Current Science International 3(4): 352-380, 2014
ISSN: 2077-4435

Application of Double Divisor Ratio Spectra Derivative Spectrophotometric [DDRS-DS], Chemometric and Chromatographic Methods for Stability Indicating Determination of Moexipril hydrochloride and Hydrochlorothiazide

[1]Engy Shokry, [2]Ahmed Emad El-Gendy, [3]Mohamed Abdel Kawy and [3]Maha Hegazy

[1]Faculty of Pharmacy, Future University in Egypt, Cairo, Egypt
[2]Faculty of Pharmacy, Misr International University, Cairo, Egypt
[3]Faculty of Pharmacy, Cairo University, Cairo, Egypt

ABSTRACT

Introduction: Moexipril hydrochloride [Mox.HCl] and Hydrochlorothiazide [HCTZ] are administered as a drug combination used in treatment of hypertension marketed under the name Uniretic®. Stability studies confirm that both actives are liable to hydrolysis yielding moxprilat [Mox-at] and 4-amino-6-chlorobenzene-1, 3 disulphonamide [DSA] respectively. Aim: To analyse a quaternary mixture of these two active ingredients of the pharmaceutical preparation simultaneously in presence of their two process degradates spectrophotometrically. Materials and Methods: In this paper, several techniques were investigated for the analysis of these actives in this complex mixture, the first is double divisor ratio derivative spectrophotometry [DDRS-DS] based on the use of transformed signals of ratio spectra of the studied drugs obtained by using double divisor. Also, three chemometric assisted techniques CLS, PCR and PLS-2 were applied. These chemometric calibrations were constructed by measuring the absorbance at full spectral points in the wavelength range of 200-350 nm for a training set composed of [2.0-6.0 $\mu g.ml^{-1}$] of the drugs and their degradates. The third method is based on the chromatographic separation on a cyanopropyl column using a mobile phase of 0.01 M aqueous potassium dihydrogen phosphate containing 0.1% triethylamine PH 6.0 with orthophosphoric acid, acetonitrile and methanol in a ratio of [70: 20: 10] , a flow rate of 1 ml/min and UV detection at 210 nm. Results: The proposed methods were validated with regard to accuracy, precision, selectivity, robustness and were successfully applied to pharmaceutical preparation with acceptable results. Conclusion: All the presented methods were found successful in the determination of the proposed drugs in presence of their degradation products.

Key words: DDRS-DS; chemometrics assisted techniques; Moexipril hydrochloride; Hydrochlorothiazide

Introduction

Mox and HCTZ are administered as a drug combination used in treatment of hypertension marketed under the name Uniretic®. Stability studies confirm that Mox is liable to hydrolysis yielding Mox-at and it was successfully determined in presence of its degradate by colorimetric methods (El Shanawane *et al.*, 2008), electrochemical methods, employing polymer membrane sensors (Belal *et al.*, 2009) and chromatographic methods namely GC (Hammes *et al.*, 1995) and HPLC (El Shanawane *et al.*, 2008; Kalász *et al.*, 2007; Kóti *et al.*, 2006). On the other hand spectrophotometric and HPLC methods were applied for determination of HCTZ in presence of its degradate [DSA] (Bebawy *et al.*, 1997; Brigante *et al.*, 2005) or in its pharmaceutical drug combination with other drugs (Erk *et al.*, 1999; Kartal *et al.*, 1999; Luis *et al.*, 2001; Martin *et al.,* 1997; Martin *et al.*, 1998; Panderi, 1999; Prasad *et al.,* 1997; Prasad *et al.,* 1998; Ulvi *et al.*, 1998; Yazbi *et al.,* 1999). Several methods have been also presented for the analysis of pharmaceutical preparations containing, mixtures of Mox and HCTZ (El Shanawane *et al.*, 2008; Sidika *et al.*, 2003).

Regarding the analysis of a quaternary mixture of these two active ingredients of the pharmaceutical preparation simultaneously in presence of their two process degradates spectrophotometrically is considered a challenge due to the complexity of the mixture and its almost completely overlapping spectra which cannot be resolved by conventional UV, derivative, ratio derivative spectrophotometry.

Three methods were proposed for the analysis of this complex mixture, the first is based on a combination of a double divisor, ratio derivative and a zero crossing method. The method was initially proposed by Dinc et al. as a new technique for simultaneous determination of three compounds in ternary mixtures (Dinç *et al.*, 1999). It is based on the use of the derivative of the ratio spectra obtained by dividing the absorption spectrum of the ternary or the quaternary mixture by a standard spectrum of a mixture of two or three components of the title mixture. The concentrations of the compounds in the title mixture are determined by using their respective calibration graphs that were obtained by measuring the amplitude at either a maximum or minimum wavelength selected.

In this method to determine species A, the absorption spectra of the mixture containing A, B and C were divided by the sum of the spectra B and C as double divisor and the ratio spectra were obtained. First derivative of the ratio spectra were calculated. The amplitudes measured at selected wavelengths λI nm and C_c^o [C^o is standard concentration], but were independent of the concentration values C_b and C_c in the ternary mixture. The

Corresponding Author: Engy Shokry, Pharmaceutical Chemistry Dept., Faculty of Pharmacy, Future University, Cairo, Egypt
E-mail: engy.shoukry@fue.edu.eg

mathematical expression of this procedure is shown in the following equation (Youssef *et al.*, 2008; Markopoulou *et al.*, 2005) :

$$\frac{d}{d\lambda}\left[\frac{\text{Aternary mix.}, \lambda i}{[\alpha\alpha B\lambda i + \beta c, \lambda i]\, C^{o}c}\right] = \frac{d}{d\lambda}\left[\frac{\gamma_A\, \lambda}{\alpha B, \lambda i + \beta c, \lambda i}\right]\frac{CA}{C^{o}c}$$

Where the amplitudes measured, $d/d\lambda$ $[A_{\text{ternary mix, }\lambda i}/_{[\alpha B,\ \lambda I\ +\ \beta c,\lambda i]}\ C^{o}_{c}]$ were drawn as a graph, versus concentration of species A, and a straight line was obtained. By using the calibration graph, one could determine the A species in the mixture of A, B and C where $\gamma_{A,\lambda I}$, $\alpha_{B,\lambda I}$ and $B_{c,\ \lambda I}$ are the molar absorbitivies of the species A, B and C respectively.

Multivariate statistical methods have been also applied in quantitative spectral analysis because of inclusion of multiple spectral intensities where by applying multivariate calibrations, models can be developed that relate the multiple spectral intensities from many calibration samples, these models can be used in the multivariate prediction analysis of spectra of unknown samples to rapidly predict analyte concentrations. From these multivariate calibration models, three models were chosen and successfully applied for the analysis of this mixture which are: classical least squares [CLS], principal component regression [PCR] and partial least squares [PLS].

The last method applied was the chromatographic separation which was considered rather difficult in this case because Mox, HCTZ and their degradates is considered a complex mixture of extremely different physicochemical properties. The cyanochemistry of cyanocolumns provides different selectivity from normal phase, phenyl and standard aliphatic [C_{18}, C_8, C_4] reversed phases where having both polar and hydrophobic properties provides combinations of weak hydrophobic interaction and polar interactions which enables successful separation even of complex mixtures which can be only separated using gradient elution.

Experimental

Instrumentation

A Shimadzu UV 1650 double beam spectrophotometer connected to a computer loaded with Shimadzu software UV probe 2.10 was used [Hiroshima, Japan]. UV spectra were recorded using a 1 cm quartz cell; the scan range was 200-350 nm with 0.2 nm intervals.

A 6410 triple quadrupole LC/MS unit equipped with binary pump, degasser, automatic injector, high energy conversion dynode and removable electron multiplier horn detector from Agilent technologies, USA. The chromatographic column from GL Sciences, Inc., USA was inertsil ODS-3® [4.6 x 50 mm i.d., 5µm particle size]. Electrospray with positive ionization mode [ESI] was used with a fragmentor voltage of 135 and collision energy of zero. Data acquisition was performed on Agilent LC chemstation software.

An HPLC unit equipped with a 20 µl loop injector and an UV detector from Agilent technologies, USA. The chromatographic column from Merck KGaA, Darmstadt, Germany was lichrospher® 100 CN [4x250 mm i.d., 5µm particle size]. Data acquisition was performed on Agilent LC chemstation software. All determinations were performed at ambient temperature.

Samples and reagents

Samples:

Raw materials:

- Moexipiril hydrochloride certified to contain 99.60% by the manufacturer method was kindly supplied by Minapharm, Egypt.
- HCTZ certified to contain 99.50% by the manufacturer method was kindly supplied by Bristol Myers squib.
- 4-amino-6-chlorobenzene-1, 3 disulphonamide [DSA] certified to contain 99.25% by the manufacturer method was supplied by Sigma-Aldrich chemie GmbH, Germany.

Market Samples:

A commercial pharmaceutical formulation [Fempress® plus] produced by [actavis Deutschland GmbH and Co.Kg, Germany, Batch no. 0003599], each tablet is labeled to contain 15 mg/25 mg of moexipiril hydrochloride/ HCTZ combination was obtained.

Reagents:

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

- Methyl alcohol used was of analytical spectroscopic grade [Sigma-Aldrich Co., USA].
- Sodium hydroxide: [E. Merck, Durmstoolt, Germany], 3.0 M aqeous solution.
- Hydrochloric acid: [BDH], 0.01 and 3.0 M aqeous solution.
- Acetonitrile and methanol used were HPLC grade [Sigma-Aldrich Co., USA.
- Potassium dihydrogen phosphate [BDH, Poole; UK], triethylamine [BDH], orthophosphoric acid [BDH] used were analytical grade.
- Distilled water was used throughout the whole work.

Standard solutions
Standard stock solutions:
Standard stock solutions of Mox.HCl, HCTZ and DSA:

Accurately weigh a portion equivalent to 100.0 mg of Mox.HCl, HCTZ and DSA, transfer into separate 100 ml volumetric flasks, dissolve in the minimum amount of methanol, sonicate for 10 minutes and make up the volume to the mark with the same solvent to give stock solutions of concentration 1.0 $mg.ml^{-1}$.

Standard stock solution of Moexipril alkaline degradate [Mox-at]

Mix a portion equivalent to 100.00 mg of Mox.HCl with 3.0 M NaOH, heat under reflux for 2 hours at 100°C, then cool the solution and neutralize with a calculated volume of 3.0 M HCl. Filter the obtained precipitate, wash, transfer quantitatively into 100 ml volumetric flask and dissolve in the least amount of methanol. Then make up the volume to the mark with distilled water to give a standard stock solution of concentration 1.0 $mg.ml^{-1}$.

Standard working solutions of Mox.HCl, Mox-at, HCTZ and DSA

Accurately transfer a portion equivalent to 10.0 mg of Mox.HCl, Mox-at, HCTZ and DSA from their standard stock solutions into four separate 100 ml volumetric flasks. Make up the volume to the mark with methanol to give standard working solutions of the studied compounds of 100.0 $\mu g.ml^{-1}$.

Procedure

Spectrophotometric method
Scanning of the absorption spectra of Mox.HCl, Mox-at, HCTZ and DSA:

Accurately transfer an aliquot equivalent to [10.0 µg] of Mox.HCl, Mox-at, HCTZ and DSA from their working standard solutions of concentration [100.0 $\mu g.ml^{-1}$] separately into four 10 ml volumetric flasks respectively, complete to the mark with 0.01 M HCl. Scan the absorption spectrum of each solution over the range of [200-350 nm] against blank, similarly prepared without the drug.

Establishment and selection of double divisor:

Prepare two separate sets of ternary mixtures containing equal portions of [Mox, Mox-at , DSA] and [HCTZ, DSA, Mox-at] and scan over the range of [200-350 nm] for proper selection of the double divisors for the estimation of Mox and HCTZ content in synthetic mixtures of the four components.

Chemometric method
Experimental design of the training set

In order to obtain a suitable calibration set; systemic experimental design was used, this work employs a multilevel partial factorial design for five concentration levels [1-5] to study a mixture, in order to obtain non correlated concentration profiles, and this calibration design was prepared to obey beer's law.

