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HPLC and chemometrics-assisted UV-spectroscopy methods for the simultaneous determination of ambroxol and doxycycline in capsule

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Abstract

High-performance liquid chromatography (HPLC) and multivariate spectrophotometric methods are described for the simultaneous determination of ambroxol hydrochloride (AM) and doxycycline (DX) in combined pharmaceutical capsules. The chromatographic separation was achieved on reversed-phase C_{18} analytical column with a mobile phase consisting of a mixture of 20 mM potassium dihydrogen phosphate, pH 6–acetonitrile in ratio of (1:1, v/v) and UV detection at 245 nm. Also, the resolution has been accomplished by using numerical spectrophotometric methods as classical least squares (CLS), principal component regression (PCR) and partial least squares (PLS-1) applied to the UV spectra of the mixture and graphical spectrophotometric method as first derivative of the ratio spectra (^{1}DD) method. Analytical figures of merit (FOM), such as sensitivity, selectivity, analytical sensitivity, limit of quantitation and limit of detection were determined for CLS, PLS-1 and PCR methods. The proposed methods were validated and successfully applied for the analysis of pharmaceutical formulation and laboratory-prepared mixtures containing the two component combination.

Keywords: Ambroxol; Doxycycline; HPLC; Multivariate spectrophotometric methods

1. Introduction

Ambroxol (AM) is a metabolite of bromhexine and is used similarly as a mucolytic. Doxycycline (DX) is a tetracycline derivative with uses similar to those of tetracycline [1].

The combination of AM and DX in capsules is used for the treatment of infections caused by susceptible strains of pathogens in acute and chronic diseases of upper and/or lower respiratory tract concomitant with formation of viscous and hardly separated expectoration.

Several analytical methods in the literature describing the determination of ambroxol hydrochloride [2–8] or doxycycline alone [9–13] or in combination with other drugs [14–23] have been reported. Only one analytical method has been reported for the simultaneous determination of AM and DX in their binary mixture by reversed-phase sequential injection chromatography (SIC) technique [24].

Chemometrics-spectrophotometry (namely calculation-spectrophotometry) is the very good combination of chemometrics with analytical chemistry, and it can enhance the ratio of signal-to-noise (S/N), improve selectivity of determination, optimize experimental conditions, raise analytical operation efficiency and provide many scientific information. Hence, it is rapidly attracting analyst attention and is used for simultaneous determination of multicomponent in recent years [25].

Among the various chemometric approaches applied to multicomponent analysis, classical least squares (CLS), principal component regression (PCR) and partial least-squares regression (PLS) have been successfully adopted in many quantitative assays of pharmaceutical formulations.

CLS is one of the simplest methods, being a multivariate least-squares procedure based directly on Beer's law [26].

PCR and PLS are factor analysis methods which allow to establish a relationship between matrices of chemical data [27]. One of the clearest explanations of these methods was given by Haaland and Thomas [28]. PLS is related to PCR in that a spectral decomposition is performed. PCR decomposition is significantly influenced by variations, which have

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no relevance to the analyte concentrations, whereas in PLS, the spectral decomposition is weighted to the concentration [29].

In this work, first derivative ratio spectra (^{1}DD), CLS, PCR, PLS-1 and HPLC methods were applied for resolution of the studied binary mixture. The proposed methods are simple, sensitive, reduce the duration of the analysis and suitable for routine determination of the components in the studied mixture.

The proposed HPLC method use C_{18} column, which is available in many laboratories; while the published sequential injection chromatographic method used monolithic column, which is relatively expensive column in comparing with commercial reversed-phase C_{18} column. In addition, the proposed method offer relatively higher sensitivity (up to 1 μg/ml), lower quantitation and detection limits. The peaks are symmetrical but in the published method the peaks are tailed with peak symmetry of 2.8 and 2.1 for AM and DX, respectively.

2. Experimental

2.1. Instrumentation

A double-beam Shimadzu (Japan) UV–vis spectrophotometer, model UV-1601 PC equipped with 1 cm quartz cells and connected to an IBM compatible computer. The bundled software was UVPC personal spectroscopy software version 3.7 (Shimadzu). The spectral bandwidth was 2 nm and the wavelength scanning speed was 2800 nm min^{-1}. PLS-1 and PCR analysis were carried out by using PLS-Toolbox software version 2.1–PC [30] for use with MATLAB5.

The HPLC (Shimadzu, Kyoto, Japan) instrument was equipped with a model series LC-10 ADVP pump, SCL-10 AVP system controller, DGU-12 A Degasser, Rheodyne 7725i injector with a 20 μl loop and a SPD-10AVP UV–vis detector. Separation and quantitation were made on a Phenomenex Luna reversed-phase C_{18} analytical column (USA) (250 mm × 4.6 mm i.d., 5 μm particle size). The detector was set at 245 nm. Data acquisition was performed on class-VP software.

