

## Spectrophotometric and Chemometric Methods for Simultaneous Determination of Two Anti-Hypertensive Drugs in their Combined Dosage Form

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### Abstract

In this study determination of Moexipril hydrochloride (MOX) and hydrochlorothiazide (HCT) were conducted by application of Spectrophotometric and Chemometric methods. Five different accurate, sensitive and reproducible methods were applied for the simultaneous determination of (MOX) and (HCT) in their bulk powder and pharmaceutical dosage form. The first method is the new absorbance subtraction (AS) method. The second method is the new amplitude modulation (AM) method. The third method is the new extended ratio subtraction (ERS) method coupled to ratio subtraction (RS) method. The fourth and fifth methods are multivariate calibration which includes Principal Component Regression (PCR) and Partial Least Squares (PLS). The suggested procedures were checked using laboratory prepared mixtures and were successfully applied for the analysis of their pharmaceutical preparations. The validity of the proposed methods was further assessed by applying the standard addition technique. The results obtained by applying the proposed methods were statistically analyzed and compared with a reported method.

### Keywords:

Moexipril hydrochloride; Hydrochlorothiazide; Absorbance subtraction; Amplitude modulation; Extended ratio subtraction; chemometric methods

### Introduction

Moexipril Hydrochloride (MOX) is chemically (3S)-2-[(2S)-2-[[[(2S)-1-ethoxy-1-oxo-4-phenylbutan-2-yl] amino] propanol]-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid, Figure (1). MOX is a non-sulphydryl containing precursor of the active angiotensin-converting enzyme (ACE) inhibitor Moxiprilate. It is used to treat high blood pressure (hypertension) and heart failure. It works by relaxing blood vessels, causing them to widen [1,2]. MOX is not official yet in any pharmacopeia

Hydrochlorothiazide (HCT) is chemically 6-chloro-3,4-dihydro-2H-1,2,4-benzothiadiazine-7-sulphonamide-1,1dioxide, (Figure 2). HCT is a first-line diuretic drug of the thiazide class used as anti-hypertensive drug that acts by inhibits active chloride reabsorption at the early distal tubule via the Na-cl co transporter, resulting in an increase in the excretion of sodium, chloride, and water. This reduces the volume of the blood, decreasing blood return to the heart and thus cardiac output and, by other mechanisms, is believed to lower peripheral vascular resistance. HCT is a calcium-sparing diuretic, meaning it can help the body get rid of excess water while still keeping calcium [3,4]. HCT is official in The British Pharmacopeia [5], The European Pharmacopeia [6] and the United States Pharmacopoeia [7].

Several analytical procedures have been proposed for the quantitative estimation of MOX and HCT separately and in combination with other drugs. Spectroscopic [8-10], High Performance Liquid Chromatography (HPLC) [11-13], Thin Layer Chromatography (TLC) [14], Electrochemical [15-17] and mass spectrometry [18] methods for estimation of MOX alone or in combination with other drugs in pharmaceutical preparation have been reported. Hydrochlorothiazide alone or in combination with other drugs is estimated by HPLC [19-29], TLC-densitometry [30-36], capillary electrophoresis [37-

39], electrochemical [40-43] and spectroscopic methods [44-53]. Simultaneous estimation of MOX and HCT has been reported by RP-HPLC [54,55], and by UV-spectrophotometric methods [54-56]. To our knowledge simple and economical analytical method for simultaneous determination of MOX and HCT has not been reported so far. In this paper five new, simple methods for simultaneous estimation of MOX and HCT in tablet formulation were developed.

### Theory of Absorbance Subtraction (AS) Method

This method based on the same principles as the absorption factor method [57,58] and it depends on that, if you have a mixture of two drugs X and Y having overlapped spectra intersect at isoabsorptive point and Y is extended more than X, while X doesn't show any absorbance ( $A_2$ ) at another wavelength ( $\lambda_2$ ).

In this method the isoabsorptive point  $\lambda_{iso}$  could be used for separate quantitative estimation of each X & Y in their mixture (X+Y). The determination can be done using mathematically calculated factor of one of these components. By simple manipulation step, we can get the absorbance value corresponding to X and Y, separately. So, the

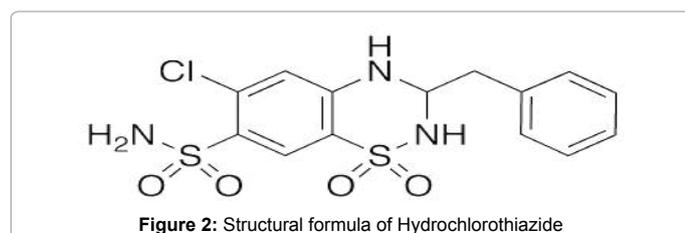
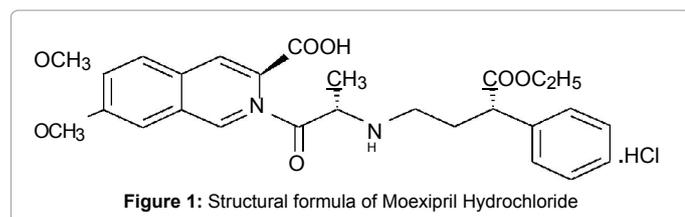
Concentration of each component could be obtained via the isoabsorptive point regression equation without any need for a complementary method.

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The absorbance values corresponding to X and Y at  $\lambda_{iso}$  were calculated by using absorbance factor  $\{A_{iso} / A_2\}$  which is a constant for pure Y representing the average of the ratio between the absorbance values of different concentrations of pure Y at  $\lambda_{iso}$

( $A_{iso}$ ) to those at  $\lambda_2(A_2)$ .

Absorbance of Y in the mixture at  $\lambda_{iso} = \text{abs}_1 / \text{abs}_2 \times \text{abs } \lambda_2(X+Y)$   
 Absorbance of X in the mixture at  $\lambda_{iso} = \text{abs } \lambda_{iso}(X+Y) - \text{abs}_1 / \text{abs}_2 \times \text{abs } \lambda_2 X+Y$

Where;  $\text{abs}_1, \text{abs}_2$  is the absorbance of pure Y at  $\lambda_{iso}$  and  $\lambda_2$ ;  $\text{abs}_1 / \text{abs}_2$  is called the absorbance factor and  $\text{abs } \lambda_{iso}(X+Y)$  and  $\text{abs } \lambda_2(X+Y)$  are the absorbance of the mixture at these wavelengths ( $\lambda_{iso}, \lambda_2$ ).

The concentration of each X or Y, separately, is calculated using the isoabsorptive point unified regression equation {obtained by plotting the absorbance values of the zero order curves of either X or Y at isoabsorptive point ( $\lambda_{iso}$ ) against their corresponding concentrations X or Y respectively}.

