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Enhanced application of square wave voltammetry with glassy carbon electrode coupled to multivariate calibration tools for the determination of B_6 and B_{12} vitamins in pharmaceutical preparations

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Abstract

Vitamins B_6 (VB_6) and B_{12} (VB_{12}) were simultaneously determined in pharmaceutical preparations by using square wave voltametry (SWV) together with artificial neural networks (ANNs). Supporting electrolyte solution, pH and voltametric technique were optimised. The calibration set was built with several artificial samples containing both active ingredients and excipients. Deviations from linearity were observed for both analytes. It is probably due to interactions among the electro active components and competition by the electrode surface, fact that supports the use of ANNs. Recoveries when analysing a nine sample validation set, of 100.2 and 96.4 were calculated for VB_6 and VB_{12}, respectively. Commercial samples were analysed with reasonably good results considering the complexity of the mixture studied.

Keywords: B_6 vitamin; B_{12} vitamin; Square wave voltammetry; Partial least squares; Artificial neural networks

1. Introduction

Vitamins constitute a group of compounds, which are essential for the normal growth and functioning of the human body. They are divided into water-soluble and fat-soluble vitamins. The well-known B complex belongs to the first group. Many diseases are related to vitamins deficiency, thus there is an increasing interest in the development of suitable and reliable methods for quantifying vitamin on complex samples in many fields (i.e. pharmaceutical and food chemistry). Many analytical methods have been proposed for individual determination of the B_1, B_6 and B_{12} vitamins. The United Stated Pharmacopeia describes a spectrofluorimetric method for the determination

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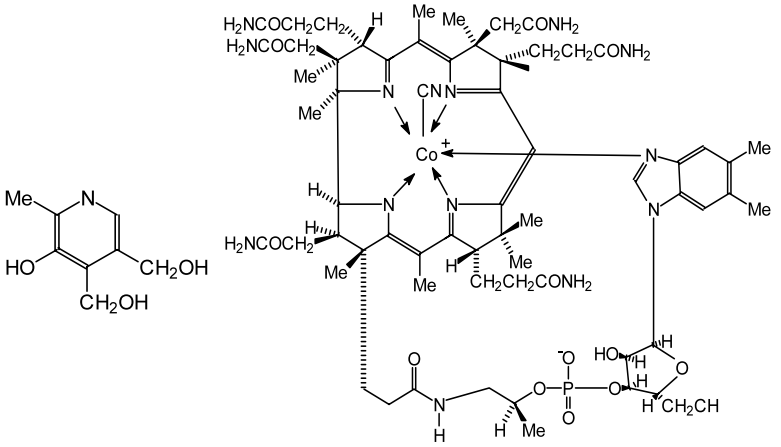
of pyridoxine or vitamin B_6 (VB_6) [1]. Other methods include gas chromatography [2] and high-performance liquid chromatography (HPLC) with electrochemical detection [3]. On the other hand, electrochemical techniques have also been reported. They include polarographic and voltammetric methods [4,5]. The former is not analytically useful due to its bad reproducibility. Alternatively, pyridoxal gives a better-defined polarographic wave than VB_6, however this chemical oxidation step makes the analytical procedure more complex and tedious [6].



Vitamin B_{12} compounds act as coenzymes in biosynthetic reactions. It is known that the reduced forms of them are involved in hydride and methyl transfer in biological systems [7]. Cyanocobalamin (VB_{12}) is the most common cobalamin form. The VB_{12} determination recommended by the British Pharmacopeia is based on a spectrophotometric UV method [8]. This vitamin can also be quantified as cobalt by voltammetric determination after UV digestion [9]. The electrochemical behaviour of VB_{12} has been widely studied in order to elucidate the vitamin reduction mechanism at both mercury and glassy carbon electrodes. These studies have demonstrated that VB_{12} and its derivatives exhibit a rich redox chemistry, centred on the cobalt atom [10–13]. Aquocobalamine VB_{12a} $(Co(III))^+$ can be reduced to VB_{12r} (Co(II)), and be further reduced to give VB_{12s} (Co(I)), in aqueous media. However, the determination of standard potentials corresponding to different couples seems to be hindered by vitamin adsorption on the electrode surface [14].

