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Simultaneous determination of levodopa and benserazide by stopped-flow injection analysis and three-way multivariate calibration of kinetic-spectrophotometric data

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Abstract

The simultaneous determination of levodopa and benserazide in pharmaceutical formulations is described, based on the application of multidimensional partial least-squares regression to the kinetic-spectrophotometric data provided by diode-array detection within a stopped-flow injection method where analytes react with periodate. Flow injection parameters were adequately optimized. Accurate analysis is performed with no sample pre-treatment steps, and with minimum experimental effort. Satisfactory recovery results were obtained on a number of synthetic and commercial samples, in the latter case including the comparison with liquid chromatography measurements.
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Keywords: Flow injection analysis; Kinetic-spectrophometric data; Multiway partial least-squares; Levodopa; Benserazide

1. Introduction

The aminoacid levodopa [3-(3,4-dihydroxyphenyl)-L-alanine] is a precursor of the neurotransmitter dopamine, which easily enters the central nervous system. It is used in the treatment of Parkinson's disease, usually associated with a peripheral aromatic-L-amino acid decarboxylase (AADC) inhibitor, such as benserazide [*N*′-(2,3,4-trihydroxybenzyl)-D,L-serine], in order to increment the proportion that enters the brain [1,2]. Scientific literature reports several methods for the determination of levodopa and benserazide in pharmaceutical preparations and in biological fluids, such as spectrophotometry [3–9], capillary zone electrophoresis [10,11], high performance liquid chromatography [12–15] and gas chromatography [16]. Flow injection analysis (FIA) has been applied to the determination of levodopa in mixtures with other drugs [17] and of benserazide in tablets [18].

Both of these drugs are readily oxidized in the presence of an excess of potassium periodate (KIO_4) in acid solution. Under these conditions, levodopa is transformed into aminochrome, and benserazide into colorless compounds, opening the possibility for the development of a kinetic method for their simultaneous determination. In fact, chemometrics-assisted kinetic-spectrophotometric methods have been reported for the determination of levodopa and benserazide in batch systems [4,5].

In view of the growing importance of the automatization of methodologies for pharmaceutical quality control, we report on a kinetic-spectrophotometric stopped-flow injection system for the chemometric-assisted simultaneous determination of levodopa and benserazide in tablets. The samples

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and the oxidant were simultaneously injected into the carrier streams which merged into a short reactor, making this procedure rapid, reproducible and consuming only small amount of drugs as compared with analogous batch systems. The flow was stopped when the reaction mixture reached a flow-cell inserted in a diode-array UV-visible detector, in order to follow the kinetic-spectrophotometric determination by measuring the complete spectral evolution with time.

Among the available multivariate calibration methodologies for processing second-order data such as kinetic spectral evolutions, we selected multiway partial least-squares (*N*-PLS), which has been previously shown to be useful in a related batch system for the determination of both analytes [4]. Although other chemometric techniques can in principle be employed for three-way data analysis, notably the parallel factor (PARAFAC) model [20], the latter is not suitable when deviations from linearity occur, as is usual when studying kinetic systems [4].

2. Experimental

2.1. Apparatus

A Gilson Minipuls three peristaltic pump was used for the propulsion of the solutions. All reference and sample solutions were manually injected into the carrier system using a dual proportional Rheodyne-type injector. UV-visible measurements were done using a spectrophotometer equipped with a Hewlett Packard 8452 A linear diode-array and a Hellma 178.712-QS flow cell (8 μL inner volume). Reactors and connectors of PTFE (0.5 mm inner diameter) were used.

The high-performance liquid chromatography (HPLC) procedure was carried out on a Merck-Hitachi-Lachrom liquid chromatograph equipped with an L-7100 pump, a Nucleosil reversed-phase C18 column (25 cm × 4 mm) with 5 μm particle size. Other parameters were: mobile phase, buffer 50 mM KH_2PO_4:H_3PO_4 (pH 2.5); flow rate, 0.8 mL min^{-1}; temperature, 35 °C; detector wavelengths, 268 nm for benserazide and 278 nm for levodopa; retention times, 2.80 min for benserazide and 4.80 min for levodopa.

