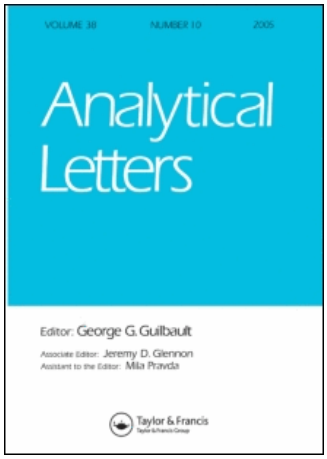

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PLS Regression in the Spectrophotometric Data for the Simultaneous Determination of Levodopa and Carbidopa in Pharmaceutical Preparations by Using an Enzymatic Stopped-Flow FIA Technique

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Abstract: An enzymatic stopped-flow-injection analysis is proposed for simultaneous determination of levodopa and carbidopa in pharmaceutical preparations. The dopaquinones obtained after the oxidation catalized by the enzyme were measured by spectrophotometric method. A reduced calibration matrix based on a central composite experimental design was built and Partial Least Squares (PLS) was applied on the spectral data after reaction with the enzyme. The LOD was 0.015 and 0.0028 mg ml^{-1}, respectively and the sample throughput was 22.5 h^{-1}. The proposed method was applied to pharmaceutical preparations and the results are in close agreement with pharmacopeial method. The recovery study and results were satisfactory.

Keywords: Levodopa, carbidopa, stopped-flow FIA, polyphenol oxidase, PLS

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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. The cause of this disease is the significant depletion of dopamine. Dopamine cannot be administered directly because it does not cross readily the blood brain barrier. Therefore, its precursor levodopa is given orally, which is easily absorbed through the bowel, there dopamine is formed by the decarboxylase action. High levels of dopamine also cause adverse reactions such as nausea, vomiting, and cardiac arrhythmias. Owing to the peripheral decarboxylation of levodopa in extracerebral tissues, it is usually associated with a peripheral aromatic-L-amino acid decarboxylase (AADC) inhibitor, 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 properly controlled. Also, it was observed that the side effects are reduced (Goodman-Hilman 1996; Osol 1975). The Fig. 1 shows the chemical structures of both compounds, LVD and CBD.

Both drugs are present in the pharmaceutical preparations therefore their determinations are extremely important in the quality control in these products.

Polyphenol oxidase (PPO; EC 1.14.18.1) is an enzyme widely distributed in the nature. This enzyme catalyzes the oxidation of LVD and CBD, to the corresponding dopaquinone, which is converted to leucodopachrome by a rapid and spontaneous auto-oxidation. Later leucodopachrome are oxidized to its corresponding dopachrome which presents hard absorption in the UV-Visible region to both LVD and CBD (Mason 1955; Vachtenheim et al. 1985; Behbahani et al. 1993)

Chemometric calibration techniques in spectral analysis are getting an increasing attention to the quality control on pharmaceutical preparations (Moreira et al. 2005; El-Gindy et al. 2006; Baratieri et al. 2006). Partial Least Squares (PLS) is an algorithm applied successfully for building regression models. PLS is a factor-based method and involve spectral decomposition relating a matrix of independent variables (e.g., spectra) to a matrix of dependent variables (e.g., concentration). The optimum number of factors is often chosen on the basis of the quality of the results for the samples in the prediction set. The combination of PLS and UV-Visible spectrophotometry has

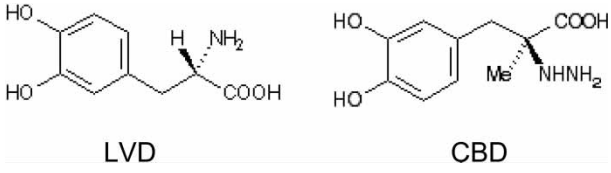


Figure 1. Chemical structures of LVD and CBD.

been reported for simultaneous determination of several common active principles in pharmaceutical preparations mainly in presence of two or more analytes with overlapping spectral (Ferraro et al. 2001; Sena et al. 2004).

