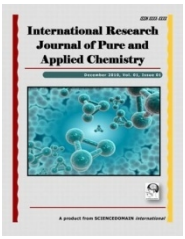

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A Comparative Study of Spectrophotometric Methods Versus Chemometric Methods; An Application on a Pharmaceutical Binary Mixture of Ofloxacin and Dexamethasone

Nagiba Y. Hassan[1], Samia M. Elgizawy[2], Hayam M. Lotfy[1] and Sarah S. Saleh[3*]

[1]Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, Kasr-El Aini Street, 11562 Cairo, Egypt.
[2]Analytical Chemistry Department, Faculty of Pharmacy, Assuit University, 71515 Assuit, Egypt.
[3]Analytical Chemistry Department, Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA), 11787 6th October City, Egypt.

Authors' contributions

"This work was carried out in collaboration between all authors. All authors contributed in literature review, practical work and statistical analysis. All authors read and approved the final manuscript."

Research Article

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ABSTRACT

Aim: To conduct a comparative study between the smart novel ratio difference spectrophotometric method (RDSM) versus four spectrophotometric methods: first derivative spectrophotometry (D^1), first derivative of the ratio spectra (^1DD), isoabsorptive point (Aiso), ratio subtraction (RS), and two chemometric techniques based on principal component regression (PCR) and partial least-squares (PLS-1) for the determination of a binary mixture of Ofloxacin (OFX) and Dexamethasone (DXM).
Study Design: The results obtained from the proposed methods were statistically compared to the reported HPLC method using student's t-test, F-test and One way ANOVA.
Methodology: (OFX) was determined by the application of direct spectrophotometry, by measuring its zero-order (D_0) absorption spectra at its λ_{max} = 296.6 nm. (DXM) was

**Corresponding author: Email: drsarahsalah@gmail.com;*

determined by (D^1) at 227.1 nm. By applying (^1DD), (DXM) was determined at 237.3. The total concentration of both (OFX + DXM) was determined at their isoabsorpative point λ_{iso}= 238.3 nm, then the concentration of (DXM) in mixtures were calculated by subtraction. (DXM) was determined using the (RS) method at its λ_{max} = 239 nm. (DXM) was determined using (RDSM) by measuring amplitude difference at two selected wavelengths (248.4 and 290 nm). A concentration of 10 $\mu g.mL^{-1}$ of OFX was used as a divisor. The linearity range was found to be (1-10 $\mu g.mL^{-1}$) and (2-14 $\mu g.mL^{-1}$) for OFX and DXM respectively.

Results: The recovery percentage for OFX was found to be 100.07 ±0.65 and for DXM was found to be 100.41 ±0.84, 100.15 ±0.97, 100.14 ±0.91, 100.54 ±0.75 and 100.11 ±.66 for the five methods, respectively.

Conclusion: The novel method showed advantages over the other proposed methods regarding simplicity, minimal data manipulation and maximum reproducibility and robustness; which enabled the analysis of binary mixtures with overlapped spectra for routine quality control testing with quite satisfactory and in lower cost.

Keywords: Ofloxacin; dexamethasone; ratio difference; isoabsorpative point; ratio subtraction; chemometric.

1. INTRODUCTION

Ofloxacin (OFX) [9-Fluoro-2, 3-dihydro-3-methyl-10-(4-methyl-1-piperazinyl)-7-oxo-7H-pyrido [1, 2, 3-de]-1, 4-benzoxazine-6-carboxylic acid], is a fluoroquinolone antibacterial agent which is active against wide range of bacteria. Dexamethasone (DXM) [9α-Fluoro-11β, 17α, 21-trihydroxy-16α-methylpregna-1, 4-diene-3, 20-Dione], is a corticosteroid with potent anti-inflammatory and immunosuppressive effects [1, 2]. Their combination is used as anti-infective eye preparation to treat acute and sub-acute conjunctivitis, keratitis and corneal ulcers.

A survey of the literature revealed the methods reported for the determination of OFX such as titrimetry [3], UV spectrophotometry [4-6], HPLC methods [7,8], HPLC stability studies [9] and TLC methods [10]. The methods reported for DXM such as UV spectrophotometry [11,12], HPLC methods [13,14], HPLC stability studies [15,16] and capillary electrophoresis method [17]. Simultaneous determination of Ofloxacin and Dexamethasone phosphate has been reported in the literature by HPLC methods [18,19] only. The first HPLC method utilized ODS column, a mobile phase of critical composition, composed of a mixture of methanol: citric acid solution (0.05 $mol.L^{-1}$): acetonitrile: ammonium acetate solution (0.5 $mol.L^{-1}$): 10 $g.L^{-1}$ phosphoric acid solution (100:75:22:1:2), flow rate of 1 $ml.min^{-1}$ and detection at 242 nm. The second HPLC method utilized C18 column, mobile phase consisting of a mixture of mixed phosphate buffer (pH 4) and acetonitrile (50:50, v/v), flow rate of 1 $ml.min^{-1}$ and detection at 236 nm. The aim of this work is to develop and validate simple, precise and low cost spectrophotometric and chemometric methods for the analysis of this binary mixture; and to conduct a comparative study between them to prove their effectiveness compared to the reported HPLC method.

