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Simultaneous determination of diazepam and pyridoxine in synthetic mixtures and pharmaceutical formulations using graphical and multivariate calibration–prediction methods

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Abstract

Several methods are reported for the simultaneous determination of diazepam and pyridoxine: two graphical methods (zero-crossing and derivative quotient spectra); and two numerical methods (multiple linear regression and partial least-squares regression). The methods have been applied in the concentration ranges 1.4–4.0 μg ml^{-1} diazepam and 4.0–12.0 μg ml^{-1} pyridoxine. The accuracy and precision of the methods have been determined and they have been validated by analysing synthetic mixtures containing the two drugs in variable proportions. The methods were also applied to the determination of diazepam and pyridoxine in pharmaceutical preparations. The analytical results were quite good in all cases.

Keywords: Derivative quotient spectra; Diazepam; Multiple linear regression; Partial least-squares regression; Pharmaceutical preparations; Pyridoxine; Zero-crossing

1. Introduction

Molecular absorption spectroscopy has been extensively used for the determination of drugs in pharmaceutical preparations (and their metabolites in plasma and urine), as well as for the analysis of synthetic mixtures, with a view to the development of analytical methods. The use of this technique for pharmaceutical analyses has the inherent constraint that most active drugs absorb in the UV region and exhibit strongly overlapped spectra that impede their simultaneous determination.

This problem has been addressed by using various methods including derivative spectroscopy and multivariate calibration–prediction methods. Prominent among the former are the zero-crossing method developed by O'Haver [1] and the derivative quotient spectrum method reported by Salinas et al. [2]. Multivariate calibration methods are powerful analytical tools in combination with

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any instrumental technique. The best choice depends on the complexity of the data to be processed: available options include multiple linear regression (MLR) [3], principal component regression (PCR) [4], partial least-squares regression (PLS) [5], curve resolution (CR) [6], iterative target transformation factor analysis (ITTFA) [7] and evolving factor analysis (EFA) [8].

This paper demonstrates the potential of two graphical methods (zero-crossing and derivative quotient spectra) and two numerical methods (MLR and PLS) for the simultaneous determination of diazepam (a widely used anti-anxiety agent) and pyridoxine (a vitamin commonly used with diazepam as it boosts the activity of the drug by its action on neural metabolism); both are present in a number of pharmaceutical preparations produced by Spanish manufacturers and have been determined simultaneously in this work.

A literature scan revealed the absence of methods for the simultaneous determination of diazepam and pyridoxine. Diazepam and its metabolites in plasma can be determined by HPLC [9], jointly with other active drugs of the benzodiazepine family by capillary gas chromatography [10], together with methaqualone by second-derivative spectrophotometry [11], and electrochemically in pharmaceutical preparations [12]. Pyridoxine has been determined spectrophotometrically in ternary mixtures [13], together with five vitamins by fluorescence spectroscopy [14], and with membrane electrodes sensitive to vitamin B_1 and pyridoxine itself [15].

The proposed methods were validated in this work by analysing synthetic mixtures containing the two drugs in variable proportions. The methods were also applied to pharmaceutical preparations.

2. Theoretical background

2.1. Zero-crossing method

The zero-crossing method is based on measurements of the derivative spectrum for a mixture at a wavelength where one component exhibits no signal. At such a wavelength, the signal is directly proportional to the concentration of the other component. This method has been well established by O'Haver [1] and applied extensively to pharmaceutical analysis [16,17].

2.2. Derivative quotient spectrum method

The derivative quotient spectrum method relies on the application of the Beer–Lambert law to a mixture M of two substances A and B at different wavelengths λ using a light pathlength of 1 cm. The absorbance for the mixture at wavelength λ is given by

$$a(\mathrm{M}, \lambda) = \epsilon(\mathrm{A}, \lambda)c_{\mathrm{A}} + \epsilon(\mathrm{B}, \lambda)c_{\mathrm{B}} \quad (1)$$

where $\epsilon(\mathrm{A}, \lambda)$ and $\epsilon(\mathrm{B}, \lambda)$ are the molar absorptivities of the analytes at wavelength λ, and c_{A} and c_{B} are the analyte concentrations. Dividing this equation by a standardized spectrum for A, $\epsilon_{\mathrm{A}}c_{\mathrm{A}}^0$ where c_A^0 is the concentration of A in the divisor spectrum) and differentiating the resulting expression with respect to λ yields:

$$\frac{\mathrm{d}}{\mathrm{d}\lambda}\left[\frac{a_{\mathrm{M}\lambda}}{\epsilon_{\mathrm{A}\lambda}}\right] = c_{\mathrm{B}}\frac{\mathrm{d}}{\mathrm{d}\lambda}\left[\frac{\epsilon_{\mathrm{B}\lambda}}{\epsilon_{\mathrm{A}\lambda}}\right] \quad (2)$$

Based on this equation, the quotient spectrum for a mixture depends exclusively on c_{B} and c_{A}^0, which allows the ready calculation of c_{B} (dividing into c_{B}^0 yields a similar expression for calculating c_{A}). This method was originally developed by Blanco et al. [18] and subsequently modified by Berzas et al. [19].

