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Flow-batch technique for the simultaneous enzymatic determination of levodopa and carbidopa in pharmaceuticals using PLS and successive projections algorithm

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Abstract

An enzymatic flow-batch system with spectrophotometric detection was developed for simultaneous determination of levodopa [(*S*)-2 amino-3-(3,4-dihydroxyphenyl)propionic acid] and carbidopa [(*S*)-3-(3,4-dihydroxyphenyl)-2-hydrazino-2-methylpropionic acid] in pharmaceutical preparations. The data were analysed by univariate method, partial least squares (PLS) and a novel variable selection for multiple lineal regression (MLR), the successive projections algorithm (SPA). The enzyme polyphenol oxidase (PPO; EC 1.14.18.1) obtained from *Ipomoea batatas* (L.) Lam. was used to oxidize both analytes to their respective dopaquinones, which presented a strong absorption between 295 and 540 nm. The statistical parameters (RMSE and correlation coefficient) calculated after the PLS in the spectral region between 295 and 540 nm and MLR-SPA application were appropriate for levodopa and carbidopa. A comparative study of univariate, PLS, in different ranges, and MLR-SPA chemometrics models, was carried out by applying the elliptical joint confidence region (EJCR) test. The results were satisfactory for PLS in the spectral region between 295 and 540 nm and for MLR-SPA. Tablets of commercial samples were analysed and the results obtained are in close agreement with both, spectrophotometric and HPLC pharmacopeia methods. The sample throughput was 18 h^{-1}.

Keywords: Flow-batch; Polyphenol oxidase; Levodopa; Carbidopa; PLS; MLR-SPA

1. Introduction

Levodopa [(*S*)-2 amino-3-(3,4-dihydroxyphenyl)propionic acid] is a precursor of the neurotransmitter dopamine used in the treatment of Parkinson's disease. It is a progressive neurological disorder that occurs when the brain fails to produce enough dopamine. This condition causes tremor, muscle stiffness or rigidity, slowness of movement and lost of balance.

Dopamine cannot be administered directly because it does not cross the blood brain barrier readily. Therefore, its precursor levodopa is given orally, which is easily absorbed through the bowel, there, the dopamine is formed by the action of the decarboxylase. High levels of dopamine also cause adverse reactions such as nausea, vomiting and cardiac arrhythmias. Usually, the peripheral decarboxylation of levodopa in extracerebral tissues is associated with an inhibitor of peripheral aromatic-L-amino acid decarboxilase, such as carbidopa [(*S*)-3-(3,4-dihydroxyphenyl)-2-hydrazino-2-methylpropionic acid]. Thus, the importance of the presence of carbidopa (CBD) together with levodopa (LVD) makes that the dopamine levels can be controlled properly. Also, it was observed that the side effects are reduced [1,2].

In order to achieve better curative effect and lower toxicity, it is very important to control the content of levodopa and carbidopa in pharmaceutical tablets. The most frequently analytical technique, used in quality control analyses of pharmaceutical products, is high-performance liquid chromatography (HPLC). However, this technique is expensive, labour-intensive task, time consuming and produces chemical waste.

The polyphenol oxidase (PPO; EC 1.14.18.1) is an enzyme widely distributed in the nature. This enzyme catalyses the oxidation of LVD and CBD to the corresponding dopaquinone

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which is converted to leucodopachrome by a rapid and spontaneous auto-oxidation. Then, the leucodopachrome is oxidized to its corresponding dopachrome. This kind of chemical reaction, where the dopachrome was formed, is produced with both analytes LVD and CBD and the products of both reactions present a strong absorption in the UV–vis spectra [3–5].

LVD and CBD determinations are commonly carried out by using high-performance liquid chromatography (HPLC) [6–8], capillary electrophoresis (CE) [9] and chemometrics-assisted spectrophotometric method [10]. Fatibello-Filho et al. [11] have published a paper on the FI spectrophotometric determination of LVD and CBD applying univariate calibration and using polyphenol oxidase. However, the mixture of both analytes shows a serious spectral overlapping, after the chemical reaction with the enzyme. Moreover, such flow manifold requires significant changes in their physical assemblies when it is necessary to analyse samples with a large variation of analyte concentration and/or physical–chemical properties.

Automated micro batch (AMBA) proposed by Sweileh and Dasgupta [12,13], and flow-batch analysers (FBA) proposed, developed and first named by Araújo et al. [14] constitute an excellent alternative to automate the quality control of pharmaceutical products because they are systems very flexible and versatile (multi-task characteristic). With AMBA or FBA, it is possible to work in wide analyte concentration range as well as to implement different analytical processes [14–25] without significant alterations on the physical configurations of the analyser. All these may be accomplished just by changing the operational parameters in their control software. These analysers have been used to implement several analytical procedures such as: liquid–liquid extraction [12], distillation of volatile analyte [13], kinetic approach [13], titrations [14,15], analyte addition [16,17], internal standard [18], screening analysis [19], exploitation of concentration gradients [20], on-line matching of pH [21] and salinity [22] and sample digestion [23].