LVD and CBD determinations are commonly carried out by using High Performance Liquid Chromatography (HPLC) (Michotte et al. 1987; Husain et al. 1994; Tolokan et al. 1997; Sagar et al. 2000) and recently Capillary Electrophoresis (CE) (Fanali et al. 2000) although another alternatives methods has been reported. Recently chemometrics-assisted spectrophotometric method using potassium periodate as an oxidant agent was proposed by Damiani (2005). Fatibello-Filho (1997) proposed a FI spectrophotometric determination of LVD and CBD applying univariate calibration and using polyphenol oxidase. However, in this paper, the simultaneous determination using univariate calibration was not clearly demonstrated owing to the sample spectrum after PPO reaction was not showed. In our knowledge, the mixture of both analytes presents a hypsochromic shift and overlapping of the spectral maxima of both analytes, after the reaction with the enzyme.

In our work, PLS was applied to spectral data of LVD and CBD for the simultaneous determination of them, in pharmaceutical preparations. A reduced calibration matrix based on a central composite experimental design and stopped-flow-injection analysis was used to develop a simple and rapid methodology without any separation process. The spectrometric 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).

EXPERIMENTAL

Apparatus and Software

Centrifugation of extracts was conducted in a refrigerated-automatic Sorvall centrifuge, provided with a type SS-34 rotor.

Spectrophotometric measurements were carried out by using a Hewlett-Packard model 8452 A UV-visible diode array spectrophotometer (Germany) with a Hellma 178-010-QS flow cell (inner volume of 18 μl).

Peristaltic pump (Gilson Minipuls 3, France) and a Rheodyne 5041 injection valve were used.

The reaction coils, sampling loop and flow lines were made of PTFE tubing (0.5 mm id).

A Spectronic 20 Genesis™ model 4001/4 was used for enzymatic activity.

An Orion model 710 A pH meter with an Orion-Ross® model 81-02 electrode was used to carry out the pH measurements.

The Savitsky-Golay algorithm and PLS algorithm incorporated in The Unscrambler software package (CAMO, Norway), version 9.1 were used for the construction of the proposed model.

Reagents and Solutions

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

A 0.1 M phosphate buffer solution of pH 7 was prepared.

A 0.05 M catechol stock solution used as substrate for enzymatic activity determination was prepared by dissolving 0.1375 g of catechol (Anedra, Argentine) in 25 ml of the buffer solution.

Stock solutions of LVD (Saporiti, Argentine) and of CBD (Saporiti, Argentine) of concentrations of 0.800 mg ml^{-1} and 0.400 mg ml^{-1}, respectively were prepared in phosphate buffer. The stock solutions were protected from light and stored at 4°C. Working standards solutions were obtained by adequate dilution of the stock solutions in phosphate buffer.

Dowex 1 × 8100-200 mesh (Fluka AG, Germany) strong basic, quaternary ammonium anion exchange resin was used as a protective and stabilizer agent in the sweet potato extract preparation. A 0.015% sodium azide (Sigma-Aldrich) solution was added to the extract as antimicrobial agent in order to extend the lifetime of it.

Sweet potato roots (*Ipomoea batatas* (L.) Lam.) acquired from a market place were washed, hand-peeled, chopped, and frozen at −18°C until they were used.

Samples, acquired in a pharmacy, of the pharmaceutical preparations Lebocar® (Pfizer, Searle Argentine) were analyzed. This preparation is presented in the form of tablets, with a nominal content of 250 mg of LVD and 25 mg CBD (Lebocar® A) or 100 mg of LVD and 25 mg CBD (Lebocar® 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 analyzed.

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

METHODS

Extraction of PPO of Sweet Potato Root

An amount of 25 g of *Ipomoea batatas* previously frozen was cut into small pieces and placed in a liquefier and added 100 ml of 0.1 M phosphate buffer (pH 7.0) and 2.5 g of Dowex 1 × 8. It was homogenized for 3 min at 4–6°C. The homogenate was rapidly filtered through two layers of cheesecloth and centrifuged at 14500 rpm for 60 min, at 2–6°C. The resulting supernatant was separated and stored at 4°C in a refrigerator with previous

addition of 0.015% sodium azide solution. This crude extract was then used as enzymatic source in the FIA procedure.

