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Green method based on a flow-batch analyzer system for the simultaneous determination of ciprofloxacin and dexamethasone in pharmaceuticals using a chemometric approach

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ABSTRACT

A green FBA method with UV detection was developed for simultaneous determination of ciprofloxacin (CIP) and dexamethasone (DEX) in ophthalmic and otic preparations. A lab-made mixing detection chamber (MDC) was designed and coupled to the spectrophotometer in order to perform the mixing of solutions and the detection in the same receptacle. Only water was used as solvent and no previous separation of the components was required. Both analytes have a strong absorption between 190 and 370 nm in aqueous medium, at pH 7. However, the spectrum of DEX is embedded in the CIP spectrum. Thus, while CIP was analyzed using univariate calibration, DEX analysis was carried out comparing partial least squares (PLS-1) and multiple linear regression (MLR). The latest required a previous variable selection step, which was performed using the genetic algorithm (GA) and the successive projections algorithm (SPA). The FBA system made it possible to automatically prepare the calibration and validation sets. The statistical parameters, in terms of relative errors of calibration and prediction, were acceptable for the determination of both CIP and DEX. Also, a comparative study of chemometric models was carried out. Commercial samples were analyzed and the obtained results are in close agreement with HPLC pharmacopeia methods. The joint interval test for the slope and the intercept was used to test for the presence of bias. There were no statistical differences between the proposed method and the reference method (α=0.05). The sample throughput was 10 h^{-1}. The combination of automation and chemometric tools allows us to develop an environmental friendly method for the quality control of CIP and DEX in pharmaceuticals.

1. Introduction

Topical antibiotic/steroid combination is a usual alternative in the treatment of a wide variety of bacterial infections. Ciprofloxacin hydrochloride (CIP) [1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(piperaziny-1-yl) quinolone-3-carboxylic acid] is a broad-spectrum antibiotic [1,2] which is often used jointly with dexamethasone (DEX, an exogenous steroid) [(11β,16α)-9-Fluoro-11,17,21-trihydroxy-16-methylpregna-1,4-diene-3,20-dione] in several anti-infective ophthalmic preparations to treat acute and sub acute conjunctivitis, keratitis and corneal ulcers caused by susceptible strains [3]. The same combination is used for the topical treatment of acute otitis media in pediatric patients with tympanostomy tubes and acute otitis externa in pediatric, adult and elderly patients [4].

The analytical determination of these drugs is normally carried out by separation techniques, such as liquid chromatography (HPLC). These techniques use organic solvents that are not environmental friendly (owing to either the type or the amount of these solvents) and are normally time-consuming. Therefore, the development of rapid, simple, inexpensive and green analytical methods for the determination of these drugs in pharmaceutical preparations is of great interest. Particularly, the amount and toxicity of wastes are important aspects to be considered. Minimization of reagent consumption contributes to reduce both operational costs and pollution. In the last years, a trend to design environmental friendly procedures in analytical chemistry has been highlighted [5–8] and the adoption of this kind of procedures is mandatory for adherence to ISO 14000 guidelines. There is therefore a real need to develop analytical methods being less harmful to humans and to the environment in accordance with the 12 principles of Green Chemistry [9].

In this sense, the flow analysis techniques have a versatility that makes them a very attractive alternative for developing green analytical procedures. Specifically, Flow-batch Analyzers (FBA) [10] join the advantages of flow and discrete (or batch) systems and programmable multi-commutation principles for carrying out sampling and analytical procedures. The main advantages derive from the complete

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programmability of the process (i.e. control of sampling, flow rates, filling of mixing chamber, detection and evacuation). Basically, a FBA system comprises a propulsion system (typically, a peristaltic pump), a transport system (i.e. PTFE tubes), a multi-commutation system (solenoid valves), mixing/reaction/detection chamber (where physicochemical process can take place) and a detection system. These characteristics give to FBA systems a great operational versatility. Since their appearance, FBA have been used to implement several analytical procedures, such as: titrations [11,12], sample pre-treatments [13,14], standard additions [15], screening analysis [16], concentration gradients [17], multi-component simultaneous analyses [18,19], and so on. From the point of view of pharmaceutical analysis, the proposed FBA systems [19,20] have demonstrated to be an interesting alternative respect to the official methods based on separation techniques, due to their simplicity, high sampling rate, less reagent and sample consumption, and low waste generation, which agrees with the principles of so called green chemistry.

These devices based on FBA are normally coupled to optical techniques, such as UV–Vis or fluorescence, whose rapid response makes them appropriated for on-line detection. Despite this fact, when mixtures of several components are simultaneously analized, the lack of selectivity of the signals often results in requiring multivariate calibration techniques. These techniques include multiple linear regression (MLR), principal component regression (PCR) and partial least squares (PLS) [21], among others. The application of these methods usually requires the selection of spectral variables for building well-fitted models [22–26]. While PCR and PLS perform the regression on latent variables without a physical meaning, MLR yields simpler and easier to interpret models because is based on real variables. However, MLR calibration is more dependent on the spectral variables selection and it can be severely affected by collinearity among the variables [27,28]. Thus, selecting the appropriate wavelengths, which redounds on a maximum accuracy, is a challenging task. This is particularly true when spectra display strong overlapping and/or show high similarity (e.g. UV–Vis spectra). In this sense, genetic algorithm (GA) [25] and the successive projections algorithm (SPA) [29] have demonstrated to be excellent variable selection strategies for MLR calibration [30,31]. GA consists of guided random search techniques inspired on natural selection mechanisms, which explore the solution space in an efficient manner and are suitable for parallel processing implementations. However, due to their stochastic nature, results are 'realization dependent' and variable selection may not be reproducible. In SPA, the selection of variables is carried out as a combinatorial optimization problem with constraints. The projections take into account the matrix of instrumental responses for choosing subsets of variables with a small degree of multi-collinearity in order to minimize redundancy and ill-conditioning problems. Then, the search is restricted to a reduced number of variable subsets.

