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Complementary use of partial least-squares and artificial neural networks for the non-linear spectrophotometric analysis of pharmaceutical samples

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Abstract The complementary use of partial least-squares (PLS) multivariate calibration and artificial neural networks (ANNs) for the simultaneous spectrophotometric determination of three active components in a pharmaceutical formulation has been explored. The presence of non-linearities caused by chemical interactions was confirmed by a recently discussed methodology based on Mallows augmented partial residual plots. Ternary mixtures of chlorpheniramine, naphazoline and dexamethasone in a matrix of excipients have been resolved by using PLS for the two major analytes (chlorpheniramine and naphazoline) and ANNs for the minor one (dexamethasone). Notwithstanding the large number of constituents, their high degree of spectral overlap and the occurrence of non-linearities, rapid and simultaneous analysis has been achieved, with reasonably good accuracy and precision. No extraction procedures using non-aqueous solvents are required.

Keywords Partial least-squares · Artificial neural networks · Chlorpheniramine · Dexamethasone · Naphazoline

Introduction

The application of multivariate calibration methods to both spectral or electrochemical data in the biomedical and pharmaceutical fields has acquired a routine nature [1, 2, 3, 4, 5, 6, 7, 8]. Partial least-squares (PLS) has become the de facto standard for multivariate calibration because of the quality of the obtained calibration models, the ease of its implementation and the availability of software [9]. It shows the advantage of using full spectra, which is critical for the spectroscopic resolution of complex mixtures of analytes. It allows for a rapid determination of components, usually with no need of a prior separation. An additional advantage of robust multivariate methods, such as PLS, 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 ophthalmic and nasal solutions as well as in syrups, whose excipients may show absorption spectra that are severely overlapped with those from the analytes. Although PLS assumes a linear relationship between the measured sample parameters and the intensity of its absorption bands, small deviations from linearity are acceptable as they can readily be suppressed by including additional modelling factors [10]. 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) [7, 11] are applicable in the latter cases.

In the present report, we discuss the possibility of analysing a three-component pharmaceutical mixture in which a large difference among the analyte concentrations exists. If all constituents are to be determined with a single calibration set, the major analytes would display concentrations which deviate from Beer's law. However, they can be determined with the aid of PLS-1, though requiring additional factors to those expected if the system were linear. On the other hand, the minor analyte requires the use of an ANN for successful prediction results. It is apparent that the non-linearities cannot be adequately modelled by PLS, a fact which may be ascribed to significant chemical interactions with the major sample components.

Specifically, the present study is concerned with common commercial drops employed for the temporary relief of nasal congestions, which contain a mixture of an antihistaminic, a vasoconstrictor, a corticoid, antibiotics and

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several excipients. A typical formulation contains chlorpheniramine [γ-(4-clorophenyl)-N,N-dimethyl-2-pyridinepropanamine (CHL)], naphazoline hydrochloride [2-(1-naphthylmethyl)-2-imidazoline monohydrochloride (NAP)], dexamethasone 21-phosphate [9 α-fluoro-16 α-methyl-11 β,17 α,21-trihydroxy-1,4-pregnadiene-3,20-dione 21-phosphate (DEX)], two antibiotics (neomycin and gramicidin) and several excipients such as sodium, potassium and calcium chlorides, thimerosal and sodium hydrogen carbonate. CHL is an antihistaminic derived from propylamine, indicated for the treatment of many allergies [12]. NAP is an imidazole-based sympathomimetic and vasoconstrictor of relatively long-lasting action that acts on the alpha-receptors of the vascular smooth muscle [12]. DEX is a synthetic glucocorticoid, typically indicated for the treatment of several pathologies, due to its anti-inflammatory and immunosuppressor effects. It leads to a symptomatic relief, but has no effects on the development of the underlying disease [12].

