, Wisconsin State – USA). Spectrum was obtained using about 30 mg of solid sample that was placed on the reflectance device. Spectra were collected in triplicate and a mean spectrum was obtained for each sample. Typical spectra of AMO and AC are shown in Figure 1.

Software

Hierarchical cluster analysis (HCA) algorithm from Pirouette® 3.11 software (http://www.infometrix.com, EUA) was used to divide the samples set in calibration and prediction sets of samples. HCA involves a measurement of the similarity between objects based on their measured properties (variables). Objects are grouped in clusters in terms of their nearness in the multidimensional space. At least one sample of each cluster was used in the calibration and prediction sets. In this work, the similarity matrix was calculated using Euclidean distances and was used to join the elements or clusters to create the dendrogram. At least one sample of each cluster was used in the calibration and prediction sets. MATLAB® software 6.5 version (The Math Works, Natick City, Massachusetts State – USA) using PLS multivariate calibration models from 'PLS Toolbox' 2.0 version was used for variables selection and multivariate models iPLS and siPLS development.[26]

Chemometric models

Initially, PLS models for CA and AMO determination in pharmaceutical preparations were built using DRIFTS without treatment and multiplicative scatter correction (MSC). Autoscalling (A) and mean centred data (MC) were used as pre-processing tools for the multivariate calibration models.

Variable selection

A widely used strategy to improve PLS regression models is the selection of one or more spectral ranges containing chemical or structural information and the elimination of spectral ranges that only contribute to the noise. In this work, the selection of variables for the PLS calibration was carried out using iPLS and siPLS.

Interval PLS models were built on spectra division in 10, 20, 30, 40, and 50 intervals. Synergy interval PLS models were also built on spectra division in 10, 20, 30, 40, and 50 intervals and with combinations of 2, 3, and 4 intervals. The sub-interval or the combined sub-intervals that presenting the minor RMSECV values were selected using iToolbox® software.

Evaluation of models

Models were evaluated according to the root mean square error (RMSE) and correlation coefficient (R) of calibration set.[20] RMSECV was used to select the number of latent variables.

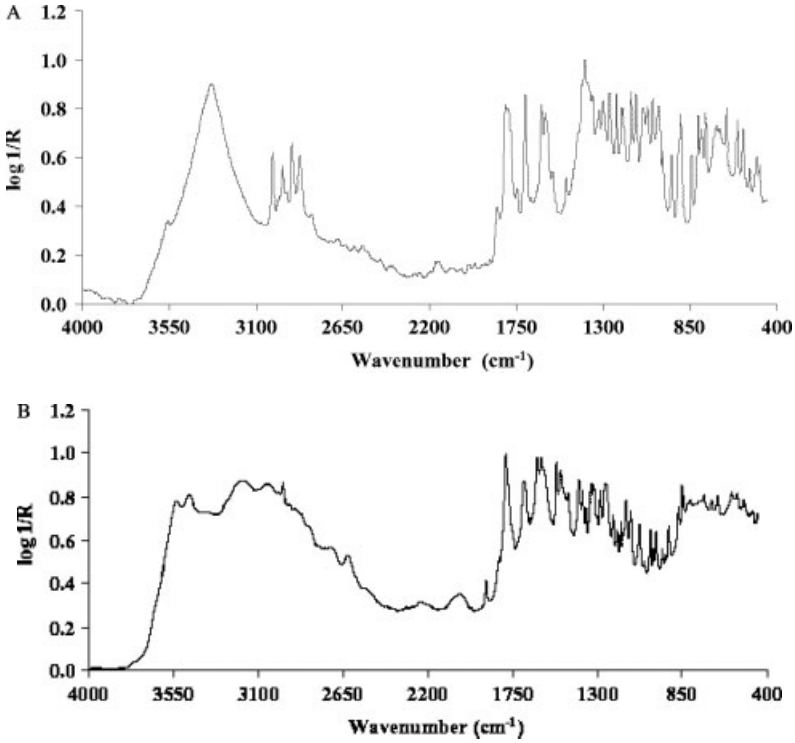

Figure 1. Active ingredients spectra used to prepare synthetic samples. A corresponds to AC and B corresponds to AMO.