1.1 Theory of the Novel Ratio Difference Spectrophotometric Method (RDSM)

LotfyandHagazy [20] introduced this method in which the amplitude difference between two points on the ratio spectra of a mixture is directly proportional to the concentration of the

component of interest; independence of the interfering component is the basic principle of the ratio difference method.

For a mixture of the two drugs (X) and (Y), X can be determined by dividing the spectrum of the mixture by a known concentration of Y as a divisor (Y'). The division will give a new curve that represents (X + Y)/Y' = X/Y' + Y/Y' = X/Y' + constant.

By selecting two wavelengths (λ_1 and λ_2) on the obtained ratio spectrum and subtracting the amplitudes at these two points the constant Y/Y' will be cancelled along with any other instrumental error or any interference from the sample matrix.Suppose the amplitudes at the two selected wavelength are P_1 and P_2 at λ_1 and λ_2, respectively; by subtracting the two amplitudes the interfering substance Y shows no interference; then;

$$P_1 - P_2 = [(X/Y')_1 + \text{constant}] - [(X/Y')_2 + \text{constant}] = (X/Y')_1 - (X/Y')_2.$$

The concentration of X is calculated by using the regression equation representing the linear relationship between the differences of ratio spectra amplitudes at the two selected wavelengths versus the corresponding concentration of drug (X). Similarly, Y could be determined by the same procedure using a known concentration of X as a divisor X'.

2. EXPERIMENTAL

2.1 Apparatus

Shimadzu - UV 1800 double beam UV-Visible spectrophotometer (Japan) with matched 1 cm quartz cells at 200-800 nm range was used for all absorbance measurements. Spectra were automatically obtained by Shimadzu UV-Probe 2.32 system software. For the chemometric methods, data analysis was performed using PLS-Toolbox 2.0 running under Matlab® Version 7.9.

2.2 Chemicals and Reagents

2.2.1Pure sample

OFX and DXM were kindly supplied by Egyptian International Pharmaceutical Industries Co. (EIPICO), Cairo, Egypt. Their purity was found to be 100.33 ±0.73 and 100.51 ±0.83 for OFX and DXM respectively by the official methods [3], respectively.

2.2.2 Market samples

Dexaflox® eye drops, labeled to contain 3mg of OFX, 1mg of DXM and 0.06mg of Benzalkonium chloride per mL (Batch number: LB060), was manufactured by Jamjoompharma, Kingdom of Saudi Arabia.

2.2.3 Solvents

Spectroscopic analytical grade pure methanol was supplied from (S.d.fine-chem limited-Mumbai).

2.3 Standard Solutions

Stock solutions of OFX and DXM of concentration 1 mg.mL^{-1} were prepared in methanol, and stored in dark bottles at 4ºC. Working solutions were freshly prepared by dilution from the stock solutions with methanol to get (20 µg.mL^{-1}) of each drugs.

2.4 Procedure

2.4.1 Linearity of Spectrophotometric methods

Separate aliquots of OFX were accurately transferred from its working solutions into a series of 10-mL volumetric flasks and then completed to volume with methanol. The zero order spectra of the prepared solutions were recorded against methanol as a blank (200 - 400 nm). A calibration curve was constructed by plotting the absorbance of zero order spectra at λ_{max}= 296.6 nm versus the corresponding concentration (1-10 µg.mL^{-1}) and the regression equation was computed.

Separate aliquots of DXM were accurately transferred from its working solution into a series of 10-mL volumetric flasks and then completed to volume with methanol. The zero order spectra of the prepared standard solutions were recorded against methanol as a blank (200 - 400 nm). Calibration curves were constructed by plotting the absorbance at λ_{max}= 239 nm and λ_{iso}= 238.3 nm versus the corresponding concentration (2-14 µg.mL^{-1}) and the regression equations were computed. The first derivative of the spectra D^1 ($\Delta\lambda$ =4) were calculated for the stored spectra of DXM and a calibration curve was constructed by plotting the peak amplitude of D^1 at 227.1 nm versus the corresponding concentration and the regression equation was computed. The stored zero order spectra of DXM were then divided by the spectrum of OFX (10 µg.mL^{-1}) as a divisor to obtain the ratio spectra. A calibration curve was constructed by plotting the difference between the amplitude ratio difference at 248.4 nm and 290 nm ($\Delta P_{248.4 - 290}$) versus the corresponding concentration and the regression equation was computed. Then the obtained ratio spectra were differentiated ($\Delta\lambda$=6) and a calibration curve was constructed by plotting the peak amplitude of ^{1}DD at 237.3 nm versus the corresponding concentration and the regression equation was computed.

