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Simultaneous spectrophotometric determination of phenobarbital, phenytoin and methylphenobarbital in pharmaceutical preparations by using partial least-squares and principal component regression multivariate calibration

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Abstract

Two multivariate calibration methods, partial least squares (PLS-2) and principal component regression (PCR) have been applied to the simultaneous spectrophotometric analysis of ternary mixtures of phenytoin (DPH), phenobarbital (PBT) and methylphenobarbital (MPBT) in the Comital-L pharmaceutical formulation. The PLS-2 and PCR procedures were employed to evaluate the data of a variable number of calibration solutions measured over the wavelength range 400–700 nm. The concentration ranges used to construct the calibration matrix were varied between 5 and 30 μg ml^{-1}. The proposed methods were validated by applying them to the analysis of the Comital-L pharmaceutical formulation and the average relative errors were less than 6% for each one of the analyzed compounds. The results obtained by both proposed methods have been compared with the results obtained by application of a RPLC reference method.

Keywords: Formulation analysis; Anticonvulsant drugs; Partial least-squares (PLS-2) regression; Principal component regression (PCR); UV spectroscopy

1. Introduction

The development of suitable methods for the routine analysis of drugs in pharmaceutical preparations is currently of particular importance in the pharmaceutical and other industries as well as government laboratories.

Multivariate calibration methods such as principal component regression (PCR) and partial least-squares (PLS) have been successfully applied to the quantitative pharmaceutical analysis, par-

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ticularly by using ultraviolet [1–4], near-infrared [5] and fluorimetric data [6]. The general goals and algorithms for PCR and PLS are similar [7–9], although they differ in the methods used to carry out the spectral decomposition and the subsequent correlation with the concentration matrix. The application of PCR or PLS, which are full-spectrum methods, is particularly well suited for systems exhibiting considerable absorption band overlap, since they circumvent some of the limitations of other methods that are frequently used in practice, such as multiple linear regression (MLR) [10,11].

Phenytoin (DPH), phenobarbital (PBT) and methylphenobarbital (MPBT) are important drugs for the treatment of epilepsy. However, because more than 25% of patients develop refractory epilepsy in the classical therapy, the use of associations of these drugs still becomes unavoidable [12]. Thus, DPH and PBT are frequently employed alone or combined in pharmaceutical preparations. Moreover, some formulations also contain MPBT such as the tablets known as Comital-L.

The simultaneous determination of binary mixtures of DPH and PBT have been performed by several methods including titrimetry, gas chromatography (GC), high performance liquid chromatography (HPLC), UV-spectrophotometry (see ref. [13] and references therein), and also, derivative spectrophotometry [14] and PLS multivariate calibration [15]. With respect to the determination of MPBT and PBT mixtures, several techniques such as GC, HPLC, UV-spectrophotometry and polarography have been described in the literature (see ref. [13] and references therein). On the other hand, only a few methods have been reported for the simultaneous determination of ternary mixtures of DPH, PBT and MPBT. These include HPLC, UV-spectrophotometry, ^{1}H-NMR spectrometry and thin layer chromatography with densitometric evaluation [13,16,17]. However, no references were found for the simultaneous determination of three aforementioned compounds by PCR or PLS multivariate calibration methods.

In this work, the PCR and PLS methods have been applied to the simultaneous spectrophotometric analysis of ternary mixtures of DPH, PBT and MPBT in the Comital-L pharmaceutical formulation. The results obtained by both proposed methods have been compared with the results obtained by the application of a HPLC reference method.

2. Theoretical background

2.1. Data analytical methods

Two multivariate projection methods were utilized for the simultaneous spectrophotometric determination of DPH, PBT and MPBT. The first method here used is called principal component regression (PCR) which is simply a principal component analysis (PCA) followed by a multiple linear regression step between the **Y** concentration matrix and the principal components of the **X** absorbance matrix. PCA works on one multivariate data matrix (the **X** matrix) which factorizes into the product of two smaller matrices, **T** and **P**′, according to:

$$\mathbf{X} = \bar{\mathbf{X}} + \mathbf{TP}' + \mathbf{E}$$

where $\bar{\mathbf{X}}$ is the mean value matrix, **T** contains the score values of the calculated principal component, **P**′ is the descriptor loadings, and **E** corresponds to a matrix of residuals.

