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Research Article

Three Spectrophotometric Methods for Simultaneous Determination of Ampicillin and Dicloxacillin in Presence of Their Major Impurity 6-Aminopenicillanic Acid

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Abstract

Three simple, selective, accurate, precise and economic spectrophotometric methods were developed for simultaneous determination of ampicillin (AMP) and dicloxacillin (DX) in presence of their major impurity namely; 6-aminopenicillanic acid (APA). Method A is derivative spectrophotometry, where DX was determined by first derivative D1 spectrophotometric method at 207.4 nm, while AMP was determined by second derivative D2 spectrophotometric method at 229.6 nm. Method B is first derivative of ratio spectra spectrophotometry which allows determination of both AMP at 265.4 nm and DX at 244.2 nm without interference of each other and in presence of 6-amino penicillanic acid (APA). Method C is the mean centering of ratio spectra spectrophotometric (MCR) method, which depends on measuring the mean centered values of ratio spectra of AMP, DX and APA at 223, 237 and 209 nm respectively. The accuracy and precision of the developed methods were determined while their selectivity was tested by their application for determination of the studied component in different synthetic prepared mixtures. Additionally, the developed methods were successfully used for determination of the studied drugs in their dosage form. Statistical comparison of the suggested methods with the reported HPLC one using F and student's t- tests showed no significant difference regarding both accuracy and precision. The specificity of the developed methods was investigated by analyzing different laboratory prepared mixtures of the AMP and DX.

Keywords: Ampicillin; Dicloxacillin; 6-Aminopenicillanic acid; First derivative spectrophotometry; Second derivative spectrophotometric; Derivative ratio spectrophotometry; Mean centring of ratio spectra spectrophotometry

Introduction

Ampicillin shown in (Figure1), is known chemically as (2S, 5R, 6R)-6-([(2R)-2-amino-2-phenylacetyl] amino)-3, 3- dimethyl-7-oxo-4-thia-1-azabicyclo [3.2.0] heptane-2-carboxylic acid. Dicloxacillin shown in (Figure 1), is known chemically as (2S, 5R, 6R)-6-{[3-(2, 6-dichlorophenyl)-5-methyl- oxazole-4-carbonyl] amino}-3, 3-dimethyl-7-oxo-4-thia-1-azabicyclo [3.2.0] heptane-2-carboxylic acid. Both of them are used individually and in fixed dose combination as antibacterial agents [1, 2]. 6-Aminopenicillanic acid shown in (Figure 1), is known chemically as (2S, 5R, 6R)-6-Amino-3, 3-dimethyl-7-oxo-4- thia-1-azabicyclo [3.2.0] heptane-2- carboxylic acid.

Ampicillin is beta-lactam antibiotic that was used extensively to treat bacterial infections since 1961. It is considered part of the amino penicillin family and is nearly equivalent to amoxicillin in terms of spectrum and level of activity.

Ampicillin is a potent antibiotic with relatively short-termed stability in aqueous solutions [3, 4]. It is used clinically to treat a broad range of bacterial infections [5-7]. With injection; ampicillin is distributed rapidly and widely, resulting in a high concentration of the drug in bile [8]. From bile, it is excreted into the gut and is known to cause disruption of the normal intestinal micro flora by diminishing the main flora and increasing the presence of yeast as well as inducing a high risk of Clostridium difficile colitis [9].

Dicloxacillin (INN) is a narrow spectrum antibiotic of the penicillin class. It is used to treat infections caused by susceptible gram-positive bacteria. It is very similar to flucloxacillin and these two agents are considered interchangeable, also dicloxacillin is more acid-stable than many other penicillins and can be given orally, in addition to parenteral routes. However, like methicillin, it is less potent than benzyl penicillin against non-β-lactamase-producing gram-positive bacteria [10].

6-Aminopenicillanic acid has a beta lactam nucleus and it is the major impurity for both of ampicillin and dicloxacillin [11].

Literature survey revealed the analytical methods for determination of ampicillin and dicloxacillin which include high performance liquid chromatography (HPLC) method for determination of ampicillin in its pharmaceutical formulation [12]. Another HPLC methods used for determination of ampicillin and dicloxacillin with other penicillins [13-15], several spectrophotometric methods were reported for the assay of ampicillin and dicloxacillin but none of them can determine AMP and DX in the prescience of APA [16-18].

