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Analytica Chimica Acta 460 (2002) 85–97

ANALYTICA CHIMICA ACTA

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Spectrophotometric determination of hydrocortisone, nystatin and oxytetracycline in synthetic and pharmaceutical preparations based on various univariate and multivariate methods

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Received 5 October 2001; received in revised form 25 January 2002; accepted 26 February 2002

Abstract

Two spectrophotometric methods are described and applied to resolve ternary mixtures of the corticosteroid hydrocortisone (HYD) and the antibiotics nystatin (NYS) and oxytetracycline (OXY). The simultaneous determination of these three compounds was firstly accomplished by a derivative method using the "ratio spectrum-zero crossing derivative" and then by multivariate methods partial least squares (PLS)-1, -2 and principal component regression (PCR). Multivariate calibration methods provide, specially PLS-2 in this case, a clear example of the high resolving powder of these techniques. The two described procedures do not require any separation step. Repeatability and reproducibility studies were achieved over two series of 10 standards for each compound showing no significant differences at 95% confidence level in the four spectrophotometric methods. A comparison of the derivative and multivariate calibration results obtained in pharmaceutical formulations was performed resulting in agreement of the values obtained and the results was confirm by a high-pressure liquid chromatography (HPLC) method.

Keywords: Corticosteroids; Derivative spectrophotometry; Partial least squares; Partial component regression; Hydrocortisone; Nystatin; Oxytetracycline

1. Introduction

Hydrocortisone (HYD) is a human corticosteroid which is usually associated with nystatin (NYS) (an antifungal polydiene antibiotic derived from *Estreptomyces noursei*) and oxytetracycline (OXY) (a tetracycline antibiotic) [1–3].

HYD is mainly determined spectrophotometrically [4,5], by reverse-phase chromatography in plasma and pharmaceuticals subsequent UV detection generally at 254 nm [6–9] and micellar electrokinetic chromatography (MEKC) in urine [10].

OXY is an important tetracycline characterised by a broad-spectrum activity against pathogenic microorganisms [11]. OXY is determined by spectrophotometric methods based on direct methods [12], complexation reactions [13,14], derivative spectrophotometry [15–17], by high-pressure liquid chromatography (HPLC) [18,19] and by capillary zone electrophoresis (CZE) [20,21] and MEKC [22] (OXY and its degradation products in synthetic and biological samples).

NYS is a polyene antifungal antibiotic that is of particular interest because it exhibits remarkable action against a wide range of pathogenic and non-pathogenic yeast and fungi. The spectrophotometric methods to determine NYS were based on direct methods [23] and

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0003-2670/02/$ – see front matter
PII: S0003-2670(02)00138-1

derivative spectrophotometry [24]. HPLC on reverse phase with UV detection at 305 nm [25–27] and CZE [28,29] are used too.

Under computer-controlled instrumentation, derivative techniques and multivariate calibrations methods are playing a very important role in the multicomponent analysis of mixtures by ultraviolet-visible molecular absorption spectrophotometry [30]. Both approaches are useful in the resolution of band overlapping in quantitative analysis. Ternary mixtures can be easily resolved by means of a spectrophotometric method, which is based on the simultaneous use of "zero-crossing" and "ratio spectra derivative" methods [31–33]. The advantage of multicomponent analysis using multivariate calibration is the speed of the determination of the components in a mixture, avoiding a preliminary separation step.

The application of quantitative chemometric methods, particularly principal component regression (PCR) and partial least squares (PLS) to multivariate method needs a calibration step where the relationship between the spectra and the component concentration is deduced from a set of reference samples, followed by prediction step in which the results of the calibration are use to determine the component concentration from the sample spectrum. The basic concept of PLS regression was originally developed by Wold [34,35] and the use of the PLS method for chemical applications was also pioneered by Wold et al. [36]. Multivariate calibration methods, in combination with several techniques, have been widely applied in analytical procedures in recent years. Optical [37,38] and electrochemical [39] signals have been analysed by these approaches.

In this work two spectrophotometric methods are reported to accomplish the simultaneous determination of HYD, OXY and NYS in mixtures without the need for prior separation: one of them is based on derivative techniques, and the other is based on the combination of multivariative calibration methods with direct spectral information. The methods were applied to mixtures of these drugs in both pure forms and pharmaceutical formulations. Satisfactory sensitivity, accuracy and precision were noted. Other advantages of the method are its simplicity and speed. In Fig. 1 we can see the chemical structure of the mixture components.

2. Experimental

2.1. Apparatus

A Beckman Instruments DU-70 spectrophotometer equipped with a 1.0 cm cell and connected to an IBM-PS model 30 computer was provided with Beckman Data Leader Software (Fullerton, CA) [40].

The Grams 386 Level 1, version 3.01, software package with the PLS plus version 2.1G application software (Galactic Industries) [41] connected to an EGA computer, which were used for statistical treatment of the data and for the application of PLS and PCR methods.

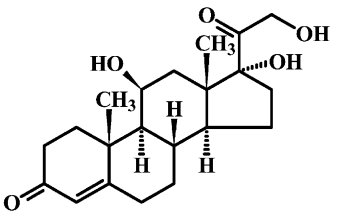
2.2. Reagents

All solvents and reagents were of analytical reagent grade unless indicated otherwise. HYD, OXY and NYS were supplied by SIGMA S.A. OXY stock aqueous solution, HYD stock hydroalcoholic (1:1) solution and NYS stock methanolic solution, with a concentration of 200 mg l^{-1} were prepared. Acetic acid/sodium acetate (0.1 M and pH = 4.5) was used as buffer solution.

