

CHEMOMETRIC METHODS FOR THE SIMULTANEOUS ASSAY OF CHLORAMPHENICOL, CHLORHEXIDINE AND METRONIDAZOLE DURING *IN VITRO* DISSOLUTION OF DRUGS FROM MUCOADHESIVE BUCCAL GELS

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Abstract

Two chemometric methods, partial least squares regression (PLS) and principal component regression (PCR), were evaluated for the simultaneous assay of chloramphenicol, chlorhexidine and metronidazole on dissolutions samples from a mucoadhesive buccal gel formulation. A calibration set consisting of 34 mixture solutions was prepared by means of an orthogonal experimental design and their absorption spectra were recorded in the spectral range of 220–390 nm.

An external HPLC validated method was carried out by applying the developed methods to the analysis of synthetic mixtures of the related compounds, obtaining successful results. The methods were finally used to assay the studied drugs for *in vitro* release studies from mucoadhesive buccal gels.

Rezumat

Au fost evaluate două metode chemometrice, regresia celor mai mici pătrate (PLS) și regresia componentelor principale (PCR) pentru dozarea simultană a cloramfenicolului, clorhexidinei și metronidazolului din probele de dizolvare ale unui gel mucoadeziv bucal. Pentru calibrare s-a folosit un format 34 de amestecuri preparate conform unui plan experimental ortogonal, iar spectrele lor au fost înregistrate în domeniul 220–390 nm.

Keywords: chemometric analysis, *in vitro* dissolution, chloramphenicol, chlorhexidine, metronidazole

Introduction

The conventional spectrophotometric methods use a discrete number of wavelengths that frequently are not enough to provide the necessary information to resolve a system with severe spectra overlapping [1-3]. Several approaches for the elaboration of spectrophotometric data have been proposed in order to extract a larger amount of analytical

information from spectra composed of unresolved bands. In the last few years chemometrics–spectrophotometry was rapidly attracting analysts' attention and is playing a very important role in the multicomponent analysis of complex drug mixtures [4-8].

The objective of this study was to develop a simple method for the *in vitro* drug release assay from a buccal mucoadhesive drug system, indented for the application in the socket of patients with risk of developing alveolar osteitis. The mucoadhesive buccal preparation contains 3 active pharmaceutical ingredients (APIs): chloramphenicol, chlorhexidine diacetate and metronidazole, all in a concentration of 1%.

A chemometric UV-Vis spectrophotometrical method for the simultaneous determination of chloramphenicol, chlorhexidine and metronidazole from the *in vitro* dissolution media without sample treatment was developed and validated.

Materials and methods

Apparatus

UV–Vis spectrophotometer: absorption spectra were recorded in a wavelength range of 190–400 nm in a 10 mm quartz cell, by a Jasco V-530 (Jasco, Japan) Spectrophotometer, in the following conditions: scan rate 100 nm min⁻¹; time response: 1 s; spectral band 1 nm. For spectral acquisition and elaboration of the data obtained with V-530 Spectrometer (Jasco, Japan) the software Spectra Manager for Windows (Jasco) was used.

HPLC system: Agilent 1100 Series (Agilent SUA) with detector VWD – UV. Chromatographic conditions: a Zorbax SB-C18 (5 µm, 4.6x150mm) column, mobile phase: acetonitrile : H₃PO₄ 0.1% (35:65 v/v), flow rate: 1.2 mL/min, injection volume: 20 µL. The detection was performed in UV at 283nm.

Materials

Chloramphenicol - CLF (Biesterfeld, Germany), chlorhexidine diacetate – CLH (Wuhan Pharmaceutical, China) and metronidazole - MET (S&D Chemicals, UK), as working standard. All the other reagents (ethanol, phosphates) were of analytical grade purity, commercially available.

Statistical analysis

The ability and the efficiency of the calibration were studied by estimating the standard variation of chemometric calibrations in the case of the investigated mixtures. The root mean square error of prediction (RMSECV) was calculated using the following formula:

$$\text{RMSECV} = \sqrt{\frac{\text{PRESS}}{n}} \quad \text{PRESS} = \sum (Y_{\text{pred}} - Y_{\text{true}})^2$$

where: Y_{true} = true concentration of the drug, Y_{pred} = predicted concentration of the drug,
 n = number of training samples

Software

Partial least squares regression (PLS) and principal component regression (PCR) analysis were performed using SIMCA P 11 software package (Umetrics Sweden). The software permits to validate the models by full cross-validation. In this procedure, iterative calibrations were performed removing in turn each standard from the training set and then predicting the excluded sample with that calibration. The calibration set (Table I), containing ternary component mixtures in a suitable combination, was built using a central composite experimental design double replicate, developed in Modde 6.0 Software (Umetrics Sweden) [2,9].