2.2. Materials and reagents

Pharmaceutical grade of AM and DX were used and certified to contain 99.7 and 99.8%, respectively.

Acetonitrile and methanol used were HPLC grade (Riedel-de Haën Laboratory Chemicals, Germany). Potassium dihydrogen phosphate, phosphoric acid used were analytical grade.

Ambrodoxy® capsules (batch no. 0502111) (The Egyptian Co. For chemicals and pharmaceuticals industries (ADWIA), 10th of Ramadan, Egypt), contains 100 mg of DX (as base) and 75 mg of AM per capsule were used.

2.3. HPLC condition

The HPLC separation and quantitation were made on a 250 mm × 4.6 mm (i.d.) Phenomenex Luna (5 μm particle size) reversed-phase C_{18} analytical column. The mobile phase was prepared by mixing 20 mM potassium dihydrogen phosphate, pH 6–acetonitrile in ratio of (50:50, v/v). The flow rate was of 1.5 ml min^{-1}. All determinations were performed at ambient temperature. The injection volume was 20 μl. The detector was set at λ 245 nm. Data acquisition was performed on class-VP software.

2.4. Standard solutions and calibration graphs

Stock standard solutions AM and DX (as base) (1 mg ml^{-1}) were prepared separately in methanol.

2.4.1. For ^{1}DD method

The standard solutions were prepared by further dilutions of the stock standard solutions with 0.1 M HCl to reach the concentration range of 1–21 μg ml^{-1} for DX and AM.

The UV absorption spectra of standard solutions of each drug were recorded in the wavelength range of 220–320 nm and divided by a normalized spectrum of coexisting drug (obtained by dividing the spectra of several standards of different concentrations by their corresponding concentrations and subsequently averaging them, in order to obtain a spectrum of unit concentration).

The first derivative was calculated for the obtained ratio spectra with $\Delta\lambda = 8$ nm. The first derivative of the ratio spectra obtained was smoothed with four experimental points and scaling factor of 10.

The amplitudes of ^{1}DD at 282.6 nm were measured and found to be proportional to the concentrations of DX and the amplitudes of ^{1}DD at 250.2 nm were measured and found to be proportional to the concentrations of AM.

2.4.2. Experimental design for CLS, PLS-1 and PCR calibration

In order to obtain a suitable calibration set; systematic experimental designs were used.

This paper employs a multilevel partial factorial design [31] for five concentration levels ($l = 5$). Mutually orthogonal designs are only possible if the number of concentration levels is a prime number or a power of a prime number. The design requires at least l^2 experiments (25 experiments) to study a mixture. After numbering the levels from −2 (lowest) to 2 (highest) the complete design was obtained using what is often described as a cyclic generator (−2, 1, 2, 1, −2), a repeater of 0 and a difference vector (0 2 3 1). In this type of design, there is no correlation between any concentrations of the compounds; hence, the correlation coefficient is zero.

A calibration set of 25 synthetic mixtures with different concentrations of each compound were prepared in 0.1 M hydrochloric acid in range of 1–21 μg ml^{-1} for AM and DX (Table 1).

The UV absorption spectra were recorded over the wavelength range of 220–320 nm. The data points of the spectra were collected every 1 nm. The computations were made using PLS-Toolbox software version 2.1. CLS, PLS-1 and PCR models

Table 1
Levels and concentration data for the AM and DX compounds in the calibration set

Experiments	Levels		Conc. ($\mu g\,ml^{-1}$)	
	AM	DX	AM	DX
1	0	0	11	11
2	0	−2	11	1
3	−2	−2	1	1
4	−2	2	1	21
5	2	−1	21	6
6	−1	2	6	21
7	2	0	21	11
8	0	−1	11	6
9	−1	−1	6	6
10	−1	1	6	16
11	1	2	16	21
12	2	1	21	16
13	1	0	16	11
14	0	2	11	21
15	2	2	21	21
16	2	−2	21	1
17	−2	1	1	16
18	1	−2	16	1
19	−2	0	1	11
20	0	1	11	16
21	1	1	16	16
22	1	−1	16	6
23	−1	−2	6	1
24	−2	−1	1	6
25	−1	0	6	11

were applied to the UV absorption spectra of this mixture using two latent variables for PLS-1 and PCR for determination of each compound.

2.4.3. For HPLC method

The standard solutions were prepared by further dilutions of the stock standard solutions with the mobile phase to reach the concentration range of 1–100 $\mu g\,ml^{-1}$ for DX and AM.

Triplicate 20 μl injections were made for each concentration and chromatographed under the specified chromatographic conditions described previously. The peak area values were plotted against corresponding concentrations.