The aim of the presented work is to develop and conduct a comparative study on three well established spectrophotometric

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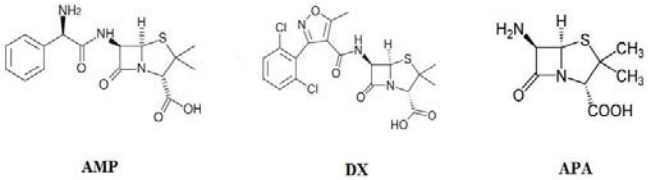

Figure 1: Chemical structures of Ampicillin (AMP), Dicloxacillin (DX) and 6-Aminopenicillanic acid (APA).

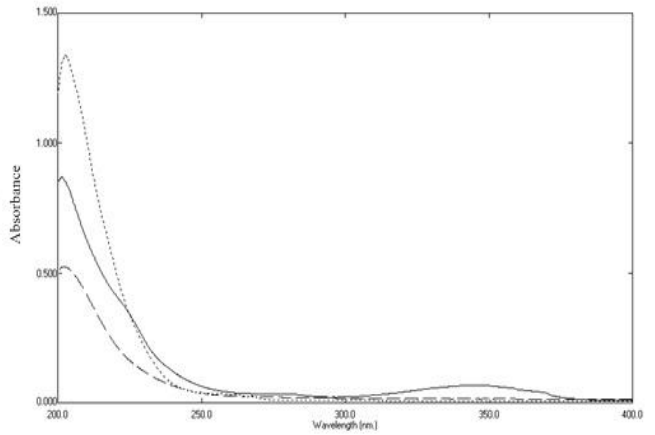

Figure 2: Zero-order absorption spectra of 28 µg mL^{-1} of AMP (……), 18 µg mL^{-1} of DX (—) and 15 µg mL^{-1} of APA(---) using 0.05 mol L^{-1} HCl as a solvent.

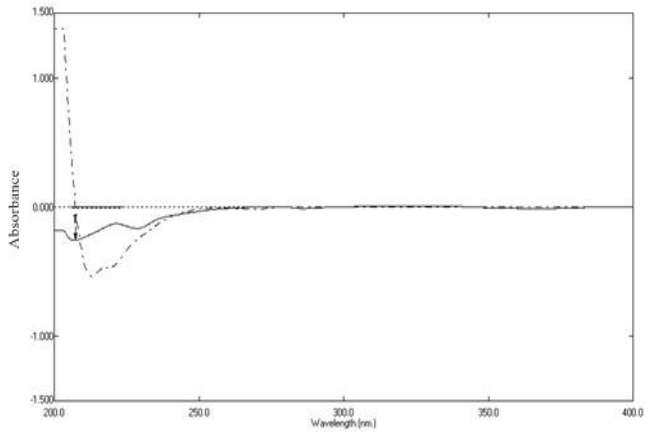

Figure 3: First derivative absorption spectra of 10 µg mL^{-1} of Dx (—), 5 µg mL^{-1} of AMP (-. -. -) and 5 µg mL^{-1} of APA (……) using 0.05 mol L^{-1} HCl as a solvent.

methods for resolving the spectral interference problem of AMP and DX mixtures in presence of 1-9 % from their impurity APA without preliminary separation. The new methods are simple, accurate, precise, stability indicating and do not require any sophisticated apparatus or computer programs. The mean centering of ratio spectra spectrophotometric (MCR) method can determine the concentrations of AMP, DX and APA mixtures without preliminary separation.

Experimental

Instruments

A double beam UV-visible spectrophotometer (SHIMADZU, Japan) model UV-1601 PC with quartz cell of 1 cm path length, connected to IBM compatible computer. The software was UVPC personal spectroscopy software version 3.7. The spectral band width was 2 nm and wavelength-scanning speed 2800 nm/min.

Matlab® version R2014a [19] was used for the proposed mean centering of ratio spectra (MCR) spectrophotometric method.

Material and reagents

Pure samples: Ampicillin was supplied by SIGMA-ALDRICH, Talat Harab, Cairo, Egypt. Its purity was found to be 99.24% according to company's analysis certificate.

Dicloxacillin was supplied by SIGMA-ALDRICH, Talat Harab, Cairo, Egypt. Its purity was found to be 99.18% according to company's analysis certificate.

6-Aminopenicillanic acid was supplied by SIGMA-ALDRICH, Talat Harab, Cairo, Egypt. Its purity was found to be 99.48% according to company's analysis certificate.