### Theory of Amplitude Modulation (AM) Method

The amplitude modulation method is a novel method of ratio spectra manipulation using a normalized spectrum as a divisor [59], if we have a mixture of X and Y where Y is extended over X and the spectra of X and Y shows isoabsorptive point at the zero spectrums and consequently retained as an isosbestic point at the ratio spectrum.

The absorbance of the zero order absorption spectrum at of mixture of X and Y at isoabsorptive point as follows:

$$[Am] = [aXCX] + [aYCY] \quad (1)$$

Dividing eq (1) with normalized spectrum of Y as a divisor ( $1\mu\text{g}/\text{mL}$ ), to get ratio spectrum with isosbestic point (at the same wavelength of the zero order) so the following equation was obtained:

$$[Am] / [aYCY'] = [aXCX] / [aYCY'] + [aYCY] / [aYCY'] \quad (2)$$

$$[Am] / [aYCY'] = [aXCX] / [aYCY'] + \text{Constant} \quad (3)$$

$$P_m = P_X + P_Y.$$

Where, ( $P_m$ ) is the amplitude of ratio spectrum of the mixture, ( $P_X$ ) is the amplitude of component X and ( $P_Y$ ) is the amplitude of component Y

i.e. the recorded amplitude at isosbestic point of the ratio spectrum is equal to the sum of amplitude corresponding to X and that corresponding to Y.

The amplitude representing the component Y ( $P_Y$ ) was the constant  $[aYCY] / [aYCY']$  and it can be measured directly from the spectrum at the straight line that is parallel to the wavelength axis in the region where Y spectrum is extended.

Since, we use normalized divisor of Y so,  $CY' = 1\mu\text{g}/\text{mL}$

$$P_Y = [aYCY] / [aYCY']$$

$$P_Y = [CY] \quad (4)$$

The recorded amplitude of the constant was modulated to concentration so it was representing the concentration of Y [ $CY$ ], ( $C$  Recorded of Y).

For determination of amplitude of X in the mixture, if we subtract the measured value of the constant from that of the mixture at isosbestic point of the ratio spectrum Eq. (2);

$$P_X = P_m - P_Y$$

$$P_X = \{[aXCX] / [aYCY'] + \text{Constant}\} - \text{Constant} \quad (5)$$

$$P_X = [aXCX] / [aYCY'] \quad (6)$$

At that isosbestic point  $aX = aY$  and normalized divisor of Y  $CY' = 1\mu\text{g}/\text{mL}$

$$P_X = [aXCX] / [aYCY'] \quad (7) \quad P_X = [CX] \quad (8)$$

This obtained amplitude of ratio spectrum was modulated to concentration and it was representing concentration of X [ $CX$ ], ( $C$  Recorded of X).

The corresponding concentration of X or Y could be calculated by using the following regression equation:

$$C \text{ Recorded} = \text{Slope } C + \text{intercept}$$

Slope was found to be approximately one and intercept almost zero

Where;  $C \text{ Recorded}$  represents the recorded amplitudes corresponding to the concentrations of either X or Y that obtained from the ratio spectrum using normalized spectrum of Y ( $1\mu\text{g}/\text{mL}$ ) as a divisor and  $C$  represents the corresponding concentration of X or Y.

### Theory of Extended ratio subtraction (ERS) Method

Extended ratio subtraction (ERS) method starts After the application of the ratio subtraction (RS) method [60-62], which depends on that, if you have a mixture of two drugs (X) and (Y) with overlapping spectra and the spectrum of (Y) is extending than (X), the determination of (X) can be done by dividing the spectrum of the mixture by a certain concentration of (Y) as a divisor ( $Y'$ ). The division will give a new spectrum that represents

$$\frac{X}{Y'} + \text{constant}$$

If we subtract this constant, then multiply the new spectrum obtained after subtraction by ( $Y'$ ) (the divisor), therefore we can obtain the original spectrum of (X)

This can summarized as follows :

$$\frac{X+Y}{Y'} = \frac{X}{Y'} + \frac{Y}{Y'} = \frac{X}{Y'} + \text{constant}$$

$$\frac{X}{Y'} + \text{constant} - \text{constant} = \frac{X}{Y'}$$

$$\frac{X}{Y'} \times Y' = X$$

The constant can be determined directly from the curve by the straight line which is parallel to the wavelength axis in the region where (Y) is extended.

The concentration of X is calculated using the regression equation representing the linear relationship between the absorbance at its  $\lambda_{max}$  versus the corresponding concentration of X.

To determine the second component Y, an extension of the already developed method has been established as a new approach in which Y could be determined by dividing the obtained D0 spectrum of X by a known concentration of X as a divisor X' to get the value of the constant X/X'. Dividing the spectrum of the mixture X+Y by the same divisor X', the division will give a new curve that represents X/X' + Y/X' where X/X' is the previously obtained constant, then multiply the obtained curve after subtraction by X' (the divisor), therefore we can obtain the zero order absorption spectrum D0 of Y (original spectrum of Y).

$$Y/X' + X/X' - X/X' = Y/X' \times X' = Y$$

The concentration of Y is calculated by using the regression equation representing the relationship between the absorbance at its  $\lambda_{max}$  versus the corresponding concentration of Y.

The correct choice of the divisor concentration is fundamental. If the concentration of the divisor is increased or decreased, the resulting constant value will be proportionally decreased or increased.

## Experimental

### Instruments

- SHIMADZU UV-1650 PC, dual beam UV-visible spectrophotometer with two matched 1 cm quartz cells, connected to an IBM compatible personal computer (PC) and an HP-600 inkjet printer. Bundled UV-PC personal spectroscopy software version (2.21) was used to process the absorption and the derivative spectra. The spectral band width was 0.2 nm with wavelength scanning speed of 2800nm min<sup>-1</sup>.

- Ultrasonic processor, sonicator (type USR3/2 907, Julabo Labortechnik, D-7633 Seelbach, West Germany).

- Shimadzu electronic balance (type AGE-220, Shimadzu, Kyoto, Japan)

### Reagents

All reagents and solvents used throughout this work were of analytical pure grade.

- Double distilled water
- Methanol used was of analytical spectroscopic grade (Sigma-Aldrich Co., USA).
- Hydrochloric acid: (E.Merck, Darmstoolt, Germany), 0.01 M aqueous solution.

### Samples

**Pure sample:** Standard MOX certified to contain 99.91 ± 0.752 according to a reported UV spectrophotometric method [13] was kindly supplied by Minapharm, Egypt, under license of Schwarz Pharma, Germany and used without further purification.