Measurement of PPO Activity

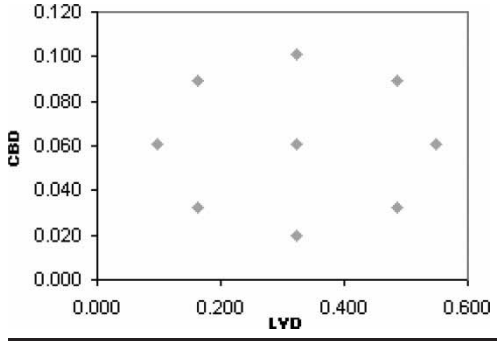
The PPO activity, that is naturally present in the crude extract of the *Ipomoea batatas*, was determined. The *o*-quinones were obtained when different volumes of PPO (0.04 to 0.2 ml) were mixed with 2.8 ml of 0.05M catechol and buffer phosphate to 5.0 ml. The classical spectrophotometric method was 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 above described.

Preparation of 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

Table 1. Concentration data (mg ml^{-1}) for the different mixtures used in the calibration matrix



Mixture	LVD	CBD
1	0.097	0.061
2	0.550	0.061
3	0.323	0.020
4	0.323	0.101
5	0.162	0.032
6	0.485	0.032
7	0.162	0.089
8	0.485	0.089
9	0.323	0.061

0.097 to 0.550 mg ml^{-1} of LVD and from 0.020 to 0.101 mg ml^{-1} of CBD. In the Table 1, the composition of the standard mixtures used in the calibration matrix are summarized. 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, another seven mixtures were prepared in concentrations that were selected at random and comprised within those of calibration.

Sample Preparation

Ten tablets of each commercial pharmaceutical preparation (A and B) were weighed, finely powered and homogenized. Equivalent to about 67 mg of the powdered was accurately weighted and dissolved in 100 ml of phosphate buffer. Then, it was filtered on-line by passing through a tubular cotton filter in FIA system.

In order to validate the proposed method a recovery study was performed by spiked samples.

FIA System and Procedure

The manifold of the stopped-flow injection system used is shown in Fig. 2. A reverse FIA manifold with spectrophotometric detection was developed. A volume of 100 μl (equivalent to 230 units of PPO) was injected in a carrier of 0.1 mol l^{-1} phosphate buffer (C1), which was flowing at 1.1 ml min^{-1}. The sample was introduced continuously through the channel C2 (flow rate of 1.1 ml min^{-1}) and was filtered on-line by passing through a tubular cotton

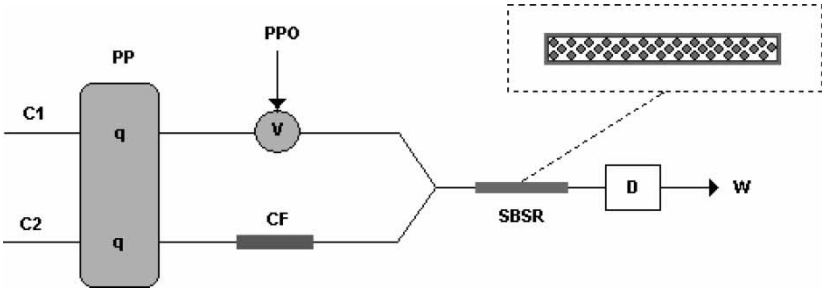


Figure 2. Flow injection system for the simultaneous determination of LVD and CBD. C1: carrier solution; C2: sample; PPO: polyphenol oxidase; PP: peristaltic pump; q: flow rate; V: injection valve; CF: cotton filter; SBSR: single beads string reactor; D: detector; W: waste.

filter (CF). The C1 and C2 streams merged into a single beads string reactor (SBSR) (100 mm length). This kind of reactor let a mixing between PPO and the analytes. When the catalyzed oxidation reaction bolus reached the flow-cell, the flow was stopped. The delay time was 40 s and the stopped flow time was 120 s (for details see below). After this time, a spectrum between 290 and 540 nm was obtained and the flow restored. The baseline was obtained in the same way by using a buffer solution stream instead of sample solution. The spectra were recorded, transferred to a microcomputer and processed by applying PLS model.