There are several analytical methods for simultaneous determination of vitamins. These include electrochemical or chromatographic with electrochemical detection techniques [15–20]. Many of these methods, however, suffer from lack of selectivity, complicated and tedious procedures, and require the use of either expensive instrumentation or dangerous reagents. The water-soluble vitamins might be lost through chemical reactions or by extraction and leaching during storage and processing of food and pharmaceuticals, consequently it is necessary to develop rapid, easy and reliable methods to analyse vitamins in food and pharmaceutical preparations.

The determination of active compounds content in pharmaceutical preparations often is made by

using HPLC. However, this technique has some disadvantages as the long time necessary to perform the analysis and the use of contaminant solvents. On the other hand, chemometric applications to improve information obtained from modern instrumental data (i.e. spectral and electrochemical data) in the biomedical and pharmaceutical fields have acquired a routine character [21–29]. Partial least-squares (PLS) has become a usual tool for multivariate calibration because of the quality of the obtained calibration models, the ease of its implementation and the availability of software [30]. It allows for a rapid determination of components, usually with no need of a prior separation for analysis. An additional advantage of such multivariate methods, is that calibration can be performed by ignoring the concentrations of all other components except the analyte of interest. This makes these methods especially appealing for the determination of the active components in complex pharmaceutical preparations, whose excipients may show analytical signals, which are severely overlapped with those from the analytes. Although PLS assumes a linear relationship between the measured sample parameters and the intensity of its signals, small deviations from linearity are acceptable as they can taken into account by including additional modelling factors [31]. However, in the presence of substantial non-linearity, PLS tends to give large prediction errors and calls for more robust models. Intrinsically non-linear calibration techniques such as artificial neural networks (ANNs) [32,33] are applicable in the latter cases. Recently we have reported the use of these kind of chemometrics tools in solving complex pharmaceutical systems with a high improvement of the results previously obtained by applying linear methods as PLS-1 [34,35].

The object of the present work was to investigate the application of square-wave voltammetry combined with chemometric tools, especially ANNs, in a simple and relatively rapid determination of these vitamins in multivitamin preparations using a glassy carbon electrode as working electrode.

2. Experimental

2.1. Reagents

B_1, B_6 and B_{12} vitamins were obtained from Sigma and were used without further purification. All other reagents were of analytical-reagent grade and used as received. Stock solutions were prepared daily by dissolving each vitamin in buffer solution.

VB_6 for electrochemical determination: a stock standard solution was prepared by dissolving 60 mg of VB_6 in buffer solution in a 10 ml volumetric flask. Prior to use, an aliquot was diluted up to appropriate concentration in supporting electrolyte solution.

Vitamin B_{12} stock solution was prepared daily by dissolving 40 mg of it in a 10 ml volumetric flask. Before completing the volume up to 10 ml with supporting electrolyte solution, this volumetric flask was placed during 20 min in an ultrasonic bath. Prior to use, an aliquot was diluted up to appropriate concentration in supporting electrolyte solution.

Vitamin B_1 (VB_1) for electrochemical determination: a stock standard solution was prepared by dissolving 50 mg of it in supporting electrolyte solution in a 5 ml volumetric flask.

Supporting electrolyte solution: buffer solution of 0.1 mol l^{-1} potassium hydrogen phthalate and 0.1 mol l^{-1} sodium chloride (pH 3).

2.2. Electrochemical measurements

Voltammetry experiments were performed using an Voltammetric Analyser (model BAS CV-50W Version 2.0 1995 from Bioanalytical Systems Inc. USA) connected to a PC Pentium II 750 MHz for data acquisition. Voltammograms were plotted by soft SIGMA PLOT version 5.0.

A conventional three-electrode system was employed, consisting of a glassy carbon as working electrode 2.5 (∅ mm), Pt wire as counter electrode and a (Ag/AgCl) silver/silver chloride in 3 mol l^{-1} NaCl solution as reference. The potential was scanned in the range, 1.2–1.4 V using 2 ml of solution containing the mentioned analytes. To obtain experimental results, it is necessary to make

measurements in a controlled atmosphere (e.g. nitrogen), therefore before each voltammetric scan, nitrogen was purged in the solution for 5 min. Prior to each determination, the glassy carbon electrode was polished using alumina powder suspension on a smooth cloth (1, 0.3 and 0.05 μm successively). Subsequently, the electrode was ultrasonicated in a water-bath and then rinsed with water.