2.3. MLR (MULTI3) [20]

This method uses several standards of the components and their mixtures. If a number of absorbance measurements of various analyte mixtures are made, the system composed of the absorbance and concentration matrices can be represented by

$$\boldsymbol{A} = \boldsymbol{KC}$$

where $\boldsymbol{A}$ is the absorbance data matrix, $\boldsymbol{K}$ the matrix from which proportionality constants are calculated on the basis of standards of known concentrations and $\boldsymbol{C}$ the concentration matrix.

The C prediction concentration matrix can be calculated from the following equation

$$C = (K'K)^{-1}K'A$$

where K' is the transpose matrix (resulting from interchange of rows and columns) of K and A is the absorbance matrix of unknown samples. Matrix K can be obtained in various ways; one involves calculating K values by regression from mixtures of known composition. Mathematically, an equation system of the following form is obtained at each wavelength

$$a(\lambda, s) = e(\lambda) + \sum_{i=1}^{n} k(\lambda, i)c_{si}; \quad \forall s = 1 \ldots n_s \qquad (3)$$

where a is the signal for mixture s at wavelength λ, $e(\lambda)$ is the independent fitting term at each λ value and c_{si} is the concentration of component i in mixture s. This equation can be solved by MLR when the number of mixtures exceeds that of components. The deviation of the fit at each wavelength, $d(\lambda)$, can be calculated from

$$d(\lambda) = \sqrt{\frac{\sum_{s=1}^{n_s} [a(s, \lambda) - \hat{a}(s, \lambda)]^2}{n_s - n}} \qquad (4)$$

$d(\lambda)$ values are re-used by adjusting them to a given weight of the initial fit, in such a way that those wavelengths with the higher deviations are assigned lower weights than the rest. The equation used for this purpose is

$$\frac{a(\lambda)}{d(\lambda)} = k'_0 + \sum_{i=1}^{n} \frac{k(i, \lambda)}{d(\lambda)} c_i; \quad \forall \lambda = 1 \ldots n_\lambda \qquad (5)$$

This method has been applied in pharmaceutical analysis [21,22].

2.4. PLS regression

The theory and applications of multivariate calibration in analytical chemistry are well established and have been described in detail in monographs [23,24].

PLS regression analysis involves resolving two matrices A and C into the product of two smaller matrices, namely T (the scores matrix, which represents the coordinates of the objects on the new axes) and either P (the A-loading matrix, which contains the directions of the principal axes or components) or Q (the C-loading matrix):

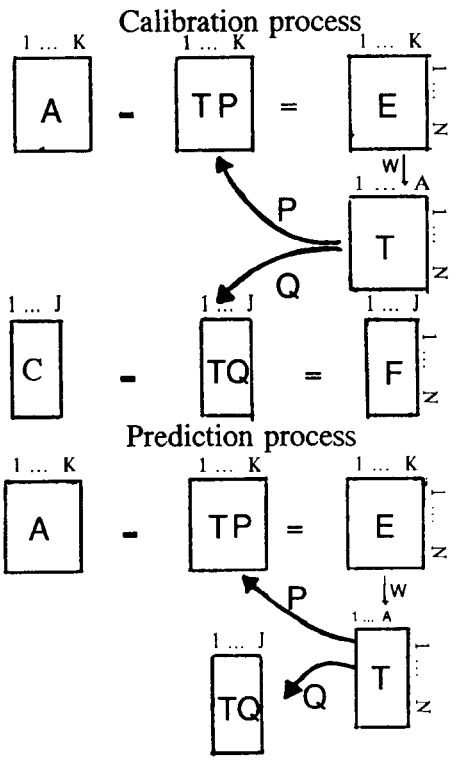
$$A = TP + E$$

$$C = TQ + F$$

where A (the absorbance matrix) consists of rows (objects) and columns (variables) that correspond to the calibration solutions and the wavelengths where measurements were made. Matric C (the concentration matrix) consists of the calibration solutions as rows and the analyte concentrations as columns. Finally, matrices E and F (the residual matrices) contain the model error and random noise respectively.