In this study, a new method based on FBA assisted by chemometric tools was developed for the simultaneous determination of CIP and DEX in ophthalmic and otic suspensions. The proposed method is an environmental friendly alternative to separation techniques. Indeed, no reagents are needed (the solvent used is water), small volumes of solutions are required and CIP and DEX standard solutions were in low concentrations. The results obtained for the determination of CIP and DEX in commercial samples were satisfactory and they are in close agreement with the ones obtained by the official method.

2. Experimental

2.1. Apparatus and software

Spectrophotometric measurements were carried out by using a Hewlett-Packard 8453 UV–Vis diode array spectrophotometer. Fluids in the flow-batch system were pumped with a Gilson Minipuls 3 peristaltic pump. Three-way solenoid valves (NResearch) were used in the system as selection valves. A mixing-detection chamber was designed in our laboratory in order to prepare the solutions and perform the detection in the same place. A lab-made stirrer system was designed to improve the mixing into the mixing-detection chamber. A Pentium 4 microcomputer furnished with a lab-made parallel interface card was used to control the peristaltic pump, valves and stirrer system, and also for data acquisition and treatment.

HPLC procedures were carried out on a Gilson liquid chromatograph equipped with a Gilson 322 pump, a Rheodyne 7725i injector and a diode array UV 170 Gilson detector. A 5 μm Restek C18 (250 mm × 4.6 mm i.d.) and a 5 μm Gemini C18 (250 mm × 4.6 mm i.d.) analytical columns were used for CIP and DEX, respectively.

The software used for controlling the flow-batch system was developed in Labview® 5.1 graphic language. PLS calculations were carried out using The Unscrambler® software (CAMO A/S) Version 9.5, MLR-GA and MLR-SPA were performed using subroutines developed in MATLAB® (The MathWorks) high-level programming language.

2.2. Reagents and solutions

Ultra pure water (18 MΩ cm) was used to prepare all solutions. Stock solutions of 240 mg L^{-1} ciprofloxacin (Saporiti, Buenos Aires, Argentina) and 80 mg L^{-1} dexamethasone (Saporiti, Buenos Aires, Argentina) were prepared in water. All stock solutions were protected from light and stored at 4 °C. Working standard solutions were prepared daily by appropriate dilutions of the stock solutions in water.

The analyzed ophthalmic suspensions were Fotamicin® (Elea), Decadron con Ciprofloxacina® (Sidus), Quidex® (Poen) and Procalm® (Atlas). The analyzed otic suspension was Otosporin Dexa® (Investi). All the pharmaceutical formulations were purchased from local pharmacy and the nominal concentrations, in all cases, were 3.0 mg mL^{-1} for CIP and 1 mg mL^{-1} for DEX.

A solution containing different excipients as benzalkonium chloride (100 mg L^{-1}), hydroxypropyl methyl cellulose (2500 mg L^{-1}), sodium acetate trihydrate (300 mg L^{-1}), acetic acid (6×10^{-3} mol L^{-1}), disodium EDTA (100 mg L^{-1}), mannitol (12,7 g L^{-1}), glycerin (15,0 g L^{-1}), Tyloxapol (500 mg L^{-1}) and sodium hydroxide (suitable volume to adjust pH) was prepared in water.

Table 1
Concentration data corresponding to the calibration and validation sets.

Type of mixture	Ciprofloxacin	Dexamethasone
Calibration mixtures		
1	0.80	0.80
2	4.00	0.80
3	2.40	0.27
4	2.40	0.33
5	1.27	0.43
6	3.53	0.43
7	1.27	1.17
8	3.53	1.17
9	2.40	0.80
Validation mixtures		
1	1.67	0.67
2	3.13	0.67
3	1.67	0.93
4	3.13	0.93

The values are expressed in mg L^{-1}.

2.3. Methods

2.3.1. Preparation of the calibration and validation sets

A calibration set consisting of nine samples was prepared in accordance with a central composite design with three central point replicates (Table 1). The concentration ranged from 0.80 to 4.00 mg L^{-1} for CIP and from 0.27 to 1.33 mg L^{-1} for DEX. The component ratios were selected considering the usual CIP/DEX relationship in the commercial pharmaceutical products. Also, a validation set with four mixtures was prepared in order to evaluate the predictive ability of the calibration models. The CIP and DEX concentrations in the validation set were comprised within the range used in the calibration set.

2.3.2. Sample preparation

The commercial pharmaceutical preparations are suspensions and they should be appropriately homogenized before they are analyzed. To do this, the recipient with the sample was inverted five times. An aliquot 200 μL of sample were immediately after took and was subsequently dissolved in 50 mL of water. All samples were analyzed by triplicate.

Samples for chromatographic CIP determination were prepared taking 1.00 mL of sample and diluting with mobile phase until 5.0 mL of final volume. For DEX determination, 1.00 mL of sample was made up with mobile phase to 25 mL. All samples were analyzed by triplicate.