Prepare a calibration set of 25 laboratory mixtures of [Mox, Mox-at, HCTZ and DSA] with different concentration of each component prepared in 0.01 M HCl in the range of [2.0-6.0μgml^{-1}] for each of Mox, HCTZ and each of the degradation products. Prepare the calibration sets by diluting different volumes of the standard working solutions of Mox, HCTZ and their degradation products of concentration 100.0 μgml^{-1} to the mark in 10-ml volumetric flasks, and complete the to the mark with 0.01M HCl to reach concentration listed in table [12]. Record the UV absorption spectra over the wavelength range of 200-350 nm. The data points of spectra were collected every 0.2 nm.

Chromatographic method

Isocratic elution technique was utilized with the column maintained at room temperature. The mobile phase used was a mixture of 10 mM potassium dihydrogen phosphate containing 0.1% triethyl amine adjusted at PH [6.0] using orthophosphoric acid, acetonitrile and methanol in a ratio [70: 20: 10].

The mobile phase was filtered through a 0.45 µm membrane filtration system [Millipore Corp., Milford, MA, USA] to remove any particulate matter then degassed by sonication for 20 minutes. The flow rate was 1 ml/min. samples of 20 µl, were injected onto the column and the detector was set at 210 nm.

All the chromatographic determinations were performed 3 times at ambient temperature.

Method validation
Spectrophotometric method:
Linearity:

Accurately transfer different aliquots equivalent to [2.0 – 12.0 µg] of Mox.HCl and [2.0 – 14.0 µg] of HCTZ from their working standard solutions of concentration [100.0 µg.ml-1] into two separate sets of 10.0 ml volumetric flasks and dilute to the volume with 0.01 M HCl. Scan the zero order absorption spectra using 0.01 M HCl as a blank. Also from the working standard solutions of Mox.HCl, Mox-at, HCTZ and DSA, prepare two ternary mixtures: [Mox-at, HCTZ and DSA] and [Mox, Mox-at and DSA], each of 10.0 µg.ml-1 and scan over the range of 200-350 nm. Divide the zero order absorption spectra of Mox.HCl by the absorption spectrum of the ternary mixture [Mox-at, HCTZ and DSA] then compute the first derivative of the obtained ratio spectra using $\Delta\lambda$ = 16.0 nm and scaling factor 100.0. Record the amplitudes at 301.8 nm.

Also, divide the zero order absorption spectra of HCTZ were by the absorption spectrum of the ternary mixture [Mox.HCl, Mox-at and DSA] then compute the first derivative of the obtained ratio spectra using $\Delta\lambda$= 16.0 nm and scaling factor 100.0. Record the amplitudes at 284.2 nm.

Construct the calibration curves of Mox.HCl and HCTZ over the range of [2.0 – 12.0 $\mu g.ml^{-1}$] and [2.0 - 14.0 $\mu g.ml^{-1}$] respectively.

Analysis of laboratory prepared mixture

Record the absorption spectra of different laboratory prepared mixtures containing different ratios of the four components were and divide by the absorption spectrum of ternary mixture [Mox alkaline degradate, HCTZ, DSA] used as a divisor then compute the first derivative of the obtained ratio spectra. Use the amplitudes recorded at 301.8 nm for determination of the Mox content in the prepared mixtures by substitution in the corresponding regression equations.

To determine HCTZ content in the same mixtures, divide the spectra of the synthetic mixtures by absorption spectrum of the ternary mixture [Mox, Mox-at, DSA] used as a divisor then compute the first derivative of the obtained ratio spectra.Then determine the HCTZ content from the corresponding regression equation by measuring the amplitudes at λ = 284.2 nm.

Chemometric method

Construction of the multivariate models:

Construction of the CLS model:

Feed the computer with the absorbance of the zero order absorption spectra and their concentration matrices. Carry out calculations to obtain the "K" matrix [absorbitivity matrix].

Construction of the PCR and PLS models:

Import recorded absorbance spectra stored in excel sheets into Unscrambler® ver. 9.8 as ASC II data and collect in a matrix [751 x 25] by the means of a spread sheet program [Quattro Pro V. 3.0, Borland International Inc., CA] and treat the concentrations of the components of the 25 mixtures of the training set in the same way to obtain a concentration matrix [25x5], then transpose the matrix of spectra, with each column of the spread sheet containing the absorptions of a spectrum to an orientation in rows [each spectrum in a row] to obtain a spectral matrix of new dimensions [25x751].

Apply Savitsky Golay smoothing for reprocessing of data in the absorbance matrix using 41 smoothing points and polynomial order = 1 [this is done using toolbox "modify→ transform → smoothing".

The absorbance at each wavelength of the absorbance matrix was mean centered. No scaling was carried out on these variables.

Regression with PCR and PLS-1 algorithm for wavelengths from 200-350 nm. The PLS and PCR calibration models relate the frequency data through a smaller set of variables, the so called latent factors or principal components [PCs].

Selection of the optimum number of factors in PCR and PLS

Determining how many factors to be used in the calibration is a key step in factor based techniques like PLS and PCR. Only those factors that contain analytical information must be kept, the discarded factors should contain only noise .

Various criteria have been developed to select the optimum number of factors. Several validation methods can be used of:

For CLS model

Since sufficient calibration samples are available, split the data into two halves, one for calibration and one for calibration validation, thus 12 samples are used for calibration and 13 samples out of the 25 samples of the training set are used as a validation set.

For PCR and PLS-2

Carry out the validation using a test set of 8 samples containing different concentrations of Mox.HCl, HCTZ and their degradation products randomly selected from the training set using unscrambler® ver. 9.8.

Predictive abilities of the proposed models

Several diagnostic tools to evaluate the predictive abilities of the suggested chemometric methods.

Predicted concentrations versus true concentration plot [Model and sample diagnostic]

Plot the predicted concentrations of the validation samples against the true concentration values. This plot is used to determine whether the model accounts for the concentration variation in the validation set. Plots are expected to fall on a straight line with a slope of one and zero intercept.

Residual concentrations versus predicted concentrations plot [model and sample diagnostics]

This tool is used to determine whether the model accounts for concentration variation in the validation set and it also, provide information about how well the method will predict future samples. The residuals [difference between known and predicted concentrations for the validation samples.

Statistical analysis of the proposed models

The predictive ability of a model can be defined using several validation diagnostics. These include the standard error of prediction [SEP], the mean squared error of prediction [MSEP], the root mean standard error of prediction [RMSEP] and the variance of prediction [S^2] (Kenneth *et al.*, 1998; Kramer *et al.*, 1998) .

$$SEP = \left[\sum_{i=1}^{n} (C_i^{True} - C_i^{Predicted})^2 / n \right]$$

$$SEP = \left[\sum_{i=1}^{n} (C_i^{True} - C_i^{Predicted})^2 / n \right]^{1/2}$$

$$S^2 = \sum_{i=1}^{n} (C_i^{Predicted} - C_i^{True} - bias)^2 /(n-1)$$

Chromatographic method

Linearity

Transfer different aliquots [2.0-12.0 µg] of Mox and [1.0-11.0 µg] of HCTZ from their working standard solutions [100 $\mu g.ml^{-1}$] into 10 ml volumetric flasks and dilute to the volume with mobile phase. Each of those dilutions were then chromatographed by injecting an aliquot of 20 µl of each into the chromatographic system three times and processed according to the method described in this work. Plot the mean peak areas of three determinations of each concentration against the same concentration and then compute the regression equations.

Laboratory prepared mixture

Prepare a laboratory prepared mixture containing 10.0 $\mu g.ml^{-1}$ of each of the four components and chromatograph by adopting the procedures mentioned under linearity [II.4.4.3.1.] Determine the concentrations by referring to the regression equations. Then calculate the percentage recoveries and standard derivations.

Results and discussion

For Chemometric method:

Many pharmaceutical compounds undergo degradation during storage or even during the different processes of their manufacture. Several chemical and physical factors can lead to degradation of drugs (Henry *et al.*, 2001). Hydrolysis and oxidation are the most famous routes of chemical degradation of drugs (Florence *et al.*, 1998; Banker *et al.*, 2002).The main classes of drugs subjected to degradation are esters, amides and lactams. Ester hydrolysis is frequently base catalyzed which makes the reaction rapid and irreversible (James, 1998). Moexipril has an ester linkage so trials were conducted for alkaline hydrolysis. It was found that the drug is liable to degradation in strong basic medium giving moexiprilat [active diacid form] by the hydrolysis of the ester group. Sodium hydroxide was the hydrolyzing agent of choice according to the ICH guidelines.In this work, moexiprilat [alkali-hydrolysis product] was prepared, then both the drug and degradate were subjected to IR^1, HNMR and mass spectral analysis for structure elucidation and the purpose of comparison. The assignment of the degradate was based on the comparison of the IR spectral data of the intact drug with the degradate. IR spectrum of the degradate showed disappearance of the carbonyl group at about 1740 cm^{-1} of the ester linkage and the appearance of a new broad band between 3100 and 3600 cm^{-1} in the IR spectrum of the degradate indicating the presence of exchangeable protons typically from a carboxylic acid which proves the hydrolysis of the ester to the corresponding acid derivative (figures 1a &1b). Further structure elucidation by mass spectroscopy showed parent mass ion peak at m/z 499.3 while mass ion peak of the degradate was at m/z 471.3 (figures 2a &2b).

On the other hand, HCTZ has one primary degradation product obtained by both acid and alkaline hydrolysis which is 4-amino-6-chlorobenzene disulphonamide [DSA] structure elucidation and confirmation of identity of both HCTZ and its degradate was carried out by IR peaks. IR spectrum of the degradate showed the appearance of two NH- stretch absorptions [3300 cm^{-1}- 3500 cm^{-1}] corresponding to the primary amine groups present in the degradate while the secondary amine group present in the intact drug produce only one and also shows the disappearance of the peak of alkyl CH_2- stretching [2950-2850 cm^{-1}] present in the IR spectrum of the intact drug (figures 3a &3b). Because the two drugs are co-formulated together and both are liable to degradation by hydrolysis, this necessitates the development of a stability indicating method for simultaneous determination of the two drugs in presence of each other and in the presence of their degradation products. Owing to the high structural similarity of the intact drugs and their corresponding degradates, absorption spectra of the two intact drugs and their degradates are extensively overlapped as well as with each other which makes it impossible to apply direct conventional for spectral resolution of this complex mixture (figure 4).

Derivative spectrophotometry from first to fourth has been attempted but also severe spectral overlapping was obtained with no zero crossing points.In this work, a new recent approach has been presented for resolving ternary and quaternary mixtures by applying the so called double divisor ratio spectra combined with derivative spectrophotometry (Ghasemi *et al*, 2004).

In this study, DDRS-DS was applied for determination of Mox and HCTZ simultaneously in the quaternary mixture with their degradates using 0.01 N HCl as the solvent system. The method is based on the use of derivative of the ratio spectra obtained by dividing the absorption spectrum of the mixture by a standard spectrum of a mixture of 3 compounds in the title mixture. The concentrations of the compounds in their mixture were determined by using their respective calibration graphs that were obtained by measuring the amplitude at either the maximum or minimum wavelengths detected for determination of Mox in this quaternary mixture DS was applied to the DDRS shown in figure (5) and DDRS first derivative spectra were obtained by using $\Delta\lambda$=16 m intervals and scaling factor 100. The application of this procedure is explained in figures (6&7). The calibration graphs for Mox at 301.8 nm were obtained by using the relation between the DDRS first derivative amplitude and concentration. The calibration graphs were applied to determine the content of Mox in the samples. HCTZ was determined by the same procedure (figures 8-10) but in this case the calibration graph at 284.2 nm was obtained. It's worthwhile to mention that trails were carried out to optimize the spectrophotometric parameters to obtain maximum resolution and sensitivity where different parameters were investigated, different smoothing factors, $\Delta\lambda$ intervals and scaling factors were tried.

Linear correlation was obtained in the region of (2.0-12.0 $\mu g.ml^{-1}$) and (2.0 -14.0 $\mu g.ml^{-1}$) for Mox and HCTZ respectively.