2.2. Reagents

The following solutions were employed for the FIA determination. The carrier solution was a 0.2 M sodium acetate/acetic acid buffer solution (pH 4.5) (Merck). The oxidant was a 0.017 M aqueous solution of potassium periodate (Fluka). A stock solution of levodopa (Roche Diagnostic GmbH from Mannheim, Germany) 2.03×10^{-2} M was prepared by dissolving an appropriate amount in 0.1N HCl. A stock solution of benserazide (Roche Diagnositc GmbH from Mannheim, Germany) 7.90×10^{-3} M was prepared by dissolving a suitable amount in 0.1N HCl. Both solutions were protected from light and stored in a refrigerator at 4–5 °C. Working solutions of both analytes were prepared by appropriate dilutions of the stock solutions with 0.1N HCl. All solutions were prepared in doubly distilled water.

2.3. Calibration set

Preliminary studies demonstrated that the FIA peaks showed large absorbances for binary mixtures containing 4.8×10^{-3} M levodopa and 8×10^{-4} M benserazide, and therefore less concentrated solutions were employed. The calibration mixtures were prepared according to a full factorial design at three equally spaced levels for each component (nine mixtures), with concentrations in the following ranges: from 4.1×10^{-4} to 2.03×10^{-3} M for levodopa and from 8.5×10^{-5} to 4.25×10^{-4} M for benserazide, which are all known to lie within the corresponding linear ranges. All mixtures were measured in random order. The component ratios were selected considering the relation of levodopa/benserazide in the pharmaceutical formulations.

2.4. Sample preparation

Five aqueous standard mixtures of both analytes were prepared, with concentration ratios different than those for the calibration curve (levodopa, 4.1×10^{-4} to 2×10^{-3} M, benserazide, 1.18×10^{-4} to 3×10^{-4} M). They were subsequently measured in random order.

Pharmaceutical samples (tablets of Madopar 250, obtained from Roche), nominally contain 200 mg of levodopa, 50 mg of benserazide and some non-specific excipients. They were processed as follows: at least 20 tablets were finely powdered and an amount of the fine powder containing 150.0 mg of levodopa and 37.5 mg of benserazide was weighed, dissolved in 200.0 mL of 0.1N HCl, sonicated during 30 min and filtered. Final dilutions with 0.1N HCl were then performed considering the calibration ranges and the contents of levodopa and benserazide in the tablets. The studied commercial sample is the only one available in the Argentinean market. Therefore, in order to expand the potentiality of the method towards commercial samples, the tablet excipients and other potential components were tested, such as microcrystalline cellulose, talc, povidone, magnessium stearate, iron oxide, gelatin, manitol and $CaHPO_4$. Artificial samples were prepared containing these components and then treated in the same manner described above for commercial tablets.

2.5. Flow injection procedure

A schematic diagram of the stopped-flow injection system employed is shown in Fig. 1. A dual proportional injector was used, with 200 μL loops both for the sample, containing a binary mixture of levodopa and benserazide, and for the oxidant 0.017 M potassium periodate, which were injected into the carrier solutions (sodium acetate buffer 0.2 M, pH 4.5). Both streams merged in the reactor R (200 mm length, Fig. 1), where the chemical reactions took place. The flow of

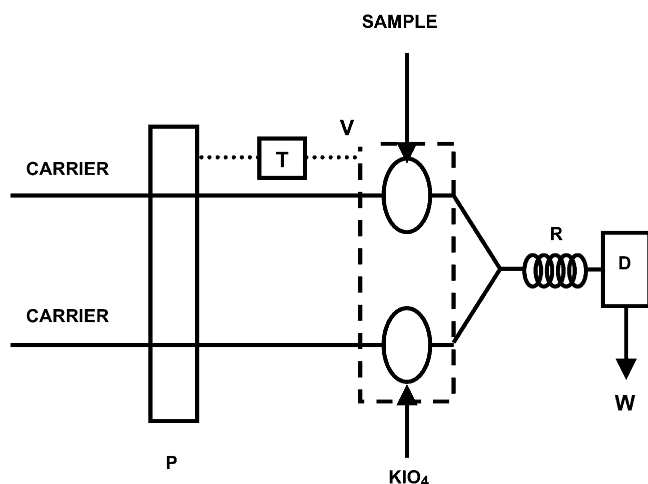

Fig. 1. Schematic diagram of the flow injection system for the chemometric-assisted kinetic-spectrophotometric determination of binary mixtures of levodopa and benserazide. The symbols have the following meanings: P: peristaltic pump, V: dual proportional injector, R: reactor, D: detector, T: timer and W: waste.