Optimization of the Time Reaction

In order to select the optimum reaction time for the oxidation of the analytes with PPO, a kinetic study was carried out. Thus, 230 units of PPO reacted in phosphate buffer medium (pH 7) with the samples corresponding to the calibration and validation sets. The spectra were registered each 60 s and during 300 s.

RESULTS AND DISCUSSION

Enzymatic Activity

The crude extract was obtained from different commercial sweet potatoes root along six months. Over five crude extracts, the mean of enzymatic activity and the standard deviation were 2997.1 UE $ml^{-1} \pm 295.4$ UE ml^{-1}. These values did not vary along four months when the extract was stored at 4°C indicating a good agreement with those cited in the literature (Da Cruz Vieira et al. 1998). Therefore, the conditions for the obtaining of crude extract are satisfactory.

Optimization of the Flow Injection System

A two-channel FIA reverse configuration was used in order to decrease the enzyme consumption, because only a small volume of it was injected.

The FIA variables of the system were optimized by univariate method, there were selected as a compromise between sensitivity and reproducibility of the analytical signals. The studied ranges and the optimum values are shown in Table 2. The kind and length of the reactor were properly selected because it was necessary a good mixture between the enzyme and the substrate.

The enzymatic activity was optimized in order to obtain the optimum spectral values for PLS model, so we tested between 150–300 UE. The selected value was 230.

Table 2. Optimization of FIA parameters

FIA parameter	Studied range	Optimum value
Flow rate (ml min^{-1})	0.8–2.2	1.1
Injected volume (μl)	50–300	100
Reactor length (mm)	100–1000	120
Delay time (s)	30–60	40
Stopped-flow time (s)	60–600	120

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-Visible region. In spite of the structural similarity between the analytes, different products are obtained from the enzymatic reaction. Figure 3 shows 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 a maximum spectral shift and overlapping spectral after the reaction with the enzyme. Therefore, the traditional methods need strictly to use a separation technique before the determination. Besides, the resolution result 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 (PLS). LVD and CBD, the spectral region between 290 and 540 nm (with data points taken every 2 nm) was selected as suitable for the analysis, which implies the use of 126 variables for each obtained

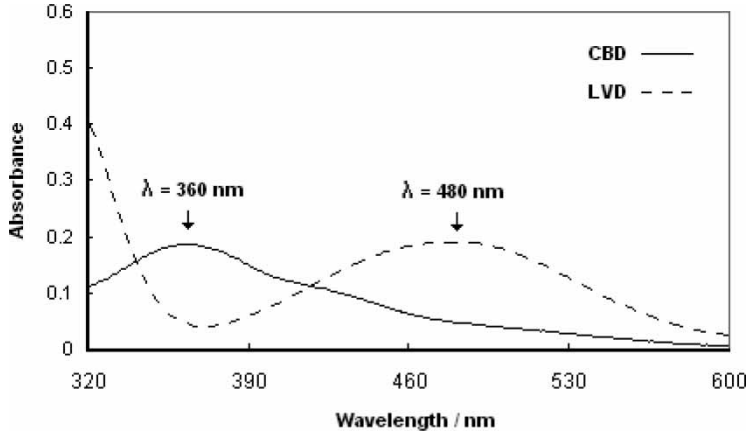


Figure 3. LVD (0.323 mg ml^{-1}) and CBD (0.061 mg ml^{-1}) spectra.

spectrum. The absorption spectra corresponding to the matrix calibration are showed in Fig. 4.

Optimization of the Time Reaction

Figure 5 shows the absorbance increase of the oxidation products of a calibration mixture after PPO reaction. The absorption of the chromophores increase significantly over the time studied. The criterion to chosen the optimum reaction time was based in the lowest RMSEP obtained in the prediction of samples (prediction set) when PLS regression was applied to the spectral data to each time studied.