2.3. Voltammetric measurements

Cyclic voltammetry was used in an attempt to elucidate vitamins behaviour at the electrode surface. Cyclic voltammetry determination of VB_1, VB_6 and VB_{12} was carried out in supporting electrolyte solution. An amount of 2 ml of supporting electrolyte solution containing each vitamin were placed into the cell, and the voltammogram was recorded by scanning at a scan rate of 50 mV s^{-1} from -1.2 to 1.4 V versus Ag/AgCl (3 mol l^{-1} NaCl solution) electrode.

Differential pulse voltammetry (DPV) and square-wave voltammetry (SWV) were carried out for each vitamin in supporting electrolyte solution at pH 3. The instrumental parameters of DPV were: scan rate 50 mV s^{-1}, pulse amplitude 50 mV, sample width 17 ms, pulse width 50 ms and pulse period 200 ms. On the other hand, the SWV instrumental parameters were: step E 4 mV, amplitude 25 mV and frequency 15 Hz. These techniques were performed with anodic sweep from -1.2 to 1.4 V versus Ag/AgCl (3 mol l^{-1}NaCl solution) electrode. With these conditions, the DPV background current is higher than the corresponding to SWV, therefore SWV was selected as analytical technique due to its better sensitivity and the best peak resolution.

2.4. Software

All voltammograms were saved in ASCII format, and transferred to a PC Pentium 550 microcomputer for subsequent manipulation. All calculations were done using MATLAB 5.3 [36]. Routines for PLS-1 and ANNs were written in our laboratory following previously known algorithms [21,51]. For model optimisation, wavelength selection was carried out by a moving-window-minimum PRESS strategy implemented with a MATLAB routine [50].

2.5. Calibration and validation sets

One fifteen sample set were built to be used as calibration set. The component concentrations correspond to a central composite design (see Table 1). Two validation sets were built with a nine sample randomised design with five concentration levels for each component (see Table 3). Both calibration and validation sets were prepared adding also VB_1 and excipients which are present in a commercial sample (see below and Table 1). One of the two validation sets was used as monitoring set and the other as test set when training the ANNs (see below). The latter was also used as validation set for PLS models. This allowed us to draw conclusions about the predictive ability of the implemented models. It is important to remark that slightly different component concentration levels were set for each design in order to take into account generalisation and interpolation ability of the models. The voltammograms for all sets were recorded in random order with respect to analyte concentrations.

2.6. Pharmaceutical preparation

A pharmaceutical preparation (Dolo-Nervobión, Merck) was analysed. It is a multivitamin injection containing per 3 ml the following amounts of vitamins: VB_1 HCl 100 mg, VB_6 HCl 100 mg and VB_{12} 10 mg, and benzilic alcohol, sodium hydroxide and potassium cyanide as excipients. In order to carry out the determination, 1 ml of injection was diluted to 5 ml with supporting electrolyte solution. An aliquot of 2 ml this solution was transferred to a voltammetric cell and was deoxygenated with pre-purified nitrogen for 5 min. Finally, the SWV was sweep from -1.2 to 1.4 V versus Ag/AgCl (3 mol l^{-1} NaCl solution) electrode, and the resulting voltammograms were saved in ASCII format to subsequent chemometric treatments.

Table 1
Composition of the calibration set built using a central composite design with seven central points

Sample	PIR (mol $l^{-1} \times 10^3$)	CIA (mol $l^{-1} \times 10^3$)	VB_1 and excipients (%)[a]
1	22.91	0.50	100.0
2	43.10	0.50	100.0
3	33.00	0.37	100.0
4	33.00	0.63	100.0
5	33.00	0.50	83.2
6	33.00	0.50	116.8
7	27.00	0.42	90.0
8	39.00	0.42	90.0
9	27.00	0.58	90.0
10	39.00	0.58	90.0
11	27.00	0.42	110.0
12	39.00	0.42	110.0
13	27.00	0.58	110.0
14	39.00	0.58	110.0
15	33.00	0.58	100.0

[a] Percentage of the target concentration.