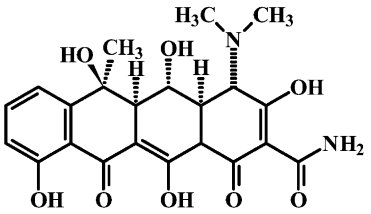
2.3. Procedure

2.3.1. Derivative methods

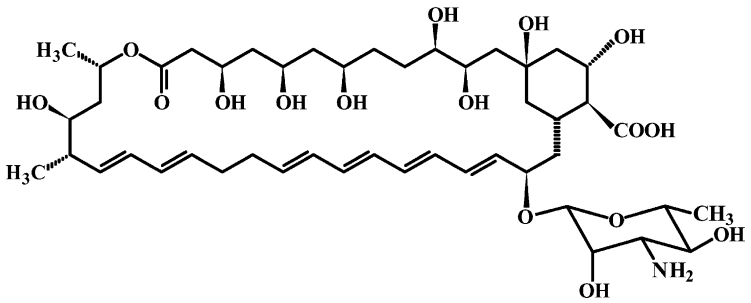
Ratio spectrum zero-crossing derivative: Samples were prepared in 25 ml calibrated flask by adding either up to 28 mg l^{-1} of HYD or up to 28 mg l^{-1} of OXY or up to 28 mg l^{-1} of NYS or their ternary mixture, 5 ml of acetate buffer solution (pH = 4.5), deionised water (Milli Q quality) and ethanol (final solution contained 16% ethanol) to the mark. The absorption spectra of the samples thus prepared were recorded between 190 and 400 nm with a scan rate of 600 nm min^{-1} against a reagent blank (the same of the samples without the compounds to be determined) using a 1.0 cm quartz cell and stored in an IBM-PS computer. We have applied this method on absorption spectra to determinate three compounds by means of a third compound in a ternary mixture, which is used as a divisor. The ratio spectrum is obtained dividing the absorption spectra of the mixture by a standard spectrum of one of the components. The first derivative of the ratio spectrum has to be obtained in order



HYDROCORTISONE



OXYTETRACYCLINE



NYSTATIN

Fig. 1. Structural formula of HYD, OXY and NYS.

to remove the spectral contribution of the compound used as divisor; the other compounds can be determined on the ratio derivative spectrum by measuring at their respective zero-crossing points (previously selected) [32,33]. The determinations carried out by this method are the following:

- When the divisor is a spectrum of NYS whose concentration is $6\,\mathrm{mg\,l^{-1}}$, HYD is determined at 256.2 nm ($^1DD_{256.2}$, zero-crossing point for ratio spectra first derivative of OXY); whereas OXY is determined by measuring at 253.4 nm ($^1DD_{253.4}$, zero-crossing points for ratio spectra first derivative of HYD), using a $\Delta\lambda = 12$ nm and a smoothing function of 15 experimental points in order to reach these first derivative spectra.
- When a standard spectrum of $6\,\mathrm{mg\,l^{-1}}$ OXY is used as a divisor, HYD is determined at 255.5 nm ($^1DD_{255.5}$, zero-crossing point for ratio spectra first derivative of NYS) whereas NYS is determined by measuring at 245 ($^1DD_{245}$, zero-crossing points for ratio spectra first derivative of HYD); and $\Delta\lambda = 8$ nm and a smoothing of 15 experimental points were applied to obtain their first derivative spectra.
- When a standard spectrum of $10\,\mathrm{mg\,l^{-1}}$ HYD is used as a divisor, OXY is determined at 286 nm ($^2DD_{286}$, zero-crossing points for ratio spectra

second derivative of NYS); whereas NYS is determined by measuring at 280.9 nm ($^2DD_{280.9}$, zero-crossing points for ratio spectra second derivative of OXY); and $\Delta\lambda = 24$ nm and a smoothing of 15 experimental points were applied to obtain their second derivative spectra.

2.3.2. Multivariate calibration

With the aim of improving the analysis for these compounds, three different chemometric approaches were evaluated. Haaland and Thomas [42] made a comparison of the different multivariate calibration methods for quantitative spectral analysis. They concluded that it is very difficult to generalise about the superiority of one method over each other, because their relative performance is often dependent on the particular data set to analyse. The best results in our particular case were for the PLS-2 method.

Experimental design of the calibration matrix and selection of the spectral zone for the analysis. As a calibration matrix a training set of 40 standard ternary mixtures samples, selected by an arbitrary design, was taken (0.0–32.0 mg l^{-1} of HYD, OXY and NYS). The spectral region between 240 and 400 nm was selected as suitable for the analysis, which implies the use of 323 experimental points for each spectrum. Selection of spectral information was made according to the spectra of the pharmaceutical products. The range of the spectrum between 190 and 240 nm was rejected due to differences between the artificial mixture spectra and the pharmaceutical spectra products at the same concentration. These differences could be due to other components of the pharmaceuticals as the excipients honey, ascorbic acid, di-sodium phosphate, sodium *meta*-bisulphite, methylparaben, propylparaben and so on.

In Fig. 2 the experimental design are given graphically. In this Figure we can see the composition of the standard mixtures used in the calibration matrix.