Several methods are available for the determination of the CHL, NAP and DEX by high-performance liquid chromatography in different pharmaceutical preparations, either alone or in combination with other active ingredients [13, 14, 15]. CHL has been determined by derivative spectrophotometry [16]. DEX and NAP have been simultaneously determined by using capillary electrophoresis in nose drops [17], while DEX has been quantified by flow-injection with chemiluminometric detection [18] and by derivative spectrophotometric determination in single formulations and also in combination with other drugs [19]. Our results are indicative that the combination of UV-visible spectroscopy with suitable chemometric techniques also constitutes a valid analytical strategy.

Materials and methods

Apparatus. Electronic absorption measurements were carried out on a Perkin-Elmer Lambda 20 spectrophotometer, using 1.00 cm quartz cells. All spectra were saved in ASCII format, and transferred to a PC Pentium 550 microcomputer for subsequent manipulation.

Software. PLS-1 was applied with the well-tested program MULTIVAR, written in Visual Basic according to algorithms already described [20]. Neural Unscrambler software version 1.02 from CAMO (Trondheim, Norway) was employed for ANN calculations and Sigma Plot 5.0 from SPSS (Chicago, USA) for statistical data processing.

Reagents. All experiments were performed with analytical-reagent grade chemicals. Stock solutions of chlorpheniramine (5.01 g L^{-1}), naphazoline (4.92 g L^{-1}) and dexamethasone disodium phosphate (1.02 g L^{-1}) were prepared by dissolving the compounds in doubly distilled water. A mixture of the excipients was also prepared in distilled water: sodium chloride (1.00 g L^{-1}), calcium chloride (50 mg L^{-1}), potassium chloride (1.00 g L^{-1}), thimerosal (100 mg L^{-1}) and sodium hydrogen carbonate 3.00 g L^{-1}. This solution also contained two antibiotics: neomycin (5.00 g L^{-1}) and gramicidin (10 mg L^{-1}).

Experimental calibration and validation sets. Two 15-sample sets were built to be used as calibration and validation sets for PLS-1. For the application of ANNs, the first 15 sample set was randomly divided into a training set (10 samples) and a monitoring set (5 samples), while the second 15 sample set was used as test set. The component concentrations within both 15 sample sets corresponded to central composite designs with different concentration levels (see Table 1). Central composite designs are formed by a three-component full-factorial design at two levels (i.e., 2^3=8 samples), a central point (1 sample), and a star design (2×3=6 samples), making a total of 15 samples. [21] All samples also contained the antibiotics neomycin and gramicidin, and excipients in the same concentrations as in the pharmaceutical preparation (i.e. 1.0 g L^{-1}). It should be noted that the concentrations of both CHL and NAP lay outside their known linear absorbance-concentration ranges, which are 5–125 mg L^{-1} and 6–130 mg L^{-1} respectively. All spectra were recorded in random order with respect to analyte concentrations, in the range 200–350 nm, every 1 nm (i.e. 151 data points per spectrum), although wavelength selection was applied before multivariate calibration (see below).

Table 1 Composition of the 15 sample central composite designs used as calibration and validation sets for applying partial least squares (PLS-1) and artificial neural networks (ANNs) methods.

CHL (mg l^{-1})		NAP (mg l^{-1})		DEX (mg l^{-1})	
Calibration	Validation	Calibration	Validation	Calibration	Validation
5.0	32.0	35.0	32.0	6.0	7.3
140.0	118.0	35.0	32.0	6.0	7.3
35.0	32.0	140.0	118.0	6.0	7.3
140.0	118.0	140.0	118.0	6.0	7.3
35.0	32.0	35.0	32.0	13.0	11.6
140.0	118.0	35.0	32.0	13.0	11.6
35.0	32.0	140.0	118.0	13.0	11.6
140.0	118.0	140.0	118.0	13.0	11.6
87.5	75.0	87.5	75.0	3.6	5.7
87.5	75.0	87.5	75.0	15.4	13
87.5	75.0	0.0	0.0	9.5	9.5
87.5	75.0	175.0	150.0	9.5	9.5
0.0	0.0	87.5	75.0	9.5	9.5
175.0	150.0	87.5	75.0	9.5	9.5
87.5	75.0	87.5	75.0	9.5	9.5