2.5. Pharmaceutical sample preparation

The contents of 20 hard capsules were emptied, weighed, mixed and finely powdered. A portion of the powder equivalent to 50 mg DX (as base) and 37.5 mg AM was accurately weighed, extracted and diluted to 50 ml with methanol. The sample solution was filtered. Further dilution was carried out with 0.1N HCl (for ^{1}DD, CLS, PLS-1 and PCR methods) or the mobile phase (for HPLC method) to reach the calibration range of each compound. The general procedures for ^{1}DD, CLS, PLS-1, PCR and HPLC methods described under calibration were followed and the concentrations of AM and DX were calculated.

3. Results and discussion

3.1. For ^{1}DD method

Fig. 1 shows the UV absorption spectra of AM and DX at their nominal concentrations. The spectra of the two coexisting drugs in binary mixture displayed considerable overlap that the conventional spectrophotometry and the application of the direct derivative technique using UV–vis spectrophotometer failed to resolve them with acceptable accuracy. However, this spectral overlap was sufficiently enough to demonstrate the advantage of the derivative ratio method.

To optimize the simultaneous determination of the two drugs by using ^{1}DD method, it is necessary to test the influence of the divisor standard concentration, the $\Delta\lambda$ and smoothing function. $\Delta\lambda = 8$ nm was selected as the optimum value. From several tests for correct choice of the divisor standard concentration, the best results in terms of signal to noise ratio, sensitivity and repeatability followed using normalized spectra [32] as divisor (obtained by dividing the spectra of several standards of different concentrations by their corresponding concentrations and subsequently averaging them, in order to obtain a spectrum of unit concentration). Due to the extent of the noise levels on the ratio spectra, a smoothing function was used and four experimental points were considered as suitable. The first derivative of the ratio peak amplitudes at the specified wavelength 250.2 and 282.6 nm was found to be proportional to the concentration of AM and DX, respectively (Fig. 2). The characteristic parameters of regression equation for the ^{1}DD method and correlation coefficient are given in Table 2.

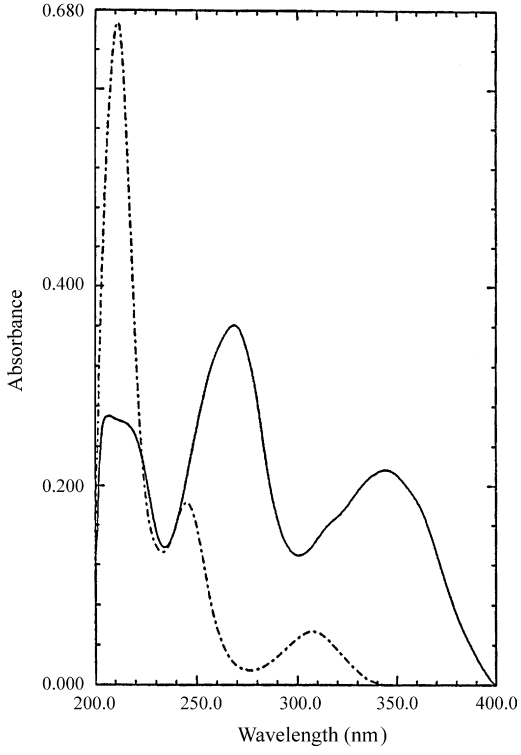

Fig. 1. UV absorption spectra of 7.5 $\mu g\,ml^{-1}$ of AM (- - -) and 10 $\mu g\,ml^{-1}$ of DX (—) in 0.1 M hydrochloric acid.