Pharmaceutical dosage form: Cloxapen® capsules batch No. 2941060404830, are labeled to contain 250 mg Ampicillin and 250 mg Dicloxacillin. It was manufactured by Misr Co. Pharmaceutical Industries, El Matareya, Cairo, Egypt.

Chemicals and reagents

All chemicals and solvents used throughout this work were of analytical grade and were used without extra purification.

Methanol HPLC grade was purchased from Sigma-Aldrich Chemie GmbH, Germany.

Hydrochloric acid analytical grade was purchased from El Nasr Co. Pharmaceutical Industries, Cairo, Egypt.

Procedure

Standard stock and working solutions: AMP, DX and APA standard stock solutions were prepared in concentration of 1mg mL^{-1} in 0.05 mol L^{-1} HCl, while AMP, DX and APA standard working solutions were prepared in concentration of 0.1 mg mL^{-1} by diluting 10 mLs of their respective stock standard solutions to100 mL in 0.05 mol L^{-1} HCl.

Spectral characteristic of AMP, DX and APA: The zero-order absorption spectra of 28 µg mL^{-1} of AMP, 18 µg mL^{-1} of DX and 15 µg mL^{-1} of APA were recorded against 0.05 mol L^{-1} HCl as a blank over the range of 200–400 nm as shown in (Figure 2).

Laboratory prepared mixtures: Mixtures containing different ratios of AMP, DX and APA were prepared using their respective working solutions in 0.05 mol L^{-1} HCl.

Construction of calibration curves: Aliquots equivalent to 3 –28 µg of AMP, 5-20 µg of DX and 5-30 µg of APA were accurately transferred from their working standard solutions (0.1 mg mL^{-1}) into two separate series of 10 mL volumetric flasks then completed to volume with 0.05 mol L^{-1} HCl. The spectra of the prepared standard solutions were scanned from 200-400 nm.

First derivative D^1 spectrophotometric method: The D^1 spectra of DX solutions in the range of 5 – 20 µg mL^{-1} were recorded using $\Delta\lambda = 4$ and scaling factor =10, then the peak amplitude was measured at 207.4 nm.

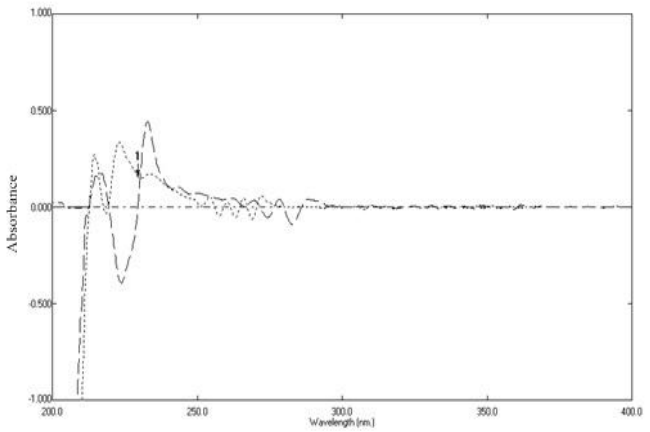

Figure 4: Second derivative absorption spectra of 28 µg mL^{-1} of AMP (.....), 20 µg mL^{-1} of DX (- - - -) and 10 µg mL^{-1} of APA (-. -. -) using 0.05 mol L^{-1} HCl as a solvent.

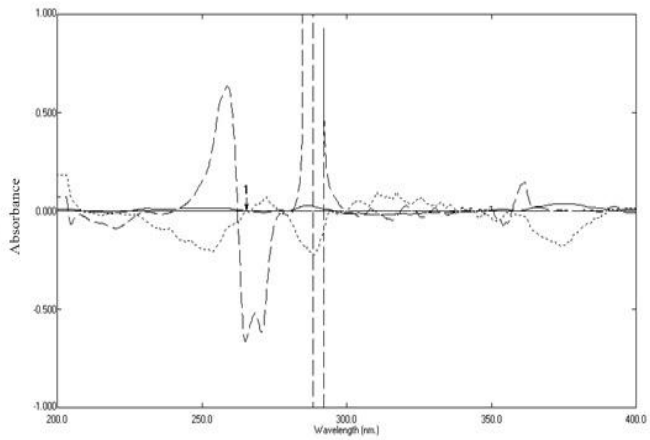

Figure 5: First derivative of ratio spectra of 28 µg mL^{-1} of AMP (- - - -),10 µg mL^{-1} of DX(……) and 5 µg mL^{-1} of APA (—) using 5 µg mL^{-1} of DX as a divisor and 0.05 mol L^{-1} HCl as a solvent.