Standard HCT certified to contain 100.02 ± 0.400 according to B.P [5] and Eur.P [6] was kindly supplied by Bristol Myers squib and used without further purification.

**Market samples:** (Fempres<sup>®</sup> Plus) produced by (ActavisDeutschland GmbH & Co.KG, Germany, Batch no. 0003599) labeled to contain 15 mg/25mg of Moexipril hydrochloride/Hydrochlorothiazide combination was obtained

### Prepared Solutions

#### Stock standard solutions

A portion equivalent to 100.00 mg of MOX and HCT was accurately weighed and transferred into two separate 100- ml volumetric flasks and dissolved in the minimum amount of methanol, sonicated for 10 minutes and the volume was made up to the mark with the same solvent to give a final concentration of 1.0 mg/ml.

#### Working standard Solutions:

A portion equivalent to 10.0 mg of MOX and HCT was transferred from their standard stock solutions into two separate 100-ml volumetric flasks. The volume was completed to the mark with methanol to give standard working solutions of 100µg/ml.

#### Laboratory-prepared mixtures

Different aliquots of MOX and HCT were accurately transferred from their standard working solutions (100 µg/ml) into a series of 10-ml volumetric flasks and the volume was made up to the mark with 0.01 N HCl to prepare mixtures containing different ratios of the two drugs.

#### Pharmaceutical dosage forms

Twenty tablets were weighed and the average weight was calculated and crushed to a fine powder. An accurately weighed powder sample equivalent to 100 mg of HCT was transferred to a 100 ml volumetric flask, dissolved in 20 ml methanol, shaken for 10 min and the volume was made up to the mark with Methanol. The solution was then filtered by WHATMAN filter paper no 41. The solution was diluted to the same concentrations of working standard solutions (100ug/ml).

## Procedures

### Linearity and construction of calibration curves

**Absorbance Subtraction (AS) method:** Standard solutions containing 1-13 µg/mL of MOX or HCT respectively were prepared separately in 0.01N HCL using their corresponding working standard solutions (100µg/ml). The absorption spectra of the resulting solution were measured and stored in the computer. The absorbance factor of pure HCT at 210 nm and 310 nm [A210 / A310] was calculated, then the concentration of either MOX or HCT, was separately calculated using the unified regression equation representing the absorbance of MOX or HCT at isoabsorptive point 210 nm and the corresponding concentration of MOX or HCT.

**Amplitude Modulation (AM) method:** Standard solutions containing 1-13 µg/mL MOX or HCT were prepared separately in 0.01N HCL using their corresponding working standard solutions (100µg/mL). The absorption spectra of the resulting solution were measured in the range of 200-400 nm. The scanned spectra of MOX or HCT were divided by the normalized absorption spectrum of HCT (1 µg/mL), then the concentration of MOX or HCT, was separately calculated using the unified regression equation representing the amplitudes of ratio spectra of either MOX or HCT at the isosbestic point 210 nm and the corresponding concentration of MOX or HCT.

**Extended Ratio Subtraction Method (ERS) coupled with Ratio Subtraction method (RS):** Aliquots containing (10-90 µg) of MOX and (10-140 µg) of HCT were transferred separately from their working standard solution (100 µg /ml) into a series of 10-ml volumetric flasks then completed to volume with 0.01N HCL; and the spectra of the prepared standard solutions were scanned. A calibration curve for MOX was constructed relating the absorbance at  $\lambda_{max}$  202 nm to the corresponding concentration and the regression equation was computed; and by the same way a calibration curve for HCT was constructed relating the absorbance at  $\lambda_{max}$  274 nm to the corresponding concentration and the regression equation was computed.

Aliquots equivalent to 20 µg of MOX and HCT were transferred separately from their working standard solution (100/ µg ml) into 10-ml volumetric flask and the volume was completed with 0.01 N HCL to be used as a divisors (2 µg/ ml).

**Multivariate spectral analysis:** Different mixtures of MOX and HCT were prepared by diluting different volumes of their working solutions into 10-ml measuring flasks. The volume was completed to the mark with 0.01N HCL to reach the concentrations listed in Table 1. The absorbencies of these mixtures were measured between 200-400 nm at 0.2 nm intervals with respect to 0.01N HCL as blank. The composition of the samples was randomly designed in order to obtain non-correlated concentration profiles and this calibration design prepared to obey Beer's law. Multivariate calibration models (PCR and PLS) were constructed using the data obtained.

## Results and Discussion

### Absorbance Subtraction (AS) method

This method is based on the absorption factor method [57,58] and its use to analysis of isosbestic point present in zero order absorption spectra known as the isoabsorptive point, where the components exhibiting this point have equal absorptivity's. The only requirements of this method (AS) are the existence of isoabsorptive point of both components and the extension of the spectra of one component.

For the determination of MOX and HCT, we will utilize their isoabsorptive point at 210 nm, Figure 3. By the analysis of the recorded absorbance at the isoabsorptive point, the absorbance corresponding to MOX or HCT, separately, at isoabsorptive point 210 nm can be calculated using absorbance factor [abs 210 / abs 310] which is the average of the absorbance of different concentrations of pure HCT using isoabsorptive point at 210 nm to that at 310 nm which shows no contribution of MOX and then the absorbance of MOX can be obtained after subtraction.

Absorbance of HCT in the mixture at  $\lambda$  210 = abs210 / abs310 (absorption factor)  $\times$  abs  $\lambda$  310(MOX+HCT).

Absorbance of MOX in the mixture at  $\lambda$  210= abs $\lambda$  210(MOX+HCT) – abs210/ abs310  $\times$  abs  $\lambda$  310 (MOX+HCT).

Where, abs  $\lambda$  MOX+HCT is the absorbance of the binary mixture at 210 nm or 310 nm and abs210/ abs310 is the absorbance factor of pure HCT at 210 nm to 310 nm and it was calculated and found to be 3.5.

The calculated absorbance value corresponding to MOX and HCT can be separately used to identify each of their concentration using the unified regression equations using isoabsorptive point 210 nm.

The advantage of the absorbance subtraction method (AS) over the conventional isoabsorptive point is that there is no need for

another complementary spectrophotometric method to measure the concentration of one of the two components to get the second by subtraction. The disadvantage of (AS) method is the increased risk of error in calculating the absorbance factor in case of low concentrations of the extended component EZ or its low value of absorbance at extension region.