The second method is partial least-squares (PLS) regression, which is used to calculate the relationship between *two* matrices; that is, the concentration matrix (denoted as **Y**) and the absorbance matrix (denoted as **X**). The method is based on the projection of the original multivariate data matrices down onto smaller matrices (**T**,**U**) with orthogonal columns, which relates the information in the response matrix Y to the systematic variance in the descriptor matrix **X**, as shown below:

$$\mathbf{X} = \bar{\mathbf{X}} + \mathbf{TP}' + \mathbf{E}$$

$$\mathbf{Y} = \bar{\mathbf{Y}} + \mathbf{UC}' + \mathbf{F}$$

$$\mathbf{U} = \mathbf{T} + \mathbf{H} \text{ (the inner relation)}$$

where $\bar{\mathbf{X}}$ and $\bar{\mathbf{Y}}$ are the corresponding mean value matrices, **T** and **U** are the matrices of scores that summarize the x and y variables respectively, **P** is

the matrix of loadings showing the influence of the x in each component, **C** is the matrix of weights expressing the correlation between **Y** and **T**(**X**) and **E**, **F**, and **H** are the corresponding residuals matrices. The PLS calculations also give an auxiliary matrix **W** (PLS weights), which expresses the correlation between **U** and **X** and is used to calculate **T**. Two PLS methods have been described, typically denoted PLS-1 and PLS-2. The difference between both types is that PLS-1 performs the optimization for only one dependent variable at a time. In the present work, the PLS-2 method is used where, the **Y** response matrix consisted of three dependent variables (the concentration values of DPH, PBT and MPBT) while the **X** matrix consisted of corresponding absorbance data. Determinations of the significant number of model dimensions was made by cross-validation [18].

PCR and PLS analysis were carried out using both, the UNSCRAMBLER® 6.11, sofware package obtained from CAMO AS, Norway, and the SIMCA 7.0 sofware package obtained from Umetri AB, Box 7960, 907 19 Umea, Sweden.

2.2. Spectrophotometric data

The method assumes that absorbance of the mixture at any wavelength is the sum of absorbances of each analyte and also, that the analytes obey Beer's Law. In the j wavelength, the absorbance addition A_j will be:

$$A_j = A_j bC_l + \ldots\ldots\ldots + A_n bC_n$$

where the subscript indicates the number of samples from *1* to *n*.

In Fig. 1, the spectra of DPH, PBT and MPBT in the 230–300 nm wavelength range are shown.

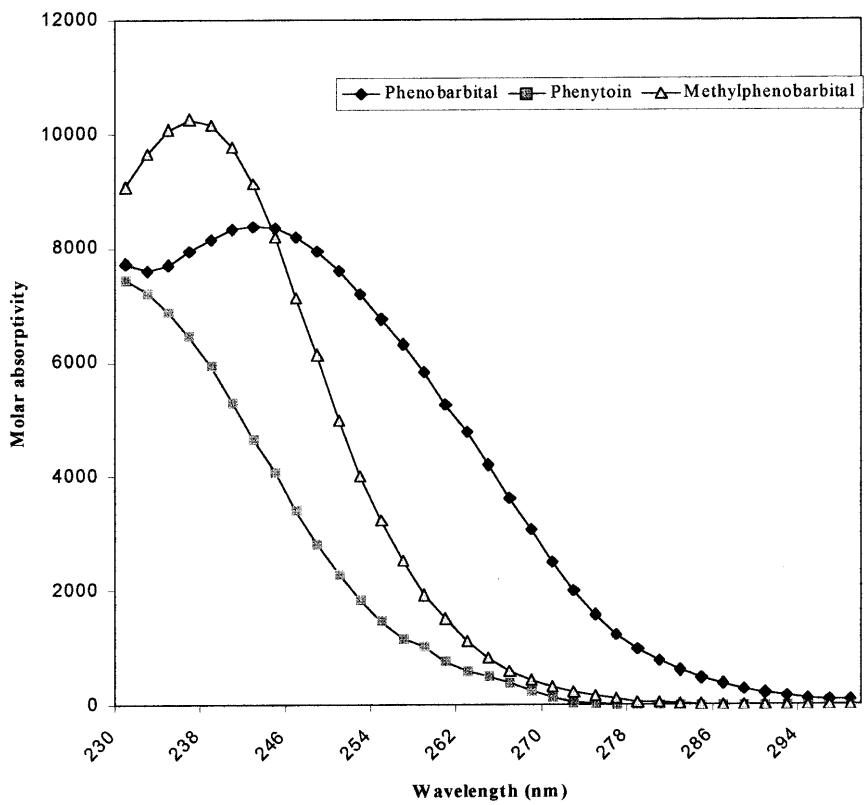

Fig. 1. Spectral curves of phenobarbital, phenytoin and methylphenobarbital obtained with ethanol:sodium hydroxide (pH 13, 0.1M) (50:50, v/v).