The calibration and prediction processes are shown in Scheme 1, where matrix W is the so-called "loading weight matrix" or "PLS weight matrix".

This methodology has been employed in multicomponent pharmaceutical analysis [25,26].



Scheme 1.

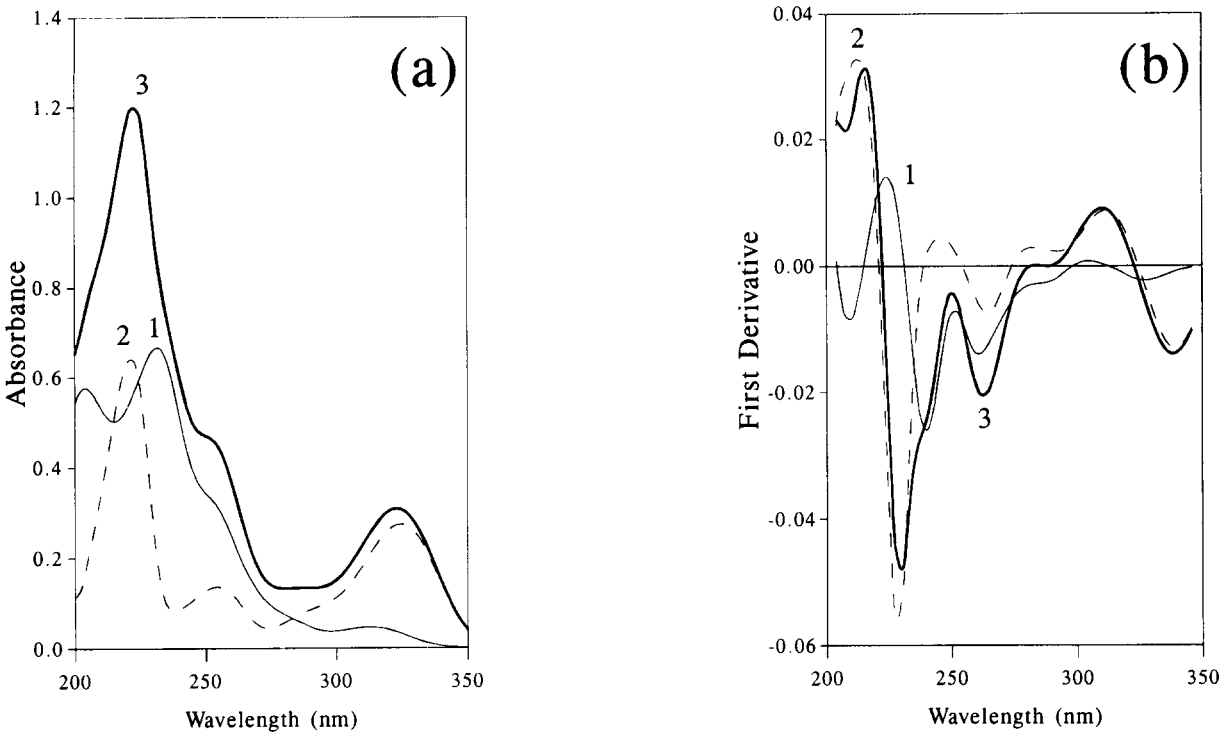


Fig. 1. (a) Absorption and (b) first-derivative spectra of (1) diazepam (5.6 μg ml^{-1}), (2) pyridoxine (8.0 μg ml^{-1}), and (3) diazepam (5.6 μg ml^{-1}) and pyridoxine (8.0 μg ml^{-1}) at pH 6.0.

3. Experimental

3.1. Apparatus

Spectra were recorded on a Hewlett-Packard HP 8452A diode-array spectrophotometer fitted with quartz cuvettes of 1 cm pathlength and interfaced to a Vectra ES computer and a Think Jet printer, also from Hewlett-Packard.

A Radiometer PHM-84 digital pH-meter fitted with a combined glass-saturated calomel electrode was used for pH measurements. The pH-meter was calibrated with at least two buffer solutions at pH 4.02 and 7.00.

An ultrasonic bath was also employed.

3.2. Computer hardware and software

Absorbance and derivative spectra were acquired and processed using the spectrophotometer's bundled software. Additional software included a MLR program (MULTI3) [20] and Unscrambler v. 5.0 [27] for calibration–prediction and experimental design. Exported data from the spectrophotometer were converted into MULTI3 and Unscrambler formats using the word processor WordPerfect 5.1 for filing in ASCII format. All software was run on a PC/AT 486 DX2 66 MHz computer.