Selection of optimum number of factors: To select of number of factors in the PLS-1 algorithm in order to model the system without overfitting the concentration data, a cross validation method leaving out one sample at a time was used [43]. The process was repeated 40 times for each tested number of factors until each calibration standard has been left out once ($n = 40$, number of calibration samples). The predicted concentration (x) of the compounds in each sample were compared with the already known concentration (x) and the prediction error sum of squares (PRESS) was

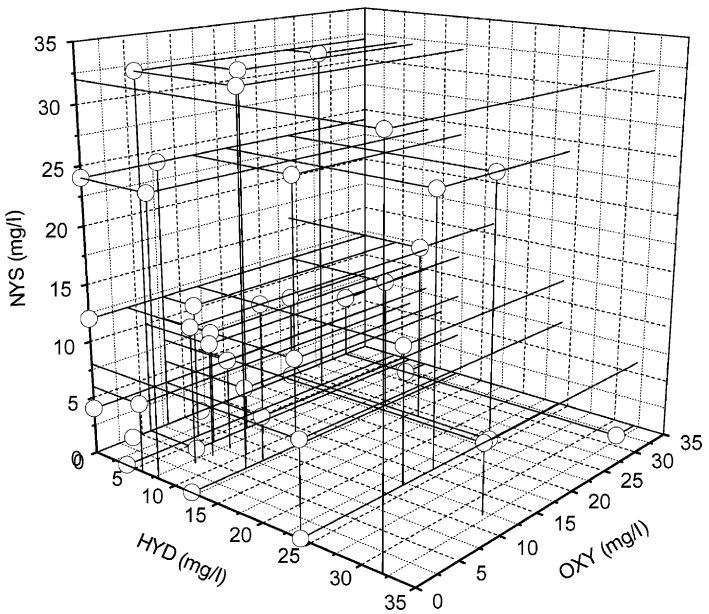


Fig. 2. Experimental design of the calibration matrix given graphically.

calculated by each number of factors:

$$\text{PRESS} = \sum_{I=1}^{n} (x_i - \bar{x}_i)^2$$

This parameter is a measure of the efficiency for a calibration fit model. The maximum number of factors used to calculate the optimum PRESS was selected as 21 (half of the numbers of standards plus one). One reasonable choice for the optimum number of factors would be the number that yielded the minimum PRESS. However, using the number of factors (h^*) that yield a minimum PRESS usually leads to some over fitting. A better criterion for calculating the optimum number of factors involves the comparison of PRESS for models with fewer than h^* factors. The model selected is that model is nor significantly greater than PRESS from the model with h^* factors. Haaland and Thomas [31] empirically determined that an F ratio probability of 0.75 is a good choice. We selected as optimum the number of which its F ratio probability drops below 0.75.

In our particular case, a number of five, nine and nine factors were obtained as optima for HYD, OXY and NYS components, respectively, by the PLS-1 method. Also the PLS-2 model was optimised by using the same set of standard samples and finding as optimum a number of eight factors for this model. The PCR model was optimised by using the same set of standard samples and finding as optimum a number of eight factors for this model.

The proposed PLS and PCR calibration models were evaluated by internal validation (prediction of compounds concentration in its own designed training set of calibration) obtaining, in general terms, recoveries between 96.5 and 104.5%.

3. Results and discussion

3.1. Influence of chemical variables

The influence of pH on the absorption spectra for solutions of HYD (20 mg l^{-1}), OXY (20 mg l^{-1}) and NYS (20 mg l^{-1}) is studied. In order to suitable pH value for this study a range between 1 and 12 pH values is examined obtaining the following results: the spectra of HYD shows a maximum at 247 nm between pH 1 and 12 remaining constant; OXY shows two maximums at 253.5 and 268 nm between pH 1 and 6 and undergoing a bathochromic shift between pH 6 and 8 and remaining constant between pH 6 and 1 showing two maximums at 357 and 278 nm and NYS shows three maximums at 320.5, 306 and 293 nm between pH 1 and 12 remaining constant. We have chosen a pH value of 4.5 as optimum.

In Fig. 3 the zero-order spectra of HYD, OXY and NYS recorded in 190–400 nm wavelengths are shown. As it can be seen the absorption spectra of the three components overlap obstructing the resolution of this ternary mixture form direct absorbance measurements. For this reason, we have proposed several spectrophotometric methods in order to resolve this ternary mixture.

3.2. Derivative spectrophotometric studies

3.2.1. Ratio spectrum-zero crossing derivative

In order to obtain the best recoveries for the three compounds, it is necessary to study and to optimise the following parameters: concentration of the standard spectrum used as divisor, $\Delta\lambda$ to obtain the first derivative, smoothing function and zero-crossing wavelengths.

Determination of HYD and OXY in presence of NYS: The above-mentioned variables were studied and the following values were chosen: a standard spectrum of NYS of 6 mg l^{-1}, an $\Delta\lambda$ of 12 nm for the first derivative spectra and a smoothing function of 15 experimental points, on the basis of the [44] and, Savitzky and Golay method [45]. In these conditions the first derivative ratio spectra of HYD/NYS and OXY/NYS are shown in Fig. 4a, where we can see that HYD content can be measured at 256.2 nm (zero-crossing point for ratio spectra of OXY), whereas OXY is determined at 253.4 nm (zero-crossing points for ratio spectra derivative of HYD). Calibration graphs were obtained at the previously selected wavelengths up 36 mg l^{-1} of HYD and OXY (Table 1). In Fig. 5a the first derivative of the ratio spectra for different concentrations of HYD.

