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Original Research Article

Novel Ratio Subtraction and Isoabsorptive Point Methods for Determination of Ambroxol Hydrochloride and Doxycycline in their Combined Dosage Form: Development and Validation

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Abstract

Purpose: *To develop and validate two innovative spectrophotometric methods used for the simultaneous determination of ambroxol hydrochloride and doxycycline in their binary mixture.*
Methods: *Ratio subtraction and isoabsorptive point methods were used for the simultaneous determination of ambroxol hydrochloride and doxycycline in their binary mixture. Linear correlations were obtained in the concentration range of 6 - 40 and 4 - 32 µg mL^{-1} for ambroxol hydrochloride and doxycycline, respectively. Ratio subtraction method was utilized for determination of ambroxol hydrochloride at 246.5 nm while isoabsorptive point method was employed for doxycycline at 244 nm (using methanol as a solvent) in mixtures as well as in their combined dosage form (Ambrodoxy capsules).*
Results: *The proposed methods were successfully applied to the analysis of the pharmaceutical capsules containing the two analytes. Recovery for ambroxol hydrochloride and doxycycline in capsules was 99.49 and 99.96 %, respectively. The relative standard deviation (% RSD) for the assay of the capsules was < 1 %. Validation of the two methods was assessed according to International Council on Harmonization (ICH) guidelines regarding linearity, accuracy, precision, specificity and range. The results of the proposed methods compared favorably with those obtained by a reported chemometrics-assisted ultraviolet (UV)-spectroscopic method.*
Conclusion: *The proposed methods are rapid, selective, simple and accurate. They also represent suitable alternatives to the chromatographic methods currently used for the analysis of the pharmaceutical mixtures in various dosage forms.*

Keywords: *Ambroxol, Doxycycline, Ratio subtraction method, Isoabsorptive point method, Binary mixture, Spectrophotometry*

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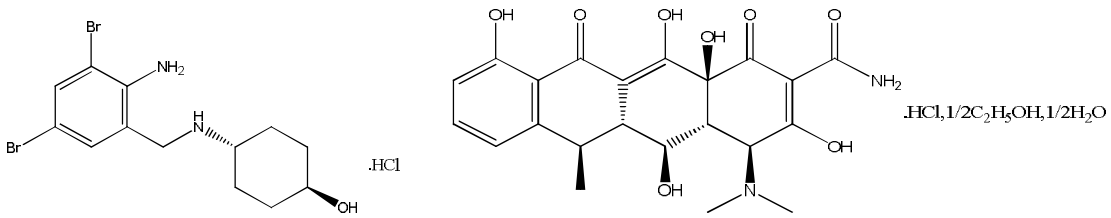
INTRODUCTION

The main problem of spectrophotometric binary mixture analysis is the simultaneous determination of the two components in the mixture without prior separation. Several spectrophotometric methods were used for resolving such mixtures with overlapping spectra. Derivative spectrophotometry [1], dual wave length spectrophotometry [2], pH-induced differential spectrophotometry [3] and chemometric methods [4] were all used for such



Ambroxol HCl (AMB) Doxycycline (DOX)

Figure 1: Chemical structures of ambroxol HCl (AMB) and doxycycline (DOX)

purpose. AMB [4-[[(2-Amino-3, 5-dibromophenyl) methyl] amino]cyclohexanol hydrochloride], is an active metabolite of bromhexine. It is used a mucolytic agent used to treat respiratory disorders associated with viscid or excessive mucus [5]. DOX [alpha-6-deoxy-5-hydroxy-tetracycline hydrochloride hemiethanolate hemihydrate], is a tetracycline that possess antibacterial and some antiprotozoal properties. It has been given in the long-term management of moderate to severe acne and has been used for the prophylaxis of malaria [5]. The chemical structures of AMB and DOX are shown in Figure 1. Both drugs are formulated together in the form of capsule dosage form (Ambrodoxy ® capsules) for the treatment of upper and / or lower respiratory tract infections accompanied by formation of viscous and hardly separated expectoration [6].