both reagents was stopped 16 s after the injection, and the FIA peak was recorded at 460 nm for a period of 168 s (for details on the optimized parameters see below). After this time, the flow was restored and the base line was obtained again. All samples were treated in the same way. Spectral data were obtained, every 2 s, from the FIA signals obtained during the time the flow was stopped. The spectra were recorded between 300 and 600 nm, transferred to a microcomputer and processed by applying *N*-PLS.

2.6. HPLC procedure

A stock solution of 5.07×10^{-4} M levodopa was prepared in 0.1N HCl. Likewise, a 3.43×10^{-4} M stock solution of benserazide was prepared. The pharmaceutical preparation was processed as follows: at least 20 tablets were finely powdered and then an accurately weighted amount (ca. 200 mg of material, equivalent to 80 mg of levodopa and 20 mg of benserazide) was dissolved in 100.00 mL of 0.1N HCl, sonicated during 30 min, centrifuged at 3000 rpm and filtered through a 0.5 μm membrane filter. This procedure was carried out in triplicate. Then, 10 μL of each test solution was injected on the HPLC system (see above) and the concentrations were calculated on the basis of the ratio of peak areas with those of the standard solutions.

2.7. N-PLS

Multiway regression methods such as *N*-PLS extend the traditional PLS algorithm to higher orders, using the multidimensional structure of the data for model building and prediction [19]. In the case of three-way data, the model is given by the following equation:

$$x_{ijk} = \sum_{f=1}^{F} t_{tf} w_{jf}^{J} w_{kf}^{K} + e_{ijk} \qquad (1)$$

where x_{ijk} is the absorbance measured for sample i at wavelength j and time k, F the number of components, t_{tf} the element of the score matrix t, w_{jf}^{J} and w_{kf}^{K} are elements of two w loading matrices and e_{ijk} is the residue not fitted by the model. The model finds the scores yielding maximum covariance with analyte concentrations as the dependent variable, in a three-dimensional sense [19]. The advantage of using *N*-PLS over bi-dimensional regression is a stabilization of the decomposition involved in Eq. (1), which potentially gives increased interpretability and better predictions.

2.8. Software

A Matlab code for *N*-PLS was applied which is freely available on the internet at http://www.models.kvl.dk/source/ [20]. An in-house additional routine for performing leave-one-out cross-validation was written and applied according to Haaland's criterion for PLS [22]. Mean-centering was applied to both concentrations and instrumental responses.

3. Results and discussion

3.1. Spectral behavior of analytes

Fig. 2 shows the spectra of both analytes in aqueous solution, in a concentration ratio, which is typical of a commercial tablet. As can be seen, both compounds absorb at wavelengths shorter than 300 nm, where tablet excipients may constitute an interference. Additionally, the overlapping is very serious in the useful spectral range. These facts hinder the application of simple direct UV-visible spectra for simultaneous determination of the analytes and also complicate the use of

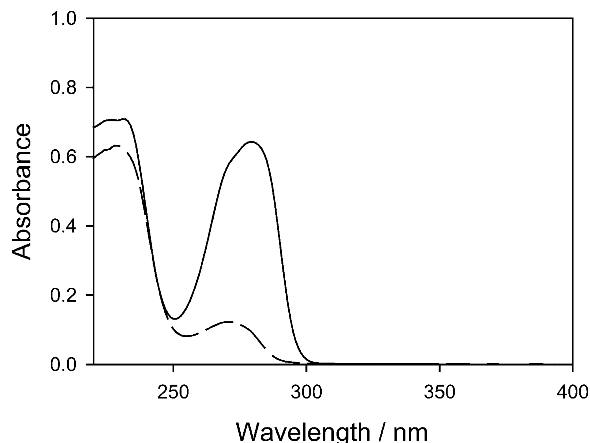

Fig. 2. Aqueous solution spectra (pH 4.5) of levodopa 3.5×10^{-4} M (solid line) and benserazide 1.4×10^{-4} M (dashed line).