### Amplitude Modulation (AM) method

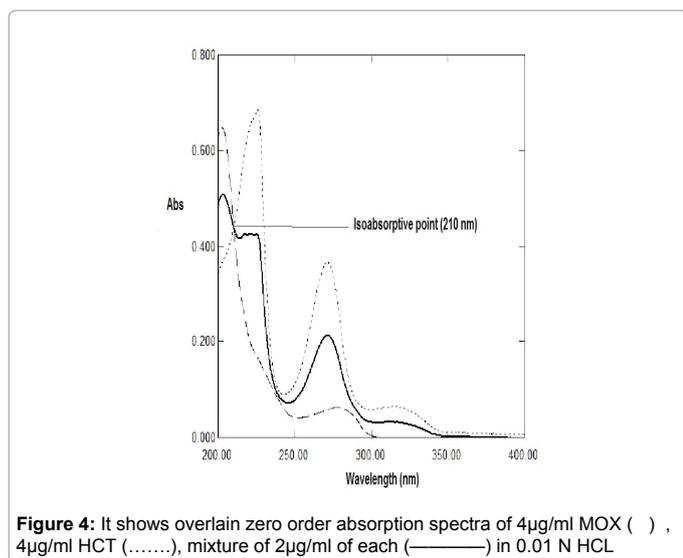
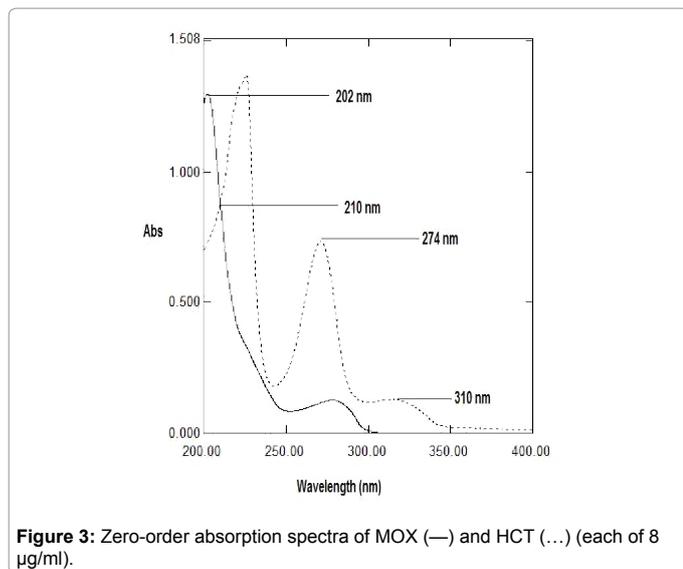
The two main requirements of this method are the existence of isoabsorptive point of both components at zero point and consequently in the ratio spectra, and the extension of the spectra of one component.

The new method is based on two facts [59]; the first that the isosbestic point whenever present in an absorption spectrum will be retained at the same point even after division by a one component as a divisor in the ratio spectrum, while the second that the results of manipulating ratio spectra techniques are greatly affected by the choice of the divisor. So to eliminate the effect of the divisor, we will use the normalized spectrum of HCT (1 µg/mL) (normalized spectrum is prepared mathematically by using sum of different spectra of HCT and divided them by total concentrations of them). Since the two components exhibiting this point have equal absorptivity's, by dividing the spectrum of the binary mixture by the normalized HCT divisor spectrum, we obtain the ratio spectra, Figures 4 and 5.

At the isosbestic point of ratio spectra the amplitude value was modulated to concentration. The amplitude value of the constant can be determined at the plateau region at 310 nm, which is equal to the amplitude constant value of HCT along the whole spectrum. At the isosbestic point ( $\lambda_{iso}$ ) at 210 nm, the amplitude of the ratio spectra at this point will be equal to the sum of the amplitudes of MOX and HCT. After the subtracting recorded amplitude at 210 nm from the previously obtained constant at 310 nm - 315 nm, we get the corresponding recorded amplitude of MOX, which is equivalent to recorded concentration of MOX in the mixture (CRecorded of MOX), while the recorded amplitude of constant value will be directly equal to the recorded concentration of HCT in the mixture (CRecorded of HCT). To eliminate any error due to signal to noise ratio, the actual concentration of MOX or HCT could be calculated by using their

Sample No.	Concentration(µg/ml)	
	MOX	HCT
1	1	1
2	3	5
3	5	3
4	2	4
5	4	2
6	4	12
7	12	4
8	6	10
10	10	6
11	5	5
12	6	12
13	12	6
14	2	10
15	10	2
16	1	10
17	10	1
18	3	6

**Table 1:** The concentrations of different mixtures of MOX and HCT, used in the training set.



corresponding unified regression equation at isosbestic point 210 nm.

$$C_{Recorded} = 1.01 C - 0.0022.$$

Where,  $C_{Recorded}$  is the recorded amplitude of ratio spectrum at 210 nm and  $C$  is the corresponding concentration of MOX or HCT. The advantage of amplitude modulation method over other mathematical techniques utilizing the constant is the reduced manipulation steps and only one divisor is needed in order to determine both components in the mixture. By using the normalized divisor, the results are not affected by the choice of divisor. This method has advantage over the isoabsorptive point at zero order that it measures the concentration of both components with no need for other complementary method to measure one of the components in the mixture. In addition, this method has advantages over the newly developed absorbance subtraction method is that by using the normalized divisor, the obtained amplitude at the ratio spectrum will directly represent the concentration of each component and the risk of error upon the determination of absorbance factor of lower absorbance as well as the manipulation steps will be reduced by elimination of the absorbance factor calculation step. That is why the sensitivity of amplitude modulation method (AM) is better

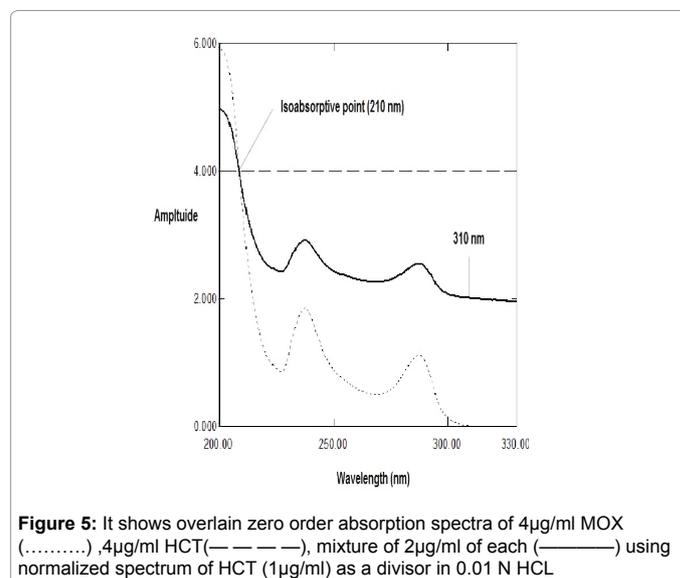
than absorbance subtraction method (AS), as shown in Table 4.