FBA and AMBA combine favorable characteristics of both flow (FA) and batch analysers (BA). As in FA, the transportation of reagents, samples or other solutions are carried out in a flow mode, and, as in BA, the sample processing is carried out into a mixing chamber (MC). In AMBA, an injecting loop is used on the sampling stage (as in FA), while in FBA the sample amounts are added into the MC by controlling the ON switching time of one solenoid valve. As most of the FA, FBA and AMBA also present good precision and accuracy, high sample throughput and low contamination, consumption, manipulation of reagents and samples, cost per analysis and waste liberation for the environment, etc. Moreover, these analysers present high sensitivity because the physical and chemical equilibria inherent to the analytical processes may be attained and the dispersion and/or dilution of the samples may be negligible. In another hand, the analytical signal measurements can be performed in flow cells or directly inside MC and the multicommutation [24,25] may be used in order to manipulate the fluids in a simultaneous and/or in an intermittent way.

The application of multivariate calibration methods, such as multiple linear regression (MLR), principal component regression (PCR) and partial least squares (PLS), to the spectrometric data may require the use of variable selection for constructing well-fitted models. Several authors have presented theoretical and empirical evidence supporting the use of variable selection to improve the predictive ability of PCR, PLS [26–28] and, principally, MLR models. MLR yields models which are simpler and easier to interpret than PCR and PLS, since these calibration techniques perform regression on latent variables, which do not have physical meaning. In another hand, MLR calibration is more dependent on the spectral variables selection. To overcome this problem, Araújo et al. proposed a novel variable selection strategy for MLR calibration, which uses the "successive projections algorithm" (SPA) to minimize collinearity problems [29,30]. SPA is a forward selection method which operates on the instrumental response. The number of variables to be selected can be optimized in order to maximize model prediction capability [29].

The aim of the present work was to propose a chemometric-assisted flow-batch method for the simultaneous spectrophotometric determination of levodopa and carbidopa in medicaments. The method was based on the enzymatic oxidation of LVD and CBD with PPO obtained from a natural source (*Ipomoea batatas* (L.) Lam), in phosphate buffer medium (pH 7.0).

2. Experimental

2.1. Apparatus and software

Centrifugation of extracts was performed in a refrigerated-automatic Sigma centrifuge. The spectrometric measurements were carried out by using a Hewlett-Packard model 8453 UV-visible diode array spectrophotometer with a Hellma flow cell (inner volume of 18 μL). A model 713 Metrohm pHmeter was used to carry out the pH measurements.

SPA and MLR calculations and pre-selection were performed using programs developed in our own laboratory with the MATLAB®, Version 5.3 high-level programming language. The UNSCRAMBLER® chemometrics software (CAMO A/S), Version 9.5, was used for PLS calculations.

2.2. Reagents and solutions

All reagents were of analytical grade. To prepare all solutions ultra pure water (18 MΩ) was used.

A 0.1 mol L^{-1} phosphate buffer solution (pH 7.0) was prepared. 0.05 mol L^{-1} catechol stock solution used as substrate for enzymatic activity determination was prepared by dissolving 0.1375 g of catechol (Anedra) in 25 mL of the buffer solution.

Stock solutions of LVD (Saporiti) and CBD (Saporiti) of 0.800 mg mL^{-1} and 0.400 mg mL^{-1} respectively were prepared in medium of phosphate buffer. All stock solutions were protected from light and stored at 4 °C. The working standard solutions were prepared by adequate dilutions of the stock solutions in medium of phosphate buffer.

Dowex 1 × 8 100–200 mesh (Fluka) strong basic, quaternary ammonium anion exchange resin was used as a protective and stabiliser agent in the sweet potato extract preparation. A 0.015%

m/v sodium azide (Sigma) solution was added to the extract as an antimicrobial agent in order to increase its lifetime.

Sweet potato roots (*I. batatas* (L.) Lam.) purchased in local supermarkets, were washed, hand-peeled, chopped and frozen at −18 °C before use.

The drug samples containing the pharmaceutical preparations Lebocar® (Pfizer, Searle) and Parkinel® (Bagó) were purchased in a local pharmacy. These preparations are presented in the form of tablets, with a nominal content of 250 mg of LVD and 25 mg CBD (Lebocar® and Parkinel® A) or 100 mg of LVD and 25 mg CBD (Lebocar® and Parkinel® B) and excipients until arriving at the weight of approximately 400 and 230 mg per tablet, respectively. In this work, both preparations (A and B) were analysed.

A solution containing different excipients as microcrystalline cellulose, maize starch, magnesium stearate and colloidal anhydride silica (Saporiti) was prepared in adequate proportions in a buffer phosphate medium.