first-order multivariate calibration methods based on spectral resolution. However, the incorporation of oxidation reactions allows for a proper spectral differentiation (see below).

3.2. Kinetic behavior of analytes

Reaction of the analytes with a suitable oxidant such as sodium periodate leads to products which: (1) absorb in the visible region, (2) do not overlap as seriously as the unreacting components and (3) yield significant absorbances when dissolved in ratios comparable to those in commercial formulations. In the presence of excess KIO_4, levodopa is oxidized to *o*-benzoquinone, and at pH > 4 the latter is further oxidized to aminochrome. Benserazide is also oxidized instantaneously to the corresponding quinone, but then decomposes to colorless products [4]. Hence, the kinetic reaction mechanisms and spectral behaviors are different for the two compounds, making kinetic-spectrophotometric measurements highly useful for their simultaneous determination [4]. The periodate excess is needed in order to obtain a reaction rate which allow to measure the product spectra in a reasonable time [4].

In principle, multivariate measurements can be carried out at fixed time periods, applying the usual PLS regression analysis. However, using the presently described stopped-flow injection system (see below), the total spectral evolution for each sample can be conveniently measured with no extra efforts. This type of data preserves the two-dimensional absorbance–time information, and is potentially richer than their bi-dimensional counterparts. As an example, Fig. 3A shows the total spectral evolution for one of the test samples using the full available time and spectral regions. For successful calibration and prediction, however, suitable wavelength regions were then restricted for each particular analyte (see below).

Other techniques, which could be applied to the presently studied system, such as PARAFAC [20] and multivariate curve resolution coupled to alternating least-squares MCR-ALS [21], rely heavily on linearity between response and concentration. Previous work has shown that these methodologies are not suitable for the quantitation of levodopa and benserazide, probably because of the presence of reaction intermediates and chemical interactions leading to deviations from linearity [4].

3.3. Optimization of the flow injection system

The FIA variables were optimized to accomplish the arrival of the reacting plug at the detector and to obtain maximum reproducibility in the analytical signal (slope of the kinetic curve). The latter parameters are influenced by the flow rate, reactor length and injected volumes. Different sample volumes were tested between 50 and 250 μL, with the optimum found at 200 μL. The reactor length was not a critical variable because the reaction is almost instantaneous. Thus, a short reactor of 200 mm length was deemed to be optimum.