In conclusion, 120 s seems to be the optimum time for the simultaneous determination of LVD and CBD and thus, it was chosen as stopped-flow time in the FIA proposed system.

Application of the PLS Method

Previous to the application of the PLS method, the data set was smoothed by using the Savitsky-Golay algorithm with a second order polynomial and overall window size of 7 points. Then, the smoothed data set and their concentrations were mean centred. This pre-processing algorithm is traditionally applied in PLS models (Del Olmo et al. 1996) and involves the subtraction of the variable mean from the individual variable values. The PLS model was developed in the PLS-1 mode where the regression is carried out for each independent variable individually.

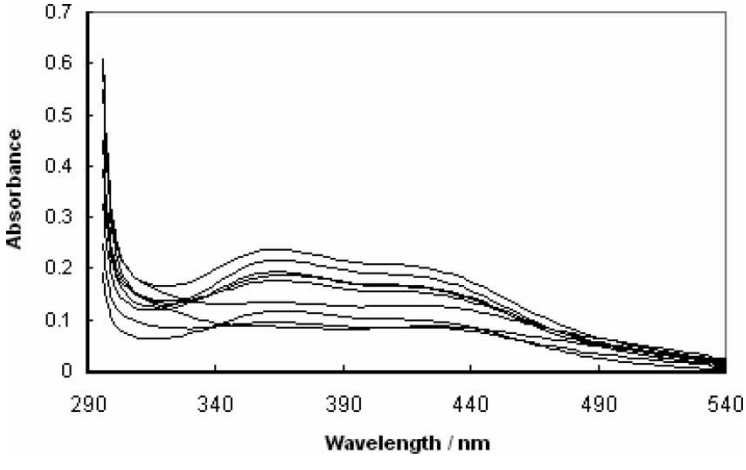


Figure 4. Spectra of 11 samples corresponding to the matrix calibration.

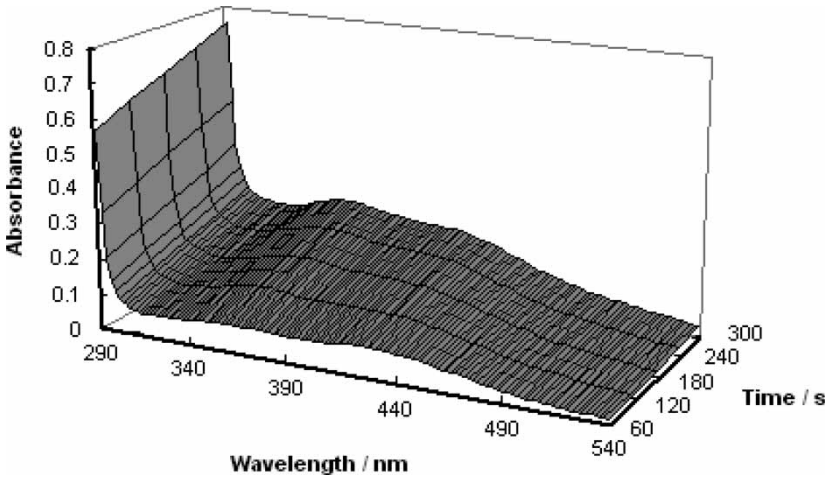


Figure 5. Spectral registers corresponding to a sample calibration (central point) at different stopped-flow times: a, 60 s; b, 120 s; c, 180 s; d, 240 s, and e: 300 s.

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 squared error of prediction by cross validation (MSECV) was not significantly different from the lowest MSECV value (Haaland et al. 1988).

$$\text{MSECV} = \frac{\sum_{i=1}^{l} (c_{nom} - c_{pred})^2}{l} \tag{1}$$

where c_{nom} and c_{pred} are the nominal and predicted concentrations, respectively, and I the total number of calibration samples.