Root mean square error of prediction (RMSEP) was employed to evaluate the prediction ability of different PLS models. F-test (95% confidence level) was used to compare the prediction errors of constructed models.[30] Systematic error ('bias') and standard deviation of validation (SDV) were calculated and systematic error was considered not significant for t systematic (t_{sist}) values lower than critical value $(t_{crit})_{\alpha} = 5\%$ and n-1 degrees of freedom.

Accuracy was evaluated for the proposed DRIFTS method based on the agreement between reference values obtained by HPLC and prediction values obtained by the calibration model. Results obtained by the proposed method were compared with reference method using HPLC by t-test paired (95% confidence level). Results obtained by DRIFTS were also evaluated taking into account the agreement to the minimum and maximum values allowed by the United States Pharmacopoeia (90 to 120% of declared value).[11]

Results and discussion

Calibration and prediction sets

The samples set was split into prediction and calibration sets using hierarchical cluster analysis. Twenty-five samples (10 commercial and 15 synthetic) were used for the calibration set with AMO concentration between 375.7 to 737.2 mg g^{-1} and CA concentration between 0 to 185.3 mg g^{-1}. For the prediction set, 15 samples (10 commercial and 5 synthetic) were used with AMO concentration between 387.5 to 686.1 mg g^{-1} and CA concentration 80.5 to 167.6 mg g^{-1} Table 1.

Results of PLS models for CA and AMO determination

Different models were obtained using the combination of treatments and a pre-processing step. Significant difference was not observed when RMSEP values of PLS models using MSC and without treatment were compared (F-test, 95% confidence level). However MSC treatment was chosen because it showed lower RMSEP values and better correlation coefficient. Therefore, MSC treatment and MC pre-processing were chosen for development of all subsequent models. The full-spectrum PLS models (MSC and MC) for CA used four latent variables and showed an RMSEP value of 14.1 mg g^{-1}. For AMO quantification full-spectrum PLS models (MSC and MC) used nine latent variables and showed an RMSEP value of 42.0 mg g^{-1}.

Interval PLS and siPLS algorithms were used for AMO and CA quantifications and also to decrease the RMSEP values. Such a fact could be explained because PLS full-spectrum models were performed with full-spectrum region (3351 variables number) and regions not correlated with CA and AMO may have been used resulting in higher RMSEP values.

Results for CA iPLS models

For iPLS the spectra were split into smaller, equidistant regions and models were developed for each sub-interval. Figure 2 shows the spectrum split in 30 different intervals and RMSECV values obtained for each interval. In this spectrum, it is possible to observe that some regions showed RMSECV values lower than the full-spectrum PLS model (e.g. interval number 26). Table 2 shows the results obtained for CA iPLS calibrations models with 10, 20, 40, and 50 intervals. All iPLS models showed RMSEP values lower than the RMSEP value of the full-spectrum PLS model, but a significant difference was not observed (F-test, 95% confidence level). The better iPLS20 model for CA determination used spectra split in 20 intervals and an RMSEP value of 19.8 mg was obtained. For this model, interval number 18 and four latent variables were used. Therefore, iPLS models can reduce noise by selecting specific spectral regions. However, useful spectral information can be lost.