Literature survey revealed that there are few methods available for the simultaneous analysis of AMB and DOX combination. These methods include reversed-phase sequential injection chromatography (SIC) technique [7], HPLC method [6], derivative spectrophotometric methods [6,8] and chemometric methods (CLS, PCR and PLS methods) [6]. The aim of this work was to develop new spectrophotometric methods for resolving binary mixture of ambroxol hydrochloride (AMB) and doxycycline hyclate (DOX) (as a case study) without preliminary separation. The proposed methods are named ratio subtraction and isobsorptive point methods, respectively.

EXPERIMENTAL

Apparatus

A dual-beam Shimadzu UV-visible spectro-photometer 1601 PC connected to an IBM compatible computer. The software was UVPC personal spectroscopy software version 3.7 (Shimadzu). The absorption spectra of the reference and test solutions were carried out in a 1 cm quartz cells over the range of 200 - 400 nm. The spectral bandwidth was 2 nm and the wavelength scanning speed was 2800 nm min^{-1}.

Materials and reagents

Pharmaceutical grade of AMB and DOX were used and certified to contain 99.69 % and 99.79 % respectively. Methanol used was of analytical grade (Merck). Ambrodoxy capsules, batch number 060643 (Adwia Pharmaceuticals and Chemical Industries Co., Cairo, Egypt) were used as reference product. Each capsule was labeled to contain 75 mg of AMB and 100 mg of DOX

Standard stock and working solutions

Stock standard solutions of AMB and DOX were prepared separately by dissolving 100 mg of AMB and 100 mg of PH separately in 100 mL methanol. Corresponding working solutions were prepared by transferring accurately 25 mL from stock standard solutions separately in 250-mL measuring flasks and volume was diluted to the mark with methanol

Spectral characteristics of AMB and DOX

Aliquots equivalent to 800 µg of AMB and DOX, respectively, were transferred separately from their stock solutions (1mg mL^{-1}) into two 25-mL measuring flasks and made up to volume with methanol. A binary mixture was prepared by transferring aliquots equivalent to 400 µg of each drug into a 25-mL measuring flask and again made up to volume with methanol. The zero order spectra of the prepared solutions from 200 to 400 nm were recorded.

Assessment of linearity

Measured portions of each of AMB and DOX solutions equivalent to 6 - 40 and 4 - 32 µg mL^{-1}, respectively were accurately transferred into a series of 25 mL measuring flasks then the volume was diluted with methanol. The spectra of the prepared standard solutions were scanned

from 200 – 400 nm and stored in the computer. A calibration curve was constructed relating the absorbance of the zero order spectra of DOX and AMB at a wavelength of 246.5 nm to their corresponding concentrations in μgmL^{-1}. Another calibration curve relating the absorbance of the zero order spectra of AMB at a wavelength of 244 nm to the corresponding concentrations in μgmL^{-1} of AMB was constructed and the regression equations computed

Assay of mixture

Into a series of 25 mL measuring flasks, aliquots equivalent to (200 - 500 µg) of AMB and DOX were transferred accurately from their working solutions (0.1 mg mL^{-1}) to prepare mixtures containing different ratios of AMB and DOX and the volume was then completed with methanol. For the determination of AMB the spectra of the laboratory prepared mixtures (absorbance at each wavelength) were divided by the spectrum of 32 µg mL^{-1} DOX to obtain the ratio spectra. The absorbance in the plateau region at a wavelength > 344 nm (the constant) was subtracted from the division spectra and the obtained curves were multiplied (absorbance at each wavelength) by the spectrum of 32 µg mL^{-1} DOX. The obtained curve was used for direct determination of the concentration of AMB from the corresponding regression equation. For the determination of DOX, the regression equation at a wavelength of 246.5 nm was used to obtain the total concentration of the mixture then the concentration of AMB was subtracted from it, to calculate the concentration of DOX.

Analysis of dosage form

The content of 10 capsules were accurately weighed and an amount of the powder equivalent to 75 mg AMB and 100 mg DOX were transferred into a 250 mL beaker, and then 50 mL methanol was added. Stirring for 20 minutes was done using a magnetic stirrer followed by filtration into a 100-mL measuring flask. The residue was washed three times each with 10 mL methanol and volume was made up with methanol. Further dilution was done by taking 10 mL of the above solution in 100-mL measuring flask. Accurately 3 mL of this solution was transferred into a 25-mL measuring flask, and the volume was complete with methanol for the determination of AMB and DOX. The spectrum of the prepared solution was recorded from (200-400 nm) and saved in the computer, then procedures were followed as described for assay of mixtures above.