For an easier comparison and interpretation of the obtained results in each one of the models tested, the root mean square error (RMSE), which is an estimate of the absolute error of prediction for the calibration and prediction sets of each analyte and relative error of prediction (REP) were calculated.

$$\text{RMSE} = \left[\frac{\sum_{i=l}^{l} (c_{nom} - c_{pred})^2}{l} \right]^{1/2} \tag{2}$$

$$\text{REP} = \frac{100}{c_{mean}} \left[\frac{\sum_{i=l}^{l} (c_{nom} - c_{pred})^2}{l} \right]^{1/2} \tag{3}$$

where c_{nom} and c_{pred} have the same meaning as in the previous Equation (1), I the total number of samples include in the different sets and c_{mean} the mean concentration.

To both analytes, 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), relative error of prediction (REP) and the squared correlation coefficients (R^2) are summarized in Table 3. Also, the validation set containing seven artificial samples was analysed by the proposed procedure. The statistical prediction results are summarized in Table 3. As can be observed, the RMSE and REP values for cross-validation and RMSE and REP values for validation set are reasonably low to both analytes, LVD and CBD.

The detection limits (LOD) and sensitivities (SEN and γ) were satisfactory (Table 3). The sample throughput was 22.5 h^{-1}.

Application to Real Samples

The proposed method was used for the simultaneous determination of LVD and CBD in pharmaceutical preparations. The results using pharmacopeial method (United States Pharmacopeia National, 1985) that detect both analytes to 280 nm and the FIA proposed method are shown in Table 4. The analysed commercial samples by the proposed method (Lebocar® A and Lebocar® B) have the concentrations of both, LVD and CBD, as they

Table 3. Statistical Results for the PLS-1 determination of LVD and CBD

	LVD	CBD
Parameter		
Concentration range (mg ml^{-1})	0.097–0.550	0.020–0.101
Spectral region (nm)	290–540	290–540
Cross-validation results		
Optimum number of factors	3	2
RMSE-CV (mg ml^{-1})	0.0059	0.0029
REP-CV (%)	1.84	4.49
R^2	0.999	0.990
Validation results		
RMSE-P (mg ml^{-1})	0.0131	0.0017
REP-P (%)	4.18	4.89
R^2	0.999	0.997
Figures of merit		
SEN[a] (ml mg^{-1})	0.36	2.82
γ[b] (ml mg^{-1})	360	2820
LOD[c] (mg ml^{-1})	0.015	0.0028

[a] $SEN_k = \frac{1}{\|\mathbf{b}_k\|}$; [b] $\gamma = \frac{SEN_k}{\|\delta \mathbf{r}\|}$; [c] $LOD_k = 3\|\delta \mathbf{r}\|\|\mathbf{b}_k\|$.

SEN_k is the sensitivity for a given analyte k and γ the analytical sensitivity $\|\cdot\|$ Euclidean norm, $\mathbf{b}_k$ is the vector of regression coefficients provided by the PLS-1 model and $\|\delta \mathbf{r}\|$ the instrumental noise.

Table 4. LVD and CBD determination in pharmaceutical preparations

	Nominal content		Pharmacopeia	FIA proposed method							
				LVD			CBD			LVD + CBD	
Sample	LVD Declared[a]	CBD Declared[a]	LVD + CBD Found[a]	Found[a]	Recovery[b]	RE[c]	Found[a]	Recovery[b]	RE[d]	Found[a]	RE[e]
Lebocar® A	250	25	278 (1)	248 (2)	99.4	−0.8	24 (1)	96.0	−4.0	272 (2)	−2.2
Lebocar® B	100	25	124 (1)	100 (1)	100.4	0.4	24 (1)	96.0	−4.0	124 (1)	−0.8

The samples were analyzed for triplicate. Standard deviations are in parenthesis.

[a]The results are given in mg per tablet.

[b]The recoveries are given in percentage and were calculated from the contents declared by the manufacturing laboratory. RE is the relative error give in percentage.

[c]RE is FIA method vs. LVD nominal content.

[d]RE is FIA method vs. CBD nominal content.

[e]RE is FIA method (LVD + CBD) vs. pharmacopeial value.

are recommended by the US Pharmacopoeia and are in close agreement with those declared by the manufacturing laboratories. On another hand, was calculated the total concentrations (LVD + CBD) of both samples using the FIA method and they are in close agreement with those obtained by pharmacopeial method.