The D^2 spectra of AMP solutions in the range of 3 – 28 µg mL^{-1} were recorded using Δλ = 4 and scaling factor =100, then the peak amplitude was measured at 229.6 nm.

First derivative of ratio spectra spectrophotometric method: The DD^1 spectra of AMP solutions in the range of 3-28 µg mL^{-1} were recorded using 5µg mL^{-1} of DX product as a divisor, Δλ = 4 and scaling factor =10, then peak amplitude was measured at 265.4 nm.

The DD^1 spectra of DX solutions in the range of 5-20µg mL^{-1} were recorded using 5 µg mL^{-1} of AMP as a divisor, Δλ = 4 and scaling factor =10, then peak amplitude was measured at 244.2 nm.

The calibration curves were constructed relating the peak amplitudes against the corresponding drug concentrations and the regression equations were calculated in the presence of APA.

Mean centering of ratio spectra (MCR) method: Aliquots of AMP equivalent to 5-35 µg mL^{-1} were accurately transferred from its standard working solution (0.1 mg mL^{-1}) into set of 10 ml measuring flasks and the volume was adjusted using 0.05 mol L^{-1} HCl. The absorption spectra of the prepared solutions were recorded in the

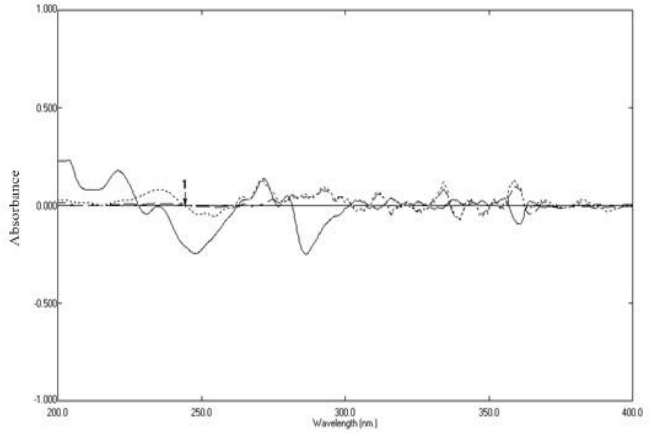

Figure 6: First derivative of ratio spectra of 10 µg mL^{-1} of AMP (—),5 µg mL^{-1} of DX(……) and 5 µg mL^{-1} of APA (- - - -) using 5 µg mL^{-1} of AMP as a divisor and 0.05 mol L^{-1} HCl as a solvent.

range of 200-255 nm and divided by the spectrum of 20 µg mL^{-1} of DX and 15µg mL^{-1} of APA then the obtained ratio spectra mean centered.

By the same way the spectra of different concentrations of standard DX 5-30 µg mL^{-1} were recorded. The stored spectra were divided by the standard spectrum of 25 µg mL^{-1} of AMP and 15 µg mL^{-1} of APA to obtain the ratio spectra then mean centering of these ratio spectra was carried out.

Finally the spectra of different concentrations of standard APA 3-28 µg mL^{-1} were recorded. The stored spectra were divided by the standard spectrum of 25 µg mL^{-1} of AMP and 20 µg mL^{-1} of DX to obtain the ratio spectra then mean centering of these ratio spectra was carried out.

Calibration curves for AMP, DX and APA were constructed by plotting the amplitudes of their respective mean centered ratio spectra from 200 to 255 nm (peak to peak) for both drugs and APA against their corresponding concentrations.

Analysis of laboratory prepared mixtures of AMP and DX in presence of APA

In two series of 10 mL measuring flasks, aliquots of AMP, DX and APA were transferred accurately from their corresponding working solutions (0.1 mg mL^{-1}) to prepare mixtures containing APA from 4-9 % of the pure AMP and DX in the mixture. The volume was completed with 0.05 mol L^{-1} HCl. The spectra of the prepared solutions were recorded from 200-400 nm and stored in the computer. Procedures under calibration for each of the proposed methods were followed. The concentrations of the AMP and DX were calculated using the specified regression equations.