### Extended Ratio Subtraction (ERS) method coupled with Ratio Subtraction (RS) method

Extended ratio subtraction (ERS) method starts after the application of the ratio subtraction (RS) method [60-62]. The (RS) method depends on that, when mixtures of MOX and HCT where the spectrum of HCT is more extended, Figure 6, the determination of MOX in the mixtures can be done by scanning the zero order spectra of the laboratory prepared mixtures (MOX and HCT), dividing them by a carefully chosen concentration of standard HCT' (2 µg/mL) as a divisor producing new ratio spectra which represent MOX / HCT' + constant as shown in Figure 7, then subtraction of the values of these constants HCT / HCT' in the plateau region (310-320nm) as shown in Figure 8, followed by multiplication of the obtained spectra by the divisor HCT' (2 µg/mL) as shown in Figure 9. Finally, the original spectra of MOX can be obtained, Figure 9 which was used for direct estimation of MOX at 202 nm and calculation of the concentration from the corresponding regression equation (obtained by plotting the absorbance values of the zero order curves of MOX at 202 nm against the corresponding concentrations).

The determination of HCT can be done by the extended ratio subtraction by dividing these obtained spectra of MOX by a carefully chosen concentration of standard

MOX' (6 µg/mL) producing ratio spectra represent the constants MOX/MOX' in plateau (200-215 nm) as shown in Figure 10. The previously scanned zero order absorption of the laboratory prepared mixtures (ALP and BENZ) were divided by standard ALP' (60µgmL<sup>-1</sup>) as a divisor producing new ratio spectra which represent HCT / MOX' + constant as shown in Figure 11, Then subtraction of these obtained constants MOX/MOX' as shown in Figure 12, which is followed by multiplication of the obtained spectra by the divisor MOX' (2 µg/ ml) as shown in Figure 13. Finally, the original spectra of HCT in Figure 13 could be obtained which are used for direct determination of HCT at 274 nm and calculation of the concentration from the corresponding regression equation (obtained by plotting the absorbance values of the zero order curves of HCT at 274 nm against the corresponding concentrations).

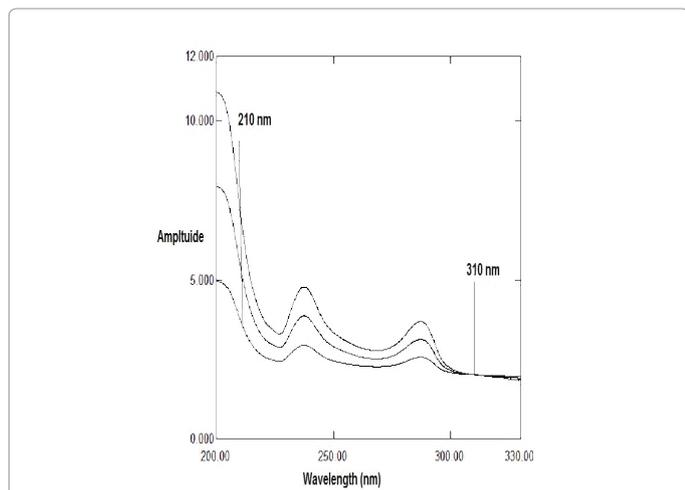


Mixture No.	Taken ( $\mu\text{g/ml}$ )		Found* ( $\mu\text{g/ml}$ )				Recovery %			
	MOX	HCT	MOX		HCT		MOX		HCT	
			PLS	PCR	PLS	PCR	PLS	PCR	PLS	PCR
1**	3	5	3.03	3.02	4.98	4.97	101.00	100.67	99.60	99.40
2	6	12	6.05	6.06	12.05	12.05	100.83	101.00	100.42	100.42
3	12	6	11.96	11.97	6.04	6.06	99.67	99.75	100.67	101.00
4**	6	10	6.04	6.02	10.1	10.08	100.67	100.33	101.00	100.80
5	4	14	3.98	3.97	13.96	13.97	99.50	99.25	99.71	99.79
6	3	6	3.04	3.04	6.02	6.03	101.33	101.33	100.33	100.50
7	5	5	5.05	5.07	4.97	4.98	101.00	101.40	99.40	99.60
8	10	6	9.97	9.97	6.09	6.07	99.70	99.70	101.50	101.17
			MEAN				100.46	100.43	100.33	100.34
			S.D				0.722	0.805	0.729	0.666
			R.S.D %				0.719	0.802	0.727	0.664

\*Average of three determinations

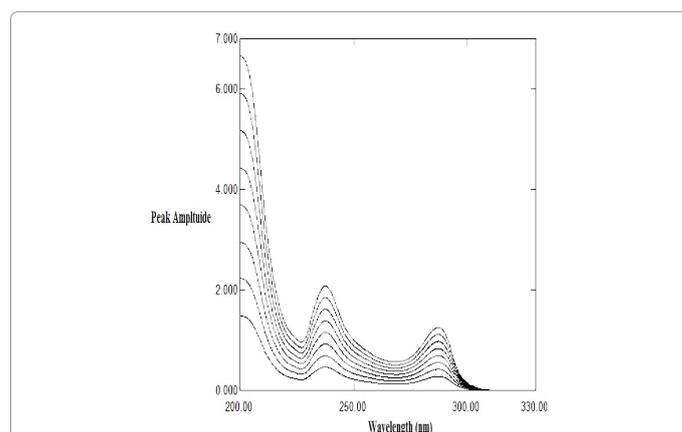
\*\* Corresponding to their ratio in pharmaceutical formulation

**Table 2:** Results obtained for the analysis of binary mixtures of MOX and (validation set) by the multivariate spectral analysis methods.

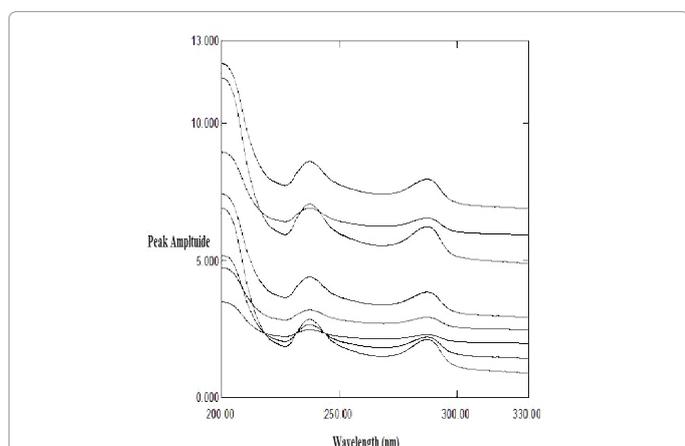


**Figure 6:** It shows divisions spectra of laboratory prepared mixtures of  $2\mu\text{g/ml}$ ,  $4\mu\text{g/ml}$ ,  $6\mu\text{g/ml}$  MOX with  $2\mu\text{g/ml}$  HCT using normalized spectrum of HCT ( $1\mu\text{g/ml}$ ) as a divisor in  $0.01\text{N HCL}$  showing two wavelength ( $210\text{ nm}$ ) and ( $310\text{ nm}$ )