2.3. Methods

2.3.1. Extraction of PPO of sweet potato root

An amount of 25 g of *I. batatas* previously frozen was cut into small pieces and placed in a liquefier and added 100 mL of 0.1 mol L^{-1} phosphate buffer (pH 7.0) and 2.5 g of resin. It was homogenized for 3 min in a temperature range of 4–6 °C. The homogenate was rapidly filtered through two layers of cheesecloth and centrifuged at 15,000 rpm for 60 min, at 4 °C. The resulting supernatant was separated and stored at −18 °C in a freezer with previous addition of 0.015% sodium azide solution. This crude extract was then used as an enzymatic source in the flow-batch procedure.

2.3.2. Measurement of PPO activity

The PPO activity in the crude extract was determined. The *o*-quinones were obtained when 0.2 mL of PPO was mixed with 2.8 mL of 0.05 mol L^{-1} catechol and buffer phosphate to 5.0 mL. The classical spectrometric method was used for the absorption measurements of the *o*-quinones at 410 nm.

One unit of PPO activity is defined as the amount of enzyme that causes an increase of 0.001 absorbance units per minute, under the conditions described above [31].

2.3.3. Preparation of the calibration and validation sets

A calibration set of nine samples was prepared following a central composite design with three central point replicates. The concentration ranges were from 0.057 to 0.553 mg mL^{-1} of LVD and from 0.021 to 0.070 mg mL^{-1} of CBD (Fig. 1). The component ratios were selected considering the usual LVD/CBD relationship in the commercial pharmaceutical products, i.e., from 4:1 to 10:1.

To evaluate the predictive capacity of the model of calibration, a validation set with eight mixtures were prepared in concentrations comprised within those of calibration.

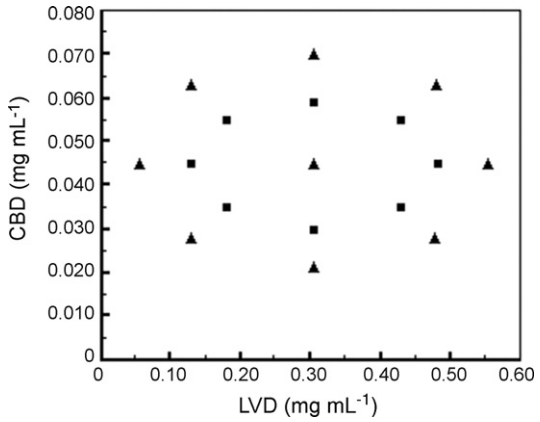

Fig. 1. Calibration (▲) and validation (■) concentrations of LDV and CBD corresponding to proposed experimental design.

2.3.4. Sample preparation

Ten tablets of each commercial pharmaceutical preparation were weighed, finely powered and homogenized, and a suitable amount of the obtained powder was weighed and dissolved in 100 mL of phosphate buffer. Then, it was filtered on-line by passing through a tubular cotton filter in the flow-batch system.

2.3.5. Flow-batch system

A schematic flow diagram of the proposed flow-batch analyser is shown in Fig. 2. Six Cole Parmer three-way solenoid valves were used: five of them (V_{LVD}, V_{CBD}, V_S, V_{BS} and V_{PPO}) to send the flows of LVD, CBD, sample, buffer solution and PPO towards mixing chamber (MC), respectively. The sixth valve ($V_{MC/W}$) was used to select the stream flowing of mixture coming from the MC or water, through the flow-cell. A Pentium 166 MHz microcomputer furnished with a laboratory-made parallel interface card was used to control the peristaltic pump and valves and to perform the acquisition and treatment of data. The software was developed in Labview® 5.1 graphic language. An electronic actuator (EA) increased the power of the microcomputer signal in order to control the valves.

A Minipuls 3 Gilson peristaltic pump, equipped with six pumping channels was used. Six Tygon® tubes of 1.29 mm i.d. were used. A home-made mixing chamber (MC) of 2 mL was constructed in Teflon®. The lines linking the valves V_{LVD}, V_{CBD}, V_S, V_{BS} and V_{PPO} to MC and $V_{MC/W}$ to detector were implemented as short as possible using 0.8 mm i.d. Teflon® tubing.

2.3.6. Flow-batch procedure

One step inherent to flow-batch technique implementation is to obtain the signals that are used to correct the responses for volume changes or the flow-rates of the channels and then performing the calibration and subsequent sample analysis. This step can be easily implemented through the procedure described by Almeida et al. [16]. As the ratio between the flow rates of two channels has varied into a range of 1.00–1.10, it was necessary to apply a correction factor on the timing control of the delivered volumes in some channels.

Before starting the procedure, all solutions in their respective channels were pumped and recycled towards their flasks (Fig. 2).