2.4.2 Application of the spectrophotometric methods for the determination of OFX and DXM in laboratory-prepared mixtures

Accurate aliquots of OFX and DXM were separately transferred from their working solutions, mixed well then completed to volume with methanol, to prepare mixtures containing different ratios of both drugs. Proceed as detailed under linearity for both drugs. The concentration of each drug was calculated using the corresponding regression equation.

2.4.3 Construction of calibration and validation sets for chemometric methods

Multilevel partial factorial design [21]was used for the construction of the calibration and validation sets. A five-level, five-factor calibration design was used. Twelve mixtures were used for building the calibration model, while eight mixtures were chosen as an external validation set. The laboratory prepared mixtures of the pure components (OFX and DXM) were prepared in a concentration range obeying Beer's law for both drugs. The absorption spectra of the prepared mixtures were recorded in the range of (200-400 nm) with 1 nm

interval; and transferred to Matlab® (version 7.9), coupled with PLS toolbox 2.0, for subsequent data manipulation.

2.4.4 Application to pharmaceutical preparation

One milliliter of Dexaflox® eye drops was transferred into 50 mL volumetric flask, the volume was completed with methanol to obtain a working solution of 60 $\mu g.mL^{-1}$ of OFX and 20 $\mu g.mL^{-1}$ of DXM, and then the prepared solution was filtered through 0.45 µm Millipore syringe membrane filter. An appropriate dilution was made with the same solvent to prepare the working solution containing of 6 $\mu g.mL^{-1}$ OFX and 2 $\mu g.mL^{-1}$ DXM. Proceed as detailed under linearity for both drugs. Six replicates for each experiment were done. The concentrations of pure OFX and DXM were calculated from their corresponding regression equations. When carrying out the standard addition technique, different known concentrations of pure standard OFX and DXM were added to the pharmaceutical formulation before proceeding in the previously mentioned methods.

3. RESULTS AND DISCUSSION

The aim of this work was to conduct a comparative study between the novel RDSM versus well-established spectrophotometric and chemometric methods for the first time to prove its validity for the analysis of binary mixtures with overlapped spectra. This was achieved through simultaneous estimation of OFX and DXM in their pure form and ophthalmic solution.

The zero-order absorption spectra (D_0) of OFX and DXM showed overlapping, Fig. 1, which allows the analysis of OFX in presence of DXM at λ_{max}= 296.6 nm, but hinders the analysis of DXM in presence of OFX. So, different spectral manipulating techniques, including the novel RDSM, have been applied to determine DXM without interference from OFX or Benzalkonium chloride.

3.1 Spectrophotometric Methods

3.1.1 D^1 method

The derivative spectrophotometry is one of the most commonly applied techniques for determination of binary mixtures. Fig. 1 showed the D^1 spectra for both drugs, where DXM was determined by measuring the peak amplitude at 227.1 nm corresponding to zero-contribution of OFX and Benzalkonium chloride.The wavelength increment (Δλ=4) gave a suitable signal to noise ratio and the spectra showed good resolution.

3.1.2 ^{1}DD method

The main advantage of the ^{1}DD method [22,23] is that the whole spectrum of the interfering substance is cancelled. Accordingly, the choice of the wavelength selected for calibration is not critical as in the D^1 methodbut the disadvantage is the multiple manipulating steps: division then calculating the derivative. DXM was determined by measuring maximum peak amplitude at 237.3 nm with no interference of OFX or Benzalkonium chloride.

In order to optimize ^{1}DD method, the selected divisors should compromise between minimal noise and maximum sensitivity. Several divisor concentrations (3, 6 and 10 $\mu g.mL^{-1}$) of OFX

were tried, the best result was obtained when using 10 μg.ml^{-1} of DXM as a divisor. Different smoothing and scaling factors were tested, where a smoothing factor Δλ= 6 and a scaling factor =10 were suitable to enlarge the signal of DXM to facilitate its measurement and to diminish error in reading the signal, Fig. 1.

3.1.3 Aiso method

Erram and Tipnis [24]developed the isoabsorpative spectrophotometric method [25] for the determination of components in binary mixture. This method could be used for simultaneous determination of OFX and DXM in their binary mixture. At the isoabsorpative point (λ_{iso}) the mixture of drugs acts as a single component and gives the same absorbance value as pure drug. Thus, by measuring the absorbance value (A_{iso}) at the chosen (λ_{iso}) 238.3 nm, the total concentration of both OFX and DXM in the mixture could be calculated, while the concentration of OFX in the mixture could be calculated without any interference, at its λ_{max} 296.6 nm, Fig. 1. Thus the concentration of DXM could be calculated by subtraction. This could be illustrated by the following equations:

$$A_{iso} = A_{OFX} + A_{DXM} = \mathcal{E}_{OFX(iso)}\, C_{OFX} + \mathcal{E}_{DXM\,(iso)}\, C_{DXM} = \mathcal{E}_{(iso)}\,(C_{OFX} + C_{DXM}) \qquad \text{at}\lambda_{iso}$$

$$A_{OFX} = \mathcal{E}_{OFX\,(max)}\, C_{OFX} \text{at}\lambda_{max}$$

$$C_{DXM} = (C_{OFX} + C_{DXM}) - C_{OFX}$$

The main advantage of this method is that the spectral measurements are done at zero order to obtain maximum accuracy and reproducibility, but the disadvantage is that it can be only applied for mixtures where one of its components can be measured singly without any interference.