Table 1
Composition of the calibration matrix for phenobarbital, phenytoin and methylphenobarbital (μg ml^{-1})

Standard	Phenobarbital	Phenytoin	Methylphenobarbital
1	5	5	5
2	7	7	7
3	10	10	10
4	15	15	15
5	17	17	17
6	20	20	20
7	22	22	6
8	30	9	9
9	6	30	8
10	8	25	14
11	9	22	18
12	13	8	22
13	19	11	25
14	11	13	30
15	16	19	19
16	24	16	23
17	14	14	27
18	18	25	10
19	17	18	12
20	16	25	15
21	15	21	24
22	14	23	8

It can be seen that all peaks of compounds are strongly overlapped with each other, which means that univariate analysis methods cannot be applied for resolving this mixture.

3. Experimental

3.1. Apparatus

The absorbance measurements were performed with a Hewlett Packard (HP-8452A model) spectrophotometer equipped with a diode array wavelenght detector and a Hewlett Packard printer (ThikJet model). All measurements were made with 1.00-cm optical-path quartz cells, and the pH adjustment was performed with an Orion EA940 pH meter digital equipped with a combined glass electrode and an internal reference Ag–AgCl electrode.

The HPLC experiments were performed with a Konic (model 500) liquid chromatograph equipped with a variable wavelength detector operated at 245 nm. The retention times were measured with a Varian 4290 integrator. A Phenosphere 5 μm ODS-2 C_{18} column (250 × 4.6 mm) was used in all experiments.

3.2. Reagents and samples

All chemicals and solvents used were of analytical and spectroscopic grades respectively. DPH and PBT were obtained from Sigma Chemical Products and MPBT was kindly supplied by Siegfried CMS AG, Switzerland. Stock solutions of DPH, PBT and MPBT with a concentration of 1000 μg ml^{-1} in absolute ethanol were prepared.

The Comital-L commercial samples with a nominal content of 50 mg of each drug, were acquired from Argentine pharmacies.

3.3. Procedure

3.3.1. Spectrophotometric multivariate calibration procedure

Appropriate volume aliquots of the stock solutions were transferred to 10 ml volumetric flasks. The volumes were made up with a mixture of ethanol–sodium hydroxide (pH 13; 0.1M) (50:50, v/v) to give a series of standard solutions in such way that their final concentrations lay within desired range. Spectra of the mixtures were recorded between 230 and 300 nm taking absorbance data at 2 nm intervals. The readings were made at a constant time and within a few minutes of preparation of the standard solutions to avoid the degradation of MPBT. The calibration procedure was carried out by using 22 calibration standards prepared using different concentrations of each compound.

The linearity of the maximal signals was examined to select an adequate concentration range suitable for spectrophotometric measurements. Thus, the DPH, PBT and MPBT concentrations were varied between 5 and 30 μg ml^{-1}. The compositions of the 22 standard mixtures used in the calibration matrix for both methods are shown in Table 1. The optimized calibration matrix (calculated by PCR and PLS-2) was applied to the assay of the three drugs in tablet form.

3.3.2. Commercial sample preparation

Ten tablets were finely powdered and an accurately weighed amount of powder, equivalent to about one tablet (labelled to contain 50 mg of each compound), was put into a 100 ml beaker. Subsequently, 50 ml of ethanol was added and the mixture was heated on a water-bath to 60°C for 10 min with constant magnetic stirring. The mixture was filtered into a 100 ml calibrated flask and diluted to volume with absolute ethanol. Aliquots of this solution were diluted with sodium hydroxide (pH13, 0.1M) and then analysed in quintuplicate at least. All aforementioned procedure was replicated three times (samples 1–3).

3.3.3. Chromatographic procedure

After the preparation of the ethanolic solution of tablets (as described before), an aliquot of this solution was tranfered into a 10 ml calibrated flask and was diluted to volume with the selected mobile phase. This solution was injected in the LC system. The chromatography was carried out at room temperature and the injection volume was 10 μl for all experiments. The flow-rate was 1 ml min^{-1}, and the mobile phase consisted of methanol–phosphate buffer (pH 3; 0.025 M) (70:30, v/v). The method here described is a modification of the one proposed [17].