The regression equations computed were found to be:

$DDRS\text{-}D^1$ = 0.0794 C +0.0902 r =0.9999 at 301.8 nm for Mox

$DDRS\text{-}D^1$ = 0.08822 C+0.0043 r=0.9999 at284.2 nm for HCTZ

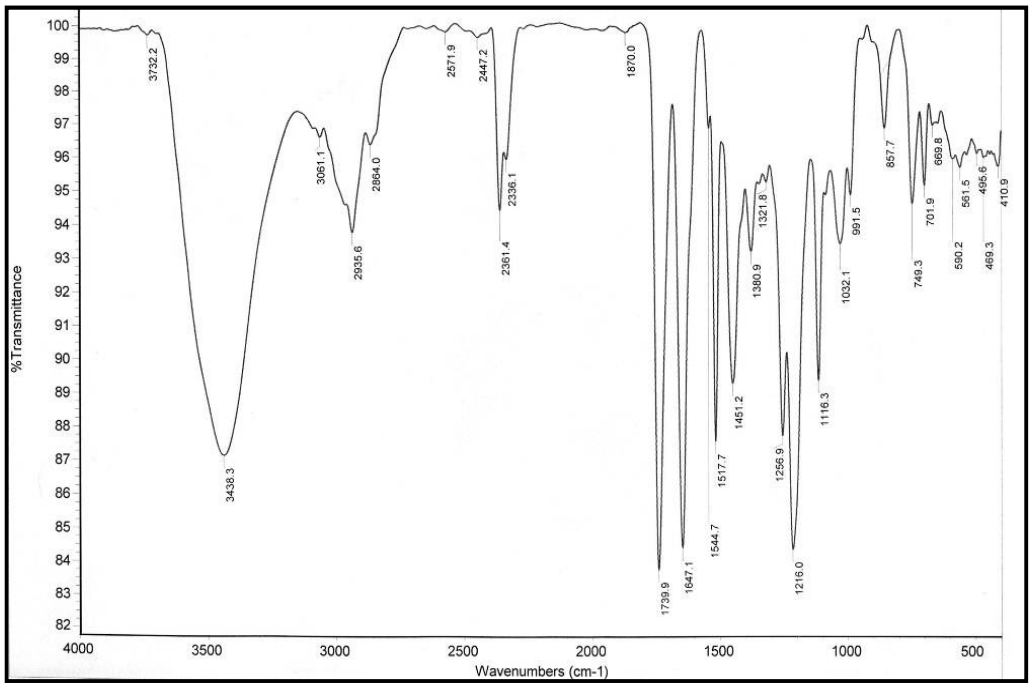
The results of accuracy of the proposed method are displayed in table (1). The mean percentage recoveries±SD were found to be 99.98±1.097 and 100.14±0.213 for Mox and HCTZ respectively.Results shown in table (2) indicate the method was found selective, valid and applicable for the determination of Mox and HCTZ in different synthetic mixtures containing different composition ratios of the intact drugs and their degradates. The proposed method were also applied for the analysis of the studied drugs in pharmaceutical preparations. The accuracy and the validity of the method were further confirmed by applying the standard addition technique. Satisfactory results were obtained and no intereference from the tablet excipients was observed. The results are presented in table (3). The assay validation parameters are tabulated in table (4). The results obtained were statistically compared to the reported methods for the analysis of the studied drugs and no significant difference was found with respect to both accuracy and precision as shown in table (5). The robustness tests examines the experimental conditions of the method and the potentially responsible factors such as experimental and environmental conditions to be taken into account during method development given that changes in the optimal conditions can result in significant error. For the proposed method, the analysis factors [wavelength range, cuvettes & room temperature] didn't have significant effect on the responses.The proposed method is simple, rapid and selective and robust therefore they can be used for routine analysis of the drugs in quality control laboratories.

For Chemometric method:

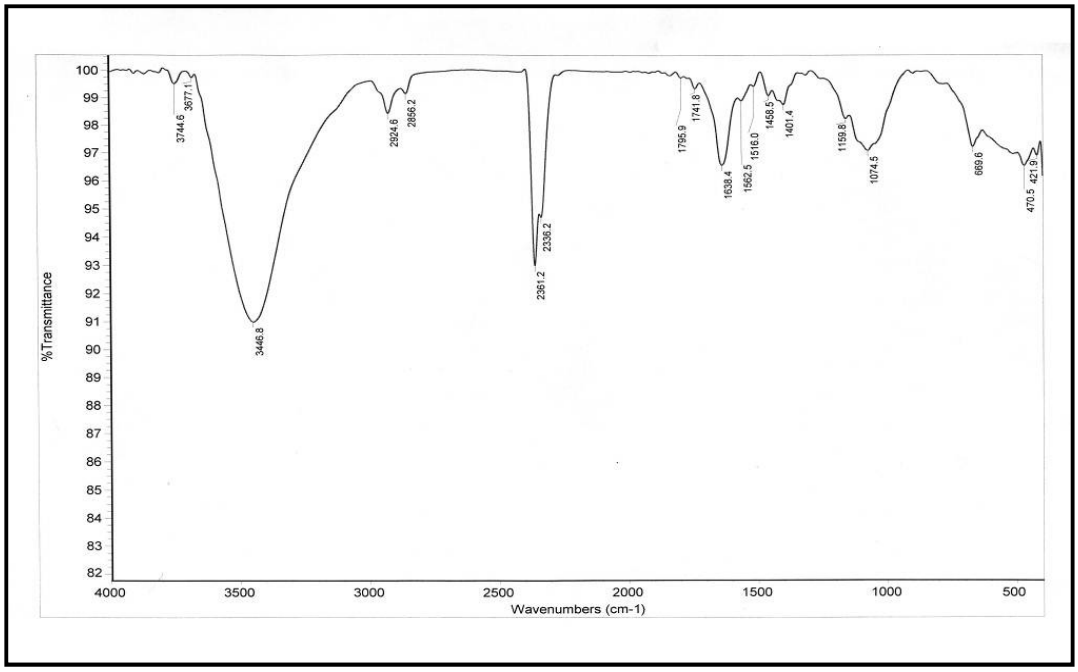
The conventional UV univariate single wavelength calibration procedures cannot provide good quantitative determination for this mixture. Reliable computational methods such as CLS, PCR and PLS-1 were applied to resolve this spectral interference because the simultaneous inclusion of many spectral wavelengths instead of single wavelength greatly improved the precision and predictive ability (Ni *et al*, 1997).

Thomas and Haaland (Thomas *et al*, 1990) made a comparison of three different multivariate calibration methods for quantitative spectral analysis. They concluded that it is difficult to generalize the superiority of one method over another, because the relative performance of the method is often dependant on particular data set being analyzed.

Curr. Sci. Int. 3(4): 352-380, 2014



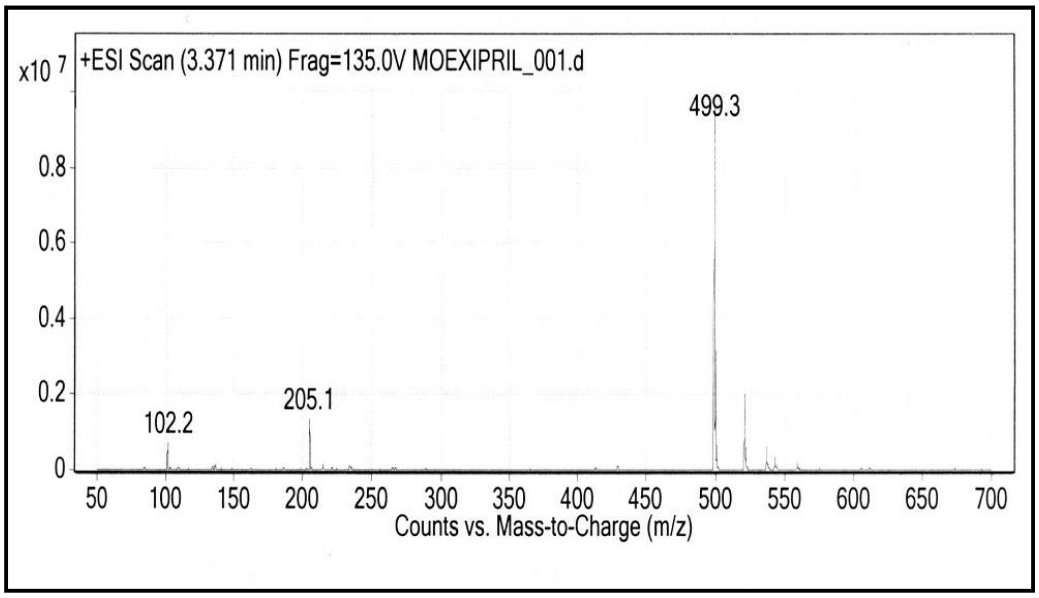
(a)



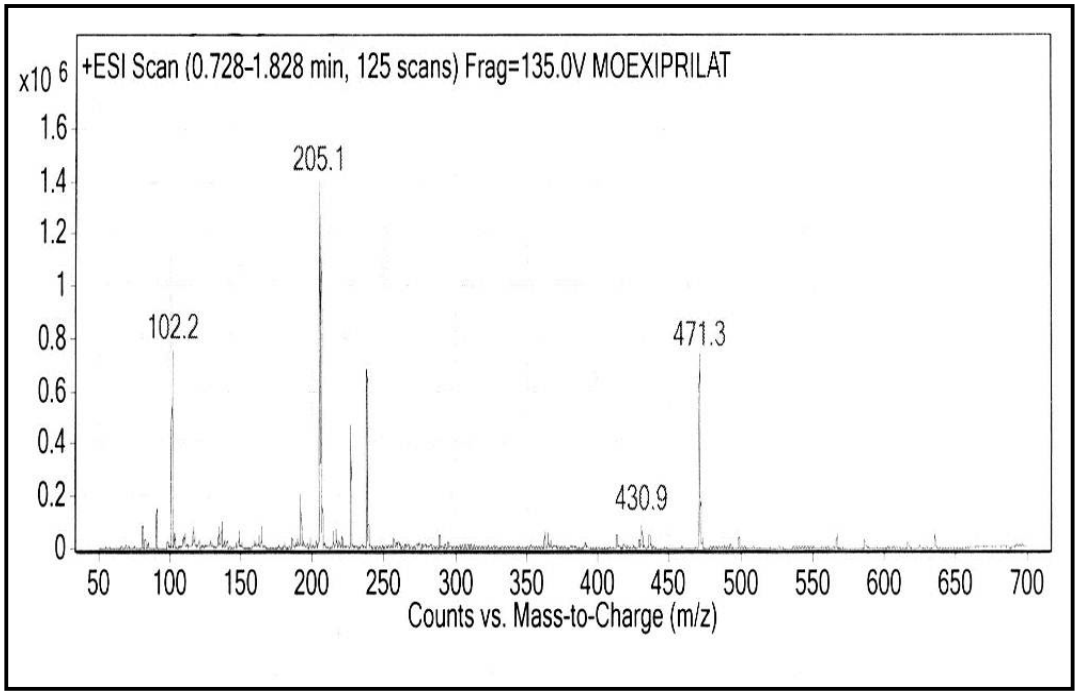
(b)

Fig. 1. IR spectrum of Mox.HCl[(a)] and its degradate mox-at (b).