3.3. Reagents

Standard solutions containing 100 μg ml^{-1} diazepam or pyridoxine were made by direct weighing of the required amount of commercially available reagent (Merck), dissolving in 0.1 M HCl and diluting to volume with 0.1 M HCl.

A NaH_2PO_4/Na_2HPO_4 buffer (pH 6.0; 1.0 M) was also prepared.

All reagents and solvents were of analytical grade and the water was doubly-distilled.

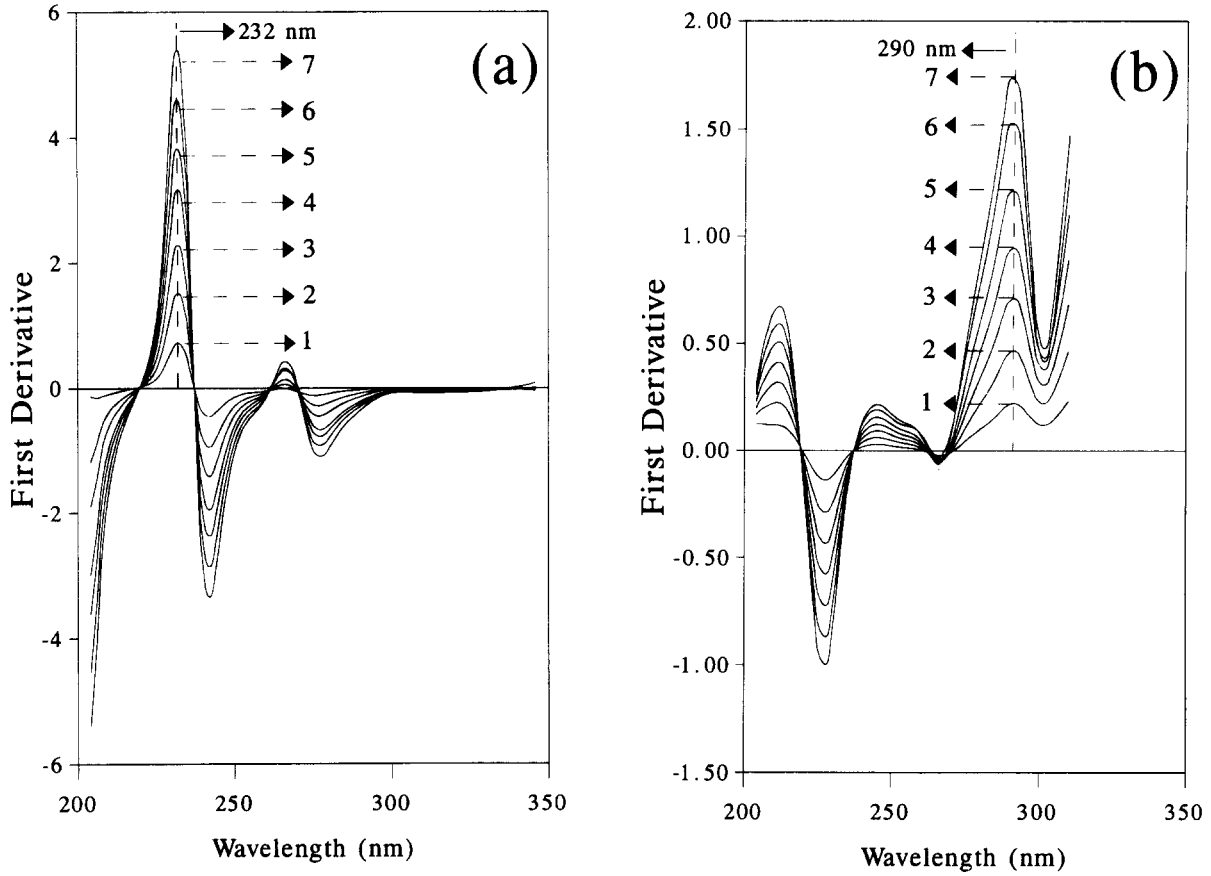


Fig. 2. First-derivative of quotient spectra when divisor was normalized. (a) Diazepam: (1) 0.8; (2) 1.6; (3) 2.4; (4) 3.2; (5) 4.0; (6) 4.8; (7) 5.6 μg ml^{-1}. (b) Pyridoxine: (1) 2.0; (2) 4.0; (3) 6.0; (4) 8.0; (5) 10.0; (6) 12.0; (7) 14.0 μg ml^{-1}.