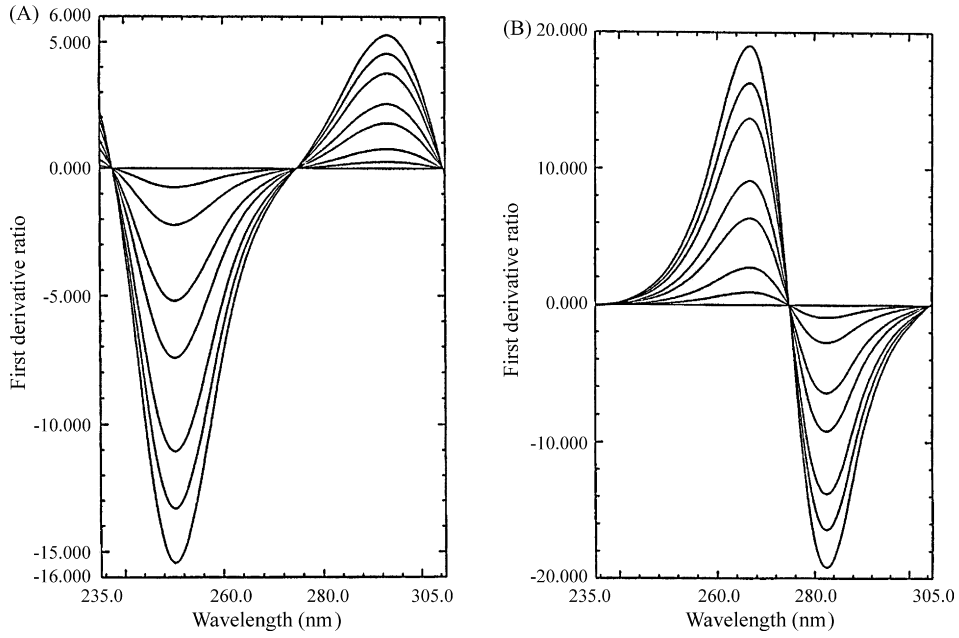

Fig. 2. First derivative ratio spectra for (A) different concentrations of AM (1, 3, 7, 10, 15, 18 and 21 μg ml^{-1}) using normalized spectrum of DX as divisor and (B) different concentrations of DX (1, 3, 7, 10, 15, 18 and 21 μg ml^{-1}) using normalized spectrum of AM as divisor in 0.1 M hydrochloric acid.

3.2. Multivariate calibration methods

3.2.1. Calibration matrix and selection of spectral zones for analysis by CLS, PLS-1 and PCR

The quality of multicomponent analysis is dependent on the wavelength range and spectral mode used [33]. PLS procedures are designated to be full spectrum computational procedures, thus wavelength selection is seemingly unnecessary, and so all available wavelengths are often used. However, measurements from spectral wavelengths that are non-informative in a model degrade performance. Original and reconstructed spectra of the calibration matrix were compared in order to select the range of

Table 2
Characteristic parameters of the calibration equations for the proposed HPLC and ^{1}DD methods for simultaneous determination of AM and DX

Parameters	HPLC		^{1}DD	
	AM	DX	AM	DX
Calibration range (μg ml^{-1})	1–100	1–100	1–21	1–21
Detection limit (μg ml^{-1})	1.98×10^{-2}	2.06×10^{-2}	1.55×10^{-2}	1.35×10^{-2}
Quantitation limit (μg ml^{-1})	6.61×10^{-2}	6.86×10^{-2}	5.18×10^{-2}	4.50×10^{-2}
Regression equation (Y)[a]				
Slope (b)	2.35×10^{4}	1.82×10^{4}	7.36×10^{-1}	9.15×10^{-1}
Standard deviation of the slope (S_b)	1.98×10^{2}	1.59×10^{2}	4.78×10^{-3}	5.26×10^{-3}
Relative standard deviation of the slope (%)	0.84	0.88	0.66	0.58
Confidence limit of the slope[b]	2.33×10^{4} to 2.37×10^{4}	1.80×10^{4} to 1.83×10^{4}	7.31×10^{-1} to 7.40×10^{-1}	9.09×10^{-1} to 9.20×10^{-1}
Intercept (a)	7.15×10^{3}	7.18×10^{3}	-3.08×10^{-2}	-3.35×10^{-2}
Standard deviation of the intercept (S_a)	11.51×10^{3}	8.61×10^{3}	6.24×10^{-2}	6.74×10^{-2}
Confidence limit of the intercept[b]	-4.03×10^{3} to 1.83×10^{4}	-1.18×10^{3} to 1.55×10^{4}	-9.14×10^{-2} to 2.99×10^{-2}	-9.90×10^{-2} to 3.20×10^{-2}
Correlation coefficient (r)	0.9999	0.9999	0.9999	0.9999
Standard error of estimation	7.05×10^{3}	1.13×10^{3}	3.42×10^{-2}	3.70×10^{-2}

[a] $Y = a + bC$, where C is the concentration of compound in μg ml^{-1} and Y is the peak area or ^{1}DD amplitude for HPLC or ^{1}DD methods.
[b] 95% confidence limit.

wavelengths. Besides, the regions in which each component of the mixture was best reconstructed were also considered. The wavelength range 220–320 nm with 1 nm intervals was selected as this range was providing the greatest amount of information about the two components.

3.2.2. Selection of the optimum number of factors

An appropriate choice of the number of principal components or factors is necessary for PCR and PLS-1 calibrations. The number of factors should account as much as possible for the experimental data without resulting in over fitting. Various criteria have been developed to select the optimum number [34]. Cross-validation methods leaving out one sample at a time was employed [35]; with a calibration set of 25 calibration spectra.

PLS-1 and PCR calibration on 24 calibration spectra were performed, and using this calibration, the concentration of the sample left out during the calibration process was determined. This process was repeated 25 times until each calibration sample had been left once. The predicted concentrations were compared with the known concentrations of the compounds in each calibration sample. The root mean squares error of cross-validation (RMSECV) was calculated for each method as follows:

$$\text{RMSECV} = \sqrt{\frac{\text{PRESS}}{n}}$$

where n is the number of training samples:

$$\text{PRESS} = \sum (Y_{\text{pred}} - Y_{\text{true}})^2$$

where Y_{pred} and Y_{true} are predicted and true concentrations in $\mu\text{g ml}^{-1}$, respectively.