Curr. Sci. Int. 3(4): 352-380, 2014

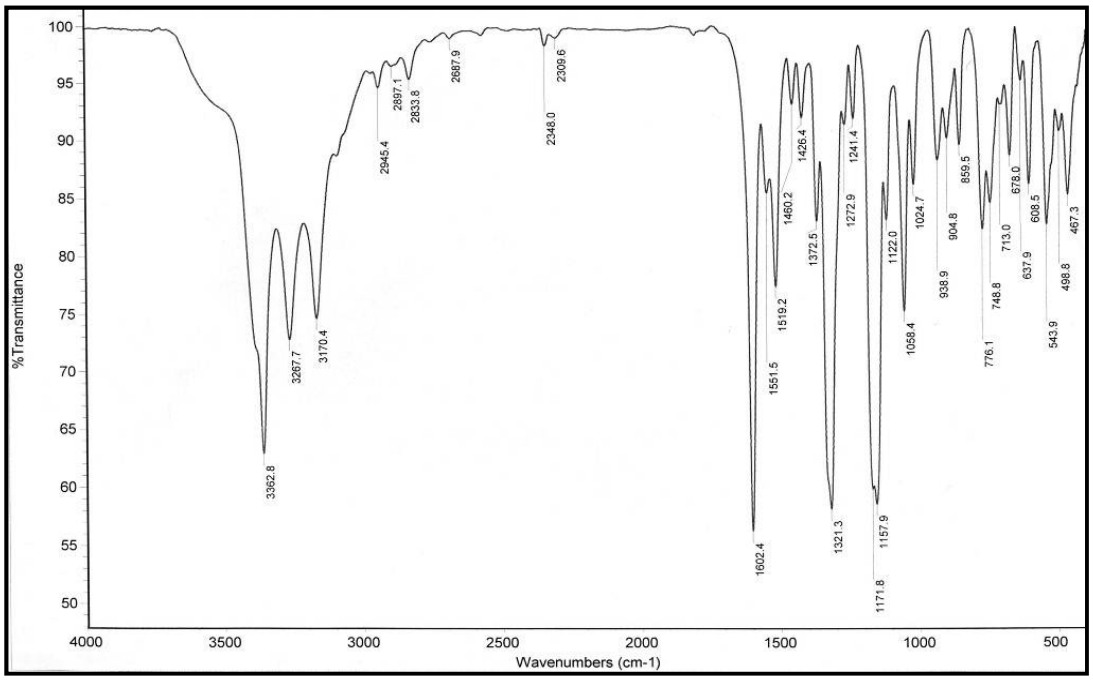


(a)

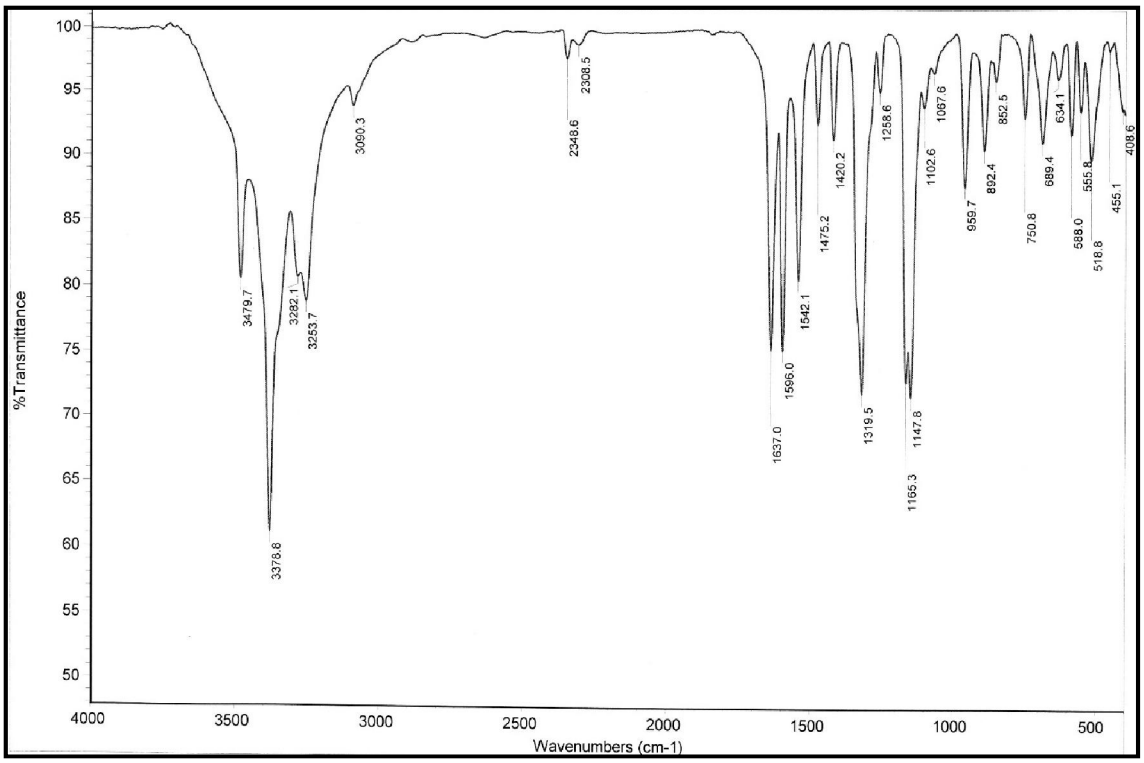


(b)

Fig. 2. LC/MS spectra of Mox.HCl (a) and its degradate moxprilat (b).



(a)



(b)

Fig. 3.IR spectrum of hydrochlorothiazide (a) and its degradate (4-amino-6-chlorobenzene (1,3) disulphonamide (DSA) (b).

Curr. Sci. Int. 3(4): 352-380, 2014

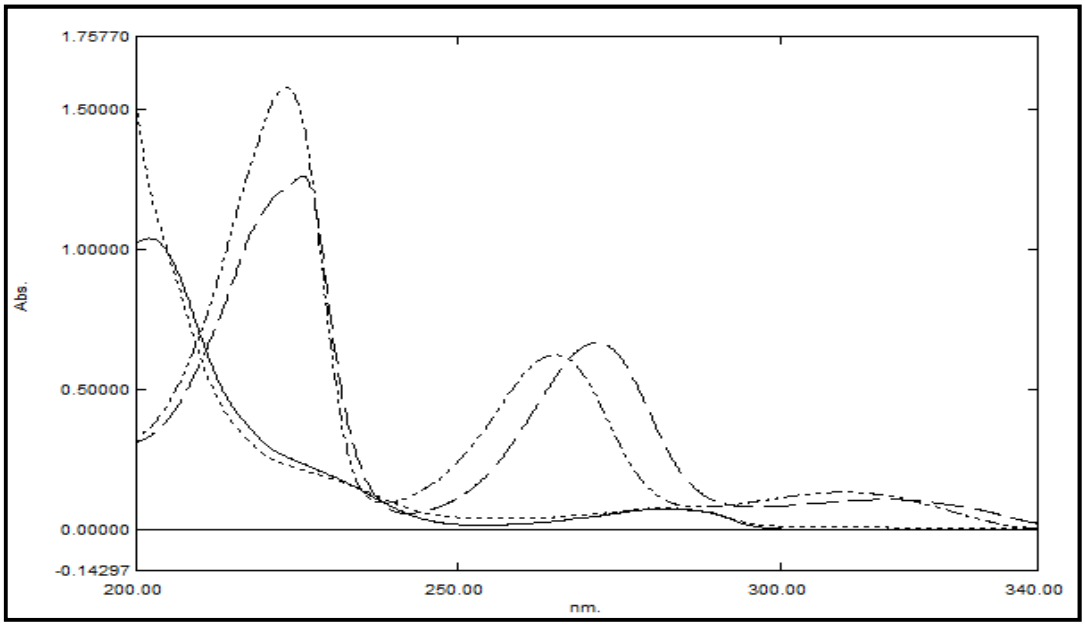


Fig. 4. Zero order absorption spectra of Mox (——), Mox-at (….), HCTZ (_.._.._) and DSA (– – –), each of (10.0 µg.ml^{-1}) using 0.01 M HCl as a blank.

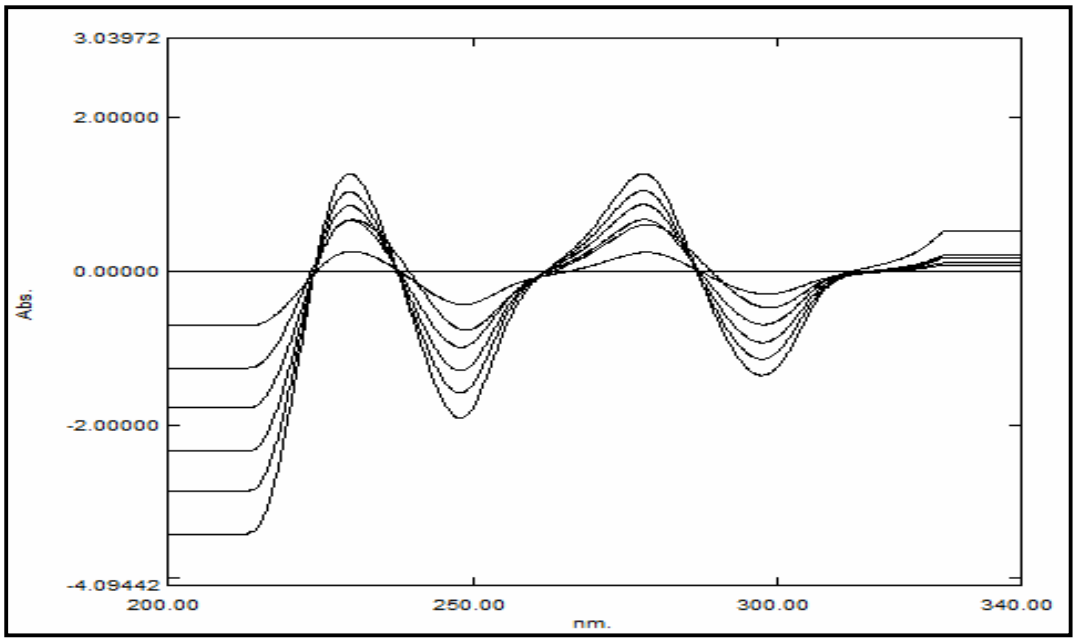


Fig. 5. First derivative of the ratio spectra of moexipril hydrochloride (2.0-12.0 µg.ml^{-1}) using 10 µg.ml^{-1} of each of Mox-at, HCTZ and DSA as a double divisor and 0.01 M HCl as blank.

Curr. Sci. Int. 3(4): 352-380, 2014

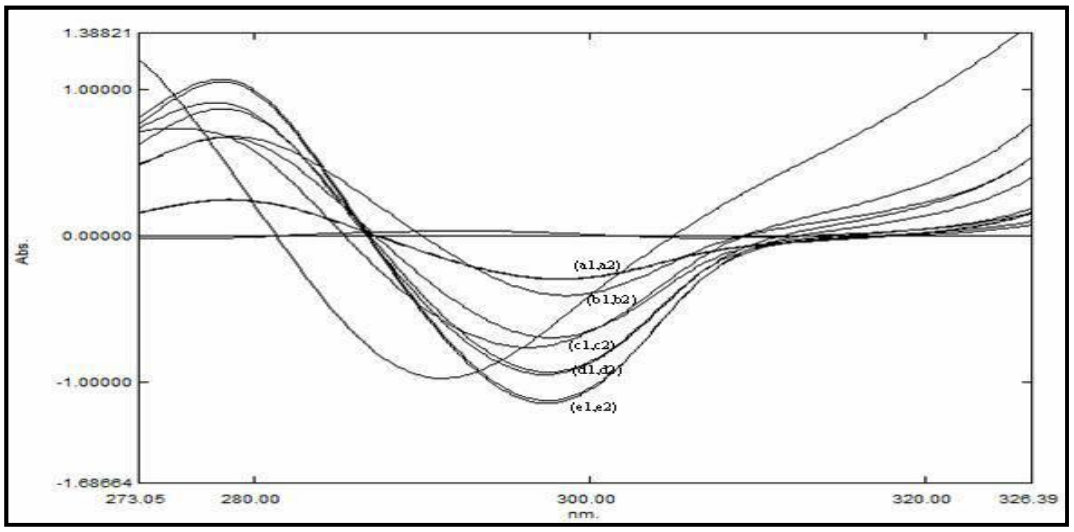


Fig. 6. The coincident spectra of the first derivative of the ratio spectra of (a1) 2.0 µg.ml^{-1} pure Mox. HCl and (a2) quaternary mixture (2.0 µg.ml^{-1} Mox.HCl, 1.80 µg.ml^{-1} Mox-at, 10.0 µg.ml^{-1} HCTZ and 1.0 µg.ml^{-1} DSA), (b1) 4.0 µg.ml^{-1} pure Mox. HCl and (b2) quaternary mixture (4.0 µg.ml^{-1} Mox.HCl, 2.80 µg.ml^{-1} Mox-at, 8.0 µg.ml^{-1} HCTZ and 2.4 µg.ml^{-1} DSA), (c1) 6.0 µg.ml^{-1} pure Mox. HCl and (c2) quaternary mixture (6.0 µg.ml^{-1} Mox.HCl, 3.0 µg.ml^{-1} Mox-at, 6.0 µg.ml^{-1} HCTZ and 3.0 µg.ml^{-1} DSA), (d1) 8.0 µg.ml^{-1} pure Mox. HCl and (d2) quaternary mixture (8.0 µg.ml^{-1} Mox.HCl, 2.40 µg.ml^{-1} Mox-at, 4.0 µg.ml^{-1} HCTZ and 2.80 µg.ml^{-1} DSA) and (e1) 10.0 µg.ml^{-1} pure Mox. HCl and (e2) quaternary mixture (10.0 µg.ml^{-1} Mox.HCl, 1.0 µg.ml^{-1} Mox-at, 2.0 µg.ml^{-1} HCTZ and 1.8 µg.ml^{-1} DSA) using 10 µg.ml^{-1} of each of Mox-at, H"CTZ and DSA and 0.01 M HCl as a blank.