3. Results and discussion

3.1. Voltammetric techniques

The electrochemical behaviour of the three mentioned vitamins at the glassy carbon electrode was initially investigated using cyclic voltammetry in buffer solution of 0.1 mol l^{-1} potassium hydrogen phthalate and 0.1 mol l^{-1} sodium chloride pH 3. Under these conditions, VB_1 was not electro active, presenting identical cyclic voltammogram to that for the supporting electrolyte solution. Typical cyclic voltammograms for VB_6 and VB_{12} are shown in Fig. 1 (A–B). The voltammogram corresponding to VB_{12} (Fig. 1A) shows a quasi-reversible well-defined couple (1) with $E_{1/2}$ −0.72 V versus Ag/AgCl ($\Delta Ep = 60$ mV, but the $I_{anodic}/I_{cathodic}$ relationship was very different to 1) and it was assigned to Co(I)/Co(II) process. Another irreversible bad-defined couple is (2), with an oxidation peak at 0.25 V and an ill-defined reduction peak. This latter couple was assigned to Co(II)/Co(III) process [10,11]. This voltammogram also presented an oxidation wave at 1.2 V, which was assigned to VB_{12} ring oxidation, probably involving the cationic radical form [37] (see Scheme 1).

The VB_6 voltammogram (Fig. 1B) presented an irreversible couple. On the anodic sweep the are two oxidation peaks: an ill-defined peak at 0.75 V

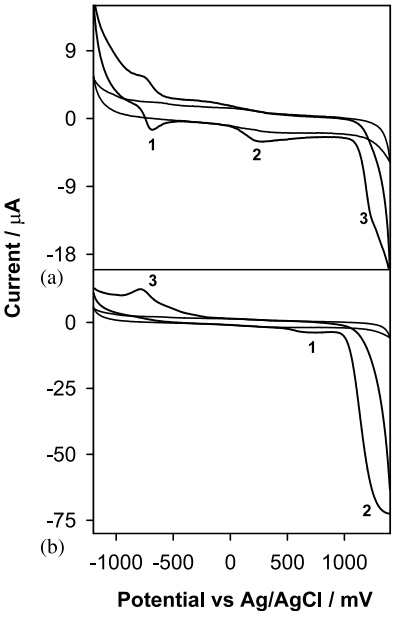
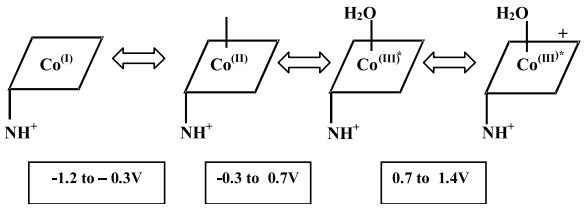


Fig. 1. Cyclic voltammograms of VB_6, VB_{12} and supporting electrolyte solution pH 3, at glassy carbon electrode. Reference electrode: Ag/AgCl (3 mol dm^{-3} NaCl), auxiliary electrode: platinum wire. (A) supporting electrolyte solution pH 3 and VB_{12} (1×10^{-3} mol l^{-1}), and (B) supporting electrolyte solution and VB_6 (2×10^{-2} mol l^{-1}). Scan rate: 50 mV s^{-1}.



Scheme 1.

(1) and a wave at 1.30 V (2) versus Ag/AgCl. The latter can be assigned to pyridoxic acid formation. On the other hand, the reduction peak at -0.8 V (3) involves a complex process. M. Blazquez et al. have documented that the latter process may involve no formation of pyridoxal aldehyde and pyridoxine. They proposed the electroreduction involving hemiacetal ring cleavage in place of aldehyde group as an intermediate step in a four-electron process [38].

The VB_6 and VB_{12} oxidation currents obtained from their individual voltammograms at 1.30 and -0.69 V potentials, respectively, showed a linear relationship with the square root of the scan rate (for scan rates ranging from 10 to 250 mV s^{-1}). This fact suggests that the VB_6 and VB_{12} oxidation are diffusion-controlled processes. On the other hand, linearity was maintained in the scan rate range between 10 and 100 mV s^{-1} when a binary mixture solution of both vitamins was processed. Therefore, electronic transfer in multivitamin solution was lower than in the individual vitamin solutions, at scan rate superior to 100 mV s^{-1}. This fact might be presumably due to interactions between the vitamins or competition by the electrode surface.