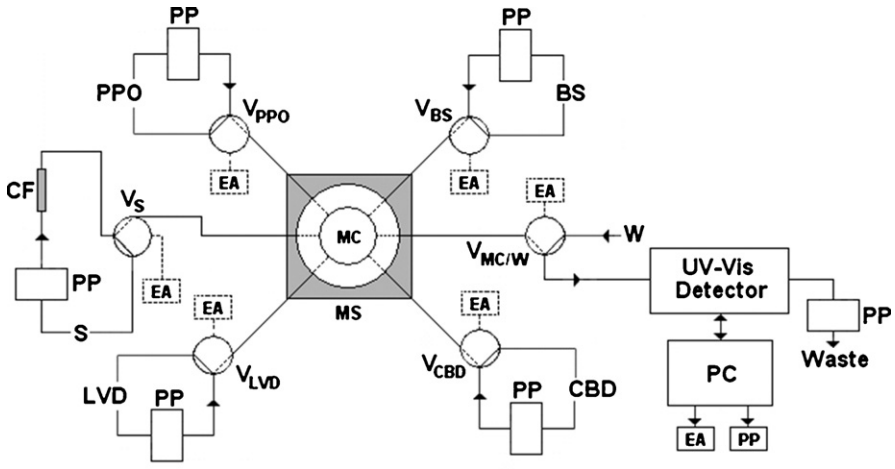

Fig. 2. Diagram of the flow-batch system at initial configuration. BS: buffer solution; CBD; carbidopa; CF: cotton filter; EA: electronic actuator; LVD: levodopa; MC: mixing chamber; MC/W: commutation MC/water; MS: magnetic stirrer; PC: microcomputer; PP: peristaltic pump; PPO: polyphenol oxidase; S: sample; V: solenoid valves; W: water. The arrows and the dotted lines indicate the direction of the fluids and the control lines, respectively.

After, each valve was switched ON during a time interval of 3 s and the solutions were pumped towards MC in order to fill the channels between the V_{LVD}, V_{CBD}, V_S, V_{BS} and V_{PPO} valves and MC. Right after, $V_{MC/W}$ was switched ON and the excess of the solutions into MC was aspirated to waste during 5 s. This operation, denominated "fill channels", consumed a total time interval of 8 s and it was always accomplished when the solution in each channel was changed.

The MC cleaning was carried out by switching ON V_{BS} valve for a time interval enough to assure the total emptying of the MC after finishing the operation. The system was cleaned always between measurements and the stirrer was ON during all the steps. After performing the filling channels and cleaning procedures, the system was ready to carry on the preparation of the standard solutions.

To perform the construction of the calibration or validation sets all the valves were initially switched OFF, so that the LVD, CBD, sample, buffer and PPO were continuously pumped into their channels, returning to their respective recipients. For the acquisition of the baseline, a blank solution was yielded by switching ON the V_{BS} and V_{PPO} valves for suitable times (t_{BS} and t_{PPO}) and afterwards it was aspirated towards the spectrophotometer. The valves V_{LVD}, V_{CBD}, V_S and V_{BS} were then simultaneously switched ON during previously defined time intervals for each valve (t_{LVD}, t_{CBD}, t_S and t_{BS}) and aliquots of each fluid were pumped towards the MC. Soon after, the V_{PPO} valve was switched ON and the reaction started (time 0 s of the reaction). Then the mixture was homogenised for 12 s and aspirated towards the spectrophotometer, by switching ON $V_{MC/W}$ for 20 s allowing the fulfilling of the flow cell. After that, the flow was stopped switching OFF the $V_{MC/W}$ valve. The delay time was 51.5 s and the stopped flow time was 128.5 s (for more details see Section 3) for each point of the calibration/validation sets and samples. Then, the spectra corresponding to each mixture yielded was recorded for posterior chemometric treatment by using PLS and MLR-SPA models. This procedure was repeated for each standard solution and sample, varying only the t_{LVD}, t_{CBD}, t_S and t_{BS} values.

In the experimental design of the multivariate calibration, the total volume that was added into MC was the same in all points of the calibration/validation sets in order to maintain the constant matrix composition or matrix effect, avoiding inaccurate results [32]. Thus, t_S and t_{PPO} were always the same and, while t_{LVD} and t_{CBD} increased, t_{BS} decreased (and vice versa).

Table 1
System operation schedule

Flow rate (mL min^{-1})	V_{LVD} 2.20	V_{CBD} 2.09	V_S 2.25	V_{BS} 2.18	V_{PPO} 2.30	$V_{MC/W}$ 2.30
Valve switching time intervals (s)						
Filling channels	3	3	3	3	3	5
Wash system						
(a) MC filling	0	0	0	47	0	0
(b) MC empting	0	0	0	0	0	50
Blank	0	0	0	27.5	19.5	0
Calibration	1.3–12.5	3.8–12.6	0	5.7–19.7	19.5	0
Validation	2.9–10.9	5.4–10.6	0	8.2–17.4	19.5	0
Samples	0	0	13.3	13.8	19.5	0
Delay (MC to spectrophotometric cell)	0	0	0	0	0	20

Table 2
Optimum values of chemical variables and flow-batch parameters

	Optimum value
Chemical variables	
Enzymatic activity (UE)	1200
Buffer concentration (mol L^{-1})	0.1
pH	7.0
Flow-batch parameters	
(a) Volume (mL)	
Sample	0.50
Buffer	0.50
Enzyme	0.75
Total volume	1.75
(b) Time (s)	
Delay	51.5
Mixture	12
Stopped-flow	128.5
Total analyses[a]	200

[a] Include times are not described in this table.