Application to pharmaceutical formulation (Cloxapen® capsule)

The content of fourteen capsules of Cloxapen® were powdered and mixed well. Accurately weighed amount of the powdered capsules equivalent to 100 mg of AMP and DX was transferred to 100 ml volumetric flask and 75 ml of 0.05 mol L^{-1} HCl was added. The prepared solution was sonicated for 15 minutes, cooled and the volume was completed to obtain 1 mg ml^{-1} stock solution and then the solution was filtered. Appropriate dilutions of the prepared

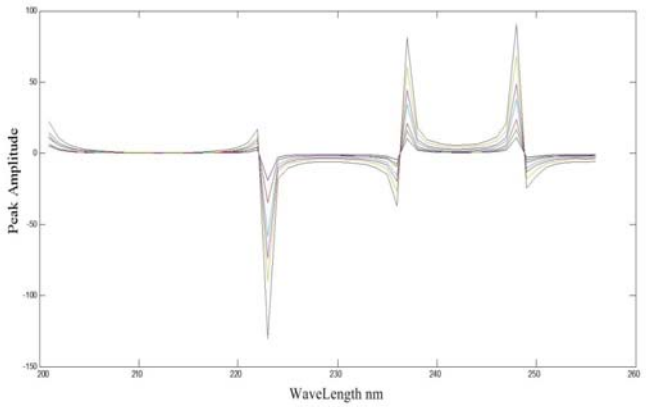

Figure 7: Mean centered ratio spectra of AMP (3-28µg mL^{-1}) using 20 µg mL^{-1} of DX and 5µg mL^{-1}of APA as a divisor and 0.05 mol L^{-1} HCl as a solvent.

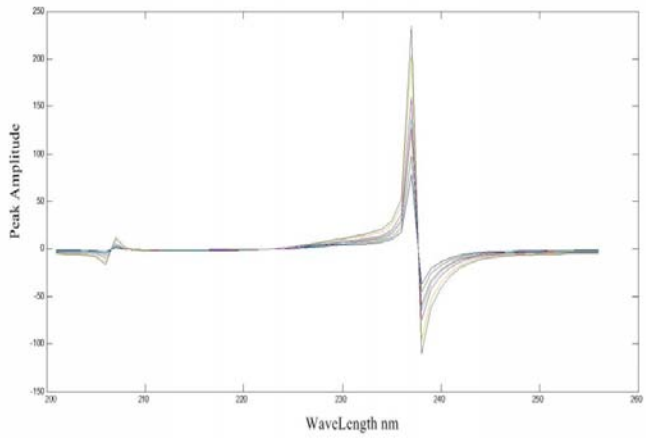

Figure 8: Mean centered ratio spectra of DX (5-20µg mL^{-1}) using 25µg mL^{-1} of AMP and 5µg mL^{-1}of APA as a divisor and 0.05 mol L^{-1} HCl as a solvent.

sample were made to prepare its working solution (0.1 mg ml^{-1}) and the procedure under linearity was followed.

Results and Discussion

First derivative D^1 spectrophotometric method used for determination of DX and D^2 spectrophotometric method used for determination of AMP in presence of APA

(Figure 2) shows the zero order absorption spectra of AMP, DX and APA which overlap seriously that direct determination of AMP and DX is not possible .The problem of overlapped spectra of AMP and DX is solved using first derivative (D^1) spectrophotometry to determine DX and second derivative (D^2) spectrophotometry to determine DX in presence of APA.

(Figure 3) shows the first derivative spectra of AMP, DX and APA. It is obvious that the overlap observed in the zero order absorption spectra was eliminated at 207.4 nm for DX, which lies at the zero crossing of DX and APA. Different factors affecting resolution including type of solvent, Δλ and scaling factor were studied to optimize resolution of drugs. The best resolution was obtained using Δλ= 4nm, scaling factor=10 and 0.05 mol L^{-1} HCl as solvent.

(Figure 4) shows the second derivative spectra of AMP, DX and APA. It is obvious that the overlap observed in the zero order absorption spectra is eliminated at 229.6 nm for AMP which lies at the zero crossing of DX and APA. Different factors affecting resolution including type of solvent, Δλ and scaling factor were studied to optimize resolution of drugs. The best resolution was obtained using Δλ= 4nm, scaling factor=100 and 0.05 mol L^{-1} HCl as solvent.