3.2. Optimisation of pH and supporting electrolyte

In order to assure the best peak resolution, the influence of pH and buffer constituents were investigated performing both CV and SWV for each vitamin. The results obtained were similar for both techniques; for this reason only SWV data were discussed. The analysed buffers were HCL, NaOH, potassium hydrogen phthalate and potassium phosphate (all at 0.1 mol l^{-1}), and potassium tetraborate 0.05 mol l^{-1}. These buffers were analysed with the addition of 0.1 mol l^{-1} NaCl

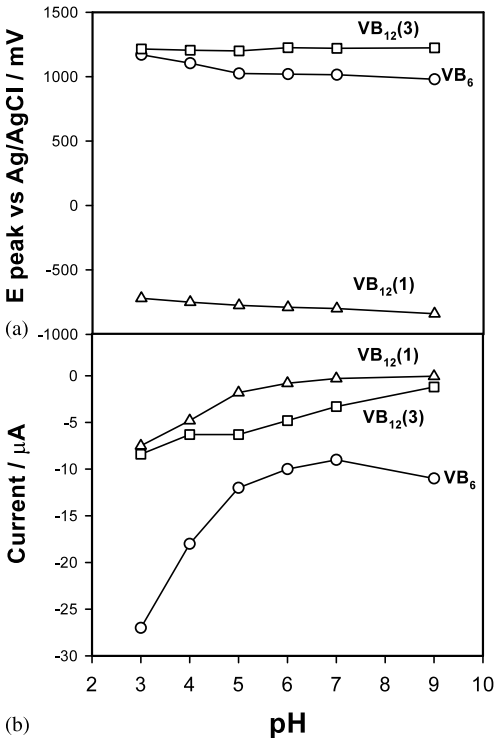
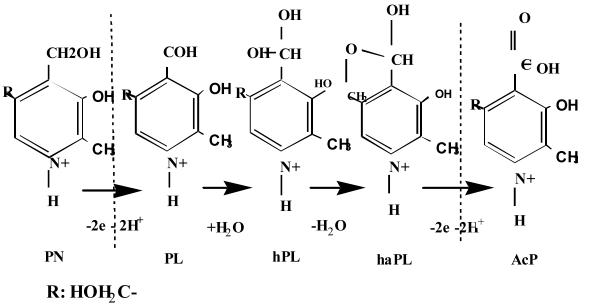


Fig. 2. Effect of pH on (A) the oxidation peak potential and (B) the peak current for VB_6 at 5×10^{-3} mol l^{-1} (circle) and two peaks of VB_{12} at 0.6×10^{-3} mol l^{-1} (triangle and square). SWV instrumental parameters: step, E 4 mV, amplitude, 25 mV and frequency, 15 Hz.

solution. pH was ranged between 3 and 9. In extreme pHs (1 and 13), vitamins were unstable and not response was observed. The SW voltammograms in the whole pH range showed three peaks for the VB_{12} and only one for VB_6. The pH change in the buffer resulted in a shift of the VB_6 oxidation peak potential and a variation in the current intensity for both vitamins. As the pH of the buffer solution increased, intensity peaks for both vitamins decreased, resulting in lower sensitivity for the analytical method. Fig. 2 (A–B) shows the Ep and Ip versus pH plots drawn for both vitamins, respectively. It known that the VB_6 electrode process is pH-dependent due to the complex distribution of species in solution provided by acid-base tautomerism and hydration equilibria [38–40]. Ep versus pH plot of VB_6 (Fig. 2A) shows the potential peak shifting to more negative potentials as the pH is increased. The



PN: pyridoxine – **PL**: pyridoxal – **hPL**: hydrate pyridoxal – **haPL**: hemiacetal pyridoxal – **AcP**: pyridoxic acid .

Scheme 2.