3.1.4 RS method

Bardicy et al. [26] introduced ratio subtraction method for the determination of binary mixtures [25] where the spectrum of one component is extended than the other. For a binary mixture of OFX and DXM, Fig. 1; DXM can be determined by dividing the spectrum of the mixture by a known concentration of OFX as a divisor (OFX' = 10 μg.ml^{-1}). The division will give a new curve that represents DXM/OFX' + [OFX/OFX' = constant]. Measure the value of OFX/OFX' constant in the plateau region. If we subtract this constant value, then multiply the obtained curve after subtraction by OFX' (the divisor), therefore we can obtain the zero order absorption spectrum D_0 of DXM (original spectrum of DXM), Fig. 2.

(OFX + DXM) / OFX' = DXM / OFX' + OFX / OFX' = DXM / OFX' + constant
DXM / OFX' + constant – constant = DXM / OFX'
And then DXM / OFX' x OFX' = DXM

The correct choice of the divisor was fundamental, so the divisor was chosen as in ^{1}DD where the best recovery percent of DXM concentrations was obtained in laboratory prepared mixtures. This method showed maximum accuracy and reproducibility over the other applied since it obtains the original zero order spectra of the component in the binary mixture and allows its determination at its λ_{max}, but the disadvantage is that it requires the extension of one of the two components in the mixture; as it is limited to determine the non-extended component only. Another drawback was observed upon analysis of mixtures containing low concentrations of the divisor, where the calculation of the constant value through plateau region was critical due to low signal to noise ratio.

3.1.5 RDSM

The most striking feature of the ratio difference method is its simplicity, rapidity and accuracy. This is a newly developed method having the ability of solving severely overlapped spectra without prior separation meanwhile it does not require any sophisticated apparatus or computer programs. For the determination of concentration of component of interest by the ratio difference method, the only requirement is the contribution of the two components at the two selected wavelengths λ_1 and λ_2 where the ratio spectrum of the interfering component shows the same amplitudes (constant) whereas the component of interest shows significant difference in these two amplitude values at these two selected wavelengths with concentration.

For the determination of DXM concentration by this method, two wavelengths were selected (248.4 and 290 nm) where the ratio spectrum of the OFX showed same amplitudes (constant) whereas the DXM showed significant amplitude difference at these selected wavelengths relative to concentration, Fig. 3. The concentration of DXM was calculated by using the regression equation representing the linear relationship between the ratio spectra amplitude difference at the two selected wavelengths versus the corresponding concentration of drug DXM. The divisors concentrations were selected as in (^{1}DD) method. The selected wavelengths were the best regarding average recovery percent when used for the prediction of DXM concentrations in bulk powder as well as in laboratory prepared mixtures.

The advantage of RDSM is the complete elimination of the interfering component in the form of constant and the difference at any two points will be equal to zero, so there is no need for critical measurements as in D^1 which leads to highly reproducible, robust results and background noise will be cancelled. Its advantage over the ^{1}DD method is the reduced manipulating steps (eliminates the step of derivative calculation, so signal to noise ratio is enhanced), so it is suitable for computer programs that cannot perform derivative or derivative ratio spectrophotometry. RDSM doesn't involve several steps to analyze each drug alone, as in isoabsorpative point method, or needs special program, such as Matlab® to analyze the data of a large number of prepared mixtures as in case of chemometric techniques. The advantage over the RS method is that the application of the last one requires the extension of one of the two components of the mixture and it is limited to determine the non-extended component only, and the critical calculation of the constant value through plateau region in lower concentrations; while RDSM can be applied for the determination of binary mixtures in different concentrations without any limitation or specified requirement except that the two spectra of the proposed drugs should be contributed at the chosen wavelengths.

All spectral measurements were done without interference of Benzalkonium chloride (preservative present in Dexaflox® eye drops) which did not show any absorption at the selected wavelengths and its contribution to the absorption of the mixture above 220 nm was considered to be negligible at a concentration up to100 µg/mL, so the ternary mixture in range (220- 400 nm) acts as a binary mixture of OFX and DXM.The corresponding concentration ranges, calibration equations and other statistical parameters for spectrophotometric methods were listed in Table 1.

3.2 Chemometric Methods

Among the different regression method existed for multivariate calibration, the factor analysis based methods including partial least squares (PLS-1) regression and principal component regression (PCR) have received considerable attention in the literature [22,23,37]. PLS-1 not only has the advantage of PCR, but also it produces more robust model as it removes noise from both absorbance and concentration data [37].