Table I
 Composition of calibration set
 (Central Composite Experimental Design double replicate)

Exp Name	X ₁	X ₂	X ₃	Exp Name	X ₁	X ₂	X ₃
N1	2.02	2.0296	2.0212	N18	20.1998	2.0296	14.1482
N2	7.0699	2.0296	2.0212	N19	2.02	7.1035	14.1482
N3	14.1398	2.0296	2.0212	N20	20.1998	14.207	14.1482
N4	20.1998	2.0296	2.0212	N21	14.1398	20.2957	14.1482
N5	2.02	7.1035	2.0212	N22	2.02	2.0296	20.2118
N6	20.1998	7.1035	2.0212	N23	2.02	2.0296	20.2118
N7	2.02	14.207	2.0212	N24	14.1398	2.0296	20.2118
N8	2.02	20.2957	2.0212	N25	20.1998	2.0296	20.2118
N9	7.0699	20.2957	2.0212	N26	14.1398	7.1035	20.2118
N10	20.1998	20.2957	2.0212	N27	20.1998	7.1035	20.2118
N11	20.1998	20.2957	2.0212	N28	7.0699	14.207	20.2118
N12	2.02	2.0296	7.0741	N29	2.02	20.2957	20.2118
N13	20.1998	2.0296	7.0741	N30	2.02	20.2957	20.2118
N14	7.0699	7.1035	7.0741	N31	20.1998	20.2957	20.2118
N15	14.1398	14.207	7.0741	N32	20.1998	20.2957	20.2118
N16	2.02	20.2957	7.0741	N33	20.1998	20.2957	20.2118
N17	7.0699	2.0296	14.1482	N34	20.1998	20.2957	20.2118

X₁ -Chloramphenicol-CLF (µg/mL); X₂ - chlorhexidine diacetate-CLH (µg/mL); X₃ - metronidazole-MET (µg/mL);

Samples preparation

Standard solutions: stock solutions of CLF, CLH and MET (5 mg/mL) in ethanol were used to set up the calibration set samples. These solutions were appropriately diluted with the phosphate buffer to give reference mixtures with concentration values within the range 2.0–20.0 µg/mL for CLF, CLH and MET. These values were chosen in agreement with the concentration ratios used during the *in vitro* dissolution studies of drugs from mucoadhesive buccal gels.

Sample solutions: in 5 mL volumetric flasks the necessary quantities of reconstituted dosage forms to obtain the same concentration as dissolution were weighed. Each sample solution was marked with CLF, CLH and MET in order to obtain the same dilution as the standard solution. The volume was completed with dissolution medium (phosphate buffer pH 7.2) and sonicated for 3 minutes. The samples were filtered (10µm, PharmaTest filter).

Dissolution samples: dissolution test on mucoadhesive buccal gel containing CLF, CLH and MET in a concentration of 1% each was performed using a dissolution apparatus (PHARMA TEST PT-DT7, paddle configuration) at 25 rpm. 1 g samples of mucoadesive buccal gel were introduced in dialysis bags (Spectra/Por Cellulose Ester Membrane MWCO: 5000-8000 Da) and placed in a dissolution medium (phosphate buffer pH 7.2, at 37±0.5°C). At prefixed time intervals, aliquots were withdrawn and the three drug substances (CLF, CLH and MET) were assayed using the methods mentioned above.

Results and discussion

Calibration matrix and selection of spectral zones for analysis by PLS and PCR

The zero order UV spectra (fig. 1) of MET, CLH and CLF completely overlap with each other making it impossible to use the conventional spectrophotometric methods to analyze MET, CLH and CLF, in mixture of each other. The simultaneous determination of such drugs in their studied mixtures by derivative and derivative ratio spectrophotometric methods is hindered by strong spectral overlap throughout the wavelength range.

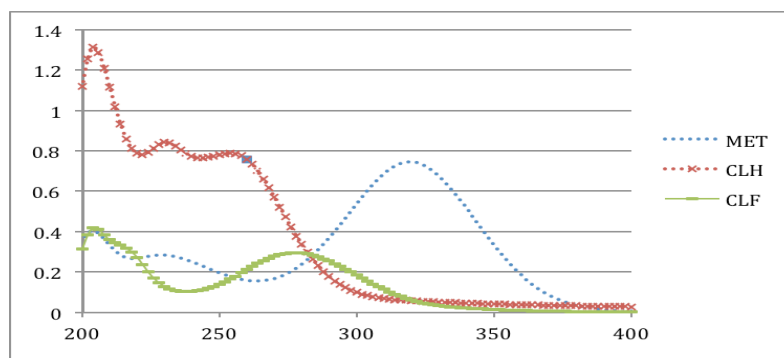


Figure 1.