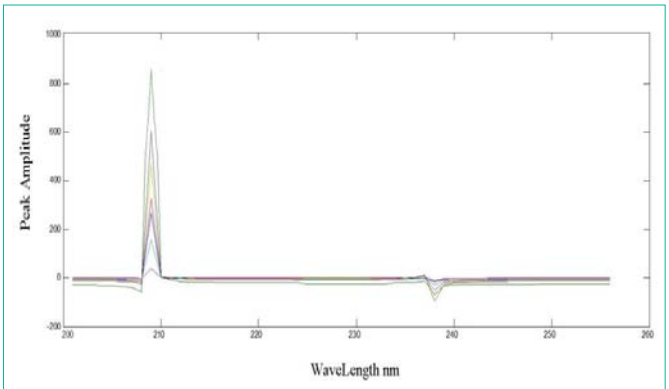

Figure 9: Mean centered ratio spectra of APA (5-30µg mL^{-1}) using 25µg mL^{-1} of AMP and 20µg mL^{-1} of DX as a divisor and 0.05 mol L^{-1} HCl as a solvent.

Linear correlations were obtained between peak amplitudes at 207.4 nm for D^1 spectra of DX in the concentration range 5–20 µg mL^{-1}, while linear correlations were obtained between peak amplitudes at 229.6 nm for D^2 spectra of AMP in the concentration range 3–28 µg mL^{-1} and the regression equation were computed as follows

$P.A_1 = 0.0260C_1 - 0.0027$ $r_1 = 0.9997$ at 207.4 nm for D^1 method of DX

$P.A_2 = 0.0049C_2 + 0.0003$ $r_2 = 0.9998$ at 229.6 nm for D^2 method of AMP

Where $P.A_1$ and $P.A_2$, are the peak amplitudes of DX using D^1 and AMP using D^2 methods, C_1 and C_2 are the concentration of DX in µg mL^{-1} and AMP in µg mL^{-1} respectively, and r_1, r_2 are the correlation coefficients.

Results described in (Table 1) showed that D^1 method and D^2 method are selective, valid and applicable for the determination of DX and AMP in presence of APA in different laboratory prepared mixtures.

First derivative of ratio spectra spectrophotometric method

(Figure 5) shows the first derivative of ratio spectra of AMP, DX and APA. As seen, the trough at 265.4 nm for AMP which lies at the zero crossing of DX and APA can be adopted for determination of AMP in presence of DX and APA. Different factors affecting resolution including divisor concentration, type of solvent, Δλ and scaling factors were studied to optimize resolution of drugs. The best resolution was obtained using 5 µg mL^{-1} of DX as a divisor, Δλ=4 nm, scaling factor=10 and 0.05 M HCl as solvent in terms of signal to noise ratio, sensitivity and selectivity. (Figure 6) shows the first derivative of ratio spectra of AMP, DX and APA. As seen, the trough at 244.2 nm for DX which lies at the zero crossing of AMP and APA can be adopted for determination of DX in presence of AMP and APA. Different factors affecting resolution including divisor concentration,

Table 1: Linear regression and analytical parameters of the proposed methods for determination of AMP and DX.

	Derivative Method		Ratio Derivative Method		Mean Centre Method		
Parameters	AMP	DX	AMP	DX	AMP	DX	APA
Linearity range	3-28 μg ml^{-1}	5-20 μg ml^{-1}	3-28 μg ml^{-1}	5-20 μg ml^{-1}	5-35 μg ml^{-1}	5-30 μg ml^{-1}	3-28 μg ml^{-1}
Slope	0.005	0.026	0.022	0.013	0.035	0.062	0.318
Intercept	0.001	-0.003	0.001	-0.001	-0.038	0.480	-0.386
Correlation coefficient	0.9998	0.9996	0.9999	0.9997	0.9998	0.9999	0.9999
Precision Repeatability(RSD%)a*	0.954	1.061	0.943	1.023	1.021	1.052	0.989
Intermediate Precision(RSD%)b*	1.353	1.152	1.724	1.162	1.326	1.134	1.298
Accuracy (Mean ± SD)	100.96 % ± 0.903	99.28 % ± 1.090	99.44 % ± 0.919	99.88 % ± 0.918	100.23 % ± 0.945	99.91 % ± 0.807	99.62 % ± 0.96
LOD**	0.80	1.30	0.91	1.38	1.33	1.28	0.87
LOQ**	2.48	4.03	2.79	4.28	4.12	3.96	2.67

(RSD %) a and (RSD %) b*; the intra- and inter-day relative standard deviation of three concentrations for each spectrophotometric method.
**Limit of detection and quantitation are determined via calculations (LOD=3.3xSD of the response/slope, LOQ=10xSD of the response/slope).

type of solvent, Δλ and scaling factors were studied to optimize resolution of drugs. The best resolution was obtained using 5μg mL^{-1} of AMP as a divisor, Δλ=4nm, scaling factor=10 and 0.05 mol L^{-1} HCl as solvent in terms of signal to noise ratio, sensitivity and selectivity.