4. Results and discussion

4.1. PLS-2 and PCR analysis: calibration set

All variables (absorbance and concentration matrices) used in PLS-2 and PCR calculations were initially autoscaled to zero mean and unit variance. The statistical significance of the screened models was judged by the correlation coefficient (r), the root mean square error (RMSE) and the F-statistic. The predictive ability was evaluated by the crossvalidation coefficient (Q) which is based on the prediction error sum of squares (PRESS).

For the PLS-2 analysis, the VIP parameter (variable importance for the projection) [19] was used to unravel which absorbance variables were the most relevant to explain the concentration matrix **Y**. Thus, preliminary analysis of the PLS-2 models obtained showed that the useful wavelength range was between 230–268 to 272–278 and 290–300 nm for the 22 prepared calibration standards. On the other hand, the PCR analysis was carried out using the same set of calibration standards and also, the same spectral zone used in the PLS-2 analysis. An interesting point to highlight is the lower obtained predictive quality for all screened models when the PCR analysis was performed on the full wavelength range (230–300). To select the number of factors in the PLS-2 and PCR algorithms, a crossvalidation method leaving out one sample at the time was used [18].

The PLS-2 analysis for the calibration matrix resulted in a significant five-component model with the following statistics: $r_{total} = 0.997$ and $Q_{total} = 0.993$ ($r_{(DPH)} = 0.998$, $Q_{(DPH)} = 0.996$; $r_{(PBT)} = 0.995$, $Q_{(PBT)} = 0.989$ and $r_{(MPBT)} = 0.998$, $Q_{(MPBT)} = 0.995$). The obtained values for root mean square error were: $RMSE_{(DPH)} = 0.392$, $RMSE_{(PBT)} = 0.598$ and $RMSE_{(MPBT)} = 0.434$. The total model accounted for 99.4% (36.1, 23.2, 33.1, 5.8, and 1.4%, respectively) of variance in the concentration **Y** matrix.

For the PCR analysis, a number of five factors was found to be optimun. The obtained PCR model resulted with the following statistics: DPH ($r = 0.998$, $Q = 0.997$), PBT ($r = 0.994$, $Q = 0.986$) and MPBT ($r = 0.998$, $Q = 0.995$). The RMSE values were: $RMSE_{(DPH)} = 0.414$, $RMSE_{(PBT)} = 0.648$ and $RMSE_{(MPBT)} = 0.480$.

In Fig. 2, the explained variance obtained in the calibration process with the PLS-2 and PCR methods are shown. In Fig. 3, the corresponding PCR and PLS-2 model loadings (each of which is related to one or more of the compounds in the mixture) are depicted. As can be seen from Fig. 3a, the loadings of first PLS component show that the 230–300 nm wavelength range correlated in the same way with this factor, while the loadings of second PLS component account for the shape of PBT spectrum. On the other hand, the loadings of the third PLS component is related to MPBT since its graphical shape partly mimics its spectral shape (to note that the 230–240nm wavelength range, which corresponds to the maximum of MPBT, have the largest contribution). Finally, a

causal interpretation of the loadings of the fourth and fifth PLS components is rather difficult. However, it should be noted that both factors probably reflect the most of the spectral difference observed between phenobarbital and the others two compounds in the mixture (see Fig. 1). Therefore, these factors reinforce the predictive ability of the derived PLS-2 model ($\mathbf{Q}^2_{[\text{comp } 4]} = 0.669$, $\mathbf{Q}^2_{[\text{comp } 5]} = 0.673$). A similar analysis can be made for the PCR model (see Fig. 3b).

The residual values obtained with both methods showed a reasonable central tendency and the agreement between the measured and calculated values is very good as shown in Table 2.

4.2. Model validation

4.2.1. Simultaneous determination of DPH, PBT and MPBT in tablet form

It is well known that the real predictive ability of any calibration PLS or PCR model cannot be