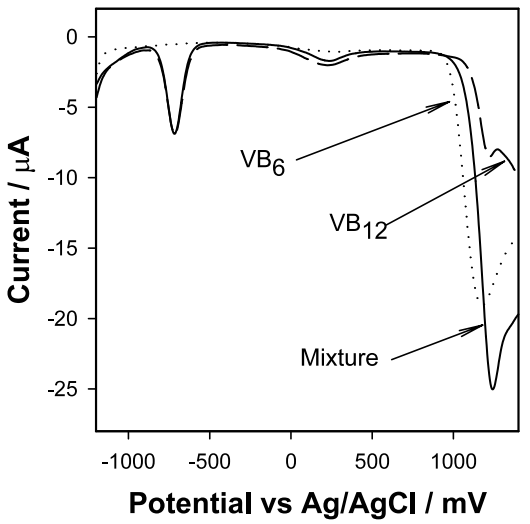


Fig. 3. SW voltammograms of VB_6 (5×10^{-3} mol l^{-1}), VB_{12} (0.6×10^{-3} mol l^{-1}) and VB_6/VB_{12} mixture (same concentrations) in supporting electrolyte solution pH 3.

curve presents a straight line with slope -65 mV pH^{-1}, in the pH range 3–5. Then, the slope changes about at pH 5. The cross-point corresponds to the first VB_6 pKa (deprotonation of ring nitrogen atom). The latter pH value is in agreement with that found in the literature [41]. On the other hand, the peak current decreases twice times as the pH changes from 3 to 5 (Fig. 2B). The pyridoxine oxidation process would involve the first step with pyridoxal formation and an ulterior step until the pyridoxic acid formation (see Scheme 2). Under our SWV experimental conditions only one overall process was observed (Fig. 3). In solution, pyridoxal is present as hemiacetal form, and the free aldehyde, which is the electroactive specie, is negligible. This fact presumably explains the first step absence in our experimental data. In order to have the electroactive specie, it is necessary a previous chemical transformation at the electrode on the time scale of the voltammetric experiments.

Fig. 2A shows that VB_{12} $peak_1$ and $peak_3$ potentials are shifted lightly to more negative potential values as the pH is increased. The Ep versus pH plots for each peak present two straight lines with similar slopes approximately 20–25 mV pH^{-1}. The first peak corresponds to the Co(I)/Co(II) process, and does not involve the participation of H^+; however this process is more reversible in acid medium. It is likely due to cobalamin molecule exists in a base-off form with the nucleotide base (5,6-dimethylbezimidazole) protonated and free from the cobalt; condition necessary for the reversibility [10,11]. The slight shift observed could indicate a slow electronic transfer, and this result is in agreement with the irreversibility increasing observed as the pH increases in the CV experiments at different pHs, experimental data not shown. On the other hand, for analogy at the metalloprotoporphyrin, the $peak_3$ might be assigned to the oxidation ring [37]. From Ip versus pH graph (Fig. 2B) we can observe that both B_{12} peaks showed better sensitivity at acid medium.

Considering the previously discussed results, the pH value of 3.0 was selected as a suitable analytical value because the investigated vitamins displayed the best peak resolution, however even it was observed overlapped peaks in the potential anodic range extreme.

One interesting aspect of the B_6 and B_{12} oxidation is the influence of the state of surface on the electrochemical process. In this sense, we have checked the reproducibility of the voltammograms after different pre-treatments of the working electrode. The procedure used in our work includes an electrochemical pre-treatment, which consists in repetitive cycling in potential range in supporting electrolyte solution.

Fig. 3 shows the SW voltammograms for each of the individual vitamins and the mixture solution at same relationship of concentrations that those

for individual solutions; we can observe the extensive degree of the overlap.