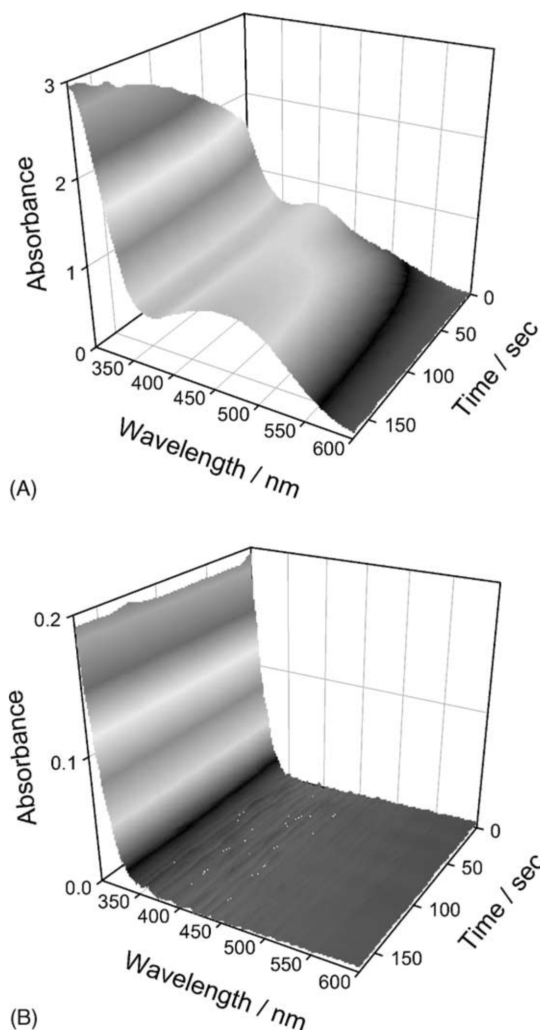

Fig. 3. (A) Total spectral evolution for one of the calibration samples in the full spectral and time range (300–600 nm and 0–168 s, respectively). (B) Total spectral evolution for a sample containing excipients.

Once the optimum residence time was established, the delay and stop times were optimized, monitoring the analytical signal between 5 and 20 s after injection, the interval during which the pump was stopped. The most favorable results were obtained at 16 s after the injection (delay time) and for a stopped-flow time of 168 s.

In order to select the optimum flow rate values, the change in absorbance with time ($\Delta A/\Delta t$) was registered for each FIA peak between 100 and 160 s, and the peak showing the maximum ratio ($\Delta A/\Delta t$) was selected. Different flow rates were tested (specific results are shown in Table 1), with an optimum value of 2.92 mL min^{-1}.

The chemical variables were also optimized, using the FIA system shown in Fig. 1, with a reactor R of length 200 mm and loops of 200 μL both for reagent and sample volumes. The effect of different concentrations of KIO_4 was tested, setting the concentrations of levodopa and benserazide in the samples at 1.2×10^{-3} and 2.0×10^{-4} M, respectively. Solutions of oxidant, which are more concentrated than 0.1 M cannot be

Table 1
Selection of the optimum concentration of KIO_4 and optimum flow rate

Effect of periodate concentration[a]	
KIO_4 (M)	$(\Delta A/\Delta t)$ (AU s^{-1})
0.017	$\mathbf{1.4 \times 10^{-3}}$
0.028	3.7×10^{-4}
0.040	3.3×10^{-4}
0.050	3.3×10^{-4}
0.080	2.0×10^{-4}

Effect of flow rate[a]	
Flow rate (mL min^{-1})	$(\Delta A/\Delta t)$ (AU s^{-1})
1.95	1.5×10^{-4}
2.43	3.7×10^{-4}
2.92	$\mathbf{4.0 \times 10^{-4}}$
3.40	3.5×10^{-4}

[a] $(\Delta A/\Delta t)$: variation of absorbance with time at 460 nm; AU: absorbance units. Optimum values are given in bold.

employed because of solubility limitations in water at room temperature. However, it was necessary to work in the presence of an excess of the oxidizing agent. Considering the 1:2 stoichiometry (levodopa/benserazide:KIO_4) and the concentrations of the drugs, solutions of at least 3×10^{-3} M had to be prepared. Several concentrations complying with the latter requirements were analyzed, and the ratio $(\Delta A/\Delta t)$ was recorded in each case, with the flow stopped and at a suitable wavelength (460 nm). Full results are shown in Table 1, where the optimum value is seen to correspond to 0.017 M KIO_4.

In the case of the carrier solutions, the working pH was established according to literature results at 4.5 (0.2 M acetate buffer) [4].

3.4. Second-order multivariate calibration

The recorded spectral evolutions were transferred to a microcomputer for subsequent multivariate calibration. Previous to the application of *N*-PLS, however, the full spectral region 300–600 nm was adequately restricted by taking into account the response of each analyte, i.e., only the absorbance values between 350 and 438 nm (with data points taken every 6 nm) were used in order to construct the *N*-PLS model for levodopa, and between 430 and 546 nm for benserazide (see Table 2). Furthermore, in both cases the time dimension was reduced by selecting one every three data points, in order to reduce the computer times required for multivariate analysis.