Linear correlations were obtained between peak amplitudes at 265.4nm for DD^1 spectra of AMP in the concentration range 3–28 μg mL^{-1} and peak amplitudes at 244.2 nm for DD^1 spectra of DX in concentration range of 5-20 μg mL^{-1} from which the regression equations were computed as follows

$P.A_1 = 0.0218\ C_1 + 0.0002$ $r_1 = 0.9999$ at 265.4 nm for DD^1 method of AMP

$P.A_2 = 0.0130\ C_2 - 0.0009$ $r_2 = 0.9998$ at 244.2 nm for DD^1 method of DX

Where $P.A_1$ and $P.A_2$, are the peak amplitudes of AMP using DD^1 and DX using DD^1 methods, C_1 and C_2 are the concentration of AMP in μg mL^{-1} and DX in μg mL^{-1} respectively, and r_1, r_2 are the correlation coefficients. Results described in (Table 1) showed that DD^1 method is selective, valid and applicable for the determination of AMP and DX in presence of each other and in presence of APA in different laboratory prepared mixtures.

Mean centering of ratio spectra (MCR) method

As shown in (Figure 1) the absorption spectra of AMP, DX and APA in 0.05 mol L^{-1} HCl overlapped in the wavelength region of 200-350 nm. To construct the calibration curves of the proposed method the absorption spectra of the standard solutions of the AMP with different concentrations were recorded in the wavelength range of 200-290 nm and divided by the standard spectrum of DX (20 μg mL^{-1}) and APA (5 μg mL^{-1}) and mean centering of the obtained ratio spectra were carried out and the concentrations of AMP were determined by measuring the amplitudes of the mean centered ratio spectra from 200 to 255 nm (peak to peak) as shown in (Figure 7). By the same way different standard solutions of the DX with different concentrations scanned then recorded spectra were divided by the standard spectrum of AMP (25 μg mL^{-1}) and APA (5 μg mL^{-1}) and the ratio spectra were obtained then mean centered. The values from 200 to 255 nm (peak to peak) in the obtained mean centered ratio spectra were used for determination of DX as shown in (Figure 8).

Finally different standard solutions of the APA with different concentrations were recorded and divided by the standard spectrum of AMP (25 μg mL^{-1}) and DX (20 μg mL^{-1}) and the ratio spectra were obtained which then mean centered. The values from 200 to 255 nm (peak to peak) in the obtained mean centered ratio spectra were used for determination of APA as shown in (Figure 9). The computed regression equation parameters for each of the studied drugs are given in (Table 1).

$P.A_1 = 0.035\ C_1 - 0.038$ $r_1 = 0.9998$ at 223 nm for AMP

$P.A_2 = 0.062\ C_2 - 0.480$ $r_2 = 0.9999$ at 237 nm for DX

$P.A_3 = 0.318\ C_3 - 0.386$ $r_3 = 0.9999$ at 209 nm for APA

Where A_1, A_2 and A_3 are the absorbance of AMP, DX and APA, respectively, C_1, C_2 and C_3 are the concentration of AMP, DX and APA in μg mL^{-1}, respectively, and r_1, r_2 and r_3 are the corresponding correlation coefficients.

To optimize the developed MCR method different parameters were tested. The wavelength range used affects greatly the obtained mean centered ratio spectra, hence different ranges were tested and the good results were obtained when using range from 200-290 nm for AMP, DX and APA. Since the divisor concentration had great effect on the selectivity of the method different divisors were tried of DX and APA such as normalized spectra of 5, 15, 18 and 20 μg mL^{-1} and 5, 10, 15 and 20 μg mL^{-1} respectively (for AMP) and different divisors were tried of AMP and APA such as normalized spectra of 3, 5, 15 and 28 μg mL^{-1} and 5, 10, 15 and 20 μg mL^{-1} respectively (for DX). Finally different divisors were tried of AMP and DX such as normalized spectra of 3, 5, 15 and 28 μg mL^{-1} and 5, 10, 15 and 20 μg mL^{-1} respectively (for APA) .The best results regarding sensitivity and selectivity were obtained when using (25 μg mL^{-1}) of AMP, (20 μg mL^{-1}) of DX and (5 μg mL^{-1}) of APA as divisors.