When the variation of SWV peak intensity with the concentration was evaluated, there were not linear relationships between current intensities at characteristic peak potentials (-0.72 and 1.18 V for VB_{12} and VB_6, respectively) and concentration. Moreover, we observed both VB_{12} and VB_6 peak intensities were proportional to concentration into range of 10^{-4}–1.2 10^{-3} mol l^{-1} for VB_{12} and 10^{-3}–3.5 10^{-2} mol l^{-1} for VB_6. When the concentrations were superior to the previously cited limits, we observed electrodic surface saturation for both vitamins. As it is well known, SWV is one of the more widely applied electrochemical techniques in quantitative analysis due to its sensitivity. Thus, very low detection limits near to 10^{-8} mol l^{-1} can be attained when applying this technique [42]. In contrast, several researchers have documented concentration dynamic ranges between 10^{-6} and 10^{-3} mol l^{-1} with quantification limits approximately 10^{-4} mol l^{-1}, using the glassy carbon as the working electrode in SWV for drug analysis [43,44]. These figures are similar to the ones achieved in the present report. As it was mentioned above, the electrodic processes for two vitamins might involve electrochemical mechanisms coupled to chemical reactions where electron transfer does not seem to be simple. On the other hand, the more irreversible redox systems, the lower and broader current peaks are obtained, compared with those found for reversible systems [45]. It might explain why in our case it was necessary higher concentrations for obtaining a good response. These results suggest that with linear univariate method it is impossible to model the system. This occurs basically because the overlapping of voltammetric peaks and non-linear response, presumably due to complex interactions between the analytes. Another interesting observation can be made in Fig. 3, it is that in presence of VB_{12}, the VB_6 peak potential was shifted to the third VB_{12} peak potential. It is known about electrocatalytic properties of VB_{12} towards the reduction and oxidation of several analytes [46–49]. This fact suggests that the complex being oxidized is an adduct between hydrate pyridoxal (hPL) or hemiacetal (haPL) and VB_{12a} $(Co(III))^+$. It might be produced by both the coordination of haPL (or another PN oxidable species) to VB_{12} and the VB_{12} (haPL) complex formation. This requires to be confirmed by ulterior investigation.

3.3. Application of PLS-1 and ANNs

PLS is a standard method for analysing multicomponent mixtures, which has been systematically applied in pharmaceutical preparations. The presence of certain types of mild non-linearities can in principle be modeled by PLS using additional spectral factors [31,32]. In order to apply PLS-1 and ANNss, voltammograms for the standard samples were recorded in the range -1.2–1.4 V (1299 points in total). These voltammograms were then subjected to PLS-1 analysis. The optimum ranges and the corresponding statistical parameters are shown in Table 2. Sensor selection is known to be a critical step for increasing the predictive ability of multivariate analysis, and should ideally eliminate both uninformative and/or highly correlated data. In the present report we have applied a moving window strategy to the calibration set itself, in order to find the most informative range in the spectra by localising the minimum calibration variance [50]. It is important to be sure that the selected regions match well with the location of component voltammogram peaks with minimum overlapping. Once the optimum ranges were obtained, the cross-validation procedure was applied to assist in the selection of the number of factors, using the criteria proposed by Haaland and Thomas [21]. In our case, the value of F corresponding to a probability smaller than 0.75 yielded the optimum numbers of factors shown in Table 2. This table also gives the values of other important statistical parameters such as the root mean square error for cross-validation (RMSECV) and prediction (RMSEP). It is important to remark that in order to use an amount of data similar to that employed for training the ANN, PLS was trained by using both the calibration and the monitoring sets (24 samples). The results obtained showed a significant increase in the quality, as compared with those obtained when we used only the fifteen calibration set As can be seen in Table 2, the calibration parameters are

Table 2
Statistical parameters when applying both PLS-1 and ANN analyses

Figures	CIA		PIR	
	PLS-1	ANN	PLS-1	ANN
Region	150–600 (sensor number)	−900–0 (mV)	600–1000 (sensor number)	0–1000 (mV)
PLS-1 factors	4	–	4	–
ANN model	–	(4,2,1)	–	(4,2,1)
RMSECV[a] (mol $l^{-1} \times 10^3$)	0.043	0.042	3.4	3.3
RMSEP[a] (mol $l^{-1} \times 10^3$)	0.055	0.053	4.3	2.0

[a] $\mathrm{RMSE} = [\frac{1}{I}\Sigma_1^I(c_{act} - c_{pred})^2]^{1/2}$, RMSECV: c_{act} corresponds to the calibration set when cross-validation is applied and RMSEP: c_{act} corresponds to the validation set.

acceptable for both analytes considering the complexity of the present system under study. An important conclusion can be reached by looking at the number of PLS factors required to adequately model both components. In both cases, they exceed in two the theoretical limit of two expected factors. This could be caused by non-linearities which are accounted for by including additional factors in the PLS calibration models.