The RMSECV was used as a diagnostic test for examining the errors in the predicted concentrations. It indicates both of the precision and accuracy of predictions. It was recalculated upon addition of each new factor to the PLS-1 and PCR models.

Appropriate selection of the number of factors to be used to construct the model is the key to achieving correct quantitation in PLS-1 and PCR calibrations. The usual procedure for this purpose involves choosing the number of factors that result in the minimum RMSECV. However, this criterion is subject to some constraints because, occasionally, the RMSECV does not reach a sharp minimum but decreases gradually above a given number of factors. On the other hand, it is calculated from a finite number of samples, so it is error-prone. For these reasons, the method developed by Haaland and Thomas [28] was used for selecting the optimum number of factors, which involves selecting that model including the smallest number of factors that result in an insignificant difference between the corresponding RMSECV and the minimum RMSECV. Fig. 3 shows the variation of RMSECV as a function of the number of factors for the determination of each component in the mixture by the PCR and PLS-1 methods. Two factors were found to be optimum for each component by the PLS-1 and PCR methods.

The proposed CLS, PLS-1 and PCR calibration models were evaluated by internal validation (prediction of drug concentrations in its own designed calibration set); recoveries between 99.39–101.28% for AM and 98.59–100.52% for DX were

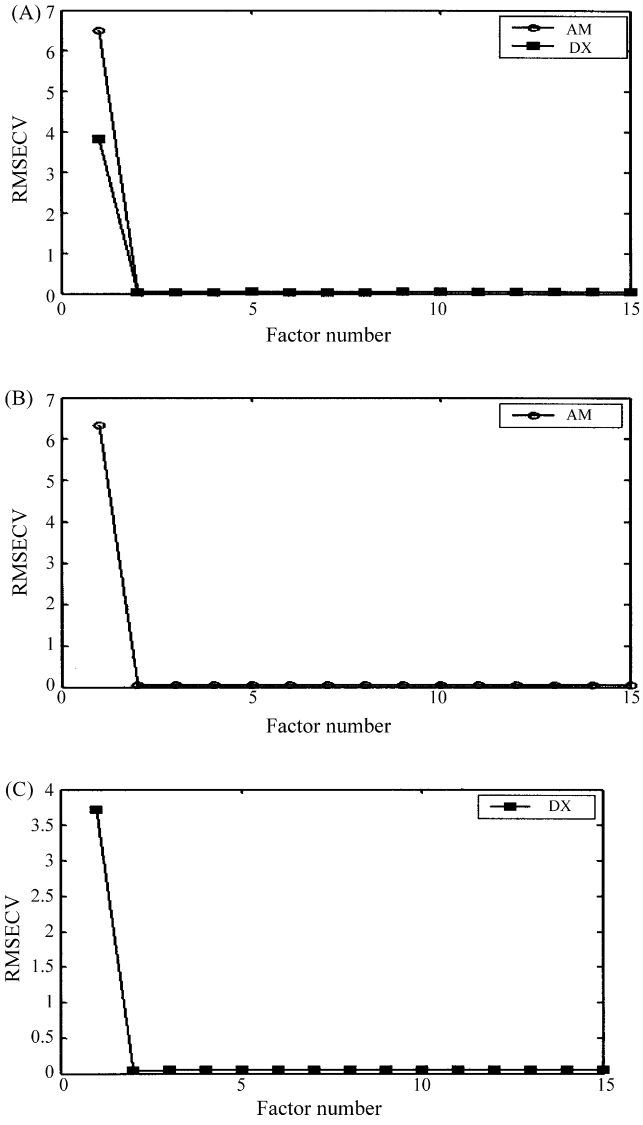

Fig. 3. Plot of RMSECV vs. factor number for a calibration set prediction using cross-validation of PCR model (A) and PLS-1 model for AM (B) and DX (C).

obtained. CLS, PLS-1 and PCR methods were evaluated, and a comparative study of the prediction capabilities of the three chemometric approaches in the particular work was undertaken.