3.4. Procedure

3.4.1. Analysis of synthetic mixtures

Binary mixtures of diazepam and pyridoxine were prepared in triplicate as follows: in a 25 ml volumetric flask were placed 5 ml of NaH_2PO_4/Na_2HPO_4 buffer (pH 6.0) and an appropriate volume of drug standard to obtain a final concentration of 1.4–4.0 μg ml^{-1} diazepam or 4.0–12.0 μg ml^{-1} pyridoxine. Spectra of the solutions were recorded over the wavelength range 200–350 nm against a blank prepared under the same conditions but containing none of the analytes. The integration time was set to 1 s in all experiments.

3.4.2. Analysis of pharmaceutical preparations

The contents of a pill, tablet or capsule were dissolved in 0.1 M HCl with the aid of ultrasonics for 15 min. The solution was filtered and the filtrate was transferred to a 100 ml volumetric flask and diluted to the mark with 0.1 M HCl. An appropriate aliquot was then taken in such a way that the final concentrations of both drugs in a 25 ml flask containing 5 ml of buffer solution (pH 6.0) lay within the range tested. Finally, the absorbance spectra for the solutions were recorded over the wavelength range 200–350 nm; samples were analysed in triplicate.

4. Results and discussion

Fig. 1a shows the absorption spectra for diazepam and pyridoxine solutions at pH 6.0. Diazepam exhibits three absorption maxima (at 204, 232 and 312 nm), and so does pyridoxine (at 222,

Table 1
Parameters of regression lines for diazepam and pyridoxine by derivative spectrophotometry

Analyte	Wavelength (nm)	Regression equation[a]	Correlation coefficient	Variance (s^2)	Detection limit ($\mu g\ ml^{-1}$)
Zero-crossing method					
Diazepam	254	$c = -622.25D_1 + 7.90 \times 10^{-5}$	0.9998	1.03×10^{-8}	0.09
Pyridoxine	312	$c = +943.40D_1 - 2.53 \times 10^{-4}$	0.9997	6.31×10^{-8}	0.32
Derivative quotient spectra method					
Diazepam	232	$c = +1.03D_1 + 3.44 \times 10^{-2}$	0.9996	1.23×10^{-2}	0.15
Pyridoxine	290	$c = +7.75D_1 + 5.97 \times 10^{-2}$	0.9988	4.22×10^{-3}	0.68

[a] Where c is the unknown concentration of the analyte in the sample ($\mu g\ ml^{-1}$), D_1 is the value of the derivative signal and $n = 7$ is the number of standard solutions.

254 and 324 nm); because the two spectra overlap strongly the simultaneous determination of the two drugs by conventional means is impeded.

In order to optimize the measurement conditions for the simultaneous determination, the effects of experimental variables affecting the absorption of both drugs were thoroughly investigated.

The study was started by testing the stability of the two drugs. Diazepam and pyridoxine solutions in 0.1 M HCl exhibited no absorbance changes for 24 h when kept at room temperature, and for 8 days when sorted refrigerated at 0–5°C.

The effect of pH on the absorbance of both drugs was studied by adding small amounts of $HClO_4$ or NaOH solutions. Diazepam was found to exhibit two spectral maxima at 232 and 312 nm at an acid pH, the latter of which decreased with increasing pH; an equilibrium between two species must therefore exist, of which only the acid form absorbs in the UV region. Pyridoxine exhibited a maximum at 290 nm at acid pH values that extensively overlapped with one of the diazepam maxima; in contrast, it showed two peaks at 220 and 235 nm at pH 5.0–6.0, which should facilitate its joint determination with the other drug. pH 6.0 was thus chosen as optimal.

Of the various buffers tested, that consisting of NaH_2PO_4 and Na_2HPO_4 was found to result in analytical signals coinciding with the signals recorded in its absence. In order to optimize the buffer concentration, its effect over the range 0.05–0.25 M was investigated. The buffer concentration was found not to influence the absorbance of the diazepam or pyridoxine solutions, so 0.20 M was selected as optimal.

4.1. Zero-crossing method

Fig. 1b shows the first-derivative spectra for diazepam and pyridoxine. As can be seen, the derivative signal for pyridoxine was zero at 254 nm, so the diazepam concentration in the mixture could therefore be readily established at this wavelength. The calibration curve for pyridoxine was run at 312 nm, where the derivative signal for diazepam was zero.

4.2. Derivative quotient spectrum method

Fig. 2a shows the derivative quotient spectra obtained by dividing the spectra for diazepam at different concentrations into the standardized spectrum for pyridoxine; the maximum signal was obtained at 232 nm. However, pyridoxine was determined at 290 nm (Fig. 2b).