2.3.3. Flow-batch analyzer

A schematic diagram of the proposed FBA is shown in Fig. 1. The system consisted of five channels, through which ciprofloxacin, dexamethasone, sample, water and waste flow. Each channel was controlled by a three-way solenoid valve. Valves V_1 to V_4 respectively allowed CIP, DEX, sample and water to flow toward the mixing-detection chamber (MDC) or recycle. The fifth valve (V_5) was used to control the emptying of the MDC. The flask of water connected to V_5 was used to ensure that the valve operated correctly when fluids were not carried from the MDC to the waste. Tygon® tubes were used in the five pumping channels. Tubes of 1.52 mm i.d. were used for CIP, DEX, sample and water, whereas a tube of 2.05 mm i.d. was used for waste. The flow rates for each channel were 3.36, 2.87, 3.31, 3.28 and 4.41 mL min^{-1} for CIP, DEX,

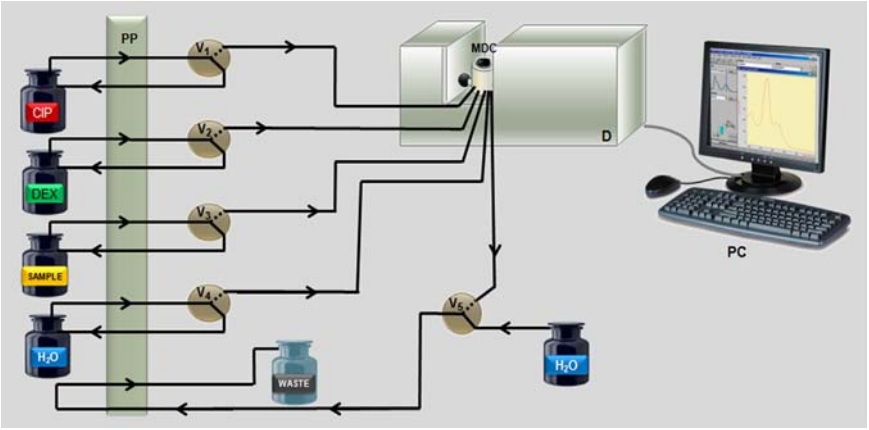

Fig. 1. Diagram of the FBA system at initial configuration. CIP, ciprofloxacin solution; D, Detector; DEX, dexamethasone solution; MDC, mixing-detection chamber; PC, microcomputer; PP, peristaltic pump; V, solenoid valves (V_1: CIP, V_2: DEX, V_3: sample, V_4: water, V_5: commutation MDC/water). The arrows indicate the direction of the fluids.

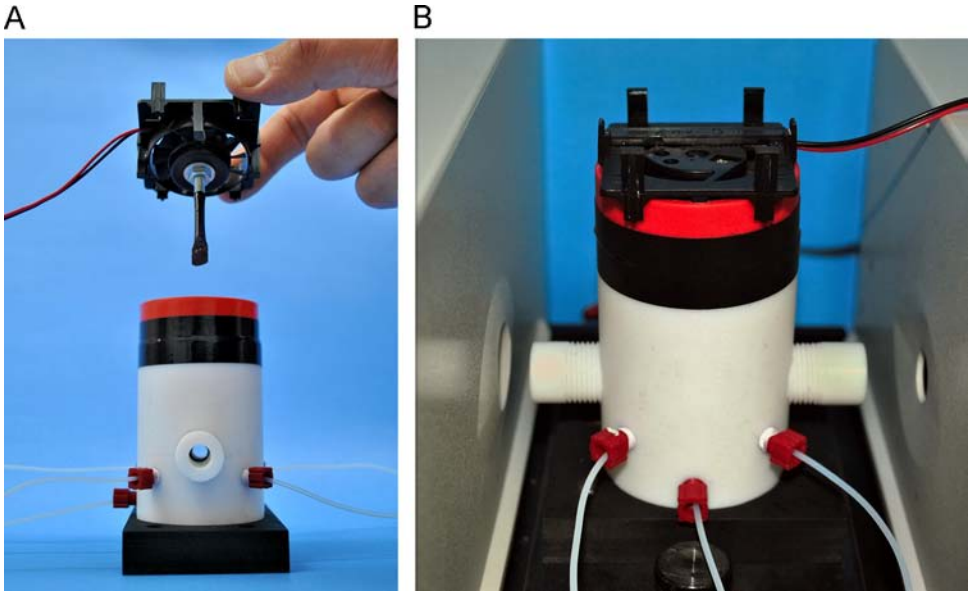

Fig. 2. (A) Lab-made mixing-detection chamber (MDC) constructed in Teflon®. The image shows the inlet connections, and the quartz windows. On the top of them there is located the stirring system with the corresponding Teflon® stirrer blade. (B) MDC coupled to the UV spectrophotometer by means of a lab-made cell holder.

sample, water and waste, respectively. The rest of tubing was of Teflon® (0.5 mm i.d.).

2.3.3.1. Mixing-detection chamber and stirring system. The MDC coupled to the stirring system was designed to obtain a rapid and efficient mixture of the solutions. The lab-made MDC was constructed with Teflon®. It had designed with four inlets and one output and it was equipped with two quartz windows, which are parallel to each other. In this way, the MDC could be used as a detection cell for UV–Vis measurements (Fig. 2a). The inner volume was 3.8 mL. In addition, a stirring system was constructed and assembled on the top of the MDC. The stirring system was made coupling a Teflon® stirrer blade to a cooler motor obtained from an Intel® microprocessor (DC 12 V, 0.06 A). This device was commanded by the electronic actuator, via software. In this way, when the motor is activated, the stirrer started the mixing process into the MDC.