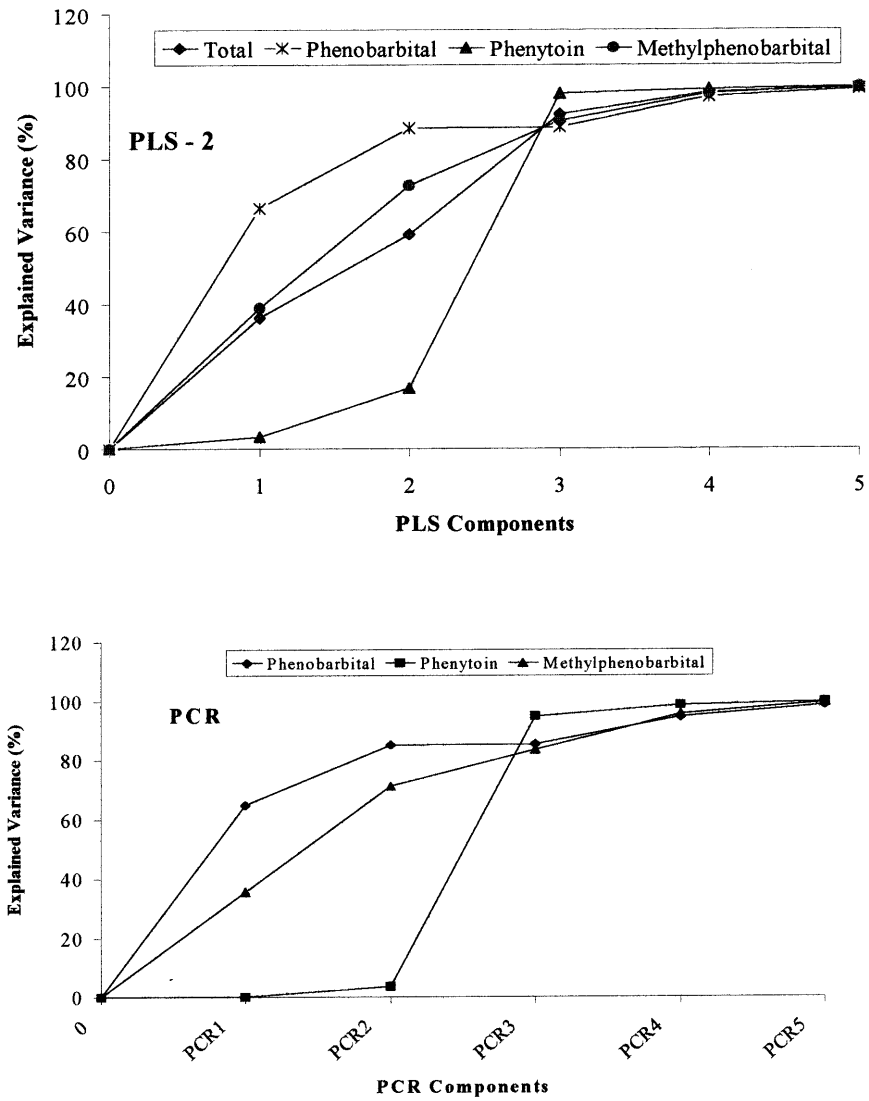

Fig. 2. Explained Variance as a function of the number of factors used in the PLS-2 and PCR calibration processes.

Table 2
Experimental and calculated concentration values for phenytoin, phenobarbital and methylphenobarbital (μg ml^{-1}) in the calibration matrix b using PLS-2 and PCR models

Std.	Phenytoin			Phenobarbital			Methylphenobarbital		
	Observed	Calculated (PLS)	Calculated (PCR)	Observed	Calculated (PLS)	Calculated (PCR)	Observed	Calculated (PLS)	Calculaed (PCR)
1	5	5.05	5.07	5	5.14	5.16	5	5.17	5.14
2	7	7.39	7.39	7	6.94	6.99	7	6.85	6.79
3	10	9.13	9.05	10	9.25	9.12	10	9.68	9.84
4	15	15.04	15.03	15	14.33	14.32	15	15.40	15.41
5	17	17.40	17.30	17	16.28	16.09	17	17.96	18.17
6	20	19.69	19.67	20	19.87	19.84	20	19.85	19.87
7	22	22.24	22.32	22	21.98	22.11	6	5.38	5.24
8	9	8.84	8.78	30	29.70	29.59	9	9.01	9.12
9	30	30.22	30.20	6	5.94	5.90	8	7.24	7.27
10	25	24.73	24.70	8	7.82	7.76	14	14.69	14.75
11	22	22.46	22.52	9	9.00	9.10	18	17.56	17.44
12	8	7.98	8.03	13	14.34	14.42	22	22.15	22.05
13	11	11.32	11.38	19	19.18	19.29	25	24.97	24.85
14	13	12.70	12.70	11	11.80	11.81	30	29.73	29.72
15	19	19.75	19.75	16	15.43	15.40	19	19.55	19.58
16	16	16.14	16.13	24	23.95	23.92	23	22.42	22.41
17	14	14.17	14.18	14	13.05	13.07	27	26.65	26.65
18	25	24.78	24.76	18	18.03	17.98	10	10.46	10.50
19	18	18.33	18.36	17	17.48	17.53	12	11.99	11.92
20	25	24.23	24.17	16	15.99	15.90	15	15.32	15.43
21	21	20.50	20.52	15	15.02	15.05	24	23.81	23.78
22	23	22.91	2297	14	15.49	15.60	8	8.17	8.06