Commercial sample. One commercial sample was tested: Dexalergin (Syncro Laboratories, Argentina), a solution containing (per 100 ml) 100 mg chlorpheniramine maleate, 100 mg naphazoline hydrochloride, and 5.0 mg dexamethasone disodium phosphate. The sample was prepared by diluting 3.00 ml of the solution with doubly distilled water in a 25.00 ml volumetric flask before measurements.

Results and discussion

Application of PLS

Figure 1 shows the aqueous solution spectra of the analytes CHL, NAP and DEX, as well as that of the mixture of excipients. As can be seen in this figure, the strong overlapping among their spectra precludes the direct determination of the components by conventional spectrophotometry. An additional disadvantage is present in the mixtures studied: the concentration of DEX is much smaller than those of CHL and NAP. In such a situation, the resolution of the three components with a single calibration set represents a difficult task for a multivariate cali-

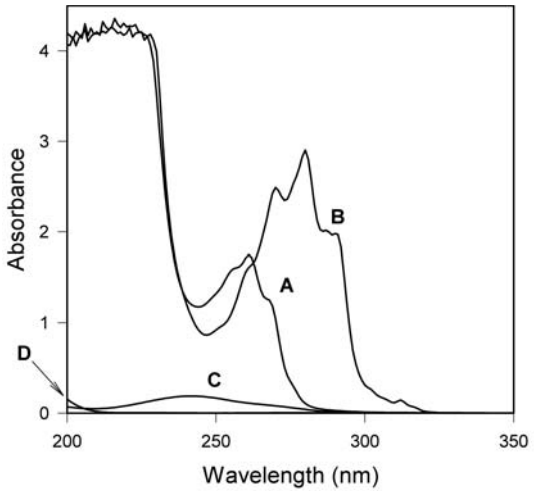

Fig. 1 Aqueous solution spectra of the analytes: *A* CHL (100.0 mg l^{-1}), *B* NAP (100.0 mg l^{-1}), *C* DEX (5.0 mg l^{-1}), *D* excipients

bration technique. In order to obtain reasonable sensitivity for the minor constituent, it is necessary to work with large concentrations of the major ones. This leads, in principle, to deviations of the signal/concentration linearity for the major analytes, but may also introduce non-linearities in the minor component if chemical interactions occur between the latter and the more concentrated compounds. In the present case, the C=O moieties of DEX may form strong hydrogen bonds with the NH protons of CHL or NAP, providing the basis for chemically induced deviations from Beer's law.

A usual method for the quantification of multicomponent mixtures, which has been thoroughly applied for analysing pharmaceutical preparations, is PLS, in which the presence of certain types of mild non-linearities can in principle be modelled by using additional spectral factors. Thus, electronic absorption spectra for the standard samples were recorded in the range 200–350 nm and subjected to PLS-1 analysis. The optimum spectral ranges (including the number of data points) and the corresponding statistical parameters are shown in Table 2. Wavelength selection is 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 [22]. However, the technique should not be blindly applied: after selecting an adequate region, care should be taken in checking whether the results are consistent with the spectroscopic properties of the analytes at hand. In our case, the selected ranges coincided with the location of component spectral peaks with minimum overlapping, a fact which strongly supports their use for multivariate regression. Once the optimum spectral ranges were obtained, the cross-validation procedure was applied to assist in the selection of the number of factors. This consists of systematically removing one of the training samples in turn, and using only the remaining

Table 2 Composition of the 15 sample central composite designs used as calibration and validation sets for applying partial least squares (PLS-1) and artificial neural networks (ANNs) methods.