The MDC/stirring system was coupled to the UV spectrophotometer by means of a lab-made cell holder (Fig. 2b).

2.3.4. Flow-batch procedure

Firstly, the time/volume relationship was evaluated for each solenoid valve in accordance with the procedure proposed by Almeida et al. [32]. Before starting the procedure, the valves were set in a way that all solutions in their respective channels were recycled toward their flasks (OFF position) (Fig. 1). Then, V_1, V_2, V_3, and V_4 valves were switched ON during 10 s and the solutions were pumped towards the MDC in order to fill the channels between the valves and the MDC. Immediately, V_5 was switched ON and the excess of the solutions contained in the MDC was aspirated to the waste during 20 s. This operation, called "filling of channels", consumed a total time of 30 s.

Then, the system was ready to carry on the preparation of the calibration and validation mixtures and the sample analysis. The blank signal was measured by switching ON the V_4 valve for 55 s. In this way, water was pumped towards the MDC and the blank was measured.

Preparation of calibration and validation mixtures were performed by sequentially switching ON the V_1, V_2 and V_4 valves during a previously defined intervals of time (t_1, t_2 and t_4 for each valve, respectively). Thus, aliquots of each standard solution and water were pumped toward the MDC. Then, calibration or validation mixtures were prepared varying only t_1, t_2 and t_4 values (Table 2). The mixture was homogenized by stirring for 5 s and remained in the MDC for 2 s. After that, the UV spectrum (232–370 nm) was recorded. Finally, the V_5 valve was switched ON during 60 s and the mixture was aspirated toward the waste. The same procedure was carried out to prepare the samples. Then, the V_3 and V_4 valves were switched ON during a t_3 and t_4 intervals of time (Table 2). In all cases, the total volume added into the MDC was the same, i.e. 3.0 mL. The system was always cleaned between measurements. The MDC cleaning procedure was carried out by switching ON V_4 valve during 55 s and activating the stirrer during 5 s. The total emptying of the MDC was assured by switching ON V_5 valve during 60 s (Table 2).

Table 2
Valve switching time intervals (expressed in seconds).

Operation	V_1	V_2	V_3	V_4	V_5
Filling channels	10.0	10.0	10.0	10.0	20.0
Wash system					
(a) MC filling	0	0	0	55.0	0
(b) MC empting	0	0	0	0	60.0
Blank	0	0	0	55.0	0
Calibration	3.6–17.9	4.2–18.4	0	22.7–43.0	0
Validation	7.5–13.9	10.3–14.9	0	27.6–38.2	0
Samples	0	0	10.9	43.9	0

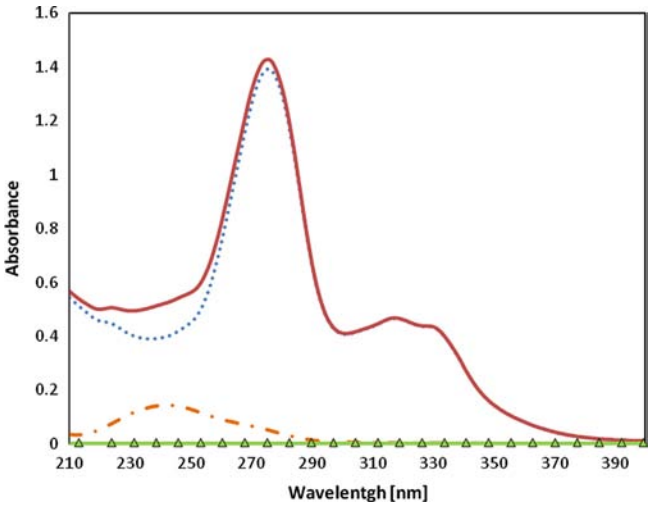

Fig. 3. Spectra of CIP (2.4 mg L^{-1}, dotted line), DEX (0.8 mg L^{-1}, dash-dotted line), CIP/DEX mixture (solid line) and a solution containing excipients (▲).

2.3.5. Data treatment

Spectra for calibration and validation mixtures and for the samples were recorded between 210 and 400 nm at steps of 1 nm (Fig. 3). Previously to the application of multivariate calibration, spectral data sets (i.e. the corresponding ones to test set, validation set and samples) were smoothed using the Savitsky–Golay algorithm [33] with a second order polynomial and an overall window size of 5 points. Then, the smoothed data sets and the corresponding concentrations matrices were mean centered.

2.3.5.1. PLS model. The PLS model was developed in the PLS-1 mode, i.e. the regression is carried out for each compound in an independent manner (in this case, only for DEX). The performance of the calibration model was evaluated using leave-one-out cross-validation. The number of significant factors was chosen using the Haaland and Thomas criterion [34]. An exploratory analysis of the spectra was carried out in order to find the suitable spectral region for PLS-1. The region between 232 and 302 nm was selected (Fig. 4a).

2.3.5.2. MLR model. The spectral region used for MRL model comprised the wavelengths between 232 and 370 nm (Fig. 4b and c). It is well-known that MLR is strongly affected by collinearity of spectral variables especially when the number of variables is high. Therefore, MLR calibration usually was performed in combination with variable selection techniques. In the current study, genetic algorithm (GA) and successive projections algorithm (SPA) were employed to select the most informative variables.

The GA algorithm was run using 80 generations of 100 chromosomes. Crossover and mutation probabilities were set at 60% and 5%, respectively. The fitness of an individual is the inverse of the root mean square error of prediction (RMSEP) for the MLR model using the wavelengths coded in its chromosome. The probability of a given individual being selected for the mating pool was proportional to its fitness. Population size was kept constant, each generation being completely replaced by its descendants and elitism was adopted.