Due to the overlapped spectra of the drugs, Fig. 1, the previous chemometric methods have been used to analyze this mixture. The first step in the determination of the cited drugs by multivariate calibration methods involved constructing the calibration matrix for the binary mixture. The calibration set was obtained using the absorption spectra set of 12 mixtures of OFX and DXM with different ratios of each component, listed in Table 2. The initial model was found to give bad results so the regions from 200 to 210 nm and above 350 nm were rejected.

An appropriate choice of the number of principal components or factors is necessary for PLS-1 calibration. Cross-validation methods leaving out one sample at a time was employed. The root mean squares error of cross-validation (RMSECV) was calculated which is used as a diagnostic test for examining the errors in the predicted concentrations. It indicated both precision and accuracy of predictions. The selected model was that with the smallest number of factors such that RMSECV for that model was not significantly greater than RMSECV from the model with additional factor. A number of factors of 3 were found to be optimum for the mixture. The root mean squares error of cross-validation (RMSECV) was calculated for each method as follows:

$$RMSECV = \sqrt{PRESS / n}$$

where n is the number of calibration set samples,

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

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

The selectivity of the proposed procedures was assessed by the analysis of laboratory prepared mixtures containing different ratios of the two drugs, where satisfactory results were obtained over the calibration ranges as shown in Table 3. The percentage recoveries of the validation set were shown in Table 4. The proposed procedures were also applied for the determination of OFX and DXM in Dexaflox® eye drops. The validity of the proposed procedures is further assessed by applying the standard addition technique which showed no interference of pharmaceutical excipients, as shown in Tables 5 and 6.

4. METHODS VALIDATION

Method validation was performed according to ICH guidelines [28]

4.1 Spectrophotometric Methods

4.1.1 Range and linearity

The linearity of both methods was evaluated by processing the different calibration curves on three different days. The calibration curves were constructed within concentration ranges that were selected on the basis of the anticipated drugs concentration during the assay of

the dosage form. The corresponding concentration ranges, calibration equations and other statistical parameters for both methods were listed in Table 1.

4.1.2 Limits of detection and quantification

The limit of detection (LOD) and limit of quantification (LOQ) were calculated respectively, for both drugs using the proposed methods with a ratio of 3.3and 10 standard deviations of the blank and the slope of the calibration line, Table 1.

4.1.3 Accuracy

To study the accuracy of the proposed methods, procedures under linearity for both drugs were repeated three times for the determination of six different concentrations of pure OFX and DXM. The accuracy expressed as percentage recoveries was shown in Table 1. The standard addition technique presented in Tables 5 and 6 showed no interference of pharmaceutical excipients. Good accuracy of the developed methods was indicated by the obtained results.

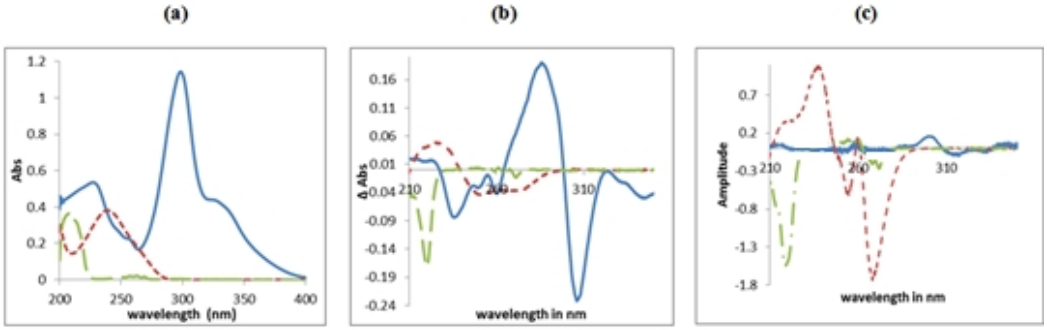

Fig. 1. (a) UV spectra of 10µg.mL^{-1} of each OFX (__), DXM (- - - -) and BNZ (__ - -), with methanol as a blank; (b) D^{1} spectra of OFX, DXM and BNZ; (c) ^{1}DD spectra of OFX and DXM in presence of BNZ, using OFX (10 µg.mL^{-1}) with methanol as a blank