Overlapping spectra of metronidazole 10 μ g/mL (MET), chlorhexidine diacetate 10 μ g/mL (CLH) and chloramfenicol 10 μ g/mL (CLF)

The quality of multicomponent analysis depends on the wavelength range and the spectral mode used [23]. PLS procedures are designated to be full spectrum computational procedures, thus the wavelength selection is seemingly unnecessary, and so all available wavelengths are often used. However, measurements from spectral wavelengths are non-informative in a model degrade performance. Original and reconstructed spectra of the calibration matrix were compared in order to select the range of wavelengths. The range was obtained by all regions in which the difference among each component of the mixture and the others was maximum. Besides, the regions in which each component of the mixture was best reconstructed were also considered. The wavelength range of 220–390 nm with 2 nm intervals was selected for chemometric analysis.

Wavelengths below 220nm were rejected due to the noise appeared and the differences between the laboratory prepared mixtures and dissolution samples spectra and wavelengths over 390nm were not used due to the neglected absorption at the concentrations used in this region. So any absorbance values obtained at these wavelengths would have introduced a significant amount of noise in the calibration matrix, thereby decreasing the precision. Multilevel experimental design [11] was used for the construction of the calibration set. A calibration set of 34 samples was prepared for calibration. A four-level, three-factor calibration design was used in concentrations ranging between 2 and 20.0 μ g/mL. The concentration details are given in Table I.

Multivariate calibration

Mathematical models based on multivariate calibration were then applied to analyse these drugs. To improve the analysis for these

compounds, two chemometric approaches based on PLS and PCR calibration were evaluated.

An appropriate choice of the number of principal components is necessary for PCR and PLS calibrations. The number of factors should account as much as possible for the experimental data without resulting in over fitting. Various criteria have been developed to select the optimum number [2, 10]. Cross-validation methods leaving out one sample at a time was employed [11]. The predicted concentrations were compared with the known concentrations of the compounds in each calibration sample.

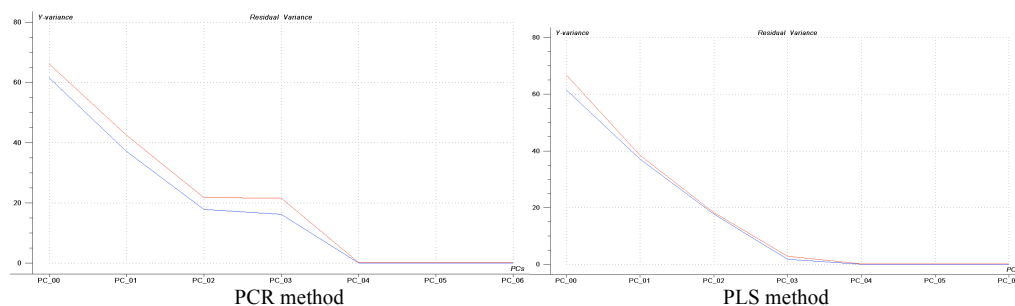
The RMSECV was used as a diagnostic test for examining the errors in the predicted concentrations. It indicates both precision and accuracy of predictions. It was recalculated upon addition of each new factor to the PLS and PCR models.

The evaluation of the predictive abilities of the models was performed by plotting the actual known concentrations against the predicted concentrations. Satisfactory correlation coefficient (r) values between actual and predicted concentrations are obtained for the studied components in the training set by PLS and PCR optimized models indicating good predictive abilities of the models. The RMSECV obtained by optimising the calibration matrix of the absorption spectra for the PLS and PCR methods are shown in Table II, indicating good accuracy and precision.

The optimum number of components was selected following the criterion of Haaland and Thomas [13]. The selected model is that with the smallest number of components such that RMSECV for that model is not significantly greater than RMSECV from the model with additional components. According to residual variance a number of 4 components were found to be optimum for using both PLS and PCR methods (figure 2).

Table II
RMSECV and statistical parameter values for simultaneous determination of MET, CLH and CLF.