ANNs are calibration methods especially created to model non-linear information, although they are also able to deal with a linear behaviour and can often improve the results in comparison with a linear model. The so-called multilayer feed-forward networks [32,51] are often used for prediction as well as for classification. In the present work we have used an ANN that consists of three layers of neurons or nodes, which are the basic computing units: the input layer with a number of active neurons corresponding to the predictor variables in regression, and one hidden layer with a number of active neurons. The input and the hidden layer numbers are optimised during training, and the output layer has just one unit. The neurons are connected in a hierarchical manner, i.e. the outputs of one layer of nodes are used as inputs for the next layer and so on. In the hidden layer the sigmoid function $f(x) = 1/(1 + e^{-x})$ is used, and the output of the hidden neuron j, O_j, is calculated as:

$$O_j = f\left[\sum_{i=1}^{m}(s_i w_{ij} + w_{bj})\right] \quad (1)$$

where s_i is the input from neuron i in the layer above, to neuron j in the hidden layer, w_{ij} are the connection weights between neurons i and j, w_{bj} is the bias to neuron j and m is the total number of neurons in the layer above. Both in the input and output layers linear functions are used. In the presently used ANN, learning is carried out through the back-propagation rule [51]. It is important to stress that ANNs trained with this rule have a remarkable advantage: there is no need to know the exact form of the analytical function on which the model should be built. Thus, neither the functional type nor the number of parameter in the model need to be given [51].

The calibration set of 15 samples was used to train the ANNs. One of the nine sample validation set was used as monitoring set. The nine sample PLS-validation set was used as the test set. The number of neurons in the input hidden layers were optimised by trial and error. The finally selected architecture for both components is displayed in Table 2. The numbers between brackets indicate how many active neurons are employed in each layer. This means that the employed architecture has four input neurons, two hidden neurons and a single output neuron for both components. In order to find the best model, each ANN was trained with the above mentioned training set, but it was subsequently stopped before it learns idiosyncrasies present in the training data [32]. This was achieved by searching the minimum value of the root mean square error for the monitoring set (RMSEMonit). On the other hand, it is important consider that the ANN should not be overtrained. Fact that can be

Table 3
Composition of validation set and results obtained when applying PLS-1 and ANNs

Sample	CIA (mol $l^{-1} \times 10^3$)			PIR (mol $l^{-1} \times 10^3$)		
	Actual	Predicted PLS-1	Predicted ANN	Actual	Predicted PLS-1	Predicted ANN
1	0.50	0.47	0.48	24.00	29.50	26.13
2	0.40	0.38	0.47	41.00	33.80	37.48
3	0.60	0.50	0.52	28.00	31.20	28.42
4	0.60	0.54	0.49	41.00	35.20	37.84
5	0.48	0.50	0.48	30.00	32.00	30.80
6	0.50	0.54	0.50	32.00	28.10	29.37
7	0.52	0.44	0.49	32.00	33.90	33.44
8	0.50	0.45	0.50	35.00	33.10	34.74
9	0.48	0.51	0.46	32.00	35.50	31.97
Average recovery (%)[a]		95.0 (9.2)	97.1 (10.0)		101.0 (13.8)	99.1 (6.2)
M1[b]	0.50	0.52(0.06)	0.49(0.03)	32.00	28.2 (1.2)	29.5(1.2)
M2[b]	0.50	0.46(0.05)	0.51(0.01)	32.00	29.2 (1.7)	29.4(1.7)

[a] Values between parenthesis correspond to S.D.
[b] Values between parenthesis correspond to S.D. for five replicates.

managed by taking into account that the number of objects should not exceed the number of adjustable weights. In the presently trained ANNs, weights were ($5 \times 3 \times 1 = 15$). These figures were obtained after considering the number of input and hidden layers plus one bias neuron on each layer.

ANNs were applied with significant improvement of the obtained results. Table 3 shows results obtained when this non-linear multivariate model was applied on the nine samples validation set and two commercial injections. As can be seen the improvement on the prediction observed when ANNs are applied explains the power of this method not only in modelling non-linearities but also in solving complex and overlapped signals.

4. Conclusions

The contents of two components present in pharmaceutical preparations, VB_6 and VB_{12}, were simultaneously determined using voltammetric data, together with PLS multivariate calibrations and ANNs. A validation set of synthetic mixtures was employed for testing the accuracy and precision of the methods, and a commercial pharmaceutical was analysed. Reasonably good recoveries and statistical indicators were obtained with an improvement on the results when ANNs were applied.

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