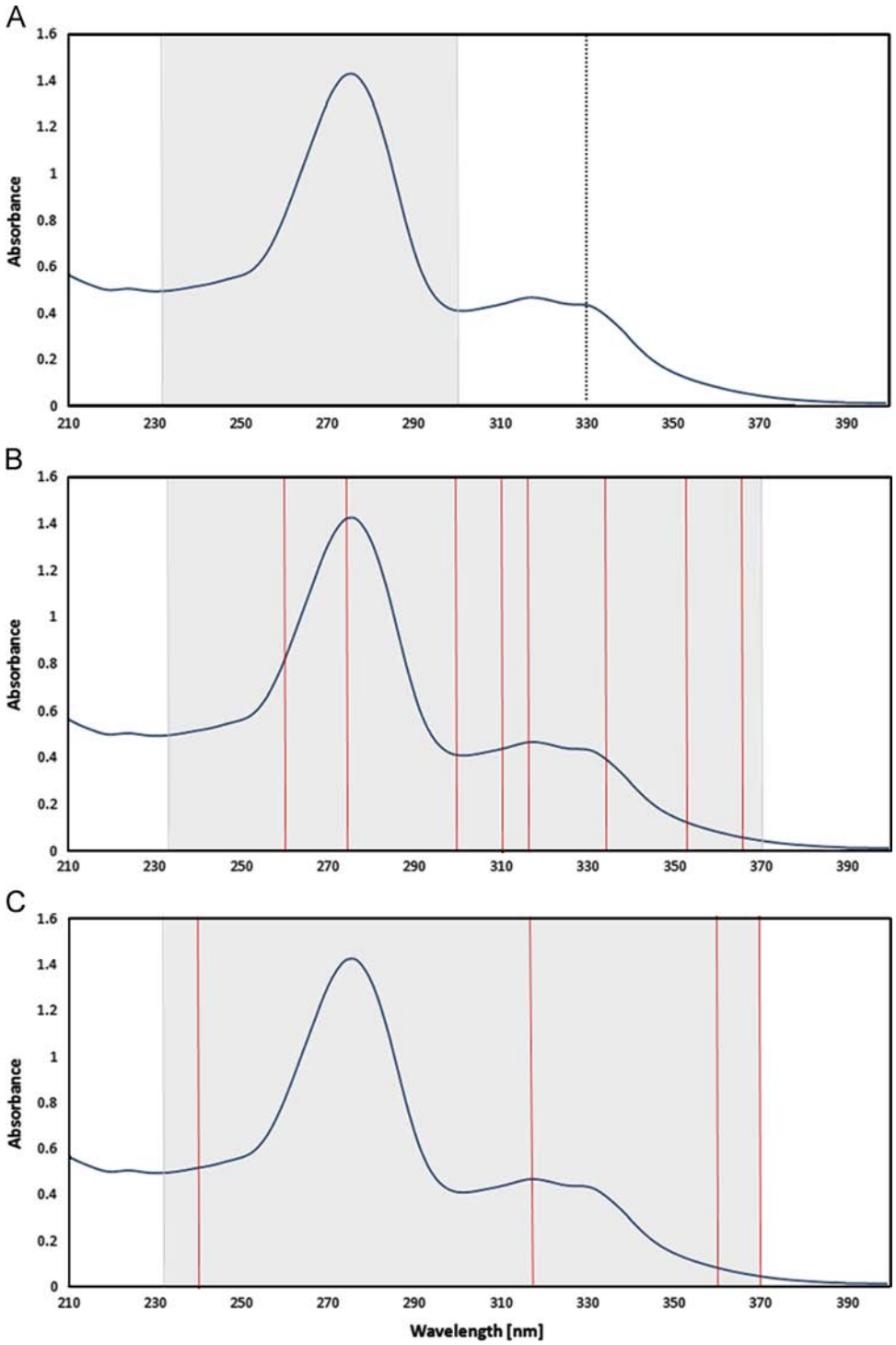

Fig. 4. (A) Selected variable (330 nm) for the determination of CIP by univariate calibration (dotted line) and spectral region used for the determination of DEX by PLS-1 (gray area). (B) Spectral region used for the application of MLR-GA (gray area) and selected variables (solid lines) for DEX determination. (C) Spectral region used for the application of MLR-SPA (gray area) and selected variables (solid lines) for DEX determination.

On the other hand, the essence of SPA consists of projection operations carried out on the calibration matrix. Starting from each of the J variables, SPA builds an ordered chain of K variables. Each new selected variable will be the one that shows the least collinearity with respect to the previous ones. The collinearity among variables is evaluated in terms of the correlation between the column vectors in the calibration matrix. In that way, a total of $J \times K$ subsets of variables can be obtained. Then, different MLR models ($J \times K$) are built with the calibration set, taking into account the subsets of variables obtained in the previous step. The more appropriate subset of variables is the one that provides the lower RMSEP when validation set is analyzed.