Determination of HYD and NYS in presence of OXY: Under the optimal conditions already described in Section 2.3, HYD content could be determined, obtaining a straight line up to 36 mg l^{-1} at 255.5 nm and NYS content could be determined, obtaining a straight line up to 36 mg l^{-1} at 245 nm. In Fig. 4b we can see

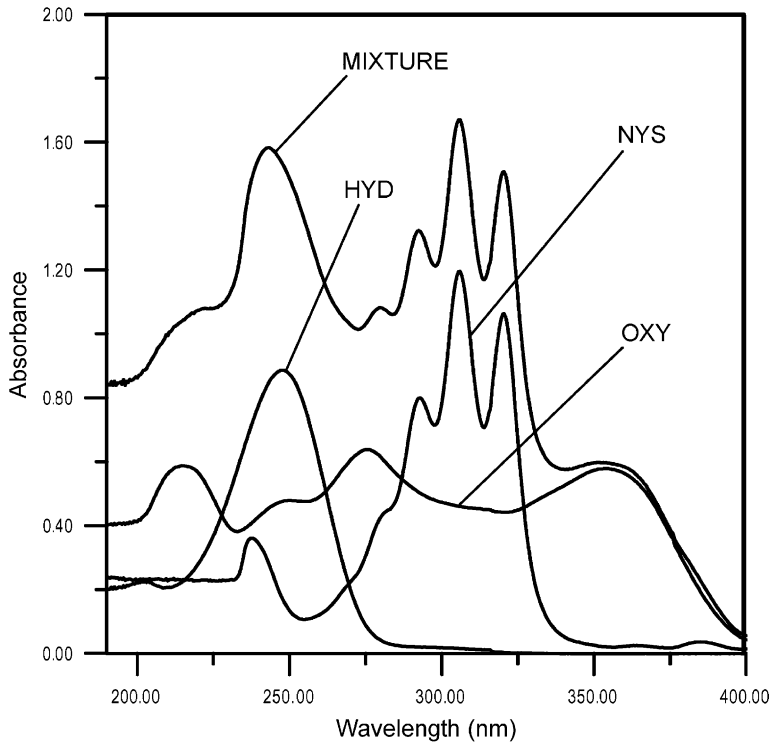


Fig. 3. Absorption spectra for solutions of mg l^{-1} of HYD, 20 mg l^{-1} of OXY, 20 mg l^{-1} of NYS and their mixture prepared in acetate buffer medium (pH 4.5) and recording against a reagent blank with a scan of 600 nm min^{-1}.

that HYD and NYS content can be measured at these wavelength. In Fig. 5b we can see the first derivative of the ratio spectra for different concentrations of NYS.

Determination of OXY and NYS in presence of HYD: Under the optimal conditions already described in Section 2.3, OXY content could be determined, obtaining a straight line up to 36 mg l^{-1} at 286 nm and NYS content could be determined, obtaining a straight line up to 28 mg l^{-1} at 280.9 nm. In Fig. 4c we can see that OXY and NYS content can be measured at these wavelength. In Fig. 5c we can see the second derivative of the ratio spectra for different concentrations of OXY. In this case we use the second derivative because in the first there are not adequate zero-crossing point for the determination.

In Table 1 the most characteristic statistical data of calibration graph for each compound by the proposed derivative method is summarised. In all cases good correlation coefficients were reached. Determination limits were calculated for each compound at its respective analytical signals according to IUPAC criterion [46,47] and they are also shown in Table 1.

3.2.2. Repeatability and reproducibility

To carry out these studies, independent series of each compound (16 mg l^{-1} of HYD, 16 mg l^{-1} of OXY and 16 mg l^{-1} of NYS) were recorded in two consecutive days. Satisfactory results for the repeatability within 1 day for each compound in terms of R.S.D. (between 0.13 and 1.96%) were obtained; with regard to reproducibility studies, the comparison between the two sets of data to detect random errors was made with the *F*-test, whereas the comparison of averages concentrations to check systematic errors was made with the Student's *t*-test; no significant differences were observed in any cases at a confidence level of 95% since the obtained experimental values of *F* and *t* were lower than their respective theoretical values ($F_{(9,9)} = 3, 18$ and $t_{20} = 2.09$).