The predictive abilities of the models was also, evaluated, by two methods. The first method was carried out by plotting the known concentrations against the predicted concentrations. A satisfactory correlation coefficient (r) value was obtained for each drug in the calibration set by the CLS, PLS-1 and PCR optimized models (Table 3), indicating the good predictive abilities of the models and the second method was carried out by calculating the standard error of prediction (SEP), which is given by the following equation:

$$\text{SEP} = \sqrt{\frac{\sum_{i=1}^{n} (\hat{c}_i - c_i - \text{bias})^2}{n-1}}$$

$$\text{bias} = \frac{\sum_{i=1}^{n} \hat{c}_i - c_i}{n}$$

Table 3
Statistical parameters and analytical figures of merit values for simultaneous determination of AM and DX using CLS, PLS-1 and PCR methods

Parameters	AM			DX		
	PLS-1	PCR	CLS	PLS-1	PCR	CLS
RMSECV	4.63×10^{-2}	4.63×10^{-2}	4.63×10^{-2}	3.57×10^{-2}	3.55×10^{-2}	3.56×10^{-2}
Intercept	1.54×10^{-2}	1.54×10^{-2}	1.51×10^{-2}	-3.35×10^{-3}	-3.37×10^{-3}	-3.53×10^{-3}
Slope	0.9990	0.9990	0.9990	1.0002	1.0002	1.0002
r	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999
S.E. of intercept	1.76×10^{-2}	1.76×10^{-2}	1.76×10^{-2}	1.37×10^{-2}	1.37×10^{-2}	1.37×10^{-2}
S.E. of slope	1.34×10^{-3}	1.34×10^{-3}	1.34×10^{-3}	1.05×10^{-3}	1.05×10^{-3}	1.05×10^{-3}
SEP	4.70×10^{-2}	4.71×10^{-2}	4.70×10^{-2}	3.68×10^{-2}	3.63×10^{-2}	3.63×10^{-2}
REP (%)	0.42	0.42	0.42	0.32	0.32	0.32
SEN	7.64	7.64	7.75	11.82	11.82	11.56
SEL	0.34	0.34	0.34	0.52	0.52	0.51
γ^{-1} (μg ml^{-1})	3.92×10^{-4}	3.92×10^{-4}	3.87×10^{-4}	2.54×10^{-4}	2.54×10^{-4}	2.60×10^{-4}
LOD (μg ml^{-1})	1.18×10^{-3}	1.18×10^{-3}	1.16×10^{-3}	7.61×10^{-4}	7.61×10^{-4}	7.79×10^{-4}
LOQ (μg ml^{-1})	3.92×10^{-3}	3.92×10^{-3}	3.87×10^{-3}	2.54×10^{-3}	2.54×10^{-3}	2.60×10^{-3}

where c_i is the reference concentration, $\hat{c}_i$ the calculated concentration, and n is the number of samples.

In addition, the relative error of prediction (REP) demonstrating the quality of fit of all the calibration data. It was calculated using the following equation:

$$\text{REP}\,(\%) = \text{RMSECV} \times \frac{100}{\bar{c}_{act}}$$

where $\bar{c}_{\text{act}}$is the average concentration in the calibration set.

The RMSECV, SEP and REP% values obtained by optimizing the calibration matrix of the absorption spectra for the CLS, PLS-1 and PCR methods are shown in Table 3, indicating good accuracy and precision.

Analytical figures of merit (FOM) are very important to quantify the quality of a given methodology or for method comparison. In multivariate calibration, several FOM have been reported, e.g., selectivity (SEL), sensitivity (SEN), the inverse analytical sensitivity (γ^{-1}), limit of quantification (LOQ) and limit of detection (LOD). Results concerning FOM are shown in Table 3. Convenient definitions for FOM can be found in the literature [36–39].

Haaland and Thomas [40] made a comparison of different multivariate calibration methods for quantitative spectral analysis. They concluded that it is difficult to generalize about the superiority of one method over another because the relative performance of methods is often dependent on the particular data set being analyzed.

3.3. HPLC method

The developed HPLC method has been applied for the simultaneous determination of DX and AM. To optimize the HPLC assay parameters, the mobile phase composition and pH of 20 mM potassium dihydrogen phosphate were studied. A satisfactory separation was obtained with a mobile phase consisting of 20 mM potassium dihydrogen phosphate, pH 6–acetonitrile (50:50, v/v). Increasing acetonitrile concentration to more than 65% led to adequate separation of AM and DX but with decreased capacity factor of AM and DX. At lower acetonitrile concentration (<30%) separation occurred but with excessive delay for AM peak.

Variation of the pH of the 20 mM potassium dihydrogen phosphate resulted in a maximum capacity factor (K') value at pH 6.5. At pH 2.7–5.0, coelution of DX with AM was observed. At pH 5.5–6.0 improved resolution for the two compounds was observed. However, at pH 6, optimum resolution with reasonable retention time was observed.

Quantitation was achieved with UV detection at 245 nm based on peak area. The specificity of the HPLC method is illustrated in Fig. 4 where complete separation of the studied compounds was noticed. The average retention time ± standard deviation for DX and AM were found to be 1.79 ± 0.01 and 5.23 ± 0.05 min, respectively, for 10 replicates.