2.3.5.3. Statistical parameters. The comparison of the results obtained from the different chemometric models was based on

different statistical parameters. The root mean square error (RMSE) was calculated as:

$$RMSE = \left[\frac{\sum_{i=1}^{I}(C_{nom}-C_{pred})^2}{I}\right]^{1/2} \quad (1)$$

where C_{nom} and C_{pred} represent the nominal and predicted concentrations, respectively, and I is the total number of samples. This parameter was calculated for both calibration (RMSEC) and validation (RMSEP) sets. The relative error of prediction (RE) was calculated as:

$$RE = \frac{RMSE}{C_{mean}} \times 100 \quad (2)$$

where C_{mean} is the mean concentration (i.e. the central point in the experimental design). Also, the relative error was calculated for both calibration (REC) and validation (REP) sets. In addition, the models were evaluated in terms of sensitivity (SEN) and limit of detection (LOD). SEN was calculated as:

$$SEN_k = \frac{1}{||b_k||} \quad (3)$$

where the denominator is the Euclidean norm of the vector b_k, which is the vector of the regression coefficients obtained for component k. The limit of detection was calculated as follows:

$$LOD_k = 3.3\ \delta_r||b_k|| \quad (4)$$

where δ_r indicates the instrumental noise.

2.3.6. Chromatographic procedure

The chromatographic procedure was carried out according to the established reference methods for CIP and DEX [35,36]. The procedure used for CIP determination was carried out in an isocratic mode. Mobile phase consisted of an 87:13 mixture of phosphoric acid (0.025 mol L^{-1}) adjusted at pH 3 with triethylamine and acetonitrile. Typically, 20 μL of sample were injected and the flow rate was 1.5 mL min^{-1}. The column was thermostated at 40 °C and the signals were recorded at 278 nm. The chromatographic determination of DEX, was performed also in isocratic mode using a 60:40 mixture of water and acetonitrile as mobile phase. The sample volume injected was 20 μL and the flow rate was 2.0 mL min^{-1}. The column was thermostated at 25 °C and the signals were recorded at 254 nm. The standard solutions were prepared by dissolving CIP and DEX in their respective mobile phases. The peaks of the analytes were well-resolved and tailless. Retention times were 12.8 min and 16.5 min for CIP and DEX, respectively. Determinations were done by triplicate.

3. Results and discussion

3.1. Spectral features

CIP and DEX aqueous solutions show a strong spectral absorption between 210–370 nm and 210–300 nm, respectively (Fig. 3). Spectra recorded at different pH values (from 3.0 to 11.0) did not show significant differences from the ones obtained in aqueous medium. Thus, the calibration and validation sets and samples were prepared in water. As can be seen, the spectra of a mixture of both analytes show a serious overlapping in the spectral region between 210 and 300 nm. In addition, in the region ranging between 300 and 370 nm CIP has two absorption bands (with maxima at 318 and 330 nm) whereas DEX and excipients do not absorb. Thus, this region could be useful for univariate determination of CIP. On the other hand, the determination of DEX by univariate calibration is not possible without performing a previous separation technique.

Moreover, in the analyzed commercial pharmaceutical preparations, DEX is a minor constituent and, at these concentration levels, DEX spectrum is completely embedded into the CIP spectrum. Thus, the quantification and resolution of mixtures results even more complex. Overcoming this problem involves using multivariate calibration techniques. In this sense, PLS-1 and MLR were applied.

3.2. Optimization of variables of the FBA system

FBA performed the automatic preparation of the calibration and validation mixtures and made it possible to significantly reduce the total time of sample analyses. The following variables were optimized: final volume in the MDC, stirring time and stabilization time (prior to detection). The optimal volume in the MDC should be the lower one that ensures the correct spectroscopic measurements, i.e. the minimum volume should surpass the quartz windows in the chamber. Thus, a volume of 3.0 mL was selected.

The stirring time was selected as the minimum time that guarantees a well mixing in the MDC, which was evaluated in terms of spectral reproducibility. The optimum time was 5 s.

The stabilization time was the interval of time elapsed between the stop of stirring and the spectroscopic measurement. The optimum value (2 s) was selected following the same criterion used in the optimization of stirring time.

Flow rates optimization was carried out in order to obtain the lower time of analysis and the maximum precision in the preparation of standard solutions and samples. Higher flow rates sensibly reduce the time of analysis but lead to high errors in the concentrations of standard solutions and low reproducibility among measurements. Thus, as a compromise solution, the flow rates for each channel were set at 3.36, 2.87, 3.31 and 3.28 mL min^{-1} for CIP, DEX, sample and water, respectively.

On the basis of the optimized variables, the calculated sample throughput was 10 h^{-1}.

3.2.1. Analytical performance for CIP determination

As was mentioned in Section 3.1, CIP was determined using univariate linear calibration that was performed by ordinary least squares (OLS). The maximum at 318 nm is slightly higher than the maximum at 330 nm (Fig. 4a). However, the signal at 330 nm was selected for further analyses because the error of prediction obtained working at this wavelength was lower than the one obtained in the determination of CIP at 318 nm. The regression line obtained was $A=(0.047\pm0.001)\times C+(0.004\pm0.003)$, where A is the absorbance and C is the concentration in mg L^{-1}. As can be seen from Table 3, determination of CIP at 330 nm presented a satisfactory analytical performance.

3.2.2. Analytical performance for DEX determination

3.2.2.1. PLS-1 model. Table 3 shows the statistical parameters for PLS-1 model. Three latent variables were used to explain a 99.98% of the data variance for the X-block and 99.96% for the Y-block. The values of REC and REP were acceptable (lower than 5%). Also, Table 3 shows satisfactory values for SEN and LOD.

3.2.2.2. MLR-GA and MLR-SPA models. Fig. 4b and c shows the variables selected by GA and SPA algorithms, respectively. GA selected eight variables. Two of them (261 and 273 nm) were selected in the spectral range in which DEX absorbs. On the other hand, SPA selected four variables and only one of them (241 nm) was chosen from the region in which DEX absorbs.