Parameters[a]	CHL	NAP	DEX
Spectral region nm	240–280	245–320	236–256
Number of data points	41	76	21
Factors	5	5	7
PRESS (g ml^{-1})^2	49.2	0.938	10.2
RMSD g ml^{-1}	1.81	0.25	0.61
REP%	2.07	0.29	6.16
R^2	0.9981	0.9999	0.9772
SEN	0.0112	0.0571	0.009
SEL	0.185	0.454	0.080
γ^{-1} (mg l^{-1})[b]	0.089	0.017	0.111

[a] $\text{PRESS} = \sum_{1}^{I} (c_{\text{act}} - c_{\text{pred}})^2$, $\text{RMSD} = \left[\frac{1}{I-1} \sum_{1}^{I} \left(c_{act} - c_{pred}\right)^2 \right]^{1/2}$,

$\text{REP\%} = \frac{100}{\bar{c}} \left[\frac{1}{I-1} \sum_{1}^{I} (c_{\text{act}} - c_{\text{pred}})^2 \right]^{1/2}$, and $R^2 = 1 - \frac{\sum_{1}^{I} (c_{\text{act}} - c_{\text{pred}})^2}{\sum_{1}^{I} (c_{\text{act}} - \bar{c})^2}$,

$\bar{c}$ is the average component concentration in the I calibration mixtures.

[b] $\delta r \approx 0.001$ (taken as the standard deviation of several blank sample signals)

ones for construction of the latent factors and regression. In order to estimate the optimum number of factors, the criteria proposed by Haaland and Thomas [1] were used. 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. Note the large number of factors required to adequately model the three components, especially in the case of DEX. This latter table also gives the values of other important statistical parameters such as the square of the correlation coefficient (R^2), the root mean square difference (RMSD) and the relative error of prediction (REP%). As can be seen in Table 2, the calibration parameters and figures of merit are excellent for CHL and NAP, but those for DEX are rather poor. This fact may be due to the lesser sensitivity towards this particular analyte, but (see below) it could also be caused by non-linearities which cannot be accounted for by including additional factors in the PLS calibration model.

Selectivity (SEL), sensitivity (SEN) and limit of determination are important figures of merit, which allow one to evaluate the performance of the analytical methodologies. They can be calculated and used for method comparison or to study the quality of a given analytical technique. The limit of determination is not strictly necessary in the present case, but only for the assessment of impurities. The SEL is a measure of the degree of overlap, and indicates the part of the total signal which is not lost due to spectral overlap [23]. In multivariate calibration it usually can be defined by resorting to net analyte signal (NAS) calculations [23]:

$$\text{SEL} = ||\mathbf{s}_k*||/||\mathbf{s}_k|| \quad (1)$$

where s_k is the spectrum of pure component k at unit concentration, and s_k^* is its corresponding NAS.

Table 3 Results obtained for CHL and NAP when applying PLS-1 analysis to the validation set and one commercial sample. The values in parentheses are the standard deviations for five replicates

Sample	CHL			NAP		
	Added mg l^{-1}	Found mg l^{-1}	Recovery %	Added mg l^{-1}	Found mg l^{-1}	Recovery %
1	32.0	31.8	99.4	32.0	32.1	100.3
2	118.0	119.0	100.8	32.0	32.0	100.0
3	32.0	32.0	100.0	118.0	118.0	100.0
4	118.0	118.0	100.0	118.0	118.0	100.0
5	32.0	32.3	100.9	32.0	32.2	100.6
6	118.0	120.0	101.7	32.0	31.9	99.7
7	32.0	32.6	101.9	118.0	118.0	100.0
8	118.0	118.0	100.0	118.0	118.0	100.0
9	75.0	76.7	102.3	75.0	74.5	99.3
10	75.0	77.1	102.8	75.0	74.8	99.7
11	75.0	76.7	102.3	0.0	0.02	–
12	75.0	78.4	104.5	150.0	148.0	98.7
13	0.0	0.03	–	75.0	74.4	99.2
14	150.0	150.0	100.0	75.0	74.5	99.3
15	75.0	77.2	102.9	75.0	74.6	99.5
Dexalergin	100.0	97.1 (7)	97.1	100.0	106.5 (7)	106.5