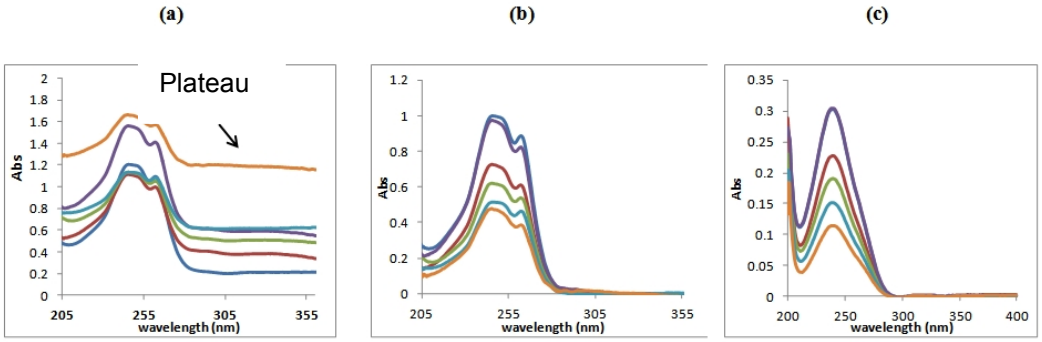

Fig. 2. (a) Ratio spectra of laboratory prepared mixtures of OFX and DXM using 10 µg.mL-^{1}of OFX as a divisor and methanol as a blank; (b) ratio spectra after constant subtraction; (c) Obtained UV spectra of DXM by the proposed ratio subtraction method for the analysis of laboratory prepared mixtures after multiplication by the divisor

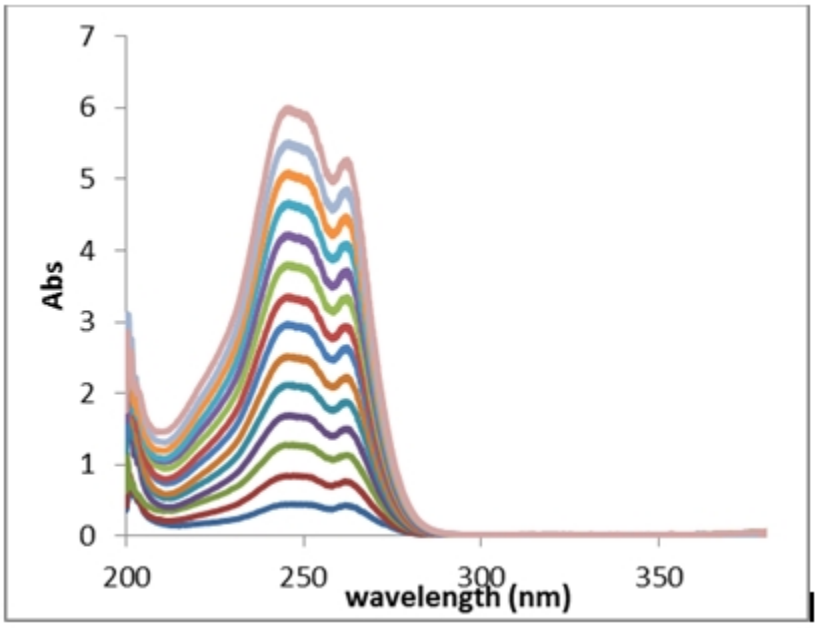

Fig. 3. Ratio spectra of DXM (2-14 µg.mL^{-1}) using 10 µg.mL^{-1} OFX as a divsor

4.1.4 Precision

The precision of the proposed methods, expressed as RSD, was determined by the analysis of three different concentrations (2, 4, 6 µg.mL^{-1}) of pure OFX and DXM within the linearity range. The intra-day precision was assessed from the results of three replicate analyses of three pure samples OFX and DXM on a single day. The inter-day precision was determined from the same samples analyzed on three consecutive days. The results of intra-day and inter-day precisions were illustrated in Table 1.

4.1.5 Selectivity

Selectivity was ascertained by analyzing different mixtures containing both drugs in different ratios within the linearity range. Satisfactory results were shown in Table 3.

4.1.6 Robustness

The spectral measurements of three concentrations of OFX and DXM (2, 4, 6 µg.mL^{-1}) were done at ±1 nm of the selected wavelengths and analyzed three times using the proposed methods. The methods proved to be robust and the percentage recoveries and standard deviation were calculated, Table 1.

4.2 Chemometric Methods

The validation of the developed PCR and PLS-1 models was assessed using several diagnostic tools. These tools were grouped into two categories: model diagnostic tools that are used to determine the quality of the model and sample diagnostic tools which are used to study the relationship between the samples and to identify unusual samples. The predicted concentrations of the validation samples were plotted against the true

concentration values. This was used to determine whether the model accounted for the concentration variation in the validation set. All plots had a slope and correlation coefficient of nearly one and an intercept close to zero. The RMSEP was another diagnostic tool for examining the errors in the predicted concentrations; it indicates both the precision and accuracy. The results in Table 6 indicated the high predictive abilities of the two models.

5. STATISTICAL ANALYSIS

Table 7 showed statistical comparison of the results obtained by the proposed methods and official methods [3]. The calculated t and F values were less than the theoretical ones indicating that there was no significant difference between the proposed and the official methods with respect to accuracy and precision. One-way ANOVA was applied for the purpose of comparison of developed methods; Table 8 shows that there was no significant difference between them for the determination of OFX and DXM.