Then, calibration models were constructed applying MLR to the selected variables. Table 3 summarizes the statistical parameters for both MLR-GA and MLR-SPA. The values of REC and REP

Table 3
Results obtained from the application of OLS, PLS-1, MLR-GA and MLR-SPA to the spectral data.

Figures of merit	CIP	DEX		
	OLS	PLS-1	MLR-GA	MLR-SPA
Spectral region (nm)	–	232–302	232–370	232–370
Number of factors	–	3	–	–
SEN (mL μL^{-1})	0.047[a]	0.235	0.008	0.015
LOD (μg mL^{-1})	0.410[a]	0.014	0.426	0.225
Selected variables (nm)	330	–	261, 273, 300, 310, 316, 335, 353, 366	241, 317, 360, 370
Calibration				
Concentration range (μg mL^{-1})	0.80–4.00	0.27–1.33		
RMSEC (μg mL^{-1})	0.101	0.016	0.010	0.010
REC (%)	4.20	1.96	1.25	1.27
Correlation	0.991	0.998	0.999_0	0.999_0
Validation				
Concentration range (μg mL^{-1})	1.66–3.13	0.67–0.93		
RMSEP (μg mL^{-1})	0.117	0.018	0.014	0.015
REP (%)	4.88	2.20	1.79	1.88

[a] For OLS, SEN and LOD was calculated as described in [38].

Table 4
Determination of CIP and DEX in pharmaceutical preparations.

Samples	Nominal		HPLC		Proposed method			
	CIP	DEX	CIP	DEX	CIP	DEX		
					OLS	PLS-1	MLR-GA	MLR-SPA
Fotamicin®	300	100	295 (2)	94 (1)	293 (3)	100 (2)	108 (1)	99 (2)
Decadron con Ciprofloxacina®	300	100	309 (2)	96 (2)	332 (3)	99 (3)	99 (3)	100 (3)
Quidex®	300	100	301 (1)	115 (2)	316 (1)	108 (1)	113 (1)	104 (1)
Procalm®	300	100	329 (2)	99 (2)	326 (3)	93 (1)	99 (3)	92 (1)
Otosporin Dexa®	300	100	314 (1)	99 (1)	321 (4)	99 (3)	101 (2)	97 (2)

The concentrations are expressed in mg/100 mL. The samples were analyzed in triplicate. Standard deviations are in parenthesis.

obtained were lower than 5% and comparable with the obtained ones for PLS. Therefore, statistical parameters indicate that MLR-GA and MLR-SPA are accurate in the prediction of synthetic samples (i.e. validation set). Also, Table 3 shows that values of SEN and LOD were satisfactory for both MLR-GA and MLR-SPA models.

3.3. Interference study

A frequent problem in the analyses of pharmaceuticals is the presence of excipients. Particularly, ophthalmic and otic suspensions contain a high number of compounds as preservatives, viscogens, antioxidants, surfactants, pH modifiers, osmotics and suspending, chelating, wetting and solubilizing agents. For this reason, a spectral analysis of these compounds was carried out. Therefore, a solution containing the excipients detailed in Section 2.2 was prepared at the concentration levels normally found in the commercial formulations. Fig. 3 shows that the excipients do not absorb in the spectral region used for the determination of both CIP and DEX (232–370 nm).

3.4. Application to real samples

The proposed method was used for the simultaneous determination of CIP and DEX in pharmaceutical preparations. Table 4 shows the results obtained for CIP univariate determination (OLS) and for the multivariate approaches used for DEX determination (i.e. PLS-1, MLR-GA and MLR-SPA). Also, Table 4 shows the results obtained from the application of the pharmacopeial method based on HPLC [35,36]. The intermediate precision was evaluated in different days (i.e. different calibration sets), and different operators. The values were expressed in terms of relative standard deviation (RSD, $n=3$) and the obtained values were 3.47% for CIP and 6.48, 8.11 and 8.40% for PLS-1, MLR-GA and MLR-SPA, respectively.

Validation of trueness was carried out by comparison of the results obtained by the proposed method with the ones obtained by HPLC, i.e. the reference method. For all analyzed samples, the obtained results for both analytes were in close agreement with those obtained by HPLC. The statistical comparison was carried out using the joint interval test for the slope and the intercept [37]. The values obtained by the proposed method were regressed against the reference values (i.e. HPLC). The estimated intercept (a) and slope (b) obtained, were compared with the ideal values of intercept=0 and slope=1. The elliptical joint confidence regions obtained (Fig. 5a and b) indicate that there is no significant statistical differences between the results obtained by both methods, considering an overall significance level of $\alpha=0.05$. From these results it can be concluded that, in comparison to the reference method, there is no bias in the results obtained for CIP and DEX.

Pharmacopeias [35,36] establish a tolerance of $\pm 10\%$ for the content in CIP and DEX respect to the declared value on the pharmaceutical label. In this sense, all the commercial samples analyzed were in agreement with the requirements of Pharmacopeias, except for DEX in Quidex® and CIP in Decadron con Ciprofloxacina®, for which the values obtained were slightly higher than the tolerance.