On the other hand, the sensitivity (SEN) tells to what extent the response due to a particular analyte varies as a function of its concentration [23], and is given by:

$$\mathrm{SEN} = ||\mathbf{s}_k*|| \quad (2)$$

Another parameter, that may be useful for method comparison, is the analytical sensitivity γ [23, 24]. It may be defined, in analogy to univariate calibration, as the quotient:

$$\gamma = (\mathrm{SEN}/\delta r) \quad (3)$$

and allows one to compare analytical methods regardless of the specific technique, equipment, and scale employed and establishes the minimum concentration difference (γ^{-1}) which is statistically discernible by the method across the dynamic range where it is applicable. In Eq. 3, r is a measure of the degree of instrumental noise.

Table 3 shows the result obtained when applying PLS-1 analysis for CHL and NAP to the validation set. As can be seen, the results are excellent for these particular analytes. The results for DEX, on the other hand, are disappointing, and will be deferred for a comparison with those provided by employing ANN.

Detection of non-linearities

The presence of non-linearity may be detected by applying both graphical and numerical detection tools. To detect the non-linear (quadratic) nature of the relationship between some of the first factors and the concentration, the Mallows augmented partial residual plot (APaRP) is recommended as the most universal diagnostic tool [10]. This procedure is implemented in the following way: individual analyte concentrations contained in the vector $\mathbf{c}_k$ are first regressed against the first A PLS-1 components (PCs) of the data matrix R and the square of the first PC [10]:

$$\mathbf{c}_k = b_0 + b_1\mathrm{PC}_1 + \ldots + b_A\mathrm{PC}_A + b_{11}(\mathrm{PC}_1)^2 + \mathbf{e}_{\mathrm{APaRP}} \quad (4)$$

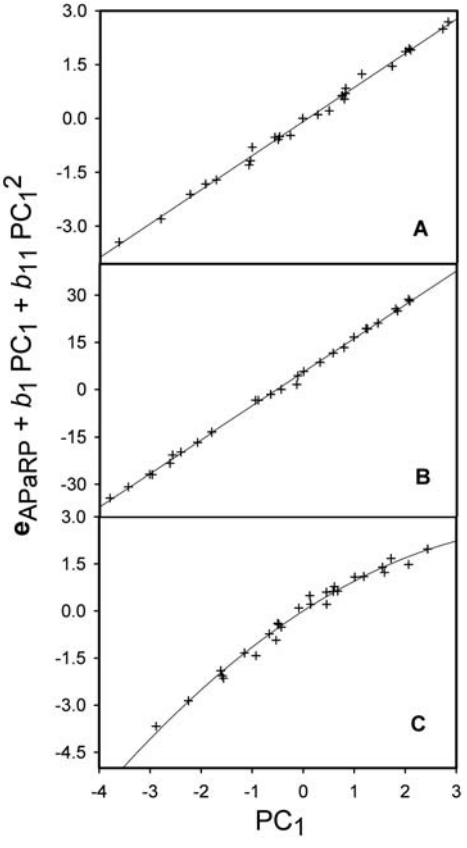

Fig. 2A-C Mallows augmented partial residual plot (*APaRP*) for CHL (5 factors) (**A**), NAP (5 factors)(**B**) and DEX (7 factors)(**C**)

where e_{APaRP} is a vector collecting the APaRP fitting residuals. The relevant plot is obtained by plotting the sum $[e_{\mathrm{APaRP}}+b_1\ \mathrm{PC}_1+b_{11}\ (\mathrm{PC}_1)^2]$ as a function of PC_1 [10].