Statistical analysis

All experiments were performed in replicates for validity of statistical analysis and the results were expressed as mean ± RSD. F test and Student's t-test were performed on the data sets with the aid of Microsoft Excel-2007 software. Differences were considered significant at $p < 0.05$.

RESULTS

Methods development

Figure 2 shows the UV absorption spectra of AMB and DOX. The overlap shown in this figure prevents application of direct spectrophotometry for the analysis of this binary mixture (especially AMB). Isoabsorptive point method was used for the determination of the total mixture concentration. Since AMB can be determined by the ratio subtraction method, therefore DOX concentration can be obtained by subtraction.

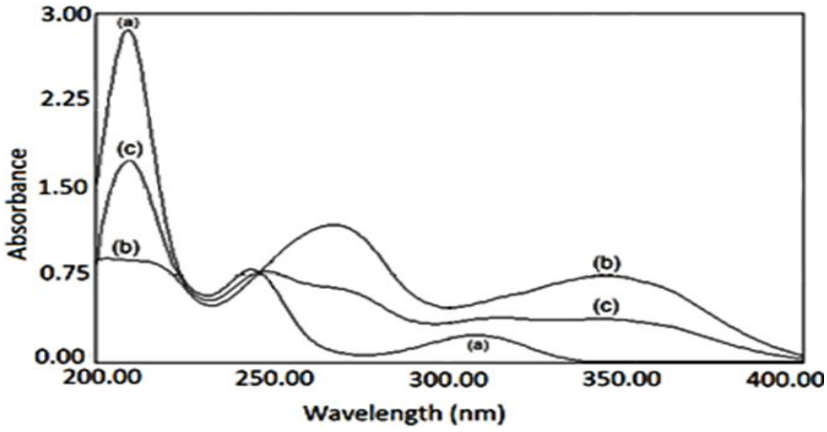


Figure 2: Absorption spectra of (a) AMB 32 µg mL^{-1}, (b) DOX 32 µg mL^{-1} and(c) mixture of 16 µg mL^{-1} of each drug, using methanol as solvent

For the determination of AMB, ratio subtraction method was applied [9] to remove DOX spectrum. The method depends on the fact that if you have a mixture of two drugs AMB (X) and DOX (Y) with overlapping spectra and the spectrum of (Y) is extended than (X) (Figure 2), the determination of (X) can be achieved by scanning the zero order absorption spectra of the laboratory prepared mixtures (DOX and AMB), dividing them by a carefully chosen concentration (32 µg mL^{-1}) of standard DOX (Y' = divisor) producing a new ratio spectra that represent as shown in Figure 3. This was followed by subtraction of the absorbance values of these constants (Y / Y') in plateau region (344 - 400 nm), followed by multiplication of the obtained spectra by (Y') the divisor. Finally, the original spectrum of (X) was obtained (Figure 4) and used for direct determination of AMB at 244 nm and calculation of the concentration from the corresponding regression equation.

For DOX assay, isoabsorptive method [10] was employed for the determination of total concentration of DOX and AMB. Absorption spectra of 32 µg mL^{-1} DOX, 32 µg mL^{-1} of AMB, and of a mixture containing equal concentration of DOX and AMB (16 µg mL^{-1} of each) showed isoabsorptive point at 246.5 nm (Figure 2). By measuring the absorbance at the chosen isoabsorptive point in the zero order absorption spectrum, the total content of DOX and AMB in the mixture can be calculated and hence DOX concentration was calculated by subtraction of AMB concentrations.