The statistical figures of merit of the calibration curves obtained with this and the previous method are given in Table 1.

4.3. MLR

The program MULTI3 was used to resolve binary calibration mixtures by using five solutions (a $2^n + 1$ design) over the concentration ranges 1.6–4.0 $\mu g\ ml^{-1}$ diazepam and 4.0–12.0 $\mu g\ ml^{-1}$ pyridoxine. The optimal wavelength range for the multidetermination was 210–350 nm.

4.4. PLS

The calibration–prediction and experimental design program Unscrambler v. 5.0 was used under the previous conditions over the same wavelength range. As can be seen from Fig. 3, two factors (the absorbances of the calibration solutions and their respective concentrations) accounted for 100% of the variance. The first factor accounted to a greater extent for the variance of the results for pyridoxine relative to diazepam; conversely, the second factor accounted for a greater fraction of the diazepam variance and the residual variance of pyridoxine.

Fig. 4 shows a plot of score 2 vs. score 1, where solutions cluster as a function of each analyte concentration. As can be seen, increased values of score 1 corresponded to solutions containing a higher concentration of pyridoxine relative to diazepam and vice versa. Therefore, the first model loading is related to diazepam since its graphical shape partly mimics its spectral shape; for the same reason, the second loading is related to pyridoxine. The third loading, however, lacks chemical significance (Fig. 5).

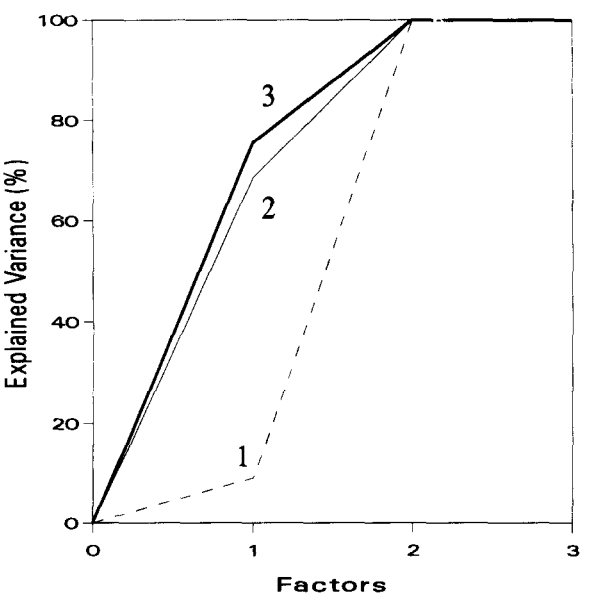


Fig. 3. Explained variance as a function of the number of factors used in the calibration process: (1) diazepam variance; (2) pyridoxine variance; (3) total variance.

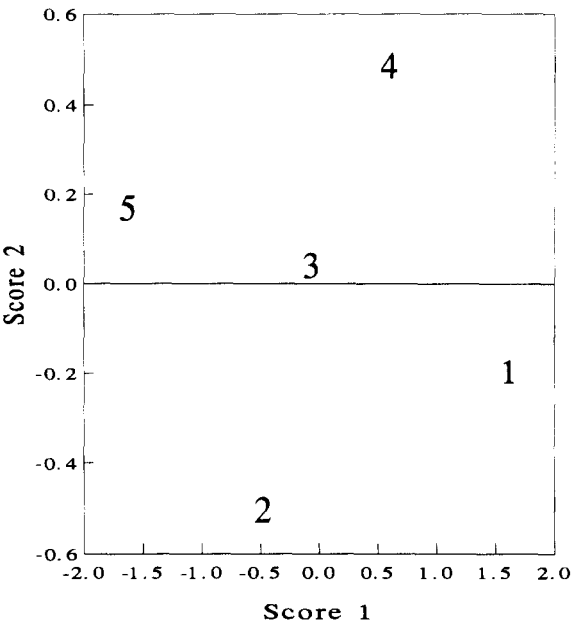


Fig. 4. Scores plot for the calibration set. Concentrations of diazepam and pyridoxine solutions are respectively: (1) 4.0 and 12.0; (2) 4.0 and 4.0; (3) 2.8 and 8.0; (4) 1.6 and 12.0; and (5) 1.6 and 4.0 $\mu g\ ml^{-1}$.

4.5. Resolution of synthetic mixtures

Table 2 shows the results obtained in the resolution of synthetic mixtures using the four methods described above. As can be seen, results for the determination of diazepam with the zero-crossing method were poor because the wavelength crossing in the derivative spectrum for pyridoxine was not an even wavelength; the diode-array spectrophotometer only allows measurements to be made at even wavelengths. Results for the other determinations were excellent for both drugs using the multivariate calibration methods and quite good using the graphical methods.