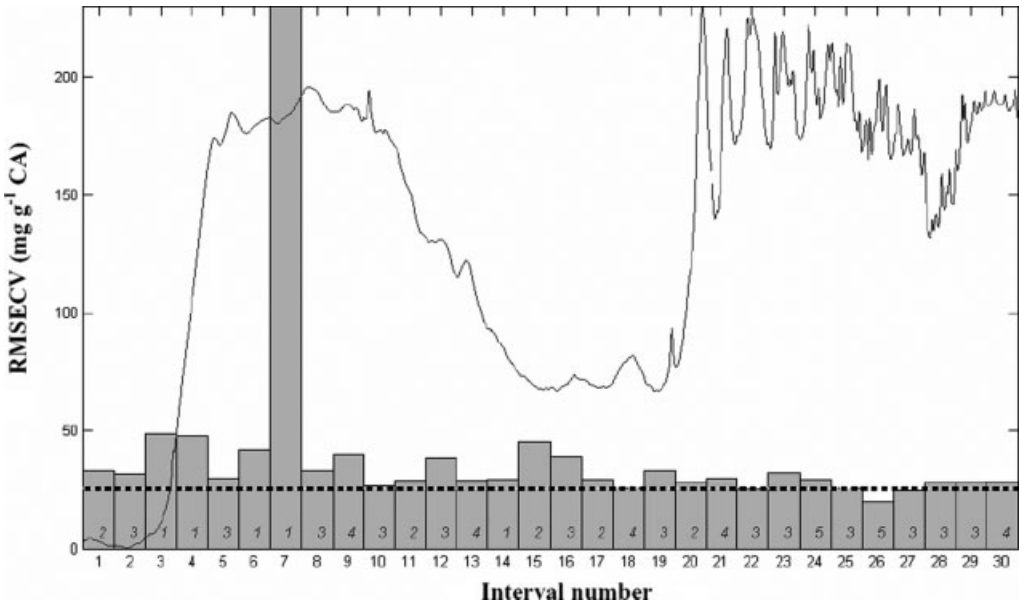

Figure 2. RMSECV values for iPLS models for the AC determination using DRIFTS spectra divided into 30 different intervals. The horizontal black line represents the RMSECV value for the full-spectrum PLS model. The numbers inside the rectangles are the optimal numbers of latent variables.

Table 1. Sample concentration for calibration and prediction set (mg g^{-1}, n = 3)

	Calibration (25 samples)		Prediction (15 samples)	
Parameters	CA	AMO	CA	AMO
Minimum value	0	375.7	80.5	387.5
Maximum value	185.3	737.2	167.6	686.1

Results of CA siPLS models

SiPLS models were evaluated to obtain RMSEP values lower than those using the full-spectrum PLS model. SiPLS models were obtained using the combination of intervals with the lowest RMSECV values. Table 2 shows the statistical results for CA obtained for siPLS calibration models. Comparing the different obtained siPLS models it was possible to conclude that the si2PLS20 and si2PLS30 models showed RMSEP values significant lower than RMSEP values of PLS full-spectrum model (F-test, 95% confidence level). However, the si2PLS30 model showed the lower RMSEP value using spectra split in 30 intervals and a combination of intervals numbered 13 and 18 (Figure 2). The selected interval 13 (2554 to 2666 cm^{-1}) and interval 18 (1994 to 2106 cm^{-1}) probably corresponds to vibrations of amine salt. This model, si2PLS30, showed a reduction of 93% of variables number when compared with the PLS full-spectrum model and showed a good correlation coefficient (0.991) between reference and predicted values. Systematic errors were not observed using the si2PLS30 model (bias = 1.28 and $t_{sist} < t_{crit}$). By using the proposed method with the si2PLS30 model, the individual error was smaller than 10% when it was compared with values obtained using pharmacopeical method (Figure 3). Results for all analyzed commercial samples using the si2PLS30 model were also in agreement with the minimum and maximum values allowed by the United States Pharmacopoeia (90 to 120% of declared value). Using siPLS models it is possible to obtain models with reduced total variable numbers (removing noisy spectral) and better predictive ability using only information correlated with AMO and CA content.

Table 2. Statistical results for CA calibration models

Model	VN[a]	Intervals	LVs[b]	R_{cal}	RMSEP (mg g^{-1})
PLS	3351	All	4	0.947	34.1
iPLS10	335	9	6	0.970	26.0
iPLS20	**168**	**18**	**4**	**0.946**	**19.8**
iPLS30	112	26	5	0.960	27.2
iPLS40	84	34	4	0.932	23.9
iPLS50	66	16	4	0.940	27.4
si2PLS10	670	4, 10	6	0.977	15.0
si3PLS10	1005	4, 7, 8	6	0.970	22.9
si4PLS10	1340	4, 7, 8, 9	7	0.983	16.5
si2PLS20	336	10, 19	10	0.997	8.9
si3PLS20	504	10, 13, 19	12	0.999	12.2
si2PLS30	**224**	**13, 18**	**7**	**0.991**	**5.1**
si3PLS30	336	17, 22, 28	8	0.987	14.5
si2PLS40	168	31, 37	9	0.988	17.5
si3PLS40	252	3, 5, 38	8	0.974	18.6

[a] VN: total variables numbers.
[b] LV: latent variables.