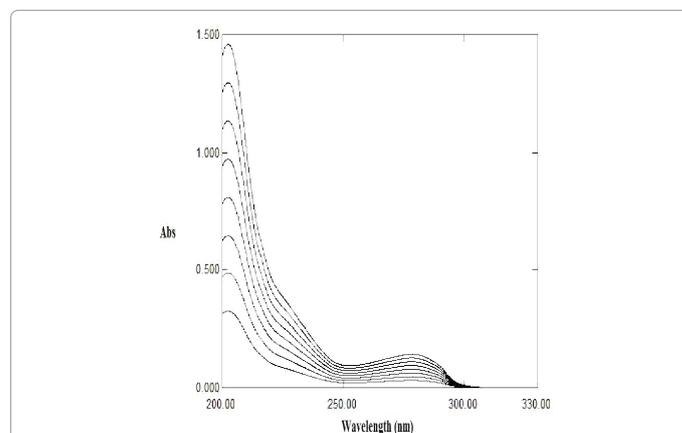
could not be achieved by the previously established ratio subtraction method [60] which had determined unextended drug only. Therefore, the two methods are considered to be complementary to each



**Figure 8:** Division spectra of laboratory prepared mixtures of MOX (X) and HCT (Y) using  $2\mu\text{g ml}$  of HCT (Y') as a divisor and  $0.01\text{N HCL}$  as a blank. After subtraction of the constant

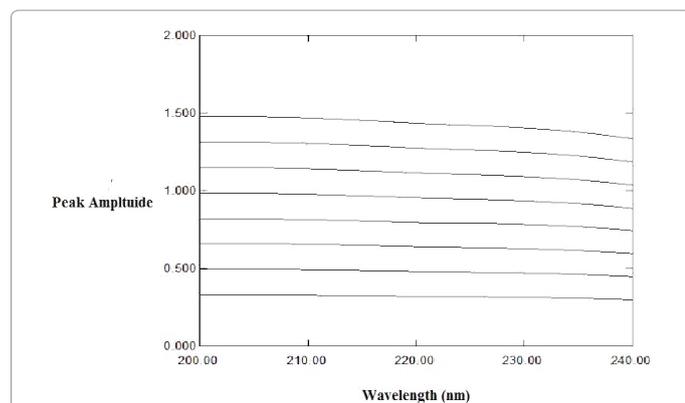


**Figure 7:** Division spectra of laboratory prepared mixtures of MOX (X) and HCT (Y) using  $2\mu\text{g/ml}$  of HCT (Y') as a divisor and  $0.01\text{N HCL}$  as a blank.

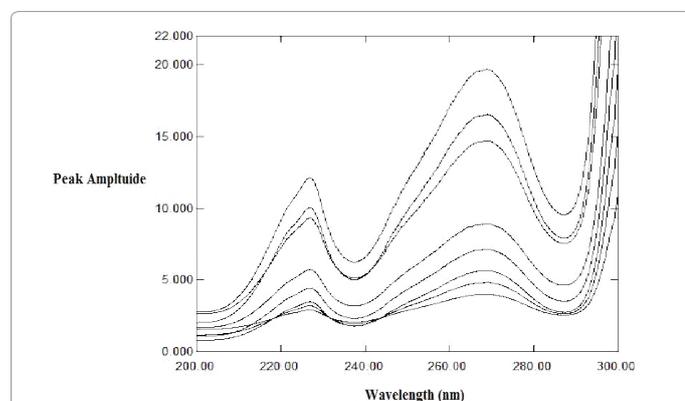


**Figure 9:** The zero order absorption spectra of MOX (X) obtained by the proposed Ratio Subtraction method for the analysis of laboratory prepared mixtures after multiplication by the divisor (Y').

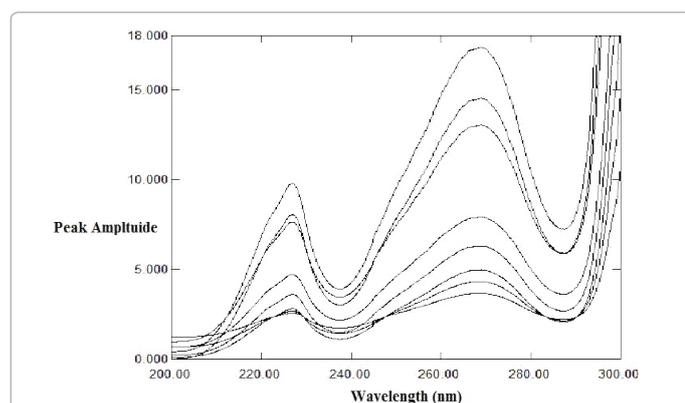
The extended ratio subtraction method has an advantage that the extended drug in the mixture could be determined at its  $\lambda_{\text{max}}$  which



**Figure 10:** Ratio spectra of obtained spectra of MOX using 6 µg/ml of MOX' as a divisor and 0.01N HCL as a blank



**Figure 11:** Division spectra of laboratory prepared mixtures of MOX (X) and HCT (Y) using 6 µg/ ml of MOX (X') as a divisor and 0.01N HCL as a blank



**Figure 12:** Division spectra of laboratory prepared mixtures of MOX (X) and HCT (Y) using 6 µg/ ml of MOX (X') as a divisor and 0.01N HCL as a blank. After subtraction of the constant

other since the two components of interest in the mixture could be determined.

### Multivariate Spectral Analysis Methods

Partial least-squares (PLS) and principal component regression (PCR) techniques are typical full-spectrum methods in which the data are fit to many data points. In these techniques, a calibration model is suggested, validated, and then used for the prediction of unknown

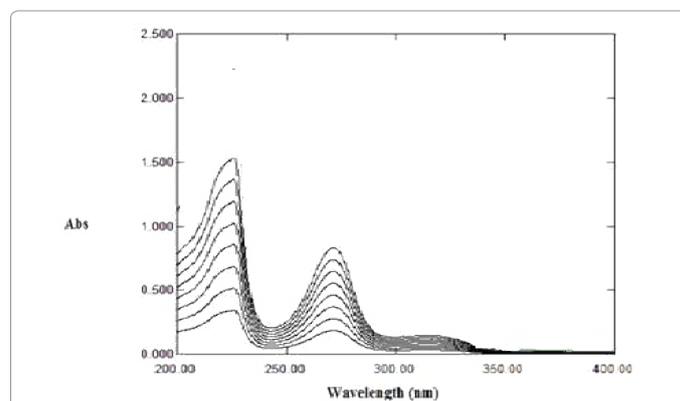
samples.