In Table 1 is summarized the schedule of procedures and the respective switching ON times of the valves to carry on the complete analysis.

2.3.7. Optimization of the reaction time

In order to obtain the optimum reaction time for the oxidation of LVD and CBD with PPO, a kinetic study was carried out. Thus, 1200 units of PPO reacted in phosphate buffer medium (pH 7) with the mixtures corresponding to the calibration and validation sets. The spectra were registered at each 60 s and during 600 s.

3. Results and discussion

3.1. Enzymatic activity

The crude extract was obtained from different commercial sweet potato roots along 2 months. The mean of enzymatic activity and the standard deviation ($n = 4$) was 4511 ± 287 UE mL^{-1}. When the extract was stored at −18 °C, these values did not vary along 1 month. Therefore, the conditions for obtaining crude extract were satisfactory.

3.2. Optimization of chemical variables and flow-batch parameters

In Table 2 are presented the optima values to chemical variables. The enzymatic activity was optimized in order to obtain the optimum spectral values for PLS and MLR-SPA models, so we tested between 1000 and 2000 UE. The selected value was 1200 UE.

In the case of the buffer solution, the working pH was established according to literature results at 7.0 (0.1 mol L^{-1} phosphate buffer) [33].

Optima values for flow-batch parameters are also shown in Table 2. The optimization was performed by using the univariate method and selected as a compromise between sensitivity and reproducibility of the analytical signals. The MC was proper enough because a good mixture between the enzyme and the substrate was obtained. Besides, the flow-batch system performed the automatic preparation of the calibration and validation samples, reducing significantly the total time of analyses and the reagents–sample consumption. The sample throughput was 18 h^{-1}.

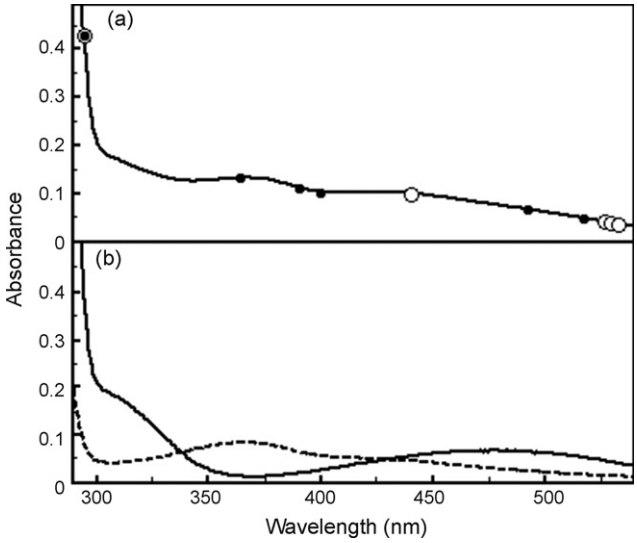

Fig. 3. (a) Spectra of LVD + CBD (0.305 mg mL^{-1} to LVD and 0.045 mg mL^{-1} to CBD). (b) Spectra of LVD (0.305 mg mL^{-1}, solid line) and CBD (0.045 mg mL^{-1}, dashed line). The circles indicate the selected variables by MLR-SPA: (◯) corresponding to LVD and (●) corresponding to CBD.

3.3. Selection of the spectral features

Both compounds are oxidized in the presence of PPO enzyme which produces *o*-quinones that have a strong absorption in the UV–vis region. In spite of the structural similarity between the analytes, different products are obtained from the enzymatic reaction. In Fig. 3b is shown the spectra of LVD and CBD when they were oxidized by PPO. It can be observed that LVD and CBD present a strong absorption around 480 and 360 nm, respectively. However, the mixtures of the analytes present serious overlapping spectra after the reaction with PPO (Fig. 3a). Therefore, the traditional methods need strictly to use a separation technique before the determination. Besides, the resolution result is more complex, taking into account that CBD is a minor constituent in the commercial pharmaceutical preparations. Nevertheless, there are some spectral differences that can be useful for multivariate calibration methods. To both analytes, the spectral region between 295 and 540 nm was selected as suitable for the analysis. Additionally, the bands of more intense absorption for the respective analytes (450–540 nm to LVD and 340–390 nm to CBD) have also been used. In all cases, the data points were taken every 1 nm.