Figure 2 shows the APaRPs for the calibration of the three studied analytes, using five PCs for both CHL and NAP, and seven PCs for DEX. It suggests that the deter-

mination of DEX should be treated as a strongly non-linear problem (Fig. 2C) and that one could expect an improvement in the results when applying non-linear calibration models such as ANNs for this particular analyte. The cases of CHL and NAP require suitable statistical tests in order to ascertain the degree of non-linearity. One commonly employed diagnostic tool to investigate the correlation in residuals is the Durbin-Watson test [25]. This test examines the null hypothesis (H_0) that successive residuals are uncorrelated, versus the alternative hypothesis that correlation occurs. The relevant statistical indicator is d, computed in the following way [25]:

$$d = \frac{\sum_{i=2}^{n} (e_i - e_{i-1})^2}{\sum_{i=2}^{n} e_i^2} \quad (5)$$

where e_i is a given residual, and e_{i-1} the preceding one (the residuals are ranked according to the associated value in the vector c_k). The value of d is compared to two critical values: d_L (lower) and d_U (upper), leading to the following conclusions: (a) if $d<d_L$ the null hypothesis is rejected, indicating correlation between residuals, (b) if $d_L<d<d_U$ the test is inconclusive, and (c) if $d>d_U$ the correlations are considered to be negligible.

An alternative is the so-called runs test, which examines the number of series of consecutive residuals with the same sign (runs). The test is based on the calculation of the indicator z [25]:

$$z = (u - \sigma + 0.5)/\sigma \quad (6)$$

where u is the number of runs, and:

$$\mu = 1 + 2n_+n_-/(n_+ + n_-) \quad (7)$$

$$\sigma^2 = 2n_+n_-(2n_+n_- - n_+ - n_-)/[(n_+ + n_-)^2(n_+ + n_- - 1)] \quad (8)$$

In Eq. 7 and Eq. 8, n_+ and n_- are the number of positive and negative residuals. Once z is obtained, it is compared with the critical value 1.96 (at 95% confidence level). If $|z|>1.96$, it can be concluded that non-linearity is present.

Table 4 shows the results obtained by analysing the APaRP plots of Fig. 2 with the above two tests. As can be seen, the results for CHL and NAP suggest the presence of only mild non-linearities, whereas those for DEX clearly indicate that this analyte behaves in a strongly non-linear manner.

Table 4 Results of the Durbin-Watson and runs tests applied to the diagnostic augmented partial residual plots, in order to detect the presence of non-linearities for the studied analytes

Component	Durbin-Watson test[a]		Runs test[a]	
	d value	Conclusion	z value	Conclusion
Chlorpheniramine	1.97	Linear	3.80	Non-linear
Naphazoline	1.31	Non-linear	1.97	Borderline
Dexamethasone	0.67	Non-linear	8.89	Non-linear

[a]The critical d and z values for the Durbin-Watson and runs tests at α=0.05 are, respectively, d_L=1.32, d_U=1.92 and z_{crit}=1.96

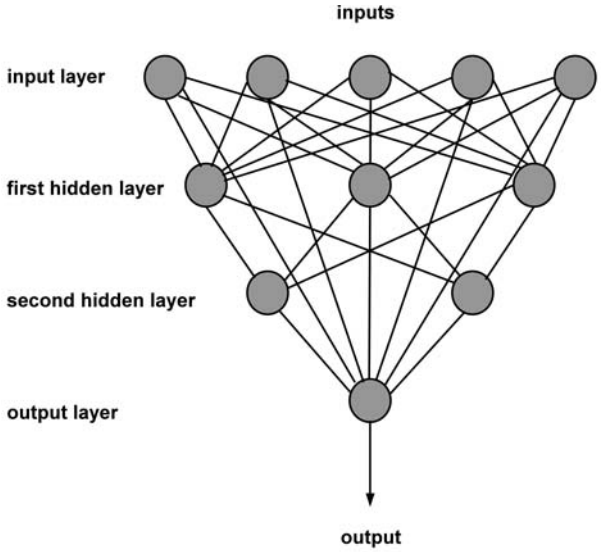

Fig. 3 Schematic representation of the unscrambler multilayer feed-forward network with direct connections used in the present work