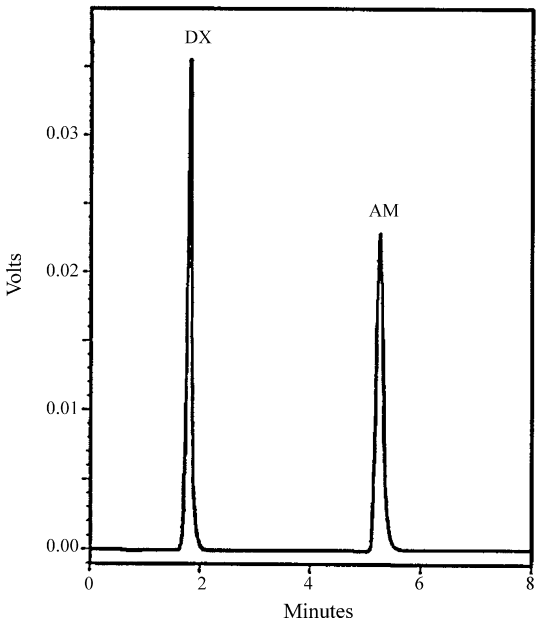

Fig. 4. Typical HPLC chromatogram of 20 μl injection of capsule sample containing 7.5 μg ml^{-1} of AM and 10 μg ml^{-1} of DX.

Table 4
Determination of AM and DX in laboratory-prepared mixtures and pharmaceutical product using the proposed methods

	Mean recovery % ±S.D.[a]				
	PLS-1	PCR	^{1}DD	CLS	HPLC
Laboratory-prepared mixtures					
For AM	100.23 ± 0.66	100.23 ± 0.66	100.14 ± 1.29	100.22 ± 0.65	100.84 ± 0.44
For DX	99.98 ± 0.50	99.98 ± 0.50	100.28 ± 0.60	99.97 ± 0.50	99.64 ± 0.57
Commercial capsule					
For AM	100.16 ± 0.57	100.16 ± 0.57	100.23 ± 1.06	100.16 ± 0.57	99.82 ± 0.66
t	1.03	1.03	0.87	1.03	(2.18)[b]
F	3.46	3.46	2.58	3.46	(4.28)[b]
For DX	101.11 ± 0.56	101.11 ± 0.56	100.56 ± 0.88	101.11 ± 0.56	101.37 ± 0.59
t	0.89	0.89	2.05	0.89	(2.18)[b]
F	1.19	1.19	2.08	1.19	(4.28)[b]
Recovery %[c]					
For AM	100.12 ± 0.70	100.12 ± 0.70	99.99 ± 1.31	100.12 ± 0.69	100.18 ± 0.55
For DX	99.80 ± 0.59	99.80 ± 0.59	100.12 ± 0.83	99.79 ± 0.59	99.82 ± 0.57

[a] Percentage recovery from the label claim amount.
[b] Theoretical values for *t* and *F* at $p = 0.05$.
[c] For standard addition of 50% of the nominal content.

3.4. Analysis of pharmaceutical products

The proposed ^{1}DD, CLS, PLS-1, PCR and HPLC methods were applied to the simultaneous determination of AM and DX in Ambrodoxy® capsules. Seven replicates determination were made. Satisfactory results were obtained for each compound in good agreement with label claims (Table 4).

Therefore, the results of the proposed ^{1}DD, CLS, PLS-1 and PCR methods were compared with those of the proposed HPLC method. Statistical comparison between the results was performed with regards to accuracy and precision using Student's *t*-test and *F*-ratio at 95% confidence level (Table 4). There was no significant difference between the results.

3.5. Validation of the methods

3.5.1. Linearity

The linearity of the ^{1}DD and HPLC methods for determination of AM and DX was evaluated by analyzing a series of different concentrations of each drug. In this study seven concentrations for AM, and DX were chosen, ranging between 1–21 and 1–100 μg ml^{-1} for ^{1}DD and HPLC methods, respectively. Each concentration was repeated three times; in order to provide information on the variation in peak area and ^{1}DD values between samples of same concentration. The linearity of the calibration graphs was validated by the high value of the correlation coefficient and the intercept value, which was not statistically ($p = 0.05$) different from zero (Table 2). Characteristic parameters for regression equations of the ^{1}DD and HPLC methods obtained by least squares treatment of the results are given in Table 2.

The linearity of the calibration graphs was checked with the lack-of-fit statistical test (Table 5). This test evaluates the variance of the residual values [41]. The calculated values were lower than the tabulated one ($\alpha = 0.05$), linearity thus being demonstrated.

3.5.2. Range

The calibration range was established through consideration of the practical range necessary, according to each compound concentration present in the pharmaceutical product, to give accurate, precise and linear results. The calibration range of the proposed ^{1}DD and HPLC method is given in Table 2.