3.4. Optimization of the time reaction

The absorption of the chromophores increased significantly over the time studied. The criterion to choose the optimum reaction time was based on the lowest RMSEV obtained in the

Table 3
Results obtained from the application of PLS-1, MLR-SPA and univariate model

Analytes	Figures of merit	Models			
		PLS-1		MLR-SPA	Univariate
LVD	Selected variables (nm)	295–540	450–540	295, 441, 527, 530, 533	500
	Number of factors	3	1	–	–
	SEN (mL mg^{-1})	1.370	1.327	0.406	0.106
	LOD (mg mL^{-1})	0.0165	0.0165	0.131	0.218
	Calibration				
	Concentration range (mg mL^{-1})			0.057–0.553	
	RMSE[a] (mg mL^{-1})	0.0047	0.0657	0.0252	0.0565
	Correlation	0.9995	0.9077	0.9876	0.9594
	Validation				
	Concentration range (mg mL^{-1})			0.129–0.481	
	RMSEV (mg mL^{-1})	0.0272	0.0959	0.0095	0.1073
	Correlation	0.9939	0.6779	0.9981	0.6957
	Prediction of real samples				
	RMSEP (mg mL^{-1})	0.0074	0.0681	0.0100	0.0662
CBD	Selected variables (nm)	295–540	340–390	295, 364, 391, 400, 493, 518	360
	Number of factors	2	2	–	–
	SEN (mL mg^{-1})	15.6	3.567	1.566	1.871
	LOD (mg mL^{-1})	0.0014	0.0022	0.040	0.015
	Calibration				
	Concentration range (mg mL^{-1})			0.021–0.070	
	RMSE[a] (mg mL^{-1})	0.0033	0.0041	0.0035	0.0039
	Correlation	0.9774	0.9645	0.9779	0.9801
	Validation				
	Concentration range (mg mL^{-1})			0.030–0.059	
	RMSEV (mg mL^{-1})	0.0030	0.0036	0.0020	0.0046
	Correlation	0.9932	0.9935	0.9916	0.9734
	Prediction of real samples				
	RMSEP (mg mL^{-1})	0.0034	0.0074	0.0038	0.0082

[a] RMSECV for PLS-1 or RMSEC for SPA-MLR and univariate.

prediction of samples of the validation set when PLS and MLR-SPA regression was applied to the spectral data to each time studied.

In conclusion, 180 s seems to be the optimum time for the simultaneous determination of LVD and CBD.

3.5. Interference study

An interference study was done by us and their results were recently published [34]. In this work a solid mixture of microcrystalline cellulose, maize starch, magnesium stearate, and colloidal anhydride silica was prepared in the amounts present in the analysed commercial samples. We demonstrated that the presence of excipients did not interfere in the spectral region (295–540 nm) used for the determination of both analytes.

3.6. Analytical performance

3.6.1. Application of univariate method

A study using univariate calibration was carried out selecting a maximum absorption of 500 nm to LVD and 360 nm to CBD, as was published by Fatibello-Filho et al. [11]. The results of predictions and figures of merit for these models were not satisfactory, as can be seen in Table 3.

3.6.2. Application of PLS and MLR-SPA

Previous to the application of the PLS and MLR-SPA methods, the data set was smoothed by using the moving average algorithm with an overall window size of 3 points. Then, the smoothed data set and their concentrations were mean centred.

3.6.2.1. PLS models. The PLS models were developed in the PLS-1 mode where the regression was carried out for each independent variable individually.

The calibration model and its performance was evaluated by leaving one out cross-validation, in which each sample was left out once, and its concentration was estimated by a model built with the remaining samples. The number of significant factors has been chosen as the lower number whose mean square error of prediction by cross-validation (MSECV) was not significantly different from the lowest MSECV value.

For an easier comparison and interpretation of the obtained results, the root mean square error (RMSE), which is an estimate of the absolute error of prediction for the calibration (with cross-validation) and validation sets of each analyte, were calculated. To both, the optimum spectral region and number of latent variables for the PLS-1 algorithm, as well as the statistical parameters: root mean square error of cross-validation (RMSECV) and the correlation coefficients are summarized in Table 3. Also, the validation set containing eight lab-made sam-

ples was analysed by the proposed procedure. The statistical validation results are summarized in Table 3. The RMSECV value for cross-validation and RMSEV values for validation set were reasonably low for both LVD and CBD when the spectral region 295–540 nm was used. The region 340–390 nm for CBD supplied correlations to the model, and the RMSECV and RMSEV were identical to those obtained in the region 295–540 nm. However, for LVD the region 450–540 nm presented a model having worse parameters.

Table 3 also gives other important statistical parameters such as the sensitivities (SEN) and detection limits (LOD). SEN for a given analyte k has been defined as

$$\mathrm{SEN_k} = \frac{1}{||\mathbf{b}_k||}$$

where $||\cdot||$ indicates the Euclidean norm and $\mathbf{b}_k$ is the vector of final regression coefficients appropriate for component k, which can be obtained by any multivariate method. In another hand, the limit of detection is

$$\mathrm{LOD_k} = 3.3||\boldsymbol{\delta}\mathbf{r}||||\mathbf{b}_k||$$

In this expression, $||\boldsymbol{\delta}\mathbf{r}||$ indicates the instrumental noise.