The statistical results for the *N*-PLS determination (calibration and prediction) of levodopa and benserazide are shown in Table 2. The required numbers of factors were estimated applying leave-one-out cross-validation [22] leading to the use of two factors for levodopa and three for benserazide (see Fig. 4A). Although cross-validation is not regularly applied for the estimation of the number latent variables in *N*-PLS, the above selection seems to be appropriate for the presently studied case. Indeed, Fig. 4B shows that the errors

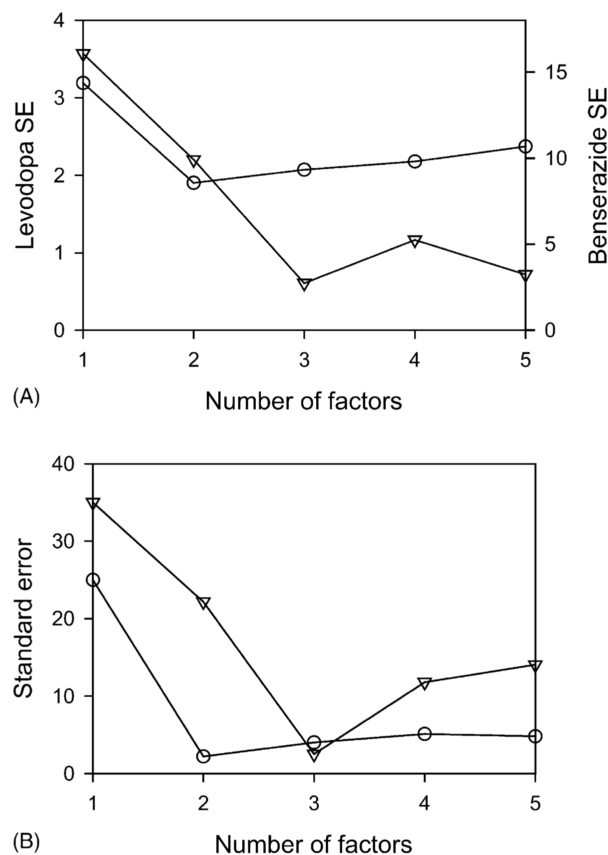

Fig. 4. Standard errors for both analytes as a function of the number of *N*-PLS latent variables. (A) Error in calibration. (B) Error in prediction for the synthetic test set. In both cases, circles are for levodopa and triangles for benserazide.

of prediction in the synthetic test parallel those for calibration, with clear minima at a number of factors equal to that from cross-validation for each analyte. The standard errors (SE) for the calibration and prediction sets were calculated as:

$$\mathrm{SE} = \left[\frac{\sum_{i=1}^{I}(c_i - \hat{c}_i)^2}{I-1}\right]^{1/2} \quad (2)$$

where c_i and $\hat{c}_i$ are experimental and calculated concentrations, respectively, and I is the number of samples in each set. It should be noticed that in a previous report based on *N*-PLS batch data for levodopa–benserazide mixtures, three and four factors were employed, respectively, for prediction [4]. The difference may be due to the selection of specific wavelength ranges in the present case.

Prediction values for commercial samples are also shown in Table 2. The recoveries were calculated considering the contents declared by the manufacturing laboratory, and are within the ranges recommended by Pharmacopeias (i.e., 90–110%).

Table 2
Statistical results for the *N*-PLS determination of levodopa and benserazide

Parameter	Levodopa	Benserazide
Cross-validation		
Concentration range × 10^4 (M)	4.1–20.3	0.85–4.25
Spectral region (nm)	430–538 nm each 6 nm (19 data points)	350–446 nm each 6 nm (17 data points)
Time region (s)	0–168 s each 6 s (29 data points)	
Total number of data points	19 × 29 = 551	17 × 29 = 493
Optimum number of factors	2	3
Calibration standard error × 10^4 (M)	1.8	2.2
Calibration relative error (%)	7.4	4.3
Prediction		
Standard error in synthetic test × 10^4 (M)	2.2	2.3
Relative error in synthetic test (%)	9.0	4.5
Commercial sample (mg per tablet)[a]	200 (2)	48 (2)

[a] Content declared by the manufacturing laboratory: levodopa, 200 mg; benserazide, 50 mg. Values in parenthesis are the S.D. for three replicate measurements.