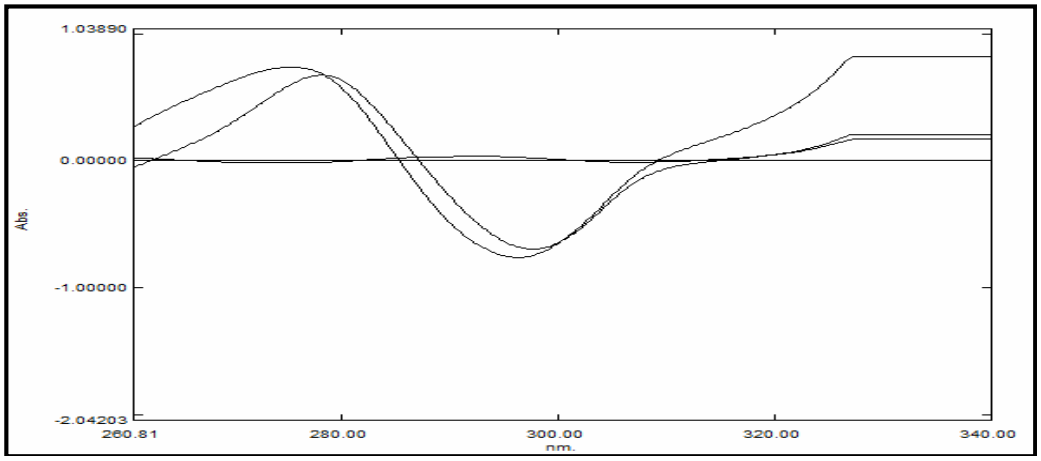


Fig. 7. The coincident spectra of the first derivative of the ratio spectra of (e1) 10.0 µg.ml^{-1} pure Mox. HCl and (e2) quaternary mixture (10.0 µg.ml^{-1} Mox.HCl, 1.0 µg.ml^{-1} Mox-at, 2.0 µg.ml^{-1} HCTZ and 1.0 µg.ml^{-1} DSA) using 10 µg.ml^{-1} of each of Mox-at, H"CTZ and DSA and 0.01 M HCl as a blank.

Curr. Sci. Int. 3(4): 352-380, 2014

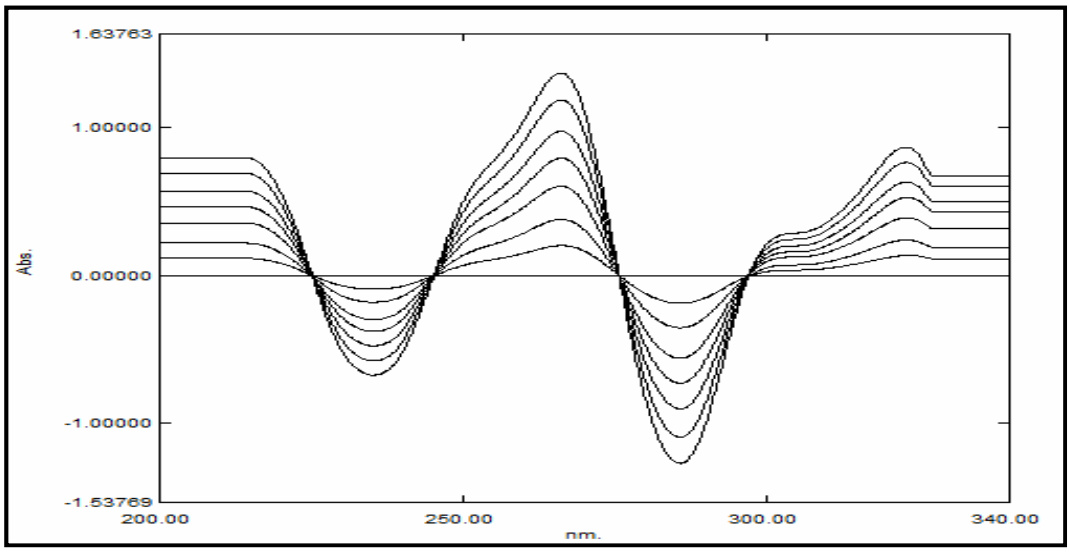


Fig. 8. First derivative of the ratio spectra of hydrochlorothiazide (2.0-14.0 µg.ml^{-1}) using (5.0 µg.ml^{-1}) of each of DSA, Mox and Mox-at as a double divisor and 0.01 M HCl as a blank.

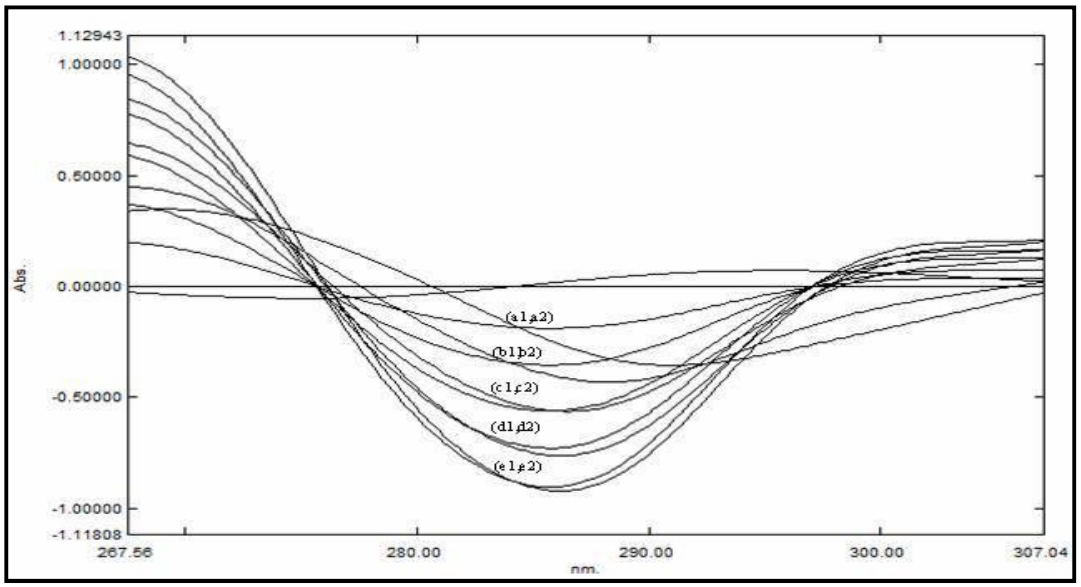


Fig. 9. The coincident spectra of the first derivative of the ratio spectra of (a1) 2.0 µg.ml^{-1} pure HCTZ and (a2) quaternary mixture (2.0 µg.ml^{-1} HCTZ, 1.8 µg.ml^{-1} DSA, 10.0 µg.ml^{-1} Mox and 1.0 µg.ml^{-1} Mox-at), (b1) 4.0 µg.ml^{-1} pure HCTZ and (b2) quaternary mixture (4.0 µg.ml^{-1} HCTZ, 2.8 µg.ml^{-1} DSA, 8.0 µg.ml^{-1} Mox and 2.4 µg.ml^{-1} Mox-at), (c1) 6.0 µg.ml^{-1} pure HCTZ and (c2) quaternary mixture (6.0 µg.ml^{-1} HCTZ, 3.0 µg.ml^{-1} DSA, 6.0 µg.ml^{-1} Mox and 3.0 µg.ml^{-1} Mox-at), (d1) 8.0 µg.ml^{-1} pure HCTZ and (d2) quaternary mixture (8.0 µg.ml^{-1} HCTZ, 2.4 µg.ml^{-1} DSA, 4.0 µg.ml^{-1} Mox and 2.8 µg.ml^{-1} Mox-at) and (e1) 10.0 µg.ml^{-1} pure HCTZ and (e2) quaternary mixture (10.0 µg.ml^{-1} HCTZ, 1.0 µg.ml^{-1} DSA, 2.0 µg.ml^{-1} Mox and 1.8 µg.ml^{-1} Mox-at) using 5 µg.ml^{-1} of each of DSA, Mox and Mox-at as a double divisor and 0.01 M HCl as a blank.

Curr. Sci. Int. 3(4): 352-380, 2014

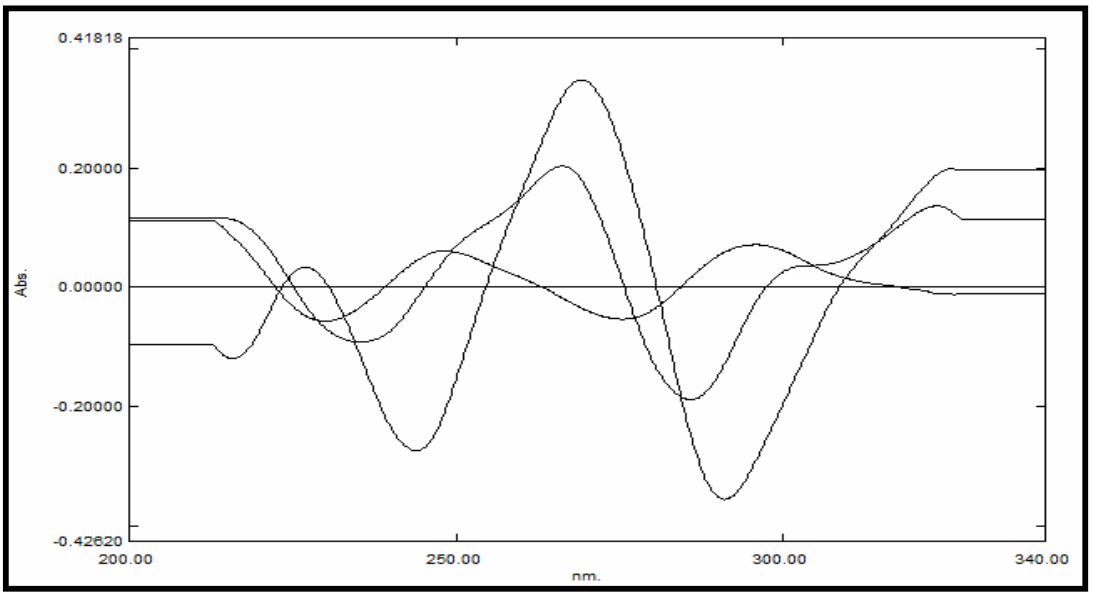


Fig. 10. The coincident spectra of the first derivative of the ratio spectra of (d1) 8.0 µg.ml^{-1} pure HCTZ and (d2) quaternary mixture (8.0 µg.ml^{-1} HCTZ, 2.4 µg.ml^{-1} DSA, 4.0 µg.ml^{-1} Mox and 2.8 µg.ml^{-1} Mox-at using 5 µg.ml^{-1} of each of DSA, Mox and Mox-at as a double divisor and 0.01 M HCl as a blank.