The specificity of the proposed methods was assessed by analysis of different laboratory prepared mixtures containing different ratios of the suggested drugs, where satisfactory results were obtained in (Table 1). The developed spectrophotometric methods were also applied for determination of AMP and DX in Cloxapen* capsules and the results obtained were acceptable. The validity of the methods was

Table 2: Determination of the studied drugs in the pharmaceutical preparations by the proposed methods and statistical comparison with the reported HPLC method.

Parameters	Derivative Method		Ratio Derivative Method		Mean Centre Method		Reported HPLC Method[13]	
	AMP	DX	AMP	DX	AMP	DX	AMP	DX
Cloxapen® capsules[a] (B.N. 2941060404830)	99.325%±0. 961	99.304%±0.962	99.116%±0.887	99.182%± 0.885	99.176%±0.863	99.222%±0.917	99.795%±1.089	99.963%±1.336
Standard addition[a]	100.541%±1.025	101.012%±1.012	100.108%±1.233	100.13%±1.308	100.621%±1.203	100.205%±1.001	-	-
F-test (5.050)[b]	1.284	1.929	1.507	2.279	1.592	2.122	-	-
Student's t-test(2.228)[b]	0.819	0.980	1.219	1.193	1.121	1.120	-	-

[a]Average of six determination.

[b]The values in the parenthesis are the corresponding theoretical values at p= 0.05.

further assessed by applying the standard addition technique and confirmed the accuracy of the methods, (Table 2). The results obtained by applying the proposed methods for determination of tablets of AMP and DX in presence of APA were statistically compared to those obtained by applying a reported HPLC method [13] and the results showed no significant differences between the proposed methods and the reported one regarding accuracy and precision, (Table 2).

On the other hand the suggested MCR method provide higher selectivity and reproducibility compared to the reported one, where it can determine AMP, DX and APA concentrations in presence of each other.

Application of methods in assay of Capsule

The proposed UV methods were applied for determination of AMP and DX in its pharmaceutical formulation Cloxapen® capsules. The results are shown in (Table 2) and compared to that of the reported method [13]. Standard addition technique was also successfully applied. The acceptable percentage recoveries values confirm the suitability of the proposed methods for the determination of these components in its pharmaceutical formulations.

Method Validation

Validation was done according to ICH recommendations [20].

Linearity

Linearity of methods was evaluated by analyzing 8 concentrations of AMP, DX and APA between 3-28 μg mL^{-1}, 5-20 μg mL^{-1} and 5-30μg mL^{-1}, respectively for derivative and ratio derivative methods and 5-35 μg mL^{-1}, 5-30 μg mL^{-1} and 3-28 μg mL^{-1}, respectively for mean center method. Each concentration was repeated three times. The assay was performed according to the experimental conditions previously mentioned.

Accuracy

Accuracy of results was checked by applying the proposed methods for determination of different blind samples of AMP, DX and APA. The concentrations were obtained from the corresponding regression equations and percentage recoveries of the proposed methods were calculated with mean percentage recovery, suggesting good accuracy as shown in (Table 1).

Range

The calibration range was established through considerations of the practical range necessary according to adherence to Beer's law and the concentration of AMP and DX present in the pharmaceutical preparations to give accurate precise and linear results, (Table 1).

Precision

Repeatability: Three concentrations of AMP, DX and APA (5, 15, 20 μg ml^{-1}) were analyzed three times intra-daily using the proposed methods. Good relative standard deviation of three concentrations was obtained confirming the repeatability of the methods.

Reproducibility (intermediate precision): The previous procedures were repeated inter-daily on three different days for the analysis of the three chosen concentrations. Good results and acceptable relative standard deviation (RSD %) are illustrated in (Table 1).

Specificity

Specificity of the methods was achieved by the analysis of different laboratory prepared mixtures of AMP, DX and APA within the calibration range. Satisfactory results are shown in (Table 1).

Conclusion

The developed derivative spectrophotometry, derivative ratio spectrophotometry and mean centring of ratio spectra spectrophotometric methods were successfully applied for simultaneous determination of AMP and DX in presence of APA in their combined sample; however mean centring of ratio spectra spectrophotometric method was the only one that could determine AMP, DX and APA simultaneously. The developed methods were found to be rapid, simple, accurate and easy to understand and apply. On the other hand mean centring of ratio spectra spectrophotometric method had the advantages of being simple, accurate and rapid. When suggested methods were completely validated they showed satisfactory data for all the method validation parameters tested. Recovery studies indicated that practically there was no interference from the capsules additives, hence, these methods can be easily and conveniently adopted for routine quality control analysis of AMP and DX.

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