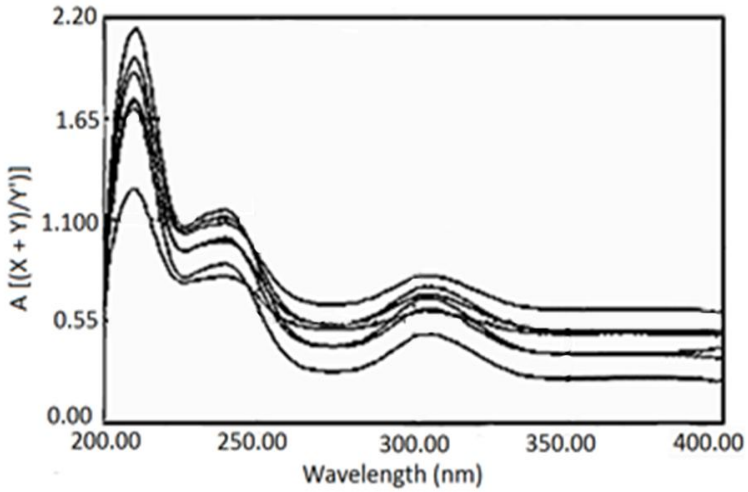


Figure 3: Ratio spectra of prepared mixtures of AMB (X) and DOX (Y) using 32 µg mL^{-1} of DOX (Y') as a divisor and methanol as a solvent

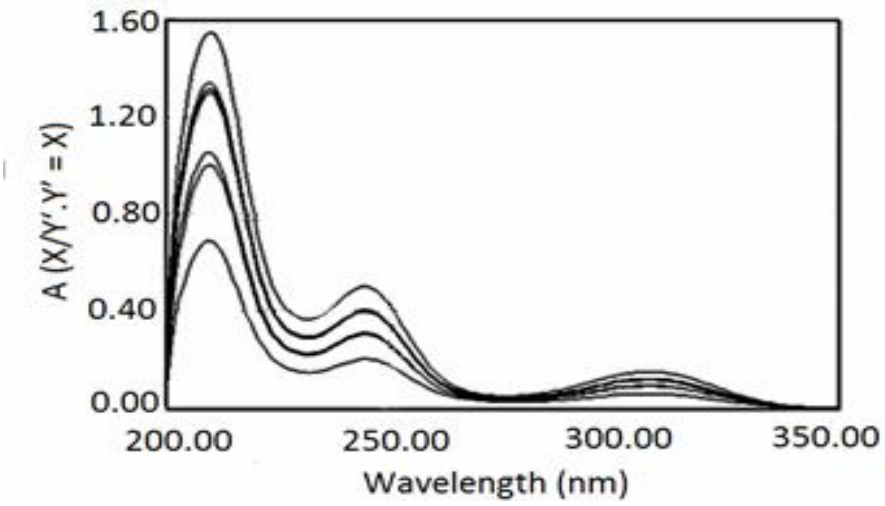


Figure 4: The obtained absorption spectra of AMB in prepared mixtures using the proposed ratio subtraction method after subtraction of the constant and multiplication by the devisor

The selectivity of the proposed procedures was assessed by the analysis of the prepared mixtures containing different ratios of the two drugs, where satisfactory results were obtained over the calibration ranges as shown in Table 1. The proposed procedures were also used for the determination of AMB and DOX in Ambrodoxy Capsules® (Table 2). The results obtained by the proposed procedures for the determination of pure samples of AMB and DOX were statistically compared to those obtained by the reported partial least squares (PLS) method [6]. It was concluded that with 95 % confidence, there was no significant difference between the proposed methods and the reported one in terms of their accuracy and precision as the calculated t and F values were less than theoretical values (Table 3).

Table 1: Recovery of AMB and DOX in prepared mixtures by the proposed methods

Concentration (µg mL^{-1})		Ratio	Recovery (%)	
			Ratio subtraction method	Isoabsorptive point method
AMB	*DOX*	*AMB: DOX*	*AMB*	*DOX*
			λ = 244 nm	λ = 244 nm
12	16	3 : 4	101.53	101.24
16	12	4 : 3	100.56	100.00
16	16	1 : 1	99.10	99.11
12	20	3 : 5	100.88	98.92
20	12	5 : 3	98.42	99.49
8	16	1 : 2	100.54	98.89
16	8	2 : 1	99.09	98.38
	Mean ± RSD.		100.02±1.144	99.43±0.949

Table 2: Application of standard addition technique for determination of AMB and DOX in Ambrodoxy Capsules (reference product) by the proposed methods