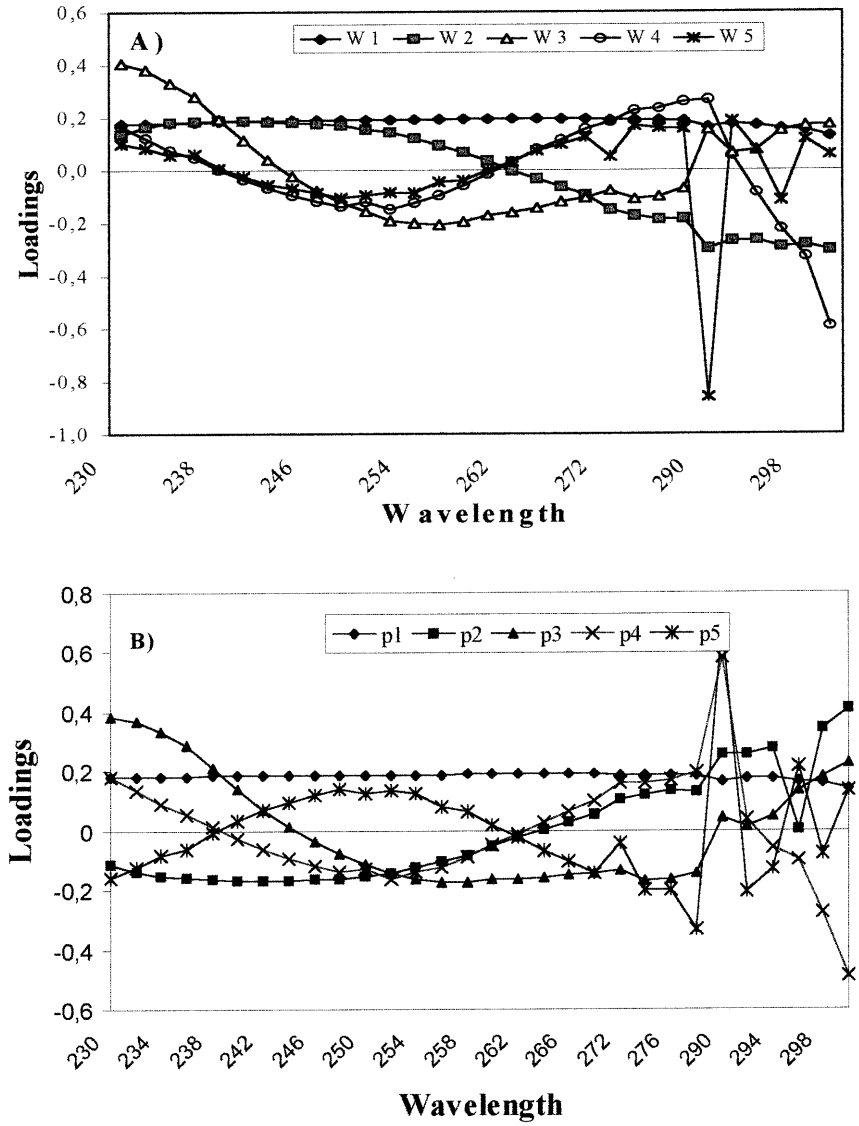

Fig. 3. Loadings plot as a function of the absorption wavelength. (A) PLS-2 loadings, (B) PCR loadings

judged solely by using internal validation (i.e., crossvalidation); it has to be validated on the basis of predictions for compounds not included in the calibration set [20]. Thus, the optimized PLS-2 and PCR matrices have been applied to the assay of the three compounds in tablet form, in order to demonstrate the applicability of the proposed methods. Table 3 and Table 4 show the results obtained by applying the developed PLS-2 and PCR models, respectively. As can be seen, the recoveries were quite acceptable as they were not greater than 6% for all samples.