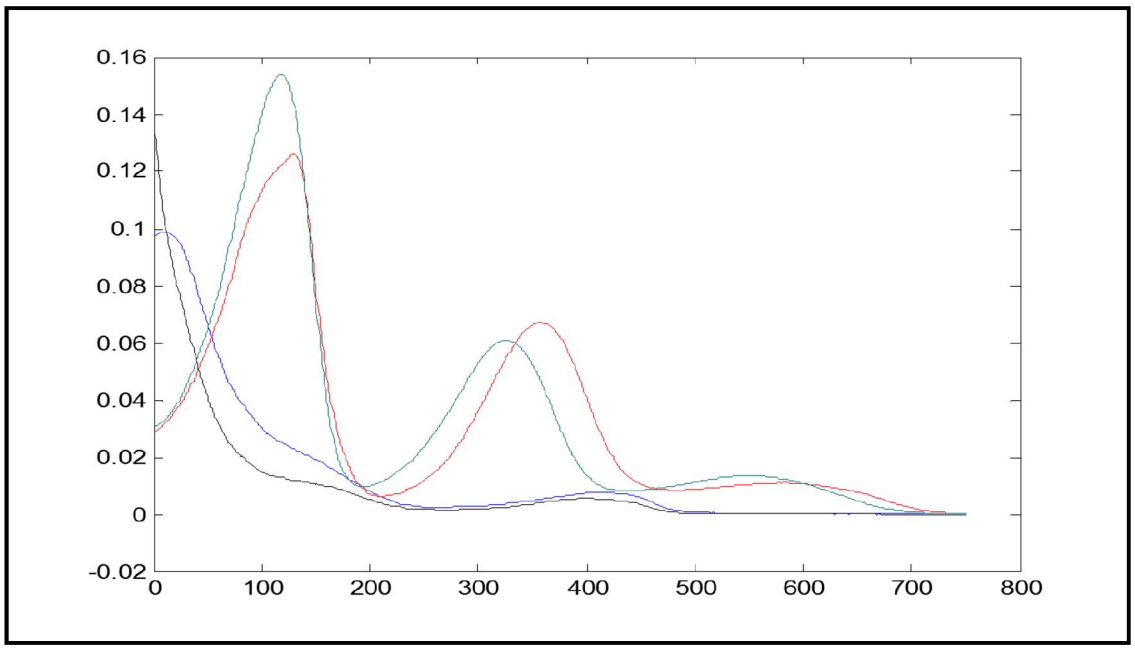


Fig. 11. Plot of the K matrix "absorbitivity matrix".

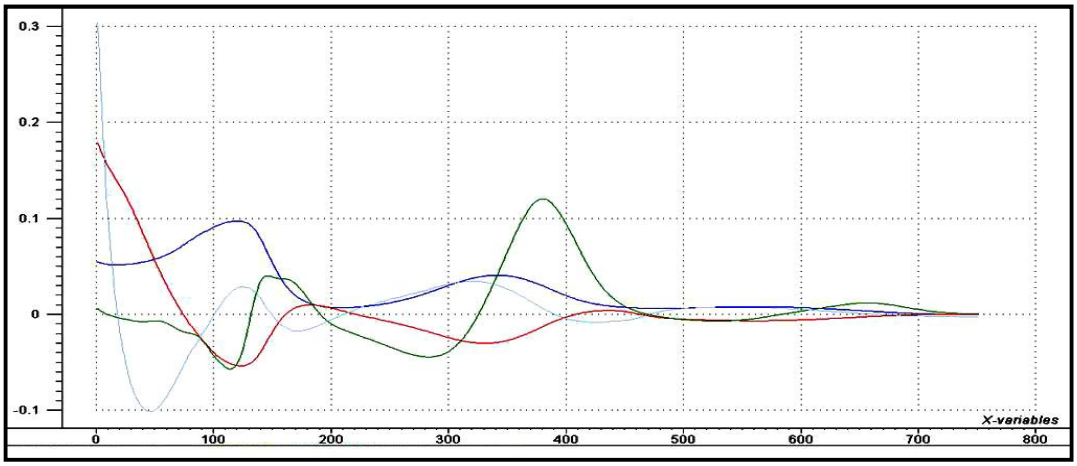


Fig. 12. Plot of the loadings obtained from the absorbance data matrix of Mox (a), Mox-at (b), HCTZ (c) and DSA (d) by principal component regression (PCR) and partial least squares (PLS) methods.

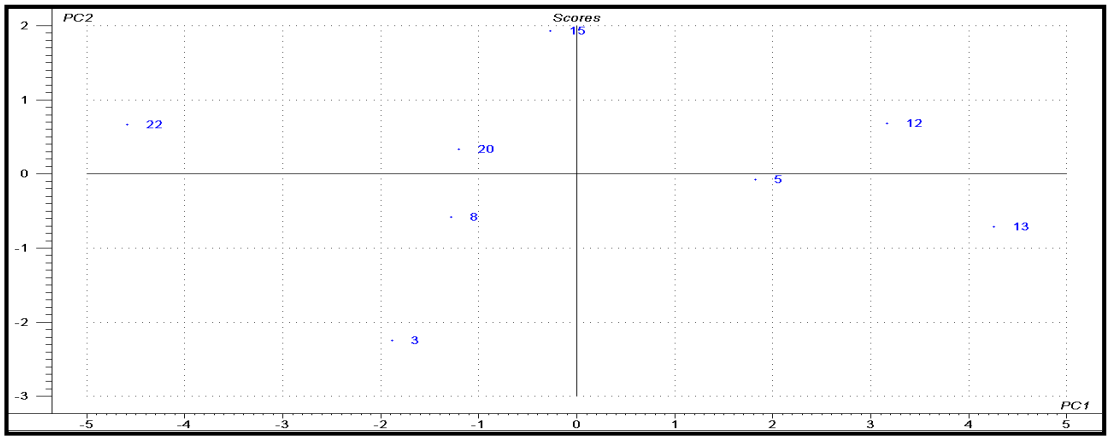


Fig. 13. Plot of score of Mox. HCl in a training set prediction using test set validation (principal component regression model).

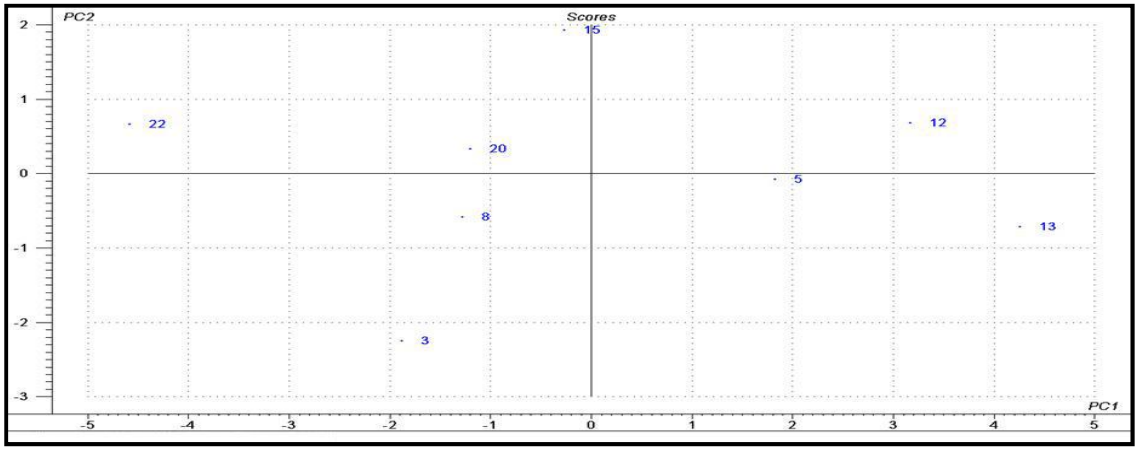


Fig. 14. Plot of score of HCTZ in a training set prediction using test et validation (principal component regression model).

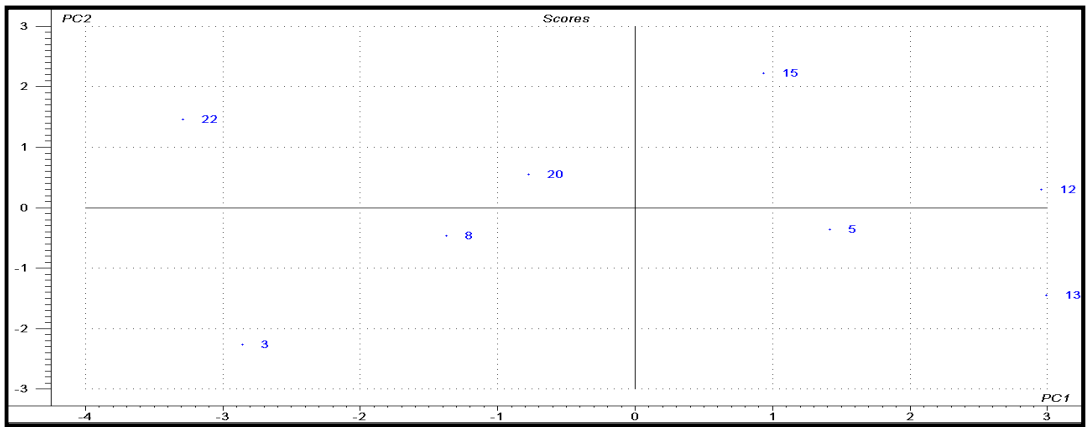


Fig. 15. Plot of scores of Mox.HCl in a training set prediction using test set validation (partial least squares model).

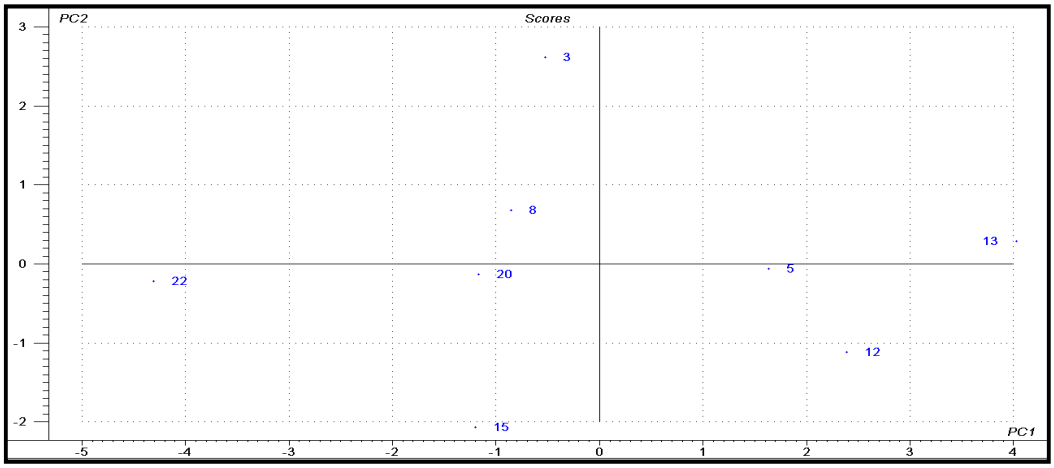


Fig. 16. Plot of scores of HCTZ in a training set prediction using test set validation (partial least squares model).

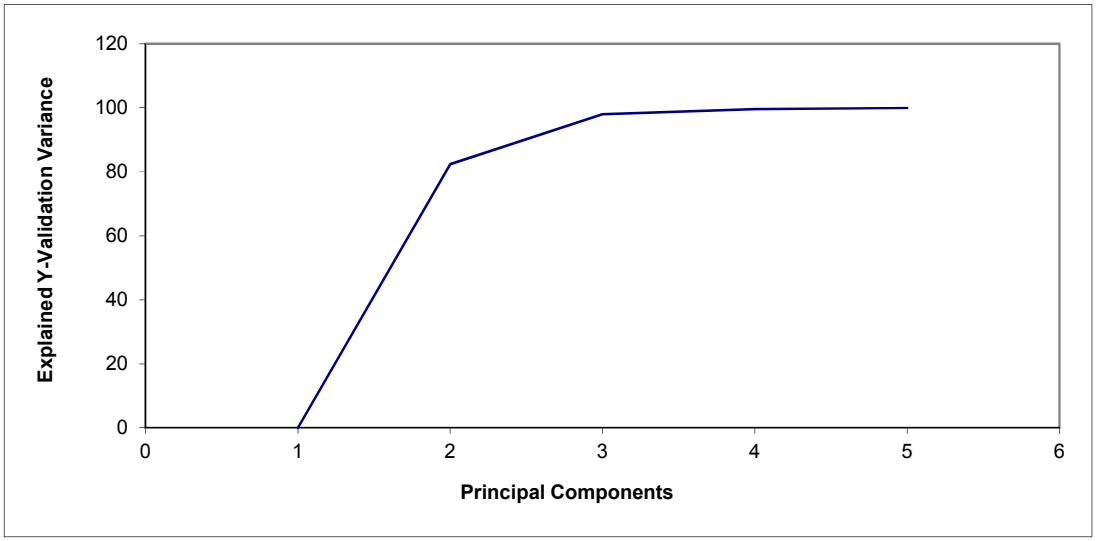


Fig. 17. Explained Y-validation variance plot of Mox. HCl in a training set prediction using test set validation (principal component regression model.