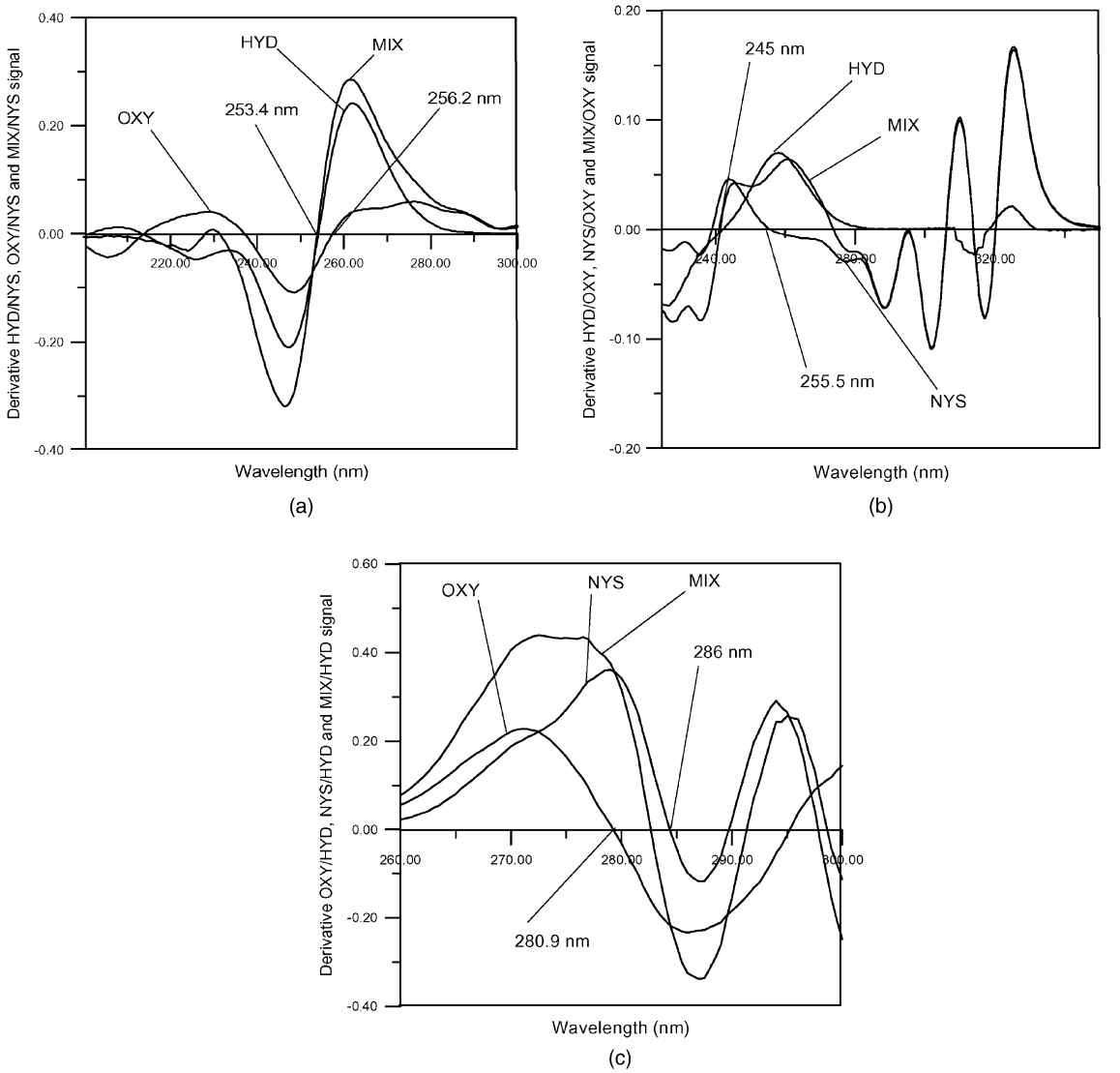


Fig. 4. (a) First derivative of the ratio spectra of $4\,mg\,l^{-1}$ of HYD, $4\,mg\,l^{-1}$ of OXY and its mixture (MIX) obtained using as divisor a spectrum of $6\,mg\,l^{-1}$ of NYS, (b) first derivative of ratio spectra of $4\,mg\,l^{-1}$ of HYD, $4\,mg\,l^{-1}$ of NYS and its MIX obtained using as divisor a spectrum of $6\,mg\,l^{-1}$ of OXY, and (c) second derivative of ratio spectra of $4\,mg\,l^{-1}$ of OXY, $4\,mg\,l^{-1}$ of NYS and its MIX obtained using as divisor a spectrum of $10\,mg\,l^{-1}$ of HYD.

3.3. Multivariate calibration studies

Three multivariate calibration methods were developed by authors in order to check the derivative method and also to confirm the derivative results in pharmaceutical mixtures. PLS and PCR methods were evaluated and a comparative study of the prediction capabilities of the three chemometric approaches in our particular work was under taken.

3.3.1. Statistical parameters of cross-validation method

Using the cross-validation method the following statistical parameters have been obtained:

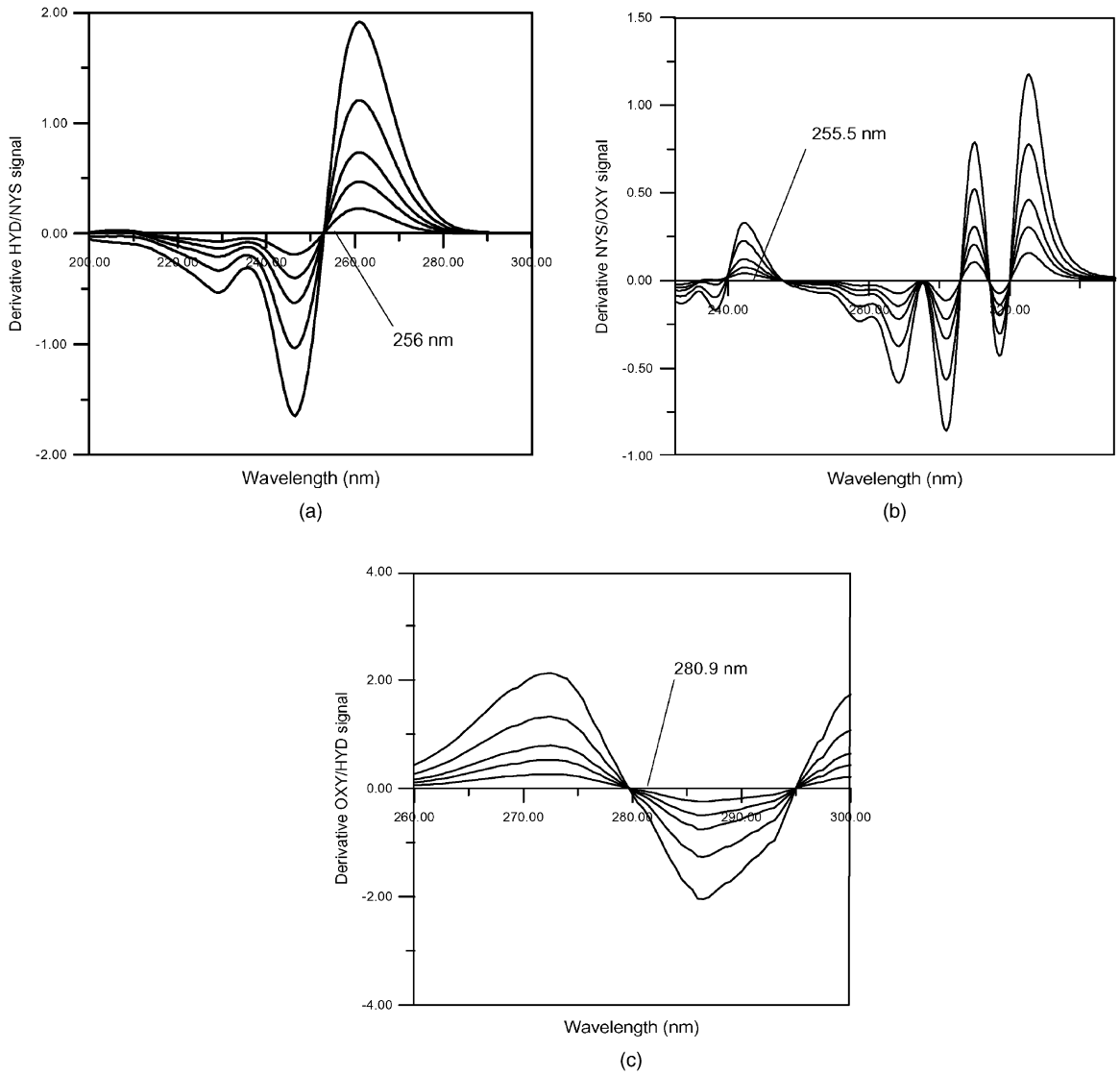


Fig. 5. (a) First derivative of the ratio spectra for different concentrations of HYD obtained using as divisor a spectrum of $6\,\mathrm{mg\,l^{-1}}$ of NYS, an $\Delta\lambda = 12\,\mathrm{nm}$ and a smoothing over 15 experimental points, (b) first derivative of the ratio spectra for different concentrations of NYS obtained using as divisor a spectrum of $6\,\mathrm{mg\,l^{-1}}$ of OXY, and $\Delta\lambda = 8\,\mathrm{nm}$ and a smoothing over 15 experimental points and (c) second derivative of the ratio spectra for different concentrations of OXY obtained using as divisor a spectrum of $10\,\mathrm{mg\,l^{-1}}$ of HYD, and $\Delta\lambda = 24\,\mathrm{nm}$ and a smoothing over 15 experimental points.

(a) The values of root mean squares difference (RSMD) that is an indication of the average error in the analysis for each component.
(b) The square of correlation coefficients (R^2), that is an indication of the quality of the straight line that fits the data.
(c) The predictive ability of each method and for each component can also be described in terms of the relative error of prediction (REP) with regard to the average valued (μ).
(d) The standard error of calibration (SEC/SEP).

Table 1
Statistical parameters of calibration graph for each compound

Regression equations	Linearity coefficient	Range ($mg\,l^{-1}$)	Standard deviation		Detection limits ($mg\,l^{-1}$)[a]
			Slope	Intercept	
Ratio spectrum-zero crossing derivative					
NYS as divisor					
$^1DD_{256.2} = 3.51708 \times 10^{-2}\ C_{HYD} + 5.046 \times 10^{-3}$	0.9997	2–36	2.08×10^{-4}	4.56×10^{-3}	3.17×10^{-2}
$^1DD_{253.4} = 1.302 \times 10^{-2}\ C_{OXY} - 7.153 \times 10^{-3}$	0.9996	2–36	9.70×10^{-5}	1.87×10^{-3}	1.85×10^{-1}
OXY as divisor					
$^1DD_{255.5} = 1.35 \times 10^{-2}\ C_{HYD} + 2.43 \times 10^{-3}$	0.9996	2–36	8.89×10^{-5}	1.75×10^{-3}	2.78×10^{-2}
$^1DD_{245} = 1.085 \times 10^{-2}\ C_{NYS} + 1.33 \times 10^{-3}$	0.9988	2–36	2.51×10^{-4}	4.90×10^{-3}	2.67×10^{-2}
HYD as divisor					
$^2DD_{286} = 6.44 \times 10^{-2}\ C_{OXY} - 2.16 \times 10^{-3}$	0.9999	2–36	4.83×10^{-4}	9.47×10^{-3}	2.16
$^2DD_{280.9} = 9.61 \times 10^{-2}\ C_{NYS} + 2.07 \times 10^{-2}$	0.9968	2–24	1.16×10^{-4}	2.23×10^{-3}	1.43

C_{HYD}, C_{OXY} and C_{NYS} are the contents of HYD, OXY and NYS, respectively, expressed as $mg\,l^{-1}$. Detection limit $= 3S.D._B/m$; where $S.D._B$ = standard deviation of blank; m = slope of calibration graph.
[a] Obtained by IUPAC criterion.

In Table 2 the results obtained for these parameters by three proposed chemometric approaches are shown. We can see that R^2 values are in all cases very nearly to 1, which is an indication of similarity between predicted and known values. On the other hand, in general terms, the obtained errors for these statistical cross-validation parameters are the same for both multivariate calibration methods, the value obtained for NYS are slightly higher than those obtained in HYD and OXY determination.