In Table 5, the results obtained by application of the HPLC method developed herein are summarized. The values indicated are the mean of five different determinations of the same commercial batch. The recoveries obtained were between 99 and 103% for the three compounds and, as can be

Table 3
Results obtained in the PLS-2 analysis of the commercial formulation Comital-L

Sample	Phenobarbital				Phenytoin				Methylphenobarbital			
	Label (mg/l)	Found (mg/l)	R.S.D. (%)	Recovery (%)	Label (mg/l)	Found (mg/l)	R.S.D. (%)	Recovery (%)	Label (mg/l)	Found (mg/l)	R.S.D. (%)	Recovery (%)
1	10	10.48 ± 0.27a[a]	2.58	104.80	10	10.52 ± 0.34[a]	3.20	105.23	10	10.55 ± 0.19[a]	1.82	105.51
2	15	14.88 ± 0.32[b]	2.12	99.20	15	15.47 ± 0.38[b]	2.42	103.13	15	15.22 ± 0.28[b]	1.81	101.47
3	20	18.84 ± 0.23[c]	1.23	94.21	20	19.30 ± 0.40[c]	2.10	96.50	20	19.88 ± 0.66[c]	3.38	99.40
Mean recovery (%)				99.40				101.62				102.13

[a] $n = 7$.
[b] $n = 8$.
[c] $n = 6$.

Table 4
Results obtained in the PCR analysis of the commercial formulation Comital-L

Sample	Phenobarbital				Phenytoin				Methylphenobarbital			
	Label (mg/l)	Found (mg/l)	R.S.D. (%)	Recovery (%)	Label (mg/l)	Found (mg/l)	R.S.D (%)	Recovery (%)	Label (mg/l)	Found (mg/l)	R.S.D. (%)	Recovery (%)
1	10	10.59 ± 0.24[a]	2.29	105.90	10	10.58 ± 0.36[a]	3.38	105.80	10	10.44 ± 0.23[a]	2.24	104.40
2	15	14.94 ± 0.27[b]	1.79	99.60	15	15.51 ± 0.35[b]	2.24	103.40	15	15.15 ± 0.27[b]	1.79	101.00
3	20	18.86 ± 0.19[c]	0.99	94.30	20	19.30 ± 0.47[c]	2.43	96.50	20	19.81 ± 0.77[c]	3.91	99.05
Mean recovery (%)				99.93				101.90				101.48

[a] $n = 7$.
[b] $n = 8$.
[c] $n = 6$.

Table 5
Results obtained in the HPLC analysis of the commercial formulation Comital-L

Sample	Phenobarbital				Phenytoin				Methylphenobarbital			
	Label (mg/l)	Found (mg/l)	R.S.D. (%)	Recovery (%)	Label (mg/l)	Found (mg/l)	R.S.D. (%)	Recovery (%)	Label (mg/l)	Found (mg/l)	R.S.D. (%)	Recovery (%)
1	6	6.18 ± 0.096[a]	1.55	103.00	6	6.09 ± 0.068[a]	1.11	101.58	6	6.14 ± 0.148[a]	2.42	102.35
2	10	10.09 ± 0.044[a]	0.44	100.86	10	10.01 ± 0.043[a]	0.43	100.08	10	10.06 ± 0.108[a]	1.08	100.56
3	14	13.99 ± 0.090[a]	0.64	99.92	14	13.91 ± 0.086[a]	0.62	99.37	14	14.10 ± 0.078[a]	0.55	100.74
Mean recovery (%)				101.26				100.35				101.22

[a] $n = 5$.