Table 1. Assay parameters and method validation obtained by applying the proposed spectrophotometric methods for determination of OFX and DXM in binary mixtures

Method	OFX	DXM				
	D0 at λ= 296.6 nm	D^1 method	^{1}DD method	Aiso method	RS method	RDSM
Calibration range (μg.mL^{-1})	1-10	2-14	2-14	2-14	2-14	2-14
(LOD)(μg.mL^{-1})	0.123	0.219	0.196	0.203	0.154	0.153
(LOQ)(μg.mL^{-1})	0.372	0.664	0.595	0.615	0.466	0.465
Slope	0.1134	0.0049	0.1085	0.0385	0.0385	0.4198
Intercept	- 0.0037	- 0.0006	+0.0036	- 0.0022	- 0.0015	-0.0020
Mean % [a]	100.07	100.41	100.15	100.14	100.54	100.11
RSD	0.65	0.84	0.97	0.91	0.75	0.66
Accuracy [b]	99.97 /0.65	100.41/1.01	99.69 / 0.83	99.65 /0.58	99.63 /0.47	99.79 /0.34
Intra-day precision [c]	100.07 / 0.54	99.88 /1.11	100.05 /0.65	99.99 / 0.79	100.04/0.77	100.55/0.38
Inter-day precision [c]	100.13 /0.71	100.06/1.17	99.38 /0.31	100.23 /0.86	100.41/0.91	100.35/0.62
Robustness	100.54 /0.25	100.76/0.75	101.03 /0.87	99.89 /0.46	100.21/0.67	100.76/0.73
Correlation coefficient (r)	0.9999	0.9998	0.9999	0.9999	0.9999	0.9999
r2	0.9999	0.9999	0.9999	0.9999	0.9999	0.9999

[a] Average of three experiments.
[b] Mean recovery percentage / RSD
[c] The mean value/ RSD of samples of OFX and DXM (2, 4, 6μg. mL^{-1}).
LOD: Limit of detection, LOQ: Limit of quantitation.

Table 2. Concentrations of OFX and DXM in the calibration and validation sets using PCR and PLS methods

Experiment no.	Levels		Conc(μg.mL^{-1})	
	OFX	DXM	OFX	DXM
1[a]	0	-2	6	2
2[a]	-2	-2	4.8	1.6
3[a]	2	-1	7.2	1.8
4[a]	2	0	7.2	2
5[a]	-1	-1	5.4	1.8
6[a]	1	2	6.6	2.4
7[a]	1	0	6.6	2
8[a]	2	2	7.2	2.4
9[a]	-2	1	4.8	2.2
10[a]	-2	0	4.8	2
11[a]	1	1	6.6	2.2
12[a]	-1	-2	5.4	1.6
1[b]	-1	2	5.4	2.4
2[b]	0	-1	6	1.8
3[b]	-1	1	5.4	2.2
4[b]	2	1	7.2	2.2
5[b]	0	2	6	2.4
6[b]	2	-2	7.2	1.6
7[b]	1	-2	6.6	1.6
8[b]	0	1	6	2.2

[a]*Calibration set*
[b]*Validation set*

Table 3. Determination of OFX and DXM in laboratory prepared mixtures by the proposed spectrophotometric methods

Mixture ratio	OFX	DXM				
	D_0 at λ= 296.6 nm	D^1 method	^{1}DD method	Aiso method	RDSM	RS method
	Recovery% [a]					
5:5	101.18	101.22	99.75	99.44	99.90	100.52
2:8	98.63	101.53	98.62	100.73	99.19	99.51
4:6	102.01	101.36	99.10	101.35	101.06	98.05
9:3 [b]	100.95	101.02	101.11	101.83	99.49	100.32
6:4	101.95	101.02	98.80	101.74	100.96	100.32
6:8	100.93	98.98	99.65	98.07	100.23	101.46
Mean	100.94	100.86	99.51	100.53	100.14	100.03
± SD	± 1.23	± 0.94	± 0.91	± 1.49	± 0.76	± 1.15

[a] *Average of three experiments.*
[b] *Ratio present in Dexaflox® eye drops.*

Table 4. Recoveries % of OFX and DXM in the validation set by PCR and PLS methods

OFX			DXM		
True concentrations	Recovery % [a]		True concentrations	Recovery % [a]	
(µg.mL^{-1})	PCR	PLS	(µg.mL^{-1})	PCR	PLS
5.4	99.67	99.67	2.4	100.51	100.50
6	99.85	99.86	1.8	98.43	98.51
5.4	99.14	99.14	2.2	99.91	99.93
7.2	99.38	99.38	2.2	100.35	100.37
6	99.42	99.42	2.4	99.96	99.95
7.2	99.83	99.83	1.6	99.66	99.63
6.6	100.80	100.80	1.6	97.10	97.10
6	99.70	99.70	2.2	99.73	99.72
Mean	99.72	99.73	Mean	99.46	99.46
±SD	± 0.50	± 0.50	±SD	± 1.14	± 1.13

[a] *Average of three experiments.*

Table 5. Application of standard addition technique to the analysis of DXM in Dexaflox ® eye drops by different spectrophotometric methods