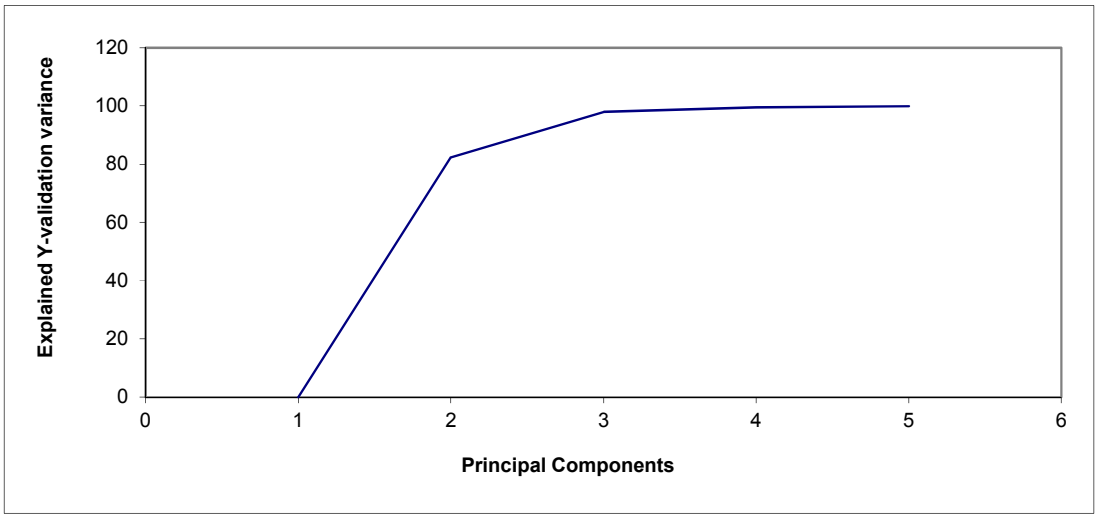


Fig. 18. Explained Y-validation variance plot of HCTZ in a training set prediction using test set validation (principal component regression model.

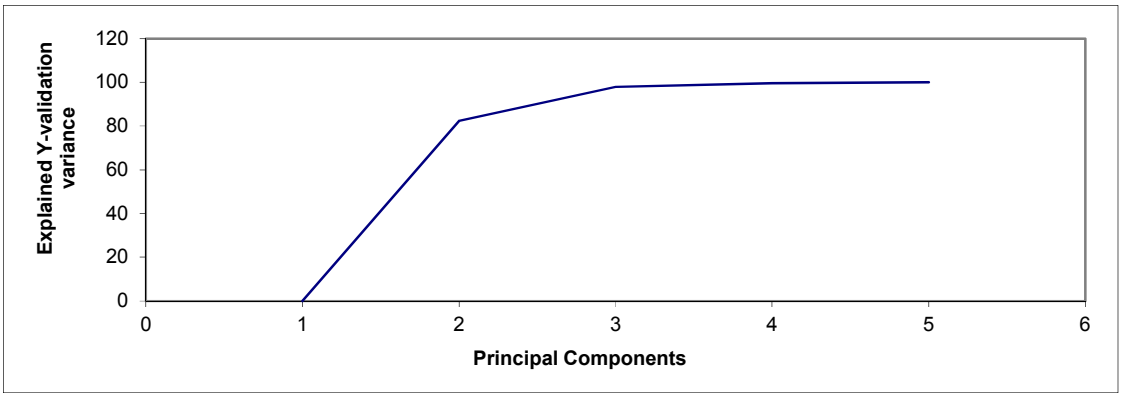


Fig. 19. Explained Y-validation variance plot of Mox.HCl in a training set prediction using test set validation (partial least squares model).

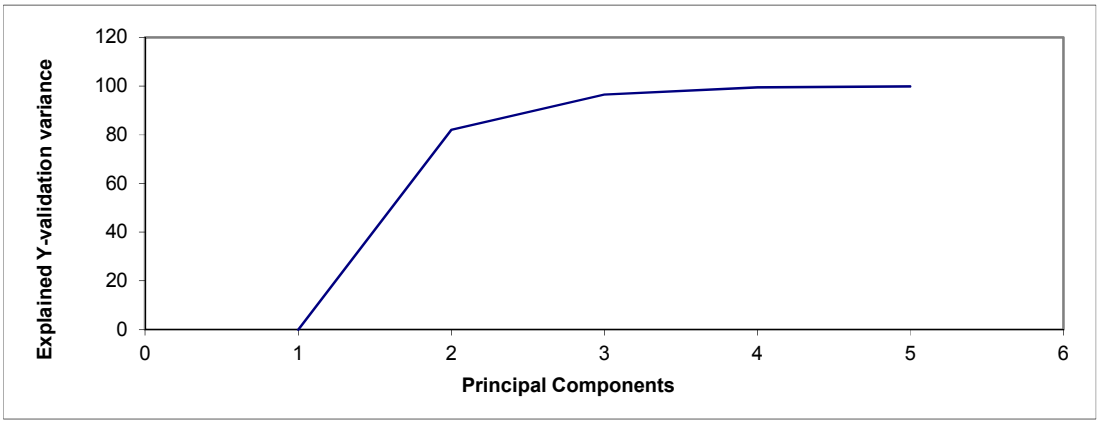


Fig. 20. Explained Y-validation variance plot of HCTZ in a training set prediction using test set validation (partial least squares model).

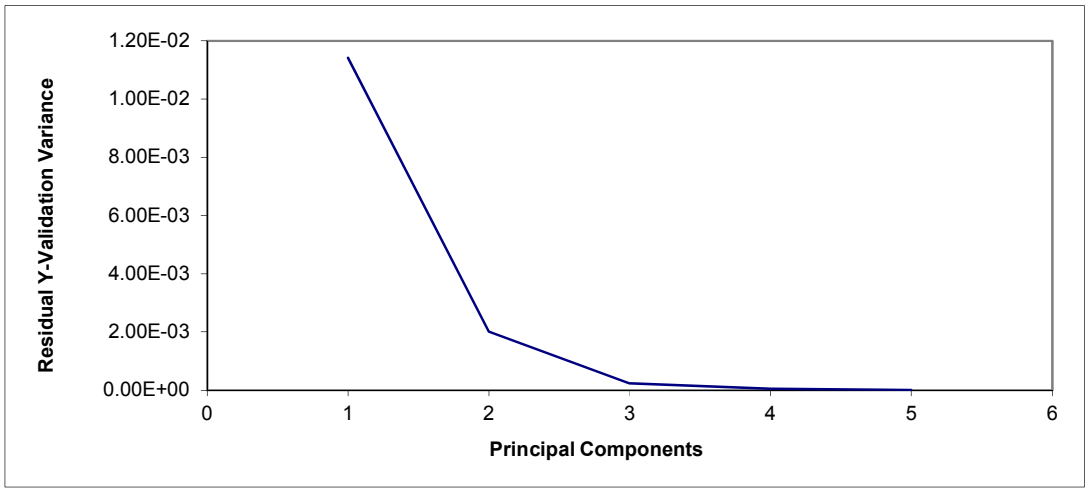


Fig. 21. Residual validation variance plot of a Mox.HCl in a training set prediction using test set validation (principal component regression model).

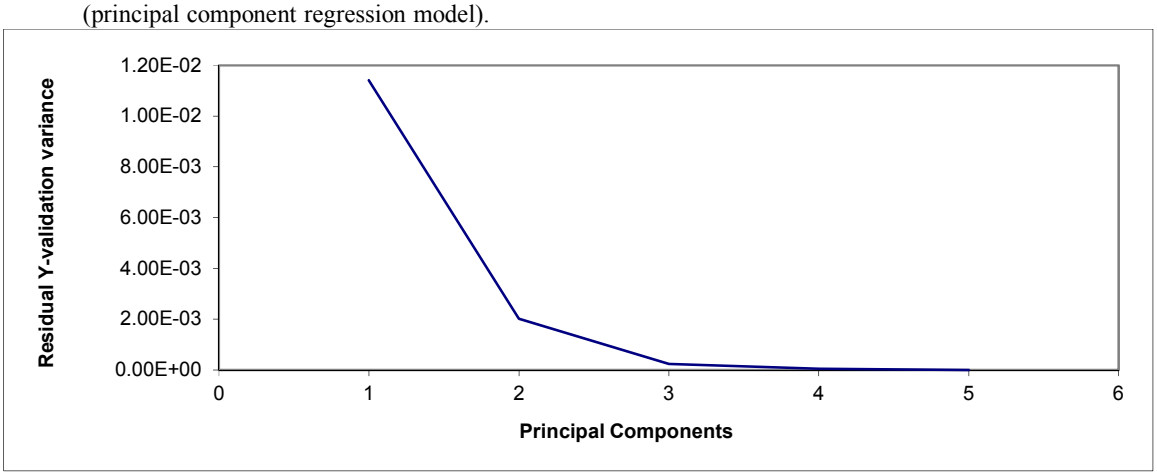


Fig. 22. Residual validation variance plot of a HCTZ in a training set prediction using test set validation (principal component regression model).

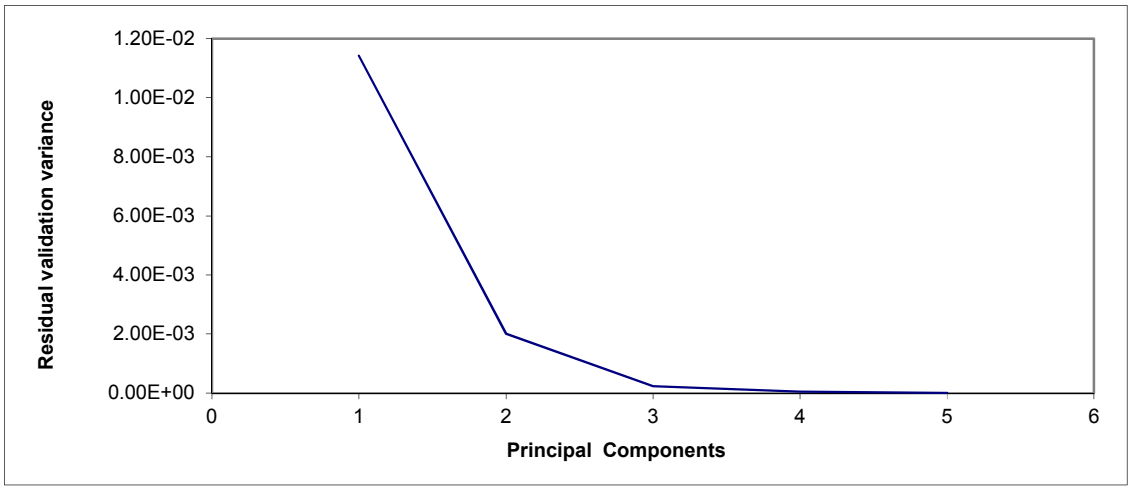


Fig. 23. Residual validation variance plot of Mox.HCl a training set prediction using test set validation (partial least squares model).

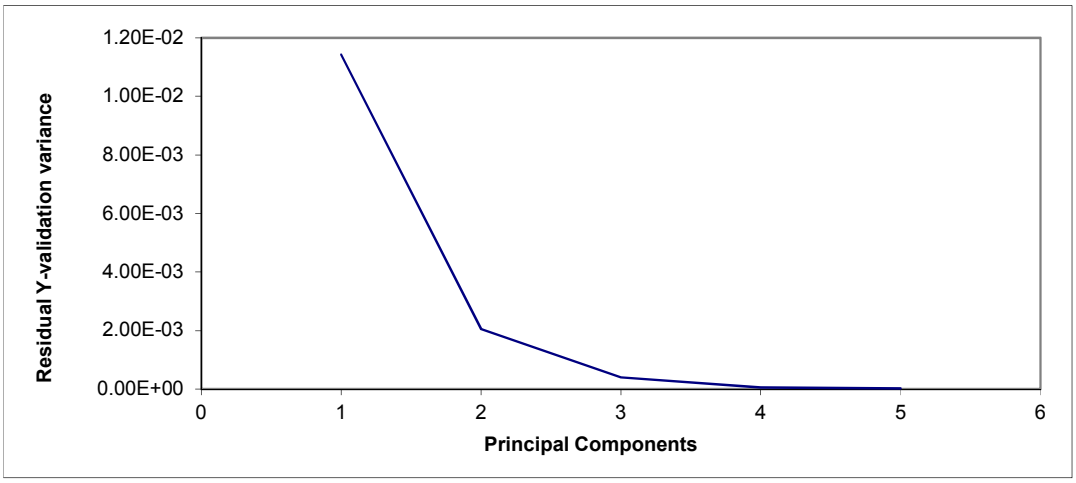


Fig. 24. Residual validation variance plot of HCTZ in a training set prediction using test set validation (partial least squares model).