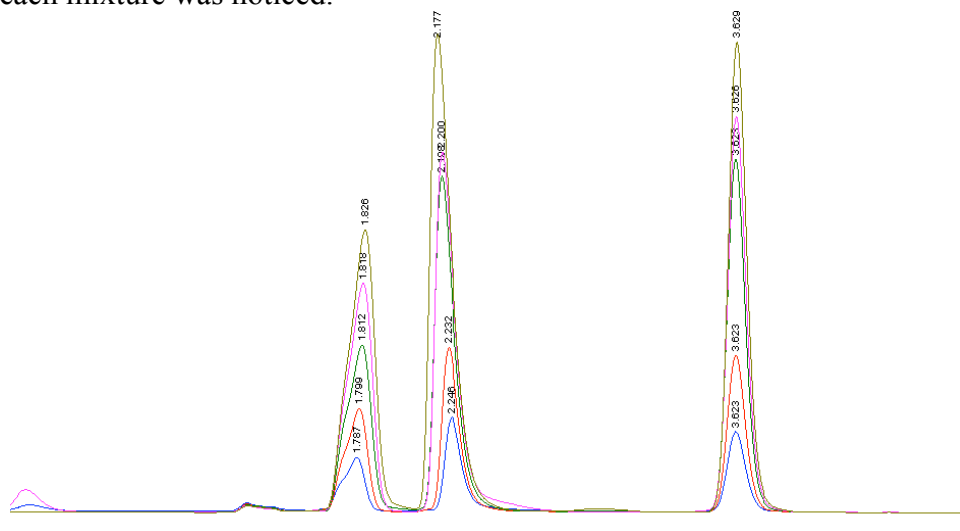
	Method		Slope	Offset	r	RMSECV	Bias
MET	PCR	Calibration	0.9998	0.00112	0.9999	0.0825	-7.36e-07
		Validation	0.9996	0.00034	0.9999	0.0967	-0.00294
	PLS	Calibration	0.9998	0.00115	0.9999	0.0823	-5.20e-07
		Validation	0.9998	0.00057	0.9999	0.0957	-0.00264
CLH	PCR	Calibration	0.9965	0.03933	0.9982	0.4711	-2.16e-07
		Validation	0.9971	0.04238	0.9974	0.5583	0.01075
	PLS	Calibration	0.9965	0.03930	0.9965	0.4637	-1.44e-08
		Validation	0.9958	0.04397	0.9950	0.5556	-0.00314
CLF	PCR	Calibration	0.9996	0.00415	0.9998	0.1466	-7.80e-07
		Validation	0.9987	0.00891	0.9994	0.1831	-0.00589
	PLS	Calibration	0.9996	0.00411	0.9998	0.1482	7.22e-07
		Validation	0.9990	0.00881	0.9995	0.1831	-0.00268

**Figure 2.**

Plotting residual variance depending on the number of components

HPLC method

A reversed-phase HPLC method was used for the validation of the proposed chemometric method. Some mobile phases and wavelengths were tested for the separation and determination of MET, CLH and CLF in the dissolution ternary mixtures. In the optimized conditions the retention times of MET, CLH and CLF were found as MET 1.826 min, CLH 2.177 min and CLF 3.629 min, respectively. The specificity of the HPLC method was illustrated in Fig. 3 where complete separation of the three compounds of each mixture was noticed.

**Figure 3.**

HPLC chromatograms of
MET (1.826 min), CLH (2.177 min) and CLF (3.629 min)

The calibration curves for the active compounds were built by plotting the concentration versus the peak area and showed a good linearity

in the concentration range established in the previous section. The representative linear equations and other validation parameters according to the ICH criteria [14] are shown in Table III.

Table III

Characteristic parameters of the calibration equations for the proposed reference HPLC method for the simultaneous determination of MET, CLH and CLF

Linearity

Test	Calculated values for standard (S.R.)			Calculated values for spiked placebo (S.R.)			Theoretical values of statistical parameters
	MET	CLH	CLF	MET	CLH	CLF	
Slope of the regression equation	34.409	43.126	39.565	34.4320	44.057	39.007	
Intercept of the regression equation	-4.693	2.247	-0.136	-4.711	-9.993	8.373	
Correlation Coefficient	0.995	0.983	0.999	0.996	0.990	0.992	
Cochran Test - comparative test of the homogeneity of variance (Ccalc)	0.383	0.324	0.605	0.678	0.337	0.383	Ct(0.05;5;2)=0.68
Fisher Test - validity of regression (Fcalc)	-1.221	0.508	-0.005	2.180	0.800	-1.230	Ft(0.05;3;10)=3.71
Fisher Test - significant slope (Fcalc)	1207.64	365.21	5474.57	1735.87	665.53	1212.81	Ft(0.05;1;13)=4.67
Student Test - comparative test of intercept with 0	-0.370	2.084	2.084	1.458	-0.437	-0.373	t(0.05;13) = 2.160
Student Test - comparative test of slope D1 and D2 (tcalc)	-0.016	-0.329	-0.019	0.517	-0.329	-0.016	t(0.05; 26) = 2.056
Student Test - comparative test of intercept for D1 and D2 (tcalc)	-0.001	-0.323	1.278	1.278	-0.323	-0.001	t(0.05; 26) = 2.056