Therefore, synergy interval PLS was suitable for CA determination in pharmaceutical formulations because it showed better prediction ability (lower RMSEP value) when compared with the PLS and iPLS models.

Results of AMO iPLS models

Figure 4 shows the spectrum split in 10 different intervals and RMSECV values obtained for each interval can be compared with full-spectrum RMSECV value. Some of these intervals showed RMSECV values lower than RMSECV value of full spectrum PLS model (e.g. intervals numbered 2 and 4). Table 3 shows the statistical results for the developed AMO iPLS calibrations models. All iPLS models did not show RMSEP values significantly different

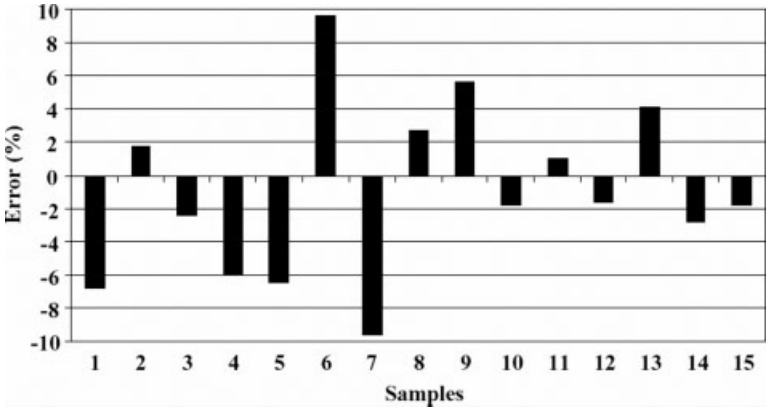

Figure 3. Individual errors for CA determination using si2PLS30 model.

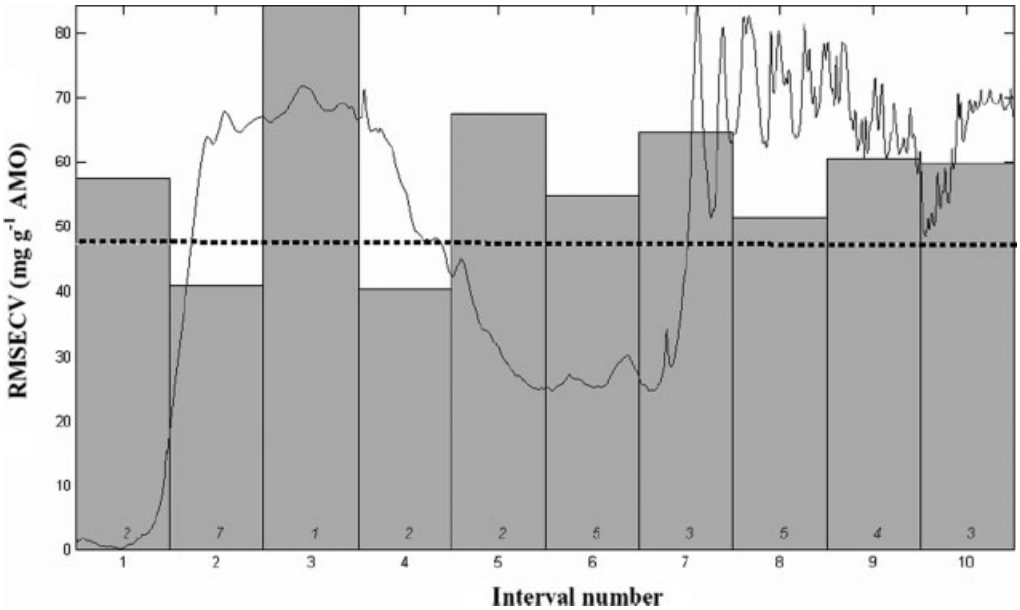

Figure 4. RMSECV values for iPLS models for the AMO determination using DRIFTS spectra divided in 10 different intervals. The horizontal black line represents the RMSECV value for the full-spectrum PLS model. The numbers inside the rectangles are the optimal numbers of latent variables.