The first step in the simultaneous determination of the two drugs by multivariate calibration methods involves constructing the calibration matrix for binary mixture. The calibration set was obtained by using the absorption spectra of a set of 18 mixtures of MOX and HCT with different ratios of each component. Initial developed models were found to have high spectral residuals in the region from 200 to 230 nm and no absorbance has been detected at region 350 to 400 nm, so these regions were rejected.

PCR and PLS are based on factor analysis. Calibration is then performed using a subset of these factors instead of the original variables. Both PCR and PLS have the signal averaging advantage of full-spectrum techniques, while they do not need the prior knowledge of the characteristics of all interfering substances [63,64].

Another advantage of PCR and PLS is the transformation of the numerous and probably correlated original variables into a smaller number of orthogonal variables (latent vectors). The first latent vectors contain the information carried in the manifest variables, where the last ones represent noise, which can be filtered away and will not be considered in the modeling [64].

Selection of the optimum number of factors for PLS and PCR techniques is a very important step before constructing the models because, if the number of factors retained is more than required, more noise will be added to the data. On the other hand, if the number retained is too small, meaningful data that could be necessary for the calibration may be discarded. To select the number of factors for the PLS and PCR methods, a cross-validation method was used in which one sample is left out in turn [65] Given a set of 18 calibration samples,



**Figure 13:** The zero order absorption spectra of HCT (Y) obtained by the proposed Extended Ratio Subtraction method for the analysis of laboratory prepared mixtures after multiplication by the divisor (X')

Parameter	Method	MOX	HCT
RMSEP	PLS	0.039	0.055
	PCR	0.041	0.051
Correlation coefficient	PLS	0.9999	0.9998
	PCR	0.9999	0.9998
Slope	PLS	0.9923	0.9993
	PCR	0.9935	0.9999
Intercept	PLS	0.0579	0.0327
	PCR	0.0532	0.0226

**Table 3:** RMSEP and statistical parameter values of MOX and HCT prediction using multivariate spectral analysis methods

Parameters	Proposed methods					
	ASM		AMM		EXRSM	
	MOX	HCT	MOX	HCT	MOX	HCT
$\lambda$ nm	210 nm, 310 nm		210 nm, 310 nm		202 nm	274 nm
<b>Linearity</b>						
Beers law range	1-13 $\mu$ g/ml	1-13 $\mu$ g/ml	1-13 $\mu$ g/ml	1-13 $\mu$ g/ml	1-9 $\mu$ g/ml	1-14 $\mu$ g/ml
Regression equation	Y = 0.1103X	Y= 0.1103X +	Y = 1.01X-	Y = 1.01X-	Y = 0.1622 X +	Y = 0.0934 X -
	+ 0.0015	0.0015	0.0022	0.0022	0.0036	0.0051
Correlation coefficient (r)	0.9999		0.9999		0.9998	0.9998
Accuracy(Mean $\pm$ S.D)	99.98	100.20	99.92	100.09	100.10	99.91
	$\pm$ 0.526	$\pm$ 0.563	$\pm$ 0.519	$\pm$ 0.529	$\pm$ 0.706	$\pm$ 0.670
LOD	0.185	0.185	0.158	0.158	0.167	0.233
LOQ	0.560	0.560	0.479	0.479	0.505	0.706
<b>Precision (R.S.D %)</b>						
Repeatability <sup>a</sup>	0.502	0.353	0.408	0.398	0.443	0.348
Intermediate precision <sup>b</sup>	0.598	0.788	0.698	0.639	0.867	0.472
<b>Specificity and selectivity</b>						
(Mean $\pm$ S.D)	99.92	99.68	100.29	100.35	100.35	99.81
	$\pm$ 1.01	$\pm$ 0.922	$\pm$ 0.819	$\pm$ 0.724	$\pm$ 0.981	$\pm$ 0.872

<sup>a, b</sup>Intraday and interday, relative standard deviations for determination of samples of ( 6,8 and 9  $\mu$ g/ml ) and ( 10,12 and 13  $\mu$ g/ml ) of MOX and HCT respectively.

**Table 4:** Validation of the results obtained by applying the proposed methods for the analysis of MOX and HCT

MIX NO	Mix Ratio	Taken ( $\mu$ g/ml)		Absorbance		Found* recovery %		Extended Ratio	
		MOX	HCT	Subtraction		Amplitude Modulation		Subtraction	
				MOX	HCT	MOX	HCT	MOX	HCT
1	1:1	7	7	100.43	100.57	99.71	100.43	99.43	100.57
2	1:1.67**	3	5	99.00	99.60	99.00	99.60	101.67	99.40
3	1.67:1	5	3	99.20	99.00	100.80	101.33	99.20	101.00
4	1:2	4	8	99.25	100.5	100.75	100.50	101.00	100.75
5	2:1	8	4	100.50	98.75	100.63	100.75	99.63	99.25
6	1:3	2	6	102.00	101.00	101.50	100.67	101.50	99.33
7	3:1	6	2	99.50	98.50	99.50	99.00	99.67	98.50
8	1:1.44	9	13	99.44	99.54	100.44	100.54	100.67	99.69
	<b>Mean</b>			99.92	99.68	100.29	100.35	100.35	99.81
	<b>S.D</b>			1.010	0.922	0.819	0.724	0.981	0.872
	<b>R.S.D %</b>			1.011	0.925	0.817	0.721	0.978	0.874

\*Average of three determinations.

\*\* Corresponding to their ratio in pharmaceutical formulation

**Table 5:** Determination of the studied drugs in the laboratory prepared mixtures by the proposed methods

the PLS and PCR calibrations were performed on 17 samples, then the concentration of the sample left out during calibration was predicted. This process was repeated 18 times, until each calibration sample had been left out once.

The predicted concentrations were then compared with the known concentrations using RMSEC (root mean square error of calibration). The optimum number of factors was selected according to the method of Haaland and Thomas [66,67]. The maximum number of factors used to calculate the optimum RMSEC was selected to be 9 (half the number of samples) [68]. Figures 14 and 15 show the RMSEC plot of the cross-validation results of the training set as a function of the number of factors used to construct the calibration. The optimum

number of factors used for building PLS and PCR models was found to be two. To validate the prediction ability of the suggested models, they were used to predict the concentration of (I) and (II) in laboratory-prepared mixtures containing different ratios of the components and satisfactory results were obtained Table 2. Another diagnostic test was performed by plotting the concentration residuals against the predicted concentrations. The residuals appear randomly distributed around zero, indicating adequate models.