3.5. Method validation

In order to validate the proposed method, robustness, repeatability, reproducibility and accuracy studies were conducted. The robustness was demonstrated by introducing changes in the pH of the carrier solution around the optimal value (i.e., pH 4.5) upon analyzing the commercial pharmaceutical Madopar 250. The tested pH values were 4.3 and 4.7. The predicted concentrations (S.D. in parenthesis) were: levodopa, 200 (1) at pH 4.3 and 201 (1) at pH 4.7; benserazide, 48 (1) at pH 4.3 and 48 (1) at pH 4.7. The comparison (including the values reported at pH 4.5 and quoted in Table 2) was based on a one-factor analysis of variance (ANOVA) treatment [23], computing the *F* values (ratios of between-groups variance to within-groups variance) as 0.17 for levodopa and 1.33 for benserazide. Since the associated probabilities (*P*) were 0.85 and 0.39, respectively, both larger than 0.05, we conclude that there is no evidence for significant differences in the predicted analyte concentrations at these pH values. This suggests that the proposed method is robust under slight pH variations.

The repeatability or inter-assay precision represents the minimum dispersion or the maximum precision of the assay. It was studied by analyzing repeatedly, in the same laboratory and on the same day, five replicate aliquots of a homogeneous pharmaceutical sample, each of which was independently prepared as described above in Section 2.4. The relative standard deviation (RSD) was 2.5% for levodopa and 4.0% for benserazide, respectively.

The reproducibility represents the maximum dispersion or the minimum precision of the assay. In the present work, five replicates of the synthetic mixtures were prepared using different stock solutions of levodopa and benserazide, and measured on different days. Under these conditions, the observed differences in the prediction of the replicates can be taken as a measure of the reproducibility, expressed in the form of the average relative standard deviation (ARSD). The latter was obtained by referring the average standard deviation to the average concentration value over the set of samples. The corresponding values for levodopa and benserazide were 3.8 and 4.0%. Both the repeatability RSD and reproducibility ARSD can be regarded as satisfactory in view of the several experimental parameters, which should be set for a successful sample analysis.

The accuracy of the proposed FIA method as regards a commercial sample was determined by comparing the obtained results with those furnished by HPLC as the analytical reference method [5]. The results are shown in Table 3, leading to the conclusion that they are statistically comparable to those provided by the combination of FIA and *N*-PLS.

As mentioned above, the analysed sample of Madopar 250 was the only one available in the local pharmaceutical market. Therefore, the tablet excipients and other compounds (microcrystalline cellulose, talc, povidone, magnessium stearate, iron oxide, gelatin, manitol and $CaHPO_4$) which could be potentially employed as tablet components or excipients were tested in the described FIA system, and confirmed to produce no significant absorbance under the present conditions. Samples were prepared by grinding together these components in proportions, usually employed in pharmaceutical formulations and treating them as described in Section 2.4. Fig. 3B displays the total spectral evolution in a typical case, showing no significant absorption in the working spectral range (430–538 nm). This would expand the range of commercial pharmaceutical with levodopa and benserazide, which could be studied by the presently developed methodology.

Table 3
Comparison of the determination of levodopa and benserazide in a commercial formulation by the proposed method and by HPLC

Parameter	Levodopa	Benserazide
Nominal composition (mg per tablet)	200	50
Composition by FIA/*N*-PLS[b]	200 (2)	48 (2)
Composition by HPLC[b]	197 (2)	49 (1)
t[a]	1.8	0.8

[a] Tabulated $t_{4,\,0.05} = 2.45$.
[b] Values given in brackets are the S.D. after three replicate determinations.

4. Conclusions

A simple, rapid and direct method for the simultaneous determination of levodopa and benserazide in tablets is possible thanks to the implementation of a stopped-flow injection system in order to follow the oxidation of the analytes with time, using diode-array UV-visible spectrophotometry. Processing the obtained second-order data with the multiway partial least-squares methodology provides accurate and precise results both on synthetic and commercial samples.

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