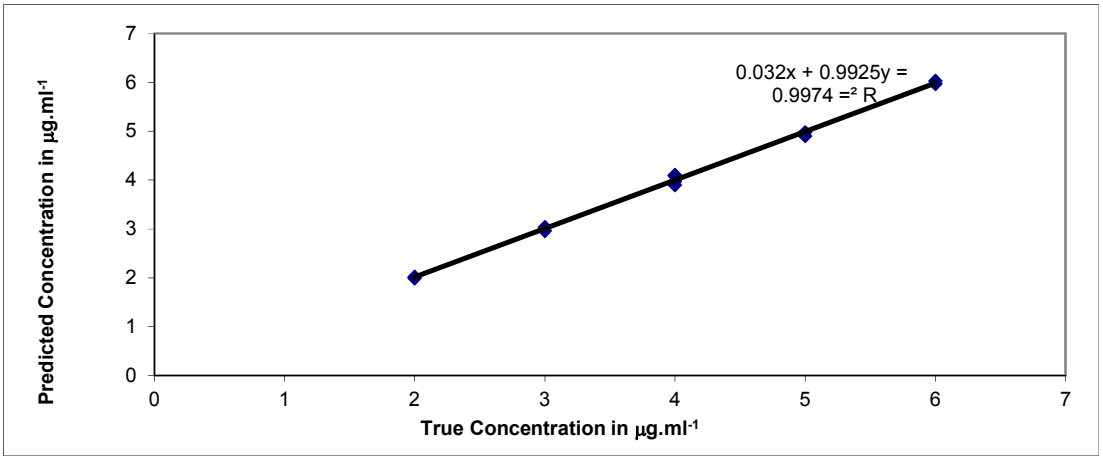


Fig. 25. Predicted concentration versus true concentration of Mox. HCl in the validation set using CLS model.

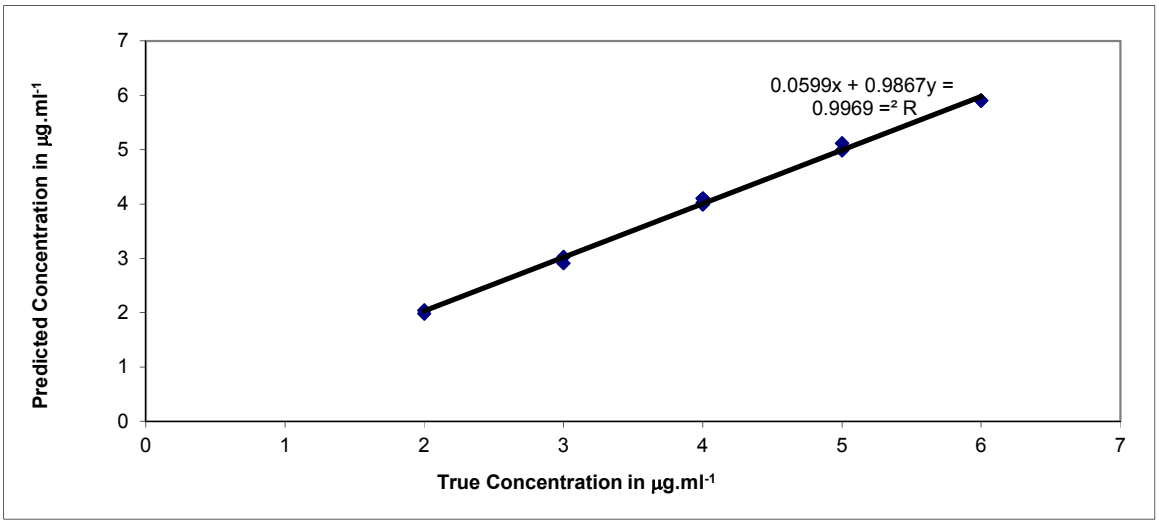


Fig. 26. Predicted concentration versus true concentration of HCTZ in the validation set using CLS model.

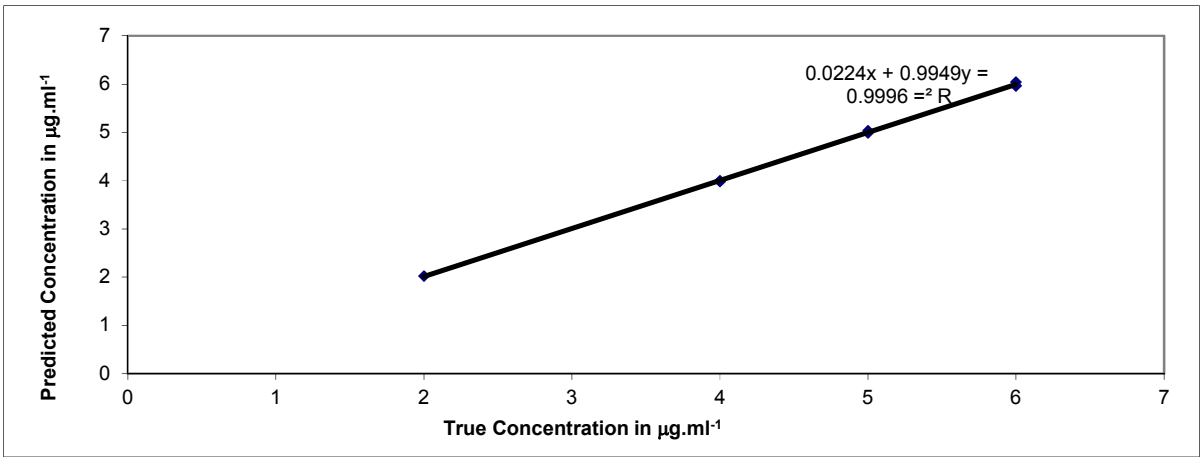


Fig.27. Predicted concentration versus true concentration of Mox.HCl in the validation set using PCR model.

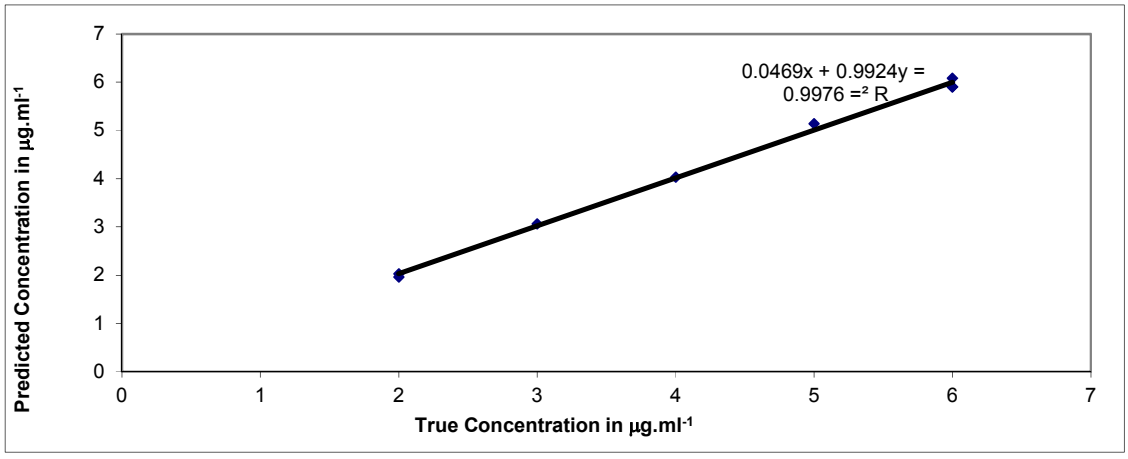


Fig. 28. Predicted concentration versus true concentration of HCTZ in the validation set using PCR model.

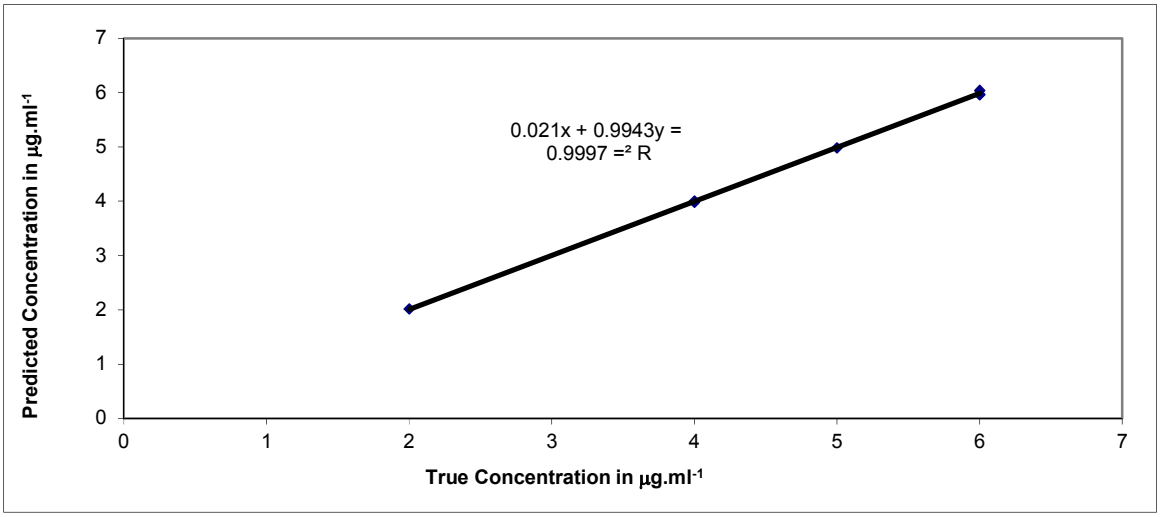


Fig. 9. Predicted concentration versus true concentration of Mox.HCl in the validation set using PLS model.

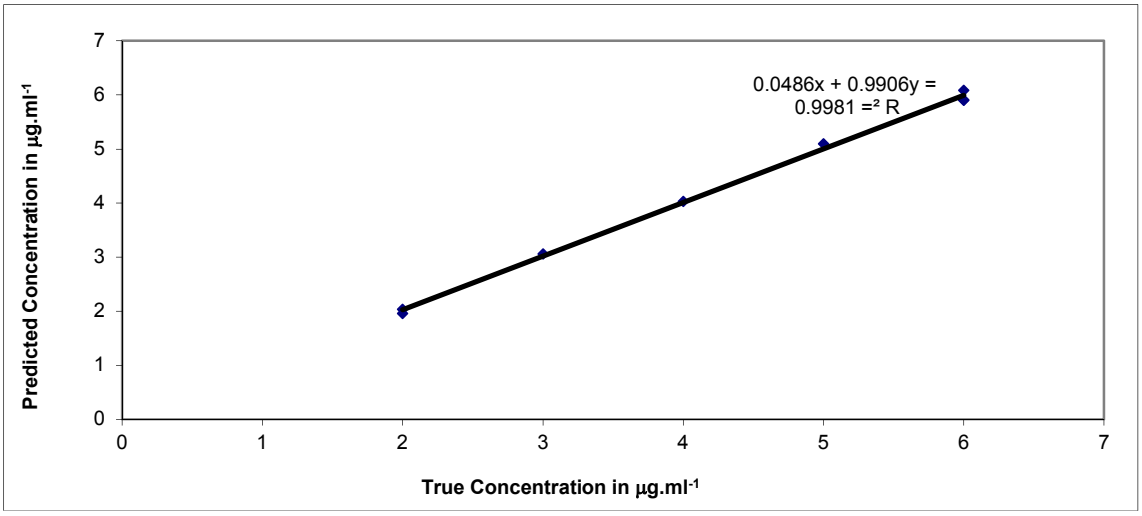


Fig. 30 Predicted concentration versus true concentration of HCTZ in the validation set using PLS model.