The applied multivariate methods gave comparable results for DEX determination in real samples. The best performance in terms of sensitivity and limit of detection was obtained for PLS-1, which showed values for these parameters that differ about one order of

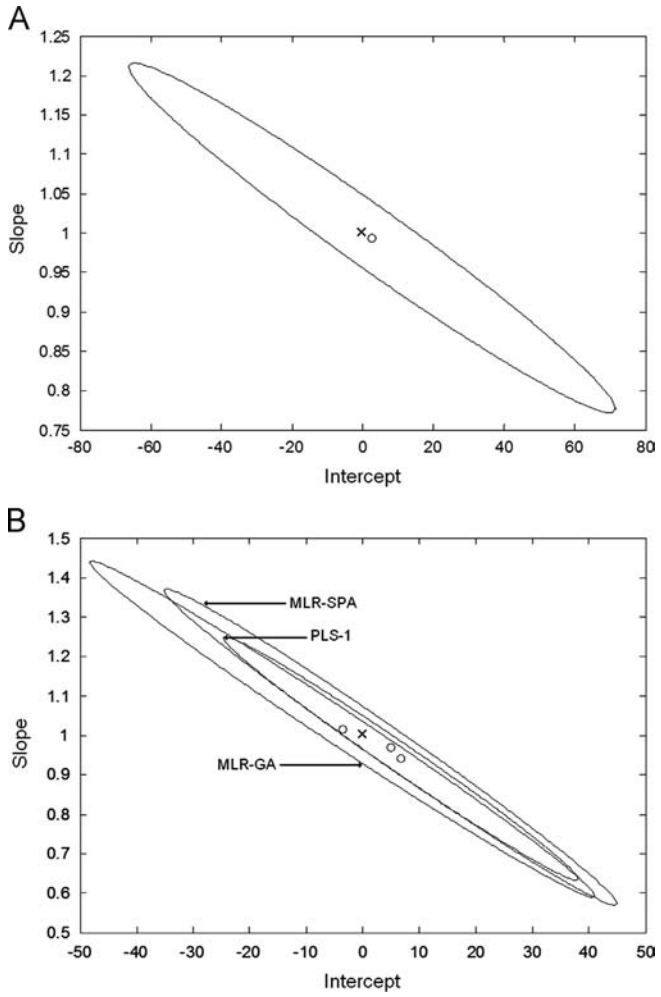

Fig. 5. Elliptical joint confidence regions for the slope (b) and intercept (a) corresponding to regressions OLS, PLS-1, MLR-GA and MLR-SPA predicted concentrations vs. the reference method (HPLC): (**A**) CIP and (**B**) DEX. The cross marks the theoretical point (intercept=0, slope=1) and the circle indicates the point (a,b).

magnitude of the obtained ones by MLR (either for GA or SPA as algorithms of variables selection). However, MLR is based on a simpler algorithm than PLS-1 which, in addition, handles latent variables that have no physical meaning. On the other hand, MLR depends on an appropriate selection of variables to minimize the errors arising from collinearity. In this sense, both GA and SPA provided satisfactory results. Nevertheless, GA selected a higher number of variables and, due to their stochastic nature, results are realization-dependent and variable selection may be not reproducible [29].

3.5. Green approach of the proposed method

In accordance with the principles of Green Analytical Chemistry, an ideal methodology should consist of a reagentless procedure, nondestructive, fast and capable of determining as many analytes as possible in a single run with a reduced energy consumption [8].

The proposed method fulfills several of the 12 principles of green chemistry [9]. The green aspects of the proposed methodology can be remarked in these points:

Reagentless procedure. An environmentally friendly analytical procedure should ideally avoid the use of hazardous chemicals. The proposed method fulfills this principle, because the only solvent used was water, no reagents were required and the standard solutions and samples were all in low concentrations. On the contrary, separation techniques normally used to determine this kind of analytes (i.e. HPLC) [35,36] often use organic solvents and some of them are not environmental friendly. From this point of view, the proposed method has an important advantage.

Analytes quantification without any chemical sample pretreatment and separation techniques. This point is closely related to the previous one. This advantage was achieved by using mathematical data treatments. The chemometric tools made it possible to reduce the time of analysis and, in many cases, to avoid using separation techniques [9].

Minimization of wastes and reuse of chemicals. In this sense, multicommutation allowed the reagents to be recycled and reused. As a consequence, reagent consumption, waste generation and, also, costs were minimized. This is in agreement with the first principle of the Green Chemistry [7,9].

In addition, potential risks for analysts were reduced by using a closed system and the automation minimizes human exposition to substances and makes the analytical operation less dependent on the operator [7].

4. Conclusion

The proposed method, based on the green chemistry approach, has been developed to simultaneously determine CIP and DEX in ophthalmic and otic suspensions. The analysis was carried out using water as solvent, without using toxic reagents or solvents. The FBA made it possible to use small volumes of CIP and DEX standard solutions and samples. Moreover, all solutions were recycling while they were not used, so the production of waste was minimal.

In addition, calibration and validation sets, based on a central composite design, were automatically prepared in the FBA system with the consequent saving of time. Furthermore, the sample throughput was 10 h^{-1}.

The CIP quantification was possible using univariate calibration at 330 nm. On the other hand, the spectral overlapping prevented the univariate determination of DEX. This hindrance was solved by application of multivariate calibration techniques. Both PLS-1 and MLR models showed a satisfactory analytical performance. Despite the fact that PLS-1 gave better sensitivity and limit of detection, the variable selection performed by SPA made it possible to obtain a MLR model with slightly better prediction errors than PLS-1 and MLR-GA, and only using four spectral variables. The validation was based on the analysis of the commercial samples by the official method (HPLC) and the obtained results were satisfactory for both CIP and DEX. The proposed method is environmental friendly, technically feasible and economically reasonable. Thus, it represents an interesting alternative for the quality control of these pharmaceutical preparations.

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