Interference and Recovery Studies

A frequent problem in the analyses of commercial samples is the presence of excipients. To analyze these interferences, a solid mixture of microcrystalline cellulose, maize starch, magnesium stearate, and colloidal anhydride silica was prepared in the amounts present in the analyzed commercial preparations. The mixture of excipients was tested in the FIA proposed system and the obtained spectrum is showed in the Fig. 6. As can be observed, the presence of excipients does not interfere in the spectral region used for the determination (290–540 nm).

The accuracy of the proposed FIA method was determined by a recovery study. The results are shown in Table 5. Known amounts of the both analytes were added at mg g^{-1} level to the two preparations tested. Recoveries from 99 to 101% for LVD and from 91 to 104% for CBD were obtained for all assayed samples demonstrating the good accuracy of the proposed method.

The precision of the method, represented by the repeatability expressed as the relative standard deviation (RSD%) was 1.4% for LVD and 0.8% for CBD.

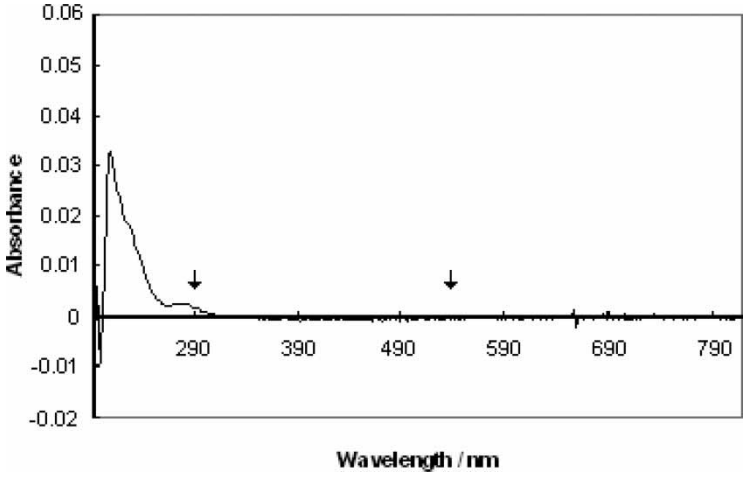


Figure 6. Spectrum of a solution containing different excipients (microcrystalline cellulose, maize starch, magnesium stearate, and colloidal anhydride silica). The rows indicate the analysed spectral region (290–540 nm).

Table 5. Results corresponding to the recovery study

	LVD			CBD		
Sample	Added[a]	Found[a]	Recovery[b]	Added[a]	Found[a]	Recovery[b]
Lebocar® A	0.432	0.426	99 (3)	0.085	0.088	104 (3)
	0.863	0.872	101 (2)	0.170	0.172	101 (4)
Lebocar® B	0.421	0.425	101 (2)	0.082	0.074	91 (1)
	0.842	0.853	101 (3)	0.165	0.151	92 (2)

The samples were analyzed for triplicate. Standard deviations are in parenthesis.
[a]The results are given in mg mg^{-1} tablet.
[b]The recoveries are given in percentage.

CONCLUSION

The simultaneous spectrophotometric enzymatic determination of LVD and CBD in pharmaceutical preparations is feasible using absorption spectral data treatment with partial least squares (PLS-1). This multivariate calibration model solved successfully the serious spectral overlapping in mixtures with high ratios of LVD/CBD concentrations (10/1; 4/1). A relatively small calibration set was required based on the experimental design. The prediction ability of the proposed method could be increased by means of an accurate selection of the number of factors, wavelengths subsets selection as well as optimum time reaction.

The mode stopped-flow in the FIA system proposed improved the analytical signal and the use of FIA reverse configuration presented a low consumption of the enzyme and this represents 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, and verified by pharmacopeial method and recoveries study.

The proposed method is rapid and of low cost and can be useful as a possible alternative method for the quality control analysis of this pharmaceutical preparations.

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