Sample no.	Authentic added *µg mL^{-1}*		*Ratio subtraction method*	Isoabsorptive point method
	AMB	*DOX*	*R% of AMB*	*R% of DOX*
1	*6*	*8*	100.13	100.96
2	*8*	*12*	99.27	99.22
3	*12*	*16*	99.08	99.70
Mean ± RSD			*99.49 ± 0.56*	*99.96 ± 0.90*
Found AMB and DOX contents of Ambrodoxy capsules* (%, ±RSD, n = 4.)			*98.66 ± 1.39*	*102.51 ± 0.85*

Table 3: Statistical comparison for the results obtained by the proposed methods and the reported method for the analysis of AMB and DOX in pure powdered form

Item	Ratio subtraction method	Isosbestic point method	Reported method*	
	AMB	*DOX*	*AMB*	*DOX*
Mean	99.97	99.86	99.98	99.96
SD	0.944	0.969	1.189	1.222
Variance	0.891	0.938	1.414	1.493
n	6	6	7	7
F-test	1.587 (4.95)	1.597 (4.95)		
Student's t-test	0.612 (2.201)	0.868 (2.201)		

*Values in parenthesis are the corresponding tabulated values at p = 0.05 [11]. *PLS method [6]*

Method validation

The proposed methods were validated according to the ICH-guidelines for validation of the analytical procedures [12] in terms of the linearity, accuracy, specificity, repeatability, reproducibility and range.

Linearity and sensitivity

A linear correlation was obtained between absorbance and the corresponding concentrations of AMB and DOX in the ranges of 6 – 40 and 4 - 32 µg mL^{-1}, respectively. The regression equations are as shown in Eqs 1 and 2.

For AMB: $A_{244} = 0.0261\ C_1 + 0.0144$ (r = 0.9998) ……. (1)
For DOX: $A_{246.5} = 0.0245\ C_2 + 0.0005$ (r = 0.9998) ….. (2)

where A is the absorbance of the zero order absorption spectra, C_1 and C_2 are the concentrations of AMB and DOX in µg mL^{-1}, respectively, and r is the correlation coefficient.

LOD and LOQ were calculated as LOD = 3.3 σ/S and LOQ = 10 σ/S, where, σ was the standard deviation of the intercept of regression line and S was the slope of regression line of the calibration curve. The results are given in Table 4.

Accuracy

The accuracy of the proposed spectrophotometric methods was tested by analyzing triplicate samples of standard AMB and DOX solutions. Recovery was 99.97 ± 0.944 and 99.86 ± 0.0.969 % for AMB and DOX, respectively (Table 4). These results revealed the high accuracy of the proposed methods. Accuracy of the methods was further assured by the use of the standard addition technique; it was performed by addition of known amounts of pure AMB and DOX to known concentrations of the pharmaceutical preparation. The resulting mixtures were assayed, and the results obtained were compared with the expected results (Table 2). The good recovery of standard addition technique suggests high accuracy of the proposed methods.

Repeatability and reproducibility

Intra-assay precision was assessed by analyzing 12 and 16 µg mL^{-1} of AMB, 24 and 30 µg mL^{-1} of DOX in triplicate in one assay batch. The inter-assay precision was assessed by analyzing the same concentrations in triplicate on 3 consecutive days. The mean recovery was approximately 100 % and low relative standard deviation (RSD) (< 1.6 % in all cases) indicated the high accuracy and precision of the proposed methods, respectively (Table 4).

Specificity

AMB and DOX were determined in the prepared mixtures containing different ratios of the two drugs. The good recovery and low RSD indicate the high specificity of the proposed methods (Table 1).

Range

The calibration range was established through consideration of the practical range necessary to achieve to adherence to Beer's law and the concentration of AMB and DOX present in the pharmaceutical preparation that would give accurate, precise and linear data (Table 4).