when compared to the RMSEP value of PLS full spectrum. However, the model with lower RMSEP (38.6 mg g^{-1}) was obtained with a spectrum divided into 30 intervals and interval number 23 selected. Four latent variables and only 112 total variables numbers were used. Interval PLS was not suitable for AMO determination in pharmaceutical formulations because it did not show good prediction ability.

Results of amoxicillin siPLS models

In order to decrease the RMSEP value, siPLS models were obtained using combinations of intervals with the lowest RMSECV values. Table 3 shows the statistical results for AMO siPLS calibration models. Comparing the different obtained siPLS models it is possible to observe that only the si4PLS10 model showed RMSEP value significantly lower than that using the full-spectrum PLS model. This si4PLS10 model showed a correlation coefficient of 0.994 between reference and predicted values when spectra were divided into 10 intervals and a combination of intervals numbered 2, 4, 5, and 6 were used. The selected intervals included regions of 3331 to 3666 cm^{-1} (interval 2), 2661 to 2996 cm^{-1} (interval 4), 2326 to 2661 cm^{-1} (interval 5), and 1991 to 2326 cm^{-1} (interval 6). Interval 2 probably corresponds to N-H stretching and in interval 4, C-H stretching vibrations can be found. Interval 5 corresponds to N-H bending of amine salt and its correspondent harmonic and in interval 6 combination bands of the aromatic ring can be found.[32] All these groups are present in the AMO chemical structure. Systematic error calculated for the si4PLS10 model was not significant (bias = 5.63 and $t_{sist} < t_{crit}$) due to the non-tendency for the prediction errors. The individual error provided by the si4PLS10 model was smaller than 8% when the AMO concentration values for the proposed method and pharmacopeical methods were compared (Figure 5). Results obtained using the si4PLS10 model were also in agreement taking in to account the minimum and maximum values allowed by the United States Pharmacopoeia (90 to 120% of declared value). Therefore, as previously found for CA determination, synergy interval PLS was also suitable for AMO determination in pharmaceutical formulations because it showed better prediction ability (lower RMSEP value) when compared with the PLS and iPLS models.

Table 3. Statistical results for AMO calibration models

Model	VN[a]	Intervals	LVs[b]	R_{cal}	RMSEP (mg g^{-1})
PLS	3351	All	9	0.993	59.0
iPLS10	335	4	2	0.917	63.3
iPLS20	168	12	8	0.995	63.3
iPLS30	**112**	**23**	**4**	**0.965**	**48.6**
iPLS40	84	13	2	0.889	62.7
iPLS50	66	38	3	0.944	39.3
si3PLS10	1005	1, 4, 6	7	0.987	46.7
si4PLS10	1340	**2, 4, 5, 6**	**9**	**0.994**	**22.3**
si2PLS20	336	2, 7	3	0.944	50.8
si3PLS20	504	7, 13, 16	10	0.993	49.5
si2PLS30	224	11, 17	5	0.981	44.4
si3PLS30	336	5, 11, 17	10	0.997	32.1
si2PLS40	168	14, 22	5	0.978	36.4
si3PLS40	252	14, 25, 37	11	0.998	37.0
si2PLS50	132	18, 32	9	0.993	44.1

[a] VN: total variables numbers.
[b] LV: latent variables.