Table 2
Statistical parameters of cross-validation process for PLS-1, -2 and PCR

Compound	Factor	PRESS	RMSD	R^2	REP (%)
PLS-1					
HYD	5	1.965	0.221	0.9995	1.401
OXY	5	2.477	0.248	0.9996	1.574
NYS	9	2.027	0.225	0.9993	1.423
PLS-2					
HYD	8	6.903	0.214	0.9998	1.356
OXY	7.526	0.234	0.9994	1.483	
NYS	5.236	0.267	0.9994	1.691	
PCR					
HYD	8	7.637	0.216	0.9995	1.367
OXY	6.526	0.235	0.9993	1.486	
NYS	7.512	0.298	0.9992	1.886	

3.3.2. Precision

Precision of the PLS-1, -2 and PCR methods, was checked by recording independent series of 10 samples for each compound ($16\,mg\,l^{-1}$ of HYD, $16\,mg\,l^{-1}$ of OXY and $16\,mg\,l^{-1}$ of NYS) in two consecutive days. Repeatability studies were satisfactory obtaining R.S.D. values of 0.68, 0.61 and 0.71 for HYD, OXY and NYS, respectively; when reproducibility studies were achieved over the two sets of 10 standards for each compound in consecutive days the following results were found: $F_{(9,9)}$ exp = 1.21, 1.49 and 2.89 for DEX, TMP and PLX, respectively, and t_{20} exp = 1.26, 1.32 and 2.21 for HYD, OXY and NYS, respectively, showing no significant differences between the two sets of 10 replicates at a confidence level of 95%.

3.4. Applications

3.4.1. Synthetic mixtures

One set of 10 synthetic mixtures containing from 4 to $32\,mg\,l^{-1}$ of HYD, OXY and NYS in different

Table 3
Recoveries found in synthetic mixtures

Contents ($mg\,l^{-1}$)			Ratio spectrum-zero crossing derivative					
HYD	OXY	NYS	NYS[a]		OXY[a]		HYD[a]	
			$H^1DD_{256.2}$	$O^1DD_{253.4}$	$H^1DD_{255.5}$	N^1DD_{245}	O^2DD_{286}	$N^2DD_{280.9}$
8	6	6	99.1	97.4	98.9	101.7	103.3	97.7
10	10	10	96.6	99.2	95.9	101.8	102.2	96.3
24	24	24	98.3	98.2	98.6	99.5	103.2	98.4
6	8	8	99.7	99.1	95.4	103.1	97.8	95.3
8	4	6	99.7	95.2	97.3	98.3	95.7	98.2
4	6	8	96.1	98.2	98.9	99.9	104.0	96.6
8	6	4	99.1	97.4	97.6	98.2	98.1	96.1
6	12	32	100.9	104.2	101.6	99.9	104.6	97.8
12	6	32	103.2	105.7	99.7	101.3	105.1	99.6
32	12	6	102.5	104.7	98.8	96.6	104.8	97.2

[a] Component used as divisor.

ratios were predicted by means of the two proposed spectrophotometric methods. The comparison of these mixtures of the recoveries obtained by derivative and multivariate calibration methods are summarised in Tables 3 and 4.

Table 3 shows that, in general terms, very good recoveries were obtained for HYD, OXY and NYS by the ratio spectrum zero-crossing derivative method. When the divisor is HYD the results are worse.

The external validation of the PLS-2 model was achieved over set of 10 synthetic ternary mixtures and the recoveries reached by the PLS-2 method can be seen in Table 4. The recoveries obtained are between 104.1 and 96.5% for most mixtures.

Table 4
Recoveries found in synthetic mixtures by PLS-2

Contents ($mg\,l^{-1}$)			PLS-2 (recovery (%))		
HYD	OXY	NYS	HYD	OXY	NYS
8	6	6	98.3	103.4	104.1
10	10	10	99.2	102.3	103.3
24	24	24	100.2	96.8	99.6
6	8	8	98.6	102.6	100.6
8	4	6	99.5	98.7	102.1
4	6	8	100.1	96.8	96.5
8	6	4	98.9	97.6	99.9
6	12	32	100.1	101.5	100.9
12	6	32	99.5	102.2	100.8
32	12	6	100.2	101.0	101.3

3.4.2. Pharmaceutical preparations

The spectrophotometric procedures were applied to the following pharmaceutical preparations:

Milrosina–Nistatina: it is a suspension by mouth with HYD and NYS. From the enterprise Biogalenica S.A.

Terra–Cortril: it is an ointment with HYD and OXY HCl. From the enterprise Farmasierra S.A.

Eoline crema 15 g. It is an ointment with HYD, OXY HCl and NYS. From the enterprise Pfizer.

The procedure for the analysis of the pharmaceutical preparation is as follows:

For Milrosina–Nistatina, once the pharmaceutical mixture was homogenised, different known aliquots were placed in 25 ml calibrated flasks, adding also 5 ml of acetate buffer 0.1 M, methanol (final solution contained 20% methanol) and deionised water to the mark.

For Terra–Cortril and Eoline, an amount of the ointment was weighed accurately into an extraction glass. A sequential extraction is made to extract all the compounds with a total volume of 100 ml. Different volumes of 20 ml are shaking and then are subjected to an ultrasonic bath for 15 min, to complete 100 ml. This total volume of the extraction was filtered and different known aliquots were placed in 25 ml calibrated flak, adding also 5 ml of acetate buffer 0.1 M, methanol (final solution contained 20% methanol) and deionised water.