DISCUSSION

Ambrodoxy ® Capsules are combined dosage form containing the mucolytic agent AMB, and the antibacterial agent DOX. It has been used in the treatment of infections caused by susceptible strains of pathogens in acute and chronic diseases of upper and/or lower respiratory tract

Table 4: Assay validation data for the proposed methods for the simultaneous determination of AMB and DOX

Parameter	Ratio subtraction method	Isoabsorptive point method
	AMB	DOX
Range (µg mL^{-1})	6.0- 40.0	4- 32
Slope	0.0261	0.0245
Intercept	0.0144	0.0005
Mean	99.97	99.86
S.D.	0.944	0.969
Variance	0.891	0.938
Correlation coefficient (r)	0.9998	0.9998
* RSD%[a]	0.371, 0.463	0.847, 0.929
*RSD%[b]	0.853, 1.150	1.066, 1.581
LOD (µg mL^{-1})	0.676	0.461
LOQ (µg mL^{-1})	2.048	1.382

**RSD %[a] and RSD %[b] are the intra-day and inter-day, respectively (n = 3) relative standard deviation of concentrations (12, 16 µg/mL) for AMB, and (24, 30 µg/mL) for DOX*

concomitant with formation of viscous and hardly separated expectoration [6]. The ratio of AMB: DOX in capsules is 3:4 respectively. This study was designed to develop simple, robust and accurate spectrophotometric methods for the simultaneous determination of two analytes in Ambrodoxy® Capsules. Because of the practical simplicity, and wide availability of spectrophotometry in quality control laboratories, it was attempted in this study.

In ratio subtraction method, the correct choice of the devisor concentration is fundamental. If the concentration of the devisor is increased or decreased, the resulting constant value will be proportionally decreased or increased. Also the constant can be determined directly from the curve [(X+Y)/Y} by the straight line which is parallel to the wavelength axis in the region where (Y) is extended.

For the isoabsorptive point method, its theory depends on the following equations:

At any λ, the absorbance can be calculated from Eqs 3 – 5.

$A = A^{1\%}_{1cm}\, bC$ …… (3)

Therefore, for drug 1:

$A_1 = A_1{}^{1\%}_{1cm}\, b_1C_1$ …… (4)

for drug 2:

$A_2 = A_2{}^{1\%}_{1cm}\, b_2C_2$ ……..…….... (5)

If $C_1 = C_2$, $A_1 = A_2$ and $b_1 = b_2$

Therefore, this λ is called the isoabsorptive point and at this λ

$A_1{}^{1\%}_{1cm} = A_2{}^{1\%}_{1cm}$ ……..…… (6)

And since for a mixture of both drugs, the absorbance at this λ can be calculated from Eq 7.

$A_M = A_1{}^{1\%}_{1cm}\, C_{1M} + A_2{}^{1\%}_{1cm}\, C_{2M}$ …… (7)

Also since $A_1 = A_2 = A_M$ and $A_1{}^{1\%}_{1cm} = A_2{}^{1\%}_{1cm}$, therefore, Eq 7 can be written as Eq 8.

$A_M = A^{1\%}_{1cm}\,(C_{1M} + C_{2M}) = A^{1\%}_{1cm}\,(C_{TM})$ ……. (8)

and therefore from Eq 8, one can conclude that

$(C_{1M} + C_{2M}) = (C_{TM})$ ……..…….. (9)

where A_1, A_2 and A_M = absorbance of drug 1, drug 2 and their mixture at isoabsorptive point, and C_{1M}, C_{2M} are the concentration of the two drugs in the mixture. Thus, if the total concentration of both drugs is known, and the concentration of one of them can be determined separately at another wavelength, then the concentration of the second drug can be calculated by subtraction.

AMB and DOX showed good linearity. Linearity was high as well as recovery of AMB and DOX, indicating high accuracy of the methods. Repeatability and intermediate precision values were within the acceptable limits. This indicates that the methods are precise. Ambrodoxy® Capsules analysis results show that there is no interference from the excipients or diluents indicating high specificity of the proposed methods. The lowest values of LOD and LOQ as obtained by the proposed methods indicate that the methods are sensitive.

CONCLUSION

The proposed methods are simple and do not require sophisticated techniques or instrumentation. They are also sensitive and selective and can be used for the routine analysis of AMB and DOX in their available dosage forms. Furthermore, the methods are suitable and valid for application in laboratories lacking liquid chromatographic instruments.

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