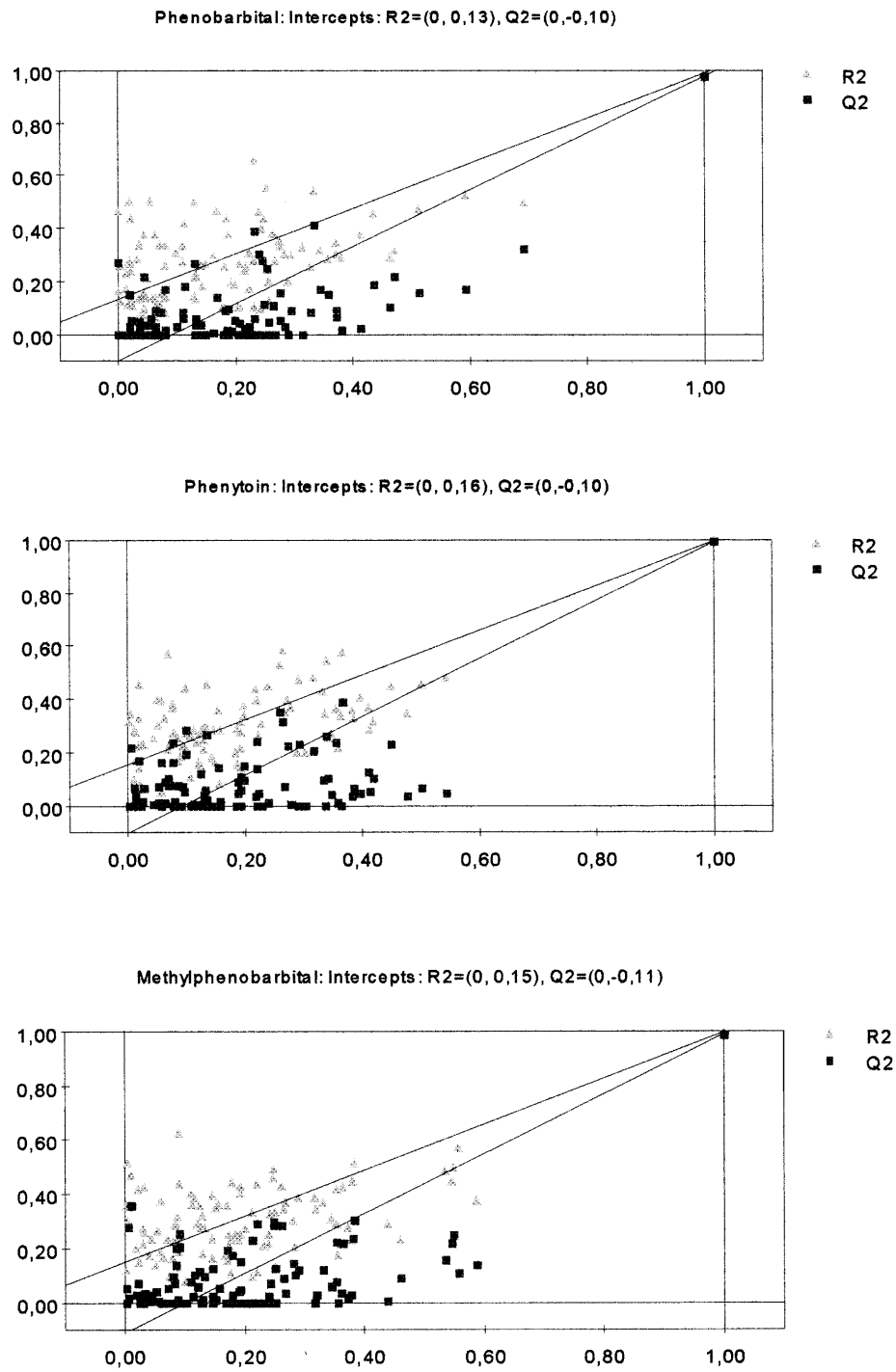

Fig. 4. Results of the permutation test. The R2 and Q2 values were obtained from 100 permutations and five PLS-2 components.

seen, these results are slightly better compared to those obtained with the PLS-2 and PCR methods. It should be noted, however, that the application of the methods developed here give rise to acceptable recovery values.

4.2.2. Response permutation test

The predictability and the degree of overfit for a PLS or PCR model may also be examined in another way; that is to apply a technique based on response permutation [21]. In the present work, this technique was applied to the PLS-2 model only. Thus, several PLS-2 models were recalculated by using the concentration data (matrix **Y**) randomly reordered. These permuted data were later related to the unperturbed absorbance data (matrix **X**) by refitting the model and including crossvalidation. In each round of calculation, pairs of R^2 and Q^2 were recorded and plotted against the absolute value of the correlation coefficient between the original response variable and its permutations. Fig. 4 shows the results obtained from 100 permutations of the calibration matrix for each one of the compounds under study. The intercepts of the two regression lines (for R^2 and Q^2) indicate the degree of overfit and overprediction. In general, intercept limits for $R^2 < 0.3$ and $Q^2 < 0.05$ indicate valid models, such as is the case of our PLS model we have developed.

5. Conclusions

Based on the results obtained in this work, the proposed spectrophotometric methods permit the simultaneous determination of phenytoin, phenobarbital and methylphenobarbital in pharmaceutical preparations. The methods proposed can be used without previous chemical separations, which provides evidence for the great potential of the PLS-2 and PCR methods for the simultaneous determination of drugs that present a substantial spectral overlap in the sample. The developed methods provide expeditious and precise results and are therefore a viable alternative to the routine analysis.

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