4.6. Accuracy and precision

The amounts obtained in the determination of synthetic samples were compared with those added to the solutions in order to evaluate the accuracy. Applicaton of the mutual confidence region test to the slope and intercept of the amounts added vs. amounts found, plotted ac-

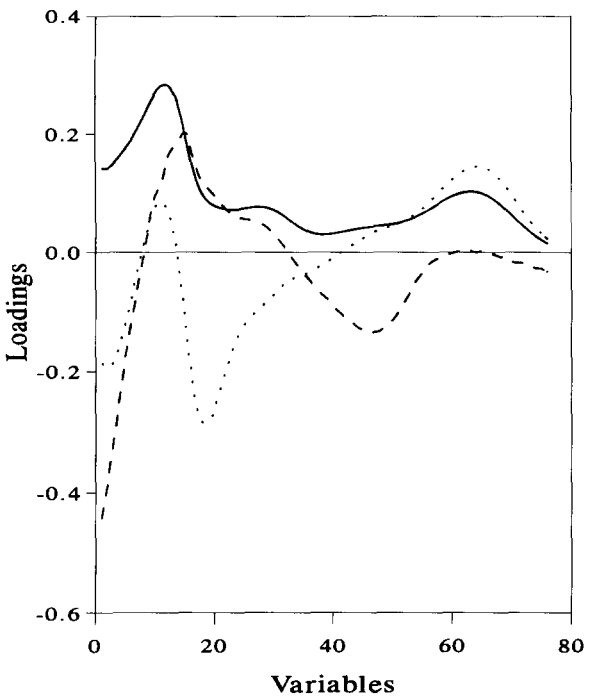


Fig. 5. Loadings plot as a function of the wavelength (variables): (—) first loading (related to diazepam spectral shape); (· · ·) second loading (related to pyridoxine spectral shape); and (– – –) third loading (lacks chemical significance).

cording to Mandel and Linnig [28], revealed the presence of a systematic error at a significance level of $\alpha = 0.01$ in the determination of both active drugs with the zero-crossing method, and at $\alpha = 0.05$ with the derivative quotient spectrum method, but not in the determinations with the multivariate calibration methods (at $\alpha = 0.05$).

The precision was determined by means of a one-way ANOVA including six replicates carried out on three successive days. Snedecor F values below the tabulated levels were obtained in all cases ($F = 3.68$, $n_1 = 2$, $n_2 = 15$; Table 3).

Application of Bartlett's test to the four methods provided M [29] values between 0.1 and 5.10 (i.e. always smaller than the tabulated value, $\chi^2_3 = 7.81$).

4.7. Applications

The proposed methods were applied to the determination of diazepam and pyridoxine in four pharmaceutical preparations from Spanish manufacturers namely: Gobanal® (pills also containing lactose and other excipients), Vincosedan® (pills including lactose as the major excipient), Aspaserine B_6 (tablets containing lactose) and Pacium® (capsules containing excipients other than lactose). These drugs are given to patients suffering from anxiety, insomnia associated with anxiety, some types of epilepsy and alcohol withdrawal syndrome.

Table 4 shows the results obtained in the determinations. As can be seen, they were quite consistent with the manufacturers' labelled contents.

Table 2
Results obtained for different synthetic mixtures using the four proposed methods

Amount added (μg ml^{-1})		Recovery (% ± RSD)[a]							
		Zero-crossing method		Derivative quotient spectra		MLR		PLS	
DIA[b]	PIR[b]	DIA	PIR	DIA	PIR	DIA	PIR	DIA	PIR
2.0	6.0	90.0 ± 1.2	101.2 ± 0.1	102.5 ± 0.5	103.0 ± 0.9	101.1 ± 0.2	99.3 ± 0.8	101.5 ± 0.5	99.7 ± 0.6
2.8	6.0	92.9 ± 0.5	101.3 ± 0.4	101.1 ± 0.3	103.0 ± 0.8	98.9 ± 0.5	99.7 ± 0.6	99.3 ± 0.4	99.5 ± 0.6
2.4	8.0	92.9 ± 0.8	100.1 ± 0.4	105.0 ± 0.9	103.1 ± 0.9	101.7 ± 0.6	99.1 ± 0.8	100.8 ± 0.6	99.8 ± 0.4
2.8	8.0	88.7 ± 1.9	100.5 ± 0.3	101.8 ± 0.6	102.4 ± 0.6	98.9 ± 0.9	100.0 ± 0.3	98.6 ± 0.8	100.0 ± 0.1
3.2	9.2	92.8 ± 0.7	99.7 ± 0.2	104.4 ± 0.8	102.9 ± 0.7	101.3 ± 0.7	100.3 ± 0.2	100.0 ± 0.2	100.4 ± 0.2
3.6	6.8	96.1 ± 0.4	100.3 ± 0.1	103.0 ± 0.5	103.4 ± 0.8	100.3 ± 0.2	99.7 ± 0.8	99.4 ± 0.5	99.7 ± 0.4
3.2	7.2	95.9 ± 0.5	100.8 ± 0.3	104.4 ± 0.8	102.9 ± 0.8	102.9 ± 0.6	100.1 ± 0.6	100.6 ± 0.5	100.0 ± 0.3