The RMSEP was used as a diagnostic test for examining the error in the predicted concentrations. Table 3 shows the RMSEP that were obtained; they are satisfactory and indicate good predictive abilities of the developed models.

Pharmaceutical preparation	Absorbance		Amplitude		Extended Ratio		Chemometry			
	Subtraction		Modulation		Subtraction		MOX		HCT	
	MOX	HCT	MOX	HCT	MOX	HCT	PLS	PCR	PLS	PCR
Fempress® tablets <sup>a</sup> (Contain 25 MG MOX+15 mg HCT) B.N 100251	99.41 ± 1.073	100.47 ± 0.880	99.76 ± 0.922	100.27 ± 0.809	99.20 ± 0.984	99.99 ± 0.936	100.15 ± 1.101	100.2 ± 1.168	100.47 ± 1.086	100.41 ± 1.027
Standard addition <sup>b</sup> technique Mean ± S.D	100.92 ± 1.283	99.92 ± 1.233	100.54 ± 1.162	99.33 ± 1.041	99.39 ± 1.136	100.75 ± 1.090	101.04 ± 1.249	101.08 ± 1.377	101.83 ± 1.258	101.75 ± 1.090

a Average of five determinations.

b Average of three determinations.

**Table 6:** Determination of MOX and HCT in their pharmaceutical formulation and application of standard addition technique

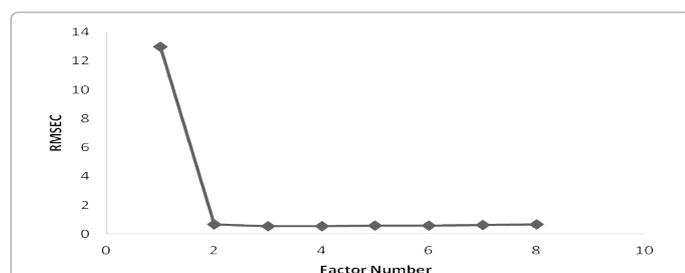
item	Absorbance Subtraction		Amplitude Modulation		Extended Ratio Subtraction		Chemometric				Reported** method	
	MOX	HCT	MOX	HCT	MOX	HCT	MOX		HCT		MOX	HCT
Drug							PLS	PCR	PLS	PCR		
Mean	99.98	100.20	99.92	100.09	100.10	99.91	100.46	100.43	100.33	100.34	99.37	100.83
S.D	0.526	0.563	0.519	0.529	0.706	0.670	0.722	0.805	0.729	0.666	1.245	1.321
R.S.D%	0.526	0.562	0.519	0.529	0.705	0.671	0.719	0.802	0.727	0.664	1.253	1.310
n	5	5	5	5	5	5	8	8	8	8	5	5
Variance	0.277	0.317	0.269	0.280	0.498	0.449	0.521	0.648	0.531	0.444	1.550	1.745
Student's t-test *	1.01 (2.306)*	0.981 (2.306)	0.912 (2.306)	1.164 (2.306)	1.141 (2.306)	1.390 (2.306)	1.781 (2.201)	1.696 (2.201)	0.776 (2.201)	0.770 (2.201)	-	-
F-value*	5.596 (6.39)*	5.505 (6.39)	5.762 (6.39)	6.232 (6.39)	3.112 (6.39)	3.886 (6.39)	2.975 (6.09)*	2.392 (6.09)	3.286 (6.09)	3.930 (6.09)	-	-

\*The values in parenthesis are corresponding to the theoretical values of t and F at (p = 0.05).

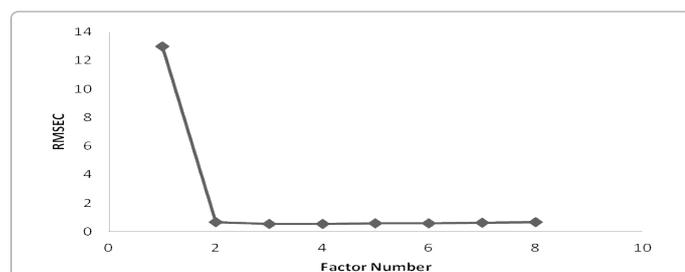
\*\*Reported method (RP-HPLC) (54) using a mobile phase of 20mM phosphate buffer (PH 4.0) and acetonitrile in a ratio of (50:50v/v)

with a flow rate of 1 ml/min. and UV detection at 212 nm.

**Table 7:** Statistical analysis of the results obtained by the proposed method and the reported method for the analysis of pure MOX and HCT



**Figure 14:** RMSEC plot of cross validation results of the training set as a function of the number of principle components used to construct the PLS calibration model.



**Figure 15:** RMSEC plot of cross validation results of the training set as a function of the number of principle components used to construct the PLS calibration model.

Plotting the known concentrations against the predicted concentrations was also performed to evaluate the predictive abilities of the models.

The corresponding concentrations ranges and calibration equations were listed in Table 4 for the proposed AS, AM and EXR methods and in Table 3 for (PLS and PCR).

The selectivity of the proposed methods was assessed by the analysis of laboratory prepared mixtures containing different ratios of the cited drugs, where satisfactory results were obtained as shown in Table 5 for the proposed AS, AM and EXR methods and in Table 2 for (PLS and PCR).

The proposed procedures were also applied for the determination of Fempress tablet; and the validity of the proposed methods was further assessed by applying the standard addition technique shown in Table 6.

### Method validation

Validation of the methods has been carried out according to ICH guidelines [69] as shown in Table 4 for (AS, AM and EXR) and Table 2 for (PLS and PCR). The data showed that the methods were accurate, precise and specific over the specified range.

### Statistical analysis

Statistical comparison of the results obtained by the proposed

methods and reported method [54] was shown in Table 7. The calculated t and F values were less than the theoretical ones indicating that there was no significant difference between the proposed and the reported method with respect to accuracy and precision.

## Conclusion

As a final conclusion, this study introduces five different methods for determination of MOX and HCT in their binary mixtures. The methods are simple, sensitive and selective could be easily applied in quality control laboratories for the routine analysis of cited drugs in their available dosage forms as it shows good accuracy and precision compared to the reported HPLC method [54], in contrast it is of lower cost. Furthermore, the proposed absorbance subtraction and amplitude modulation methods can be applied for determination of both components in the binary mixtures using a unified regression equation. Moreover the extended ratio subtraction method could determine the extended spectrum which can't be determined by the ordinary ratio subtraction method. On the other hand Partial least-squares (PLS) and principal component regression (PCR) techniques are typical full-spectrum methods in which the data are fit to many data points. All The proposed methods are also suitable and valid for application in laboratories lacking liquid chromatographic instruments.

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