Accuracy

Test	MET	CLH	CLF	Theoretical values
Cochran Test - comparative test of the homogeneity of variance	Ccalc = 0.367	Ccalc = 0.655	Ccalc = 0.547	C(0,05;5,2)=0.68; nj = 3
Fisher test - validity of average recovery	Ccalc = 0.881	Ccalc = 0.046	Ccalc = 1.225	F(0,05;4;10)=3.48
Confidence interval	96.39-104.79	96.61-104.29	96.01-104.09	t (0,05; 14)=2.145

Reliability

Parameter	MET	CLH	CLF
Cochran test - comparative test of the homogeneity of variances intra-group	0.659	0.476	0.476
Intra - day precision	2.20	1.75%	1.75%
Inter -day precision	2.19	1.72%	1.72%

Analysis of synthetic mixtures and dissolution samples

Quantitative analysis of the same synthetic mixtures and dissolution samples of MET, CLH and CLF was performed by the chemometric methods and references HPLC method. The results for synthetic mixtures and dissolution samples determination were listed in Table IV.

For the synthetic mixture the recovery was calculated reported at theoretic taken quantity and for dissolution samples the recovery was calculated reported at practical assay (found) quantity by HPLC.

Table IV

Determination of MET, CLH and CLF in laboratory prepared mixtures using the proposed methods

Synthetic mixtures	taken	PCR		PLS		HPLC	
		found	recovery	found	recovery	found	recovery
MET	2.021	1.934	95.69	2.066	102.23	2.050	101.43
	12.127	11.898	98.11	12.176	100.40	12.096	99.74
	20.212	20.980	103.80	20.313	100.50	20.033	99.12
CLH	2.123	2.123	100.52	2.172	102.82	2.050	101.43
	10.445	10.445	98.90	10.525	99.65	12.096	99.74
	21.343	21.343	101.04	20.850	98.71	20.033	99.12
CLF	2.020	2.092	103.58	2.033	100.63	2.016	99.82
	10.100	10.002	99.03	9.817	97.20	9.982	98.83
	20.200	20.682	102.39	20.863	103.28	20.263	100.31
Dissolution samples		found	recovery*	found	recovery*	found	
MET		8.029	97.78	8.012	97.57	8.212	
		14.149	100.16	14.494	102.60	14.127	
		18.023	99.48	18.103	99.92	18.118	
CLH		2.144	101.52	2.112	99.99	2.112	
		10.956	98.32	10.910	97.91	11.143	
		16.098	98.85	16.653	102.26	16.286	
CLF		2.692	97.25	2.772	100.14	2.768	
		6.269	103.44	6.169	101.79	6.061	
		10.222	100.53	10.034	98.68	10.168	

* in comparison with HPLC reference method

The assay results on dissolution samples obtained by applying chemometric methods were compared with the HPLC reference method. A good similarity among the results from the three proposed methods was observed.

Conclusions

For analytical purposes it is always important to establish methods capable of analyzing a large number of samples in a short period of time with acceptable accuracy and precision. Spectroscopic techniques can generate large amounts of data within a short period of analysis; however, when coupled with chemometric tools, the quality of the spectral

information can be markedly increased, converting this combined technique into a powerful and highly convenient analytical tool.

A comparative study of the use of HPLC and multivariate calibration (PLS and PCR) methods for the resolution of MET, CLH and CLF has been accomplished, showing that multivariate calibration methods provide, with adequate software support, a clear example of the high resolving power of this technique. Although the HPLC method is more specific than the multivariate calibration methods, it needs expensive equipment and materials. Multivariate calibration methods are less expensive when compared to the HPLC method and they do not require sophisticated instrumentation and any prior separation steps. The proposed chemometric methods were found to be suitable for the assay of chloramphenicol, chlorhexidine and metronidazole during *in vitro* dissolution studies of drugs release from buccal mucoadhesive preparations, intended for the local treatment of alveolar osteitis.

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