3.5.3. Detection and quantitation limits

According to the International Conference on Harmonization (ICH) recommendations [42], the approach based on the standard deviation (S.D.) of the response and the slope was used for determining the detection and quantitation limits. The theoretical values for ^{1}DD and HPLC methods were assessed practically and given in Table 2.

3.5.4. Precision

For evaluation of the precision estimates, repeatability and intermediate precision were performed at three concentration levels for each compound. The data for each concentration level were evaluated by one-way ANOVA. An 8 days × 2 replicates design was performed. Statistical comparison of the results was performed using the *p*-value of the *F*-test. Three univariate analyses of variance for each concentration level were made. Since the *p*-value of the *F*-test is always greater than 0.05, there is no statistically significant difference between the mean results obtained from one level of day to another at the 95% confidence level.

3.5.5. Selectivity

Methods selectivity was achieved by preparing eight laboratory-prepared mixtures of the studied compounds

Table 5
ANOVA (showing lack of fit calculation) for AM and DX

Method	Comp.	Source of variation	Sum of squares	d.f.	Mean sum of squares	F-Ratio
HPLC	AM	Total	4.88×10^{12}	21	2.32×10^{11}	2.53
		Regression	4.88×10^{12}	2	2.44×10^{12}	
		Residual	1.33×10^{9}	19	6.99×10^{7}	
		Replicate	2.48×10^{8}	7	3.55×10^{7}	
		Lack of fit	1.08×10^{9}	12	8.99×10^{7}	
	DX	Total	3.15×10^{12}	21	1.50×10^{11}	2.01
		Regression	3.15×10^{12}	2	1.57×10^{12}	
		Residual	7.69×10^{8}	19	4.05×10^{7}	
		Replicate	1.73×10^{8}	7	2.47×10^{7}	
		Lack of fit	5.96×10^{8}	12	4.97×10^{7}	
^{1}DD	AM	Total	2.10	21	1.00×10^{-1}	2.05
		Regression	2.10	2	1.05	
		Residual	5.56×10^{-4}	19	2.93×10^{-5}	
		Replicate	1.23×10^{-4}	7	1.76×10^{-5}	
		Lack of fit	4.33×10^{-4}	12	3.61×10^{-5}	
	DX	Total	4.17	21	1.98×10^{-1}	1.57
		Regression	4.17	2	2.08	
		Residual	7.71×10^{-4}	19	4.06×10^{-5}	
		Replicate	2.09×10^{-4}	7	2.99×10^{-5}	
		Lack of fit	5.62×10^{-4}	12	4.68×10^{-5}	

The critical value of F-ratio is 3.57 at $\alpha = 0.05$.

at various concentrations within the linearity range. The laboratory-prepared mixtures were analyzed according to the previous procedures described under the proposed methods. Satisfactory results were obtained (Table 4) indicating the high selectivity of the proposed methods for simultaneous determination of AM and DX.

3.5.6. Accuracy

The interference of excipients in the pharmaceutical formulations was studied in detail by ^{1}DD, CLS, PCR, PLS-1 and HPLC methods. For this reason, standard addition method was applied to the pharmaceutical formulation containing these compounds. In application of standard addition method to the pharmaceutical formulation, the mean percentage recoveries and their standard deviation for the proposed methods for six replicate were calculated (Table 4). According to the obtained results a good precision and accuracy was observed for these methods. Consequently, the excipients in pharmaceutical formulation do not interfere in the analysis of these compounds in the pharmaceutical formulation.

3.5.7. Robustness

Variation of the pH of the 20 mM potassium dihydrogen phosphate of the mobile phase by ±0.2 units and the acetonitrile concentration of the mobile phase by ±2% did not have significant effect on chromatographic resolution in HPLC method. Variation of strength of hydrochloric acid by ±0.02 M did not have significant effect on spectrophotometric methods.

3.5.8. Analytical solution stability

The analytical solutions of the studied compounds in mobile phase or 0.1 M hydrochloric acid exhibited no chromatographic or absorbance changes for 4 h when kept at room temperature, and for 1 day when stored refrigerated at 5 °C.

4. Conclusion

For routine analytical purposes, it is always of interest to establish methods capable of analyzing a large number of samples in a short time period with due accuracy and precision. Spectrophotometric techniques can generate large amounts of data within a short period of analysis; however, when coupled with chemometrics tools, the quality of the spectral information can be markedly increased, converting this combined technique into a powerful and highly convenient analytical tool.

In this paper, a comparative study of the use of HPLC and graphical and numerical spectrophotometric methods for the resolution of AM and DX in their multicomponent mixtures have been accomplished, showing that multivariate calibration methods provide, with adequate software support, a clear example of the high resolving power and low cost of this technique. Although the HPLC method is more specific than the multivariate calibration methods, it needs expensive equipment and materials.

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