Validation of the best calibration models obtained for CA and AMO

The proposed method was validated in accordance with The American Society for Testing and Material (ASTM) E1655-05, International Conference on Harmonisation (ICH) and the European Medicines Agency (EMEA) using parameters usually recommended: precision or repeatability, linearity, and accuracy.[32–34]

Precision

Precision was evaluated by repeatability (or intra-day precision). Repeatability of the proposed method was performed with the same analyst and applying the method to the same production sample three times on the same day. In this work the repeatability was calculated by three replicates of three different concentrations, using Eqn 1:

$$precision = \sqrt{\frac{\sum_{i=1}^{n}\sum_{j=1}^{m}(\hat{y}_{ij} - \hat{\bar{y}}_i)}{n(m-1)}} \quad (1)$$

where m is the replicate number carried out, n the samples number, $\hat{\bar{y}}_i$ is the prediction mean values mean of each replicate $\hat{y}_{ij}$.[35] Thus, values for precision were 5.7 mg g^{-1} for CA and 19.3 mg g^{-1} for AMO that correspond to 4.7% and 3.7%, respectively. The values for RSD were lower than the widely accepted value for this type of determination (5%).[13]

Linearity

Correlation between the values using the best siPLS model and the reference method was evaluated. Ideally the intercept (a), and slope (b), should be zero and one, respectively, if there was no systematic error in the calibration equation.[13,36,37] Linear regression test was performed between the two methods and the agreement of results was evaluated by Eqn 2:

$$y = bx + a \quad (2)$$

where y is the DRIFTS predicted value, x the obtained reference value, a the intercept and b the slope values. For CA determination the regression equation was $y = 0.9820x + 1.8007$, being the confidence interval for the slope [0.93;1.03] and for the intercept [−4.1;7.7] included 1 and zero, respectively. For AMO determination the regression equation was $y = 0.9880x + 6.2714$, being the confidence interval for the slope [0.94; 1.03] and for the intercept [−17.04; 29.59] included 1 and zero, respectively. Therefore, the proposed method for both drugs allowed a suitable linearity when it is compared to the reference method, within the concentration range of the calibration set.

Accuracy

Accuracy was evaluated by comparison of results obtained using the proposed method with those obtained by HPLC for prediction set.[34] Paired t-test was performed between the DRIFTS proposed method and HPLC reference method. This t-test allowed checking if the results obtained for proposed and reference methods showed a significant difference. For CA and AMO determinations, the obtained experimental t value was smaller than the critical t value. Thus, the proposed method can be considered a suitable alternative for the reference method HPLC for investigated pharmaceutical formulations.

Conclusion

Overall results demonstrated that DRIFTS associated with multivariate analysis is a convenient method for quality control of CA and AMO in pharmaceutical formulations. Results obtained using siPLS models for simultaneous determination of CA and AMO in powder pharmaceutical formulations showed suitable prediction

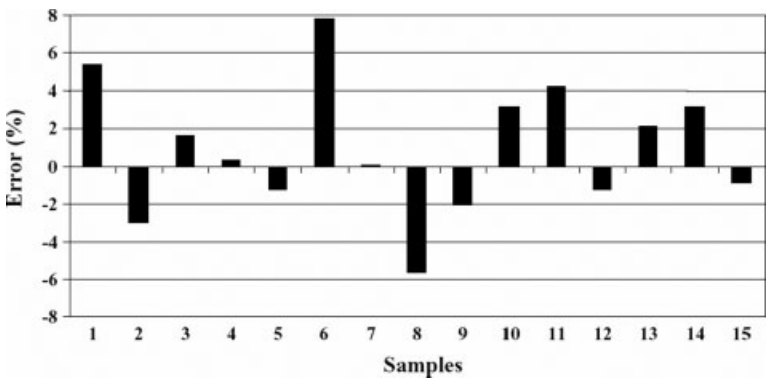

Figure 5. Individual errors for AMO determination using si4PLS10 model.

capacity (lower RMSEP). Variable selection methods were able to produce better models in comparison to the PLS full-spectrum model. Results obtained for CA and AMO using the better siPLS models were in agreement with results obtained by reference method. The proposed procedure using DRIFTS and PLS algorithms is faster, less expensive and minimizes solvent use when compared to HPLC method (Pharmacopeical method).

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