Application of ANNs

When non-linearities are significant and cannot be properly modelled by PLS, one can apply ANNs [11]. These are calibration methods especially created to model non-linear information, although they are also able to deal with linear behaviour and can often improve the results in comparison with a linear model. These so-called multilayer feed-forward networks [26] are often used for prediction as well as for classification. The unscrambler multilayer feed-forward network (UMLF) consists of four layers of neurons or nodes which are the basis computing unit: the input layer with a number of active neurons (up to 16) corresponding to the predictor variables in regression, two hidden layers with a number of active neurons (up to 8 in the first and 3 in the second hidden layer) which are optimised during the training, and the output layer with just one unit (Fig. 3).

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. Direct connections from the input layer to the output layer are also available. In an ANN using direct connections, the weighted input data are directly added to the output neuron. This increases the training speed, but its use is inadvisable for modelling systems with strong non-linearities.

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:

$$O_j = f[\sum_{i=1}^{m} (s_i w_{ij} + w_{bj})] \quad (9)$$

Table 5 Comparative prediction of DEX on the validation set and one commercial sample by applying PLS-1 and ANNs methods

Validation sample	DEX mg l^{-1}			Recovery %	
	Added	Found ANNs	Found PLS-1	ANNs	PLS-1
1	7.3	8.7	4.1	118.6	56.1
2	7.3	6.3	3.8	86.7	51.5
3	7.3	6.1	3.7	83.4	50.6
4	7.3	6.3	3.4	86.3	46.9
5	11.6	12.1	8.6	104.3	73.8
6	11.6	9.7	8.2	83.8	70.8
7	11.6	9.6	8.3	82.4	71.4
8	11.6	8.1	8.2	70.2	70.4
9	5.7	5.7	2.1	99.9	36.7
10	13.0	11.5	9.9	88.8	76.0
11	9.5	10.0	6.4	105.6	67.2
12	9.5	7.7	5.7	81.2	60.4
13	9.5	9.6	6.2	101.1	65.4
14	9.5	8.2	6.0	85.8	63.1
15	9.5	7.7	6.2	81.1	65.6
Dexalergin	5.0	4.9	–3.3	98.0	–

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. Linear functions are used in both the input and output layers. In the UMLF the learning is carried out through the back-propagation rule.

In the present work we applied UMLF to the determination of DEX. The calibration set of 15 samples was divided into two randomised subsets: a training and a monitoring set, formed by 10 and 5 samples respectively. The 15-sample validation set was used as the test set. The number of neurons in the input and first and second hidden layers were optimised by trial and error. The finally selected architecture was (5, 4, 0, 1)+ (the numbers indicate how many active neurons are employed in each layer, and '+' stands for the presence of direct connections): this means that the employed architecture had five input neurons, four neurons in a single hidden layer and a single output neuron. The results when analysing the test set with both PLS and ANNs are given in Table 5, which also shows the results obtained for a real sample. As can be seen, the results are reasonably good when the ANNs are used, whereas very poor recoveries are obtained with PLS-1. This fact suggests that the simultaneous determination of all three analytes is possible using PLS-1 for CHL and NAP and ANNs for DEX, employing a single calibration set.

To conclude, the contents of several components usually present in nasal solutions, chlorpheniramine, naphazoline and dexamethasone were simultaneously determined using electronic absorption data, together with PLS multivariate calibration for the former two and an ANN for the latter. 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, statistical indicators and figures of merit (SEL, SEN and analytical sensitivity) were obtained with PLS-1 for the two major analytes, chlorpheniramine and naphazoline. The use of an ANN allowed the determination of dexamethasone, a minor component which could not be adequately modelled by PLS-1.

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