The spectra of the commercial samples thus prepared were recorded against a reagent blank (the same

Table 5
Contents found of HYD, OXY and NYS in pharmaceutical preparations

Pharmaceutical preparation	Nominal contents ($mg\,l^{-1}$)			Found contents ($mg\,l^{-1}$)					
	HYD	OXY	NYS	Ratio spectrum-zero crossing derivative					
				HYD (%)	OXY (%)	NYS (%)			
Milrosina–Nistatina	2.40	0	25.69	3.17 (132.1)[a]	–	26.7 (103.9)[b]			
Milrosina–Nistatina[d]	2.40	7.02	25.69	3.21 (133.7)[a]	10.60 (151.0)[c]	26.5 (103.2)[b]			
Terra–Cortril	7.73	23.18	0	7.44 (96.4)[a]	23.19 (100.1)[c]	–			
Terra–Cortril[e]	2.90	8.69	28.19	2.85 (98.3)[a]	8.80 (101.2)[c]	27.68 (98.2)[b]			
Eoline	8.0	19.0	25.0	7.65 (95.6)[a]	18.20 (95.8)[c]	23.95 (95.8)[b]			
				PLS-2 (%)			HPLC		
				HYD	OXY	NYS	HYD	OXY	NYS
Milrosina–Nistatina	2.40	0	25.69	2.81 (115.7)	–	26.5 (103.2)	2.31 (96.2)	–	25.08 (97.6)
Milrosina–Nistatina[d]	2.40	7.02	25.69	2.74 (114.1)	16.88 (118.2)	24.90 (97.1)	2.30 (95.8)	7.03 (100.1)	24.78 (96.5)
Terra–Cortril	7.73	23.18	0	7.45 (96.4)	23.11 (99.7)	–	7.35 (95.1)	23.39 (100.9)	–
Terra–Cortril[e]	2.90	8.69	28.19	2.79 (96.2)	8.79 (101.2)	27.69 (98.2)	2.76 (95.4)	9.21 (103.1)	29.10 (103.2)
Eoline	8.0	19.0	25.0	7.69 (96.1)	18.30 (96.3)	24.05 (96.2)	7.75 (96.9)	18.40 (96.8)	24.25 (97.0)

[a] $^1DD_{256.2}$.
[b] $^1DD_{245}$.
[c] $^1DD_{253.4}$.
[d] Added compound. The original product do not contain OXY.
[e] Added compound. The original product do not contain NYS.

of the samples without the compounds to be determined and with some of the excipients indicated to the enterprise), with a scan speed of 600 nm min^{-1} between 400 and 190 nm. The contents of HYD, OXY and NYS were calculated by analysing the recorded spectra with the derivative procedure and with PLS-2 chemometric approach. The predicted concentrations expressed as mass/volume ratio (mg of compound l^{-1} of commercial product) are summarised in Table 5, where the contents supplied by the manufacturer are also shown.

The Table 5 displays a acceptable agreement between the results obtained by the two methods an these values are also close to the values provided by the manufacturer with relative error below 4%. We think there is a negative effect of the excipients in the correct determination in this pharmaceutical preparations, especially for HYD and OXY in Milrosina–Nistatina. This products has a lot of excipients and they absorb in the region between 190 and 240 so there is a extra signal in this zone and as a result of that The recoveries are higher than 100% in ratio zero-crossing derivative and multivariante methods.

The determination of HYD, OXY and NYS in the two pharmaceutical products was also verified by HPLC method using a diode-array detector with measurements at 247, 350 and 306 nm (wavelength where the absorbance was maximal for each compound). Separation was carried out at 25 °C by using a LichrCART® C 18 column and a methanol–NaH_2PO_4 (0.1 M) (pH 4.5) buffer solution as mobile phase in gradient mode: (50–70 (v/v) in 4 min; 70 (v/v) during 8 min and 70–50 (v/v) in 4 min). The mobile phase flow-rate and sample volume injected were 1 ml min^{-1} and 20 μl, respectively. Under these conditions the retention times were 1.7 min for OXY, 6.2 min for HYD and 11.3 min for NYS, and calibrations graphs were established for each compounds at wavelengths corresponding to its maximum. Chromatographic results are also summarised in Table 5, where it can be seen that the results and recoveries show agreement between claimed and found values by calibration multivariate method for the three compounds in all pharmaceutical preparation analysed, showing the highest deviation for HYD and OXY by derivative method from its respective PLS- 2 and chromatographic values.

4. Conclusions

Ultraviolet-visible spectra usually contain non-specific data, which can be converted into useful information by multivariate calibration methods. In order to achieve this, chemometrics has found much interest in analytical molecular spectroscopy. Clear explanations of the different chemometric approaches and properly designed software should provide a bridge between chemometrics, mathematicians and spectroscopic technicians, enabling them to make successful use of these powerful tools.

A comparative study of the use of derivative and multivariate calibration methods for the resolution of ternary mixtures of HYD, OXY and NYS has been accomplished, showing that multivariate calibration methods provide, with adequate software support, a clear example of the high resolving powder of these techniques. In general terms, the results are better by PLS-2, reaching agreement in pharmaceutical products between the chemometric data and those obtained by a HPLC method. On the other hand, satisfactory results were obtained when the precision of chemometric approaches was checked at a confidence level of 95% for the three compounds.

The proposed methods provide satisfactory results in all cases (except by ratio zero-crossing derivative method when the matrix of pharmaceutical samples is very complex).

According to these studies, multivariate calibration methods (PLS-2) using direct spectra signal are recommended as a very suitable choices to resolve accurately overlapped absorption spectra of mixtures of the compounds HYD, OXY and NYS, due to the simplicity of the determination, the inexpensive, the easy treatment of pharmaceutical samples and the acceptable results obtained in the analysis of these products.

Acknowledgements

The wish to thank the DGES of the Ministerio de Educación y Ciencia for the financial support (Project PB-97-0431).

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