The LODs and SENs obtained for LVD, when PLS was applied in the two studied regions, 295–540 nm and 450–540 nm, were comparable and satisfactory. However, for CBD the results were better when PLS was applied in the region 295–540 nm (Table 3).

3.6.2.2. MLR-SPA model. MLR-SPA uses a calibration (Xcal) and a validation (Xval) set consisting of instrumental response data and parameter values measured by a reference method (y). The essence of SPA consists of projection operations carried out on the calibration matrix. A detailed explanation of the projection operations is given elsewhere [29,30]. Starting from each of the J variables (columns of Xcal) available for selection, SPA builds an ordered chain of K variables where each element is selected in order to present the least collinearity with the previous ones. The collinearity between variables is assessed by the correlation between the respective column vectors of Xcal. It is worth to point out that, according to this selection criterion, no more than K variables can be included in the chain [29,30]. It is possible to extract K subsets of variables from each of the J chains constructed by using one up to K elements in the order in which they were selected. Thus, a total of $J \times K$ subsets of variables can be formed. In order to choose the most appropriate subset $J \times K$, MLR models are built using the calibration samples set and compared in terms of the root mean square error obtained for the validation samples set.

Table 3 presents selected variables and the figures of merit for models constructed with MLR-SPA. The selected variables are also shown in Fig. 3a. In Fig. 3 it can verify that the selected variables for SPA agree with the regions of higher absorbance for each analyte. Thus, CBD appears to be better predicted if absorptions at 364, 391 and 400 nm are included, while LVD is better predicted if absorptions at 295, 527, 530 and 533 nm are considered. However, some variables outside these regions are important and have also been identified by SPA. For example, the absorption of LVD at 295 nm was also selected for CBD model. We believe that this must be the way in which SPA solves the spectral overlapping. The regression coefficient at 295 nm in the CDB model was negative, corroborating this assumption.

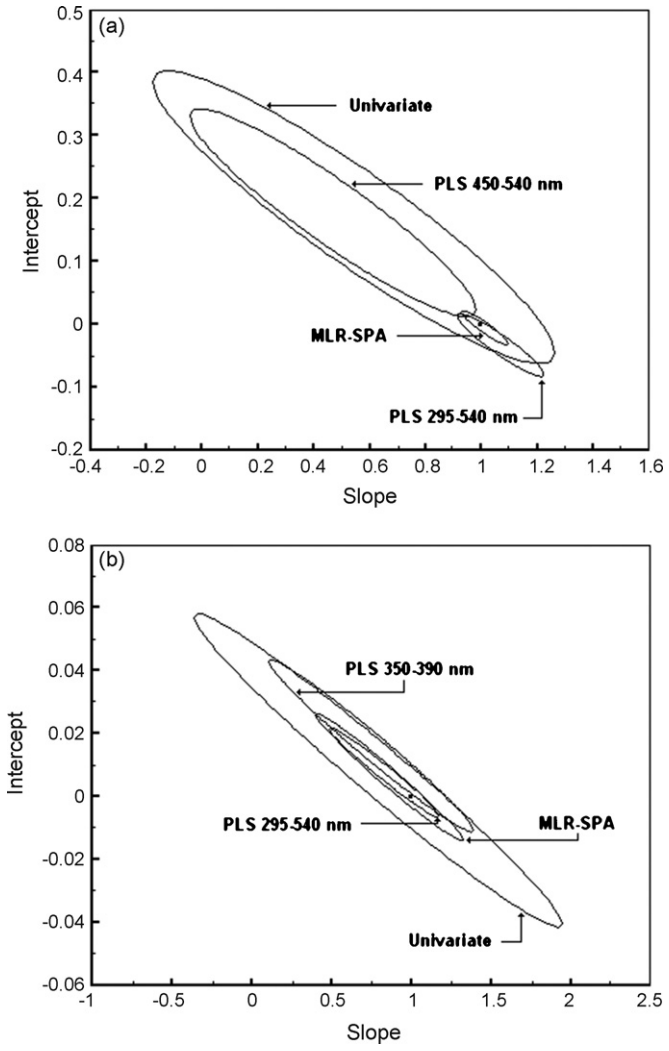

Fig. 4. Elliptical joint confidence regions for the slope (b) and intercept (a) corresponding to regressions of actual concentrations vs. univariate, PLS and MLR-SPA predicted concentrations for: (a) LVD and (b) CBD. The point marks the theoretical point ($a = 0$, $b = 1$).

The LOD and SEN obtained for LVD and CBD when MLR-SPA was applied are shown in Table 3. The model supplied a higher LOD and a lower SEN than PLS model for both, LVD and CBD.