[a] Mean and relative standard deviation for five determinations.
[b] DIA, diazepam; PIR, pyridoxine.

Table 3
Analysis of variance (ANOVA) for the proposed methods

Parameter	Zero-crossing		Derivative quotient spectra		MLR		PLS	
	DIA[a]	PIR[a]	DIA	PIR	DIA	PIR	DIA	PIR
Between-days variance	1.6×10^{-1}	1.3×10^{-2}	2.2×10^{-2}	1.1×10^{-2}	7.2×10^{-3}	5.2×10^{-2}	7.2×10^{-3}	7.4×10^{-2}
Within-days variance	9.6×10^{-2}	4.1×10^{-2}	1.3×10^{-2}	8.8×10^{-2}	5.9×10^{-3}	6.0×10^{-2}	2.0×10^{-2}	8.6×10^{-2}
F ratio[b]	1.68	0.33	1.65	0.13	1.22	0.86	0.36	0.44
Mean value	4.85	10.07	5.10	10.22	5.06	9.90	4.98	9.94
Between-days RSD (%)	8.29	1.17	2.89	1.06	1.68	2.30	1.70	2.73
Within-days RSD (%)	6.39	2.03	2.25	2.91	1.52	2.47	2.83	2.96

[a] DIA = diazepam; PIR = pyridoxine.
[b] Between-day and within-day degrees of freedom 2 and 15 respectively. The critical F ratio value for 2 and 15 degrees of freedom and a confidence level of 95% is 3.68.

Also, the amount of pyridoxine in Pacium® determined by the four methods was below the nominal content whereas that of diazepam was slightly above the nominal content in most of the formulations.

5. Conclusions

The joint use of spectrophotometry and graphical methods or multivariate calibration for the resolution of mixtures of analytes with overlapped spectra is an effective choice for developing new analytical methods as well as for the quality control of pharmaceutical preparations and for the avoidance of some steps (e.g. separation, extraction and pre-concentration) of classical determination processes. The methods used in this work allow the satisfactory simultaneous determination of diazepam and pyridoxine in synthetic mixtures and pharmaceutical preparations of widespread use in Spain.

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Table 4
Determination of diazepam and pyridoxine in pharmaceutical preparations using the four proposed methods

Pharmaceutical formulations analysed	Recovery (% ± RSD)[a]							
	Zero-crossing method		Derivative quotient spectra		MLR		PLS	
	DIA[b]	PIR[b]	DIA	PIR	DIA	PIR	DIA	PIR
Gobanal® (pills)	106.7 ± 0.9	93.5 ± 0.6	118.2 ± 1.2	110.1 ± 1.5	114.2 ± 0.8	102.1 ± 1.1	114.9 ± 1.0	104.3 ± 1.4
Vincosedan® (tablets)	101.9 ± 0.5	94.4 ± 4.2	109.6 ± 0.9	107.8 ± 5.8	108.4 ± 0.9	102.0 ± 4.6	107.3 ± 1.2	100.6 ± 5.5
Pacium® (capsules)	103.7 ± 9.3	99.3 ± 0.8	112.6 ± 8.7	112.6 ± 8.7	107.6 ± 0.8	95.6 ± 0.0	99.4 ± 0.9	94.9 ± 1.2
Aspaserine B_6® (tablets)	92.5 ± 2.5	99.9 ± 0.8	109.5 ± 6.8	109.6 ± 2.6	102.7 ± 2.5	107.2 ± 0.6	101.4 ± 2.9	104.8 ± 1.7

[a] Mean and relative standard deviation for five determinations. Recovery relative to nominal content.
[b] DIA, diazepam; PIR, pyridoxine. Label claims of diazepam and pyridoxine are 5 and 10 mg respectively for tablet, pill or capsule.

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