OFX Zero order absorption		**DXM D^1 method**		**^{1}DD method**		**RDSM**		**Aiso method**		**RS method**		**Reported method**	
												OFX	**DXM**
Recovery % of pure [a]	Recovery % of added [b]	Recovery % of pure [a]	Recovery % of added [b]	Recovery % of pure [a]	Recovery % of added [b]	Recovery % of pure [a]	Recovery % of added [b]	Recovery % of pure [a]	Recovery % of added [b]	Recovery % of pure [a]	Recovery % of added [b]	101.32 ±0.65	99.43 ±0.76
(6 µg.mL^{-1})		(2 µg.mL^{-1})		(2 µg.mL^{-1})		(2 µg.mL^{-1})		(2 µg.mL^{-1})		(2 µg.mL^{-1})			
101.14 ±0.56	100.56	100.13 ±0.39	99.75	99.96 ±0.85	100.64	99.58 ±0.66	100.85	99.78 ±0.43	100.84	100.06 ±0.29	100.31		
	100.56		100.86		100.09		100.43		99.81		99.50		
	100.09		99.96		100.04		100.78		99.72		100.03		
	99.76		100.05		100.02		99.85		100.18		100.32		
	100.24 ±0.39		100.16 ±0.49		100.20 ±0.30		100.48 ±0.46		100.14 ±0.51		100.04 ±0.38		

[a] *Average of six experiments.*

[b] *Average of three experiments (2, 4, 6, 8 µg.mL^{-1}) respectively.*

Table 6. Summary of results obtained by applying the diagnostic tools for model validation of the chemometric methods

Validation parameters	OFX		DXM	
	PCR	PLS-1	PCR	PLS-1
a) Predicted vs. known conc. plot:				
	1.0005	1.0005	1.027	1.026
1.slope	- 0.0291	- 0.0292	- 0.060	- 0.059
2.Intercept	0.9998	0.9998	0.9995	0.9995
3.Correlation coefficient "r"				
b) RMSEP	0.0279	0.0278	0.0124	0.0120
Analysis of pharmaceutical dosage form				
Mean ± SD [a]	101.15±0.61	101.10±0.55	99.99±0.44	99.99±0.44
Standard addition				
Mean ± SD [b]	99.65±0.47	99.63±0.51	100.34±0.28	100.37±0.62

[a] *Average of six experiments.*

- [b] *Average of three experiments (2, 4, 6 8 μg.mL^{-1}) respectively.*

Table 7. Statistical comparison of the results obtained by the spectrophotometric, chemometric and the official methods [3] for the determination of OFX and DXM in pure powder form

Items	OFX				DXM							
	Spectro-photometric	Chemometric		Official	Spectrophotometric					Chemometric		Official
	D_0 at λ= 296.6 nm	PCR	PLS-1	BP Method [a]	D^1 method	^1DD method	Aiso method	RS method	RDSM	PCR	PLS-1	BP Method [a]
No. of experiments	8	8	8	5	8	8	8	8	8	8	8	5
Mean %	100.07	99.35	99.38	100.33	100.41	100.15	100.14	100.54	100.11	100.60	100.52	100.51
RSD	0.65	0.43	0.48	0.73	0.39	0.97	0.91	0.75	0.66	0.60	0.64	0.83
SEM	0.309	0.121	0.097	0.328	0.161	0.349	0.177	0.121	0.234	0.18	0.179	0.369
Student's *t*-test (2.201)[b]	0.672	1.795	1.790		0.220	0.833	0.745	0.684	0.958	1.786	1.782	
***F* value (6.094)[b]**	1.275	2.174	2.173		1.028	1.220	1.212	1.204	1.553	1.900	1.873	

[a] *BP methods for OFX is a non-aqueous potentiometric titrimetric method, while for DXM is absorbance method.*
[b] *Figures between parentheses represent the corresponding tabulated values of t and F at P=0.05*

Table 8. Results of ANOVA (single factor) for comparison of the proposed methods and the reported HPLC method [19] for determination of OFX and DXM in pharmaceutical dosage form

Source of variation		Degree of freedom	Sum of squares	Mean square	F	P-value
OFX	Between columns	3	2.207	0.7356	1.648 [a]	0.2102 [a]
	Within columns	20	8.929	0.4465		
	Total	23	11.136			
DXM	Between columns	7	1.172	0.1674	0.6213 [a]	0.7351 [a]
	Within columns	40	10.780	0.2695		
	Total	47	11.953			

[a] *There was no significance difference between the methods using one-way ANOVA at P =0.05.*

6. CONCLUSION

From the previous discussion, the novel ratio difference spectrophotometric method showed advantages over the other proposed methods regarding simplicity, minimal data manipulation and maximum reproducibility as it does not need critical measurement at fixed wavelengths or any derivative calculation, hence signal to noise ratio is enhanced. It does not need any special program or sophisticated apparatus as HPLC methods, so it can be easily applied in quality control laboratories without any preliminary separation steps for binary mixture of OFX and DXM; in contrast it is of lower cost.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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