3.6.2.3. Comparative study of chemometric models. In order to get further insight into the precision ability of both chemometric methods herein analysed, linear regression analysis of actual concentration values versus PLS and MLR-SPA predictions was applied. The estimated intercept and slope (a and b, respectively) were compared with their ideal values of 0 and 1 using the elliptical joint confidence region (EJCR) test, in this case by using an ordinary least squares fitting (OLS) of the actual versus the simultaneously 12 predicted values for each method (8 of the validation sets more 4 real samples) by each

Table 4
Determination of LVD and CBD in pharmaceutical preparations

	Nominal (mg per tablet)		Pharmacopeia (mg per tablet)			Proposed method (mg per tablet)					
	LVD	CBD	HPLC		λ = 280 nm	PLS-1 (295–540 nm)			MLR-SPA		
			LVD	CBD	LVD + CBD	LVD	CBD	LVD + CBD	LVD	CBD	LVD + CBD
Sample											
Lebocar® A	250	25	235 (2)	24 (1)	276 (2)	238 (1)	24 (1)	262 (1)	237 (1)	23 (1)	260 (1)
Lebocar® B	100	25	104 (1)	25 (1)	123 (1)	100 (2)	23 (1)	123 (2)	100 (1)	23 (1)	123 (1)
Parkinel® A	250	25	243 (1)	23 (1)	272 (2)	242 (1)	25 (1)	267 (1)	249 (2)	24 (1)	273 (2)
Parkinel® B	100	25	87 (1)	21 (1)	122 (1)	92 (2)	21 (1)	113 (2)	94 (1)	22 (1)	116 (1)
t calculated[a]						0.3	0.3	2.4	1.2	0.9	1.35
t critical[b]							3.18			3.18	
n							4			4	

Significance test (*t*-test) for comparison between pharmacopeial and proposed methods. The samples were analysed for triplicate. Standard deviations are in parenthesis.

[a] For LVD and CBD: HPLC vs. proposed method and for LVD + CBD: spectrophotometric vs. proposed method.

[b] Tabulated 95% confidence limit.

analyte, as recommended by Martinez et al. [35]. The ellipses that contain the theoretical point ($a = 0$, $b = 1$) are indicative that proportional and constant errors are not present. On the other hand, the size of the joint confidence region for a given level of significance α depends directly on the estimate of the experimental error $\hat{s}^2$. In this way, when few experimental data are available, the values of $\hat{s}^2$ are usually overestimated. This increase in uncertainty is due to the lack of information inherent to a small number of data pairs, or in some cases, to the lack of fit of the experimental data to the OLS regression line. In these cases, the joint confidence region is oversized [35]. Fig. 4(a) and (b) shows the EJCR plots for the four studied chemometric-assisted methods. As can be seen, PLS in the region 295–540 nm and MLR-SPA presents an excellent predictive ability to both LVD and CBD, compared with the other studied chemometrics methods.

3.6.3. Application to real samples

The proposed method was used for the simultaneous determination of LVD and CBD in pharmaceutical preparations. Table 4 shows a comparative study of the obtained results of the following methods: flow-batch method (with spectral data analysed by PLS in the region 295–540 nm and MLR-SPA) and pharmacopeial methods (the spectrophotometric at 280 nm [36] and the HPLC [37]).

It is important to say that, for all analysed samples, the obtained concentrations for both analytes by proposed methods were in close agreement with those obtained by HPLC. On another hand, the obtained concentrations, when PLS and MLR-SPA were applied, were used to calculate the total concentrations (LVD + CBD), in order to compare with the results obtained from the spectrophotometric method.

Results of significance test (test "*t*") for comparison between proposed methods and pharmacopeial methods are shown in Table 4. Additionally, the root mean square error of prediction (RMSEP) and the correlation coefficients for PLS-1 and MLR-SPA models are present in Table 3.

The repeatability expressed as the relative standard deviation (R.S.D. %) was 1.3% for LVD and 2.1% for CBD when PLS-1 was applied and 3.6% for LVD and 3.7% for CBD when MLR-SPA was applied ($n = 4$).

4. Conclusion

The simultaneous spectrophotometric enzymatic determination of LVD and CBD in pharmaceutical preparations is feasible using absorption spectral data with PLS in the region 295–540 nm and MLR-SPA. Both multivariate calibration models solved successfully the serious spectral overlapping in mixtures with high ratios of LVD/CBD concentrations (10/1; 4/1). A relatively small calibration set, based on the central composite design, was required. MLR-SPA models have been constructed with five wavelengths for LVD and six for CBD and presented results of prediction with performance comparable to PLS models.

The proposed system improved the analytical signal and allowed the automatic preparation of calibrations and validation samples and this was an important advantage. The excipients were retained successfully in the cotton filter and did not interfere in the determination.

Precise and accurate results were obtained based on the estimation of figures of merit. The validation was done on real samples against the official methods of the pharmacopeia.

The proposed flow-batch method is of low cost and fast, it can be useful as a possible alternative method for the quality control analysis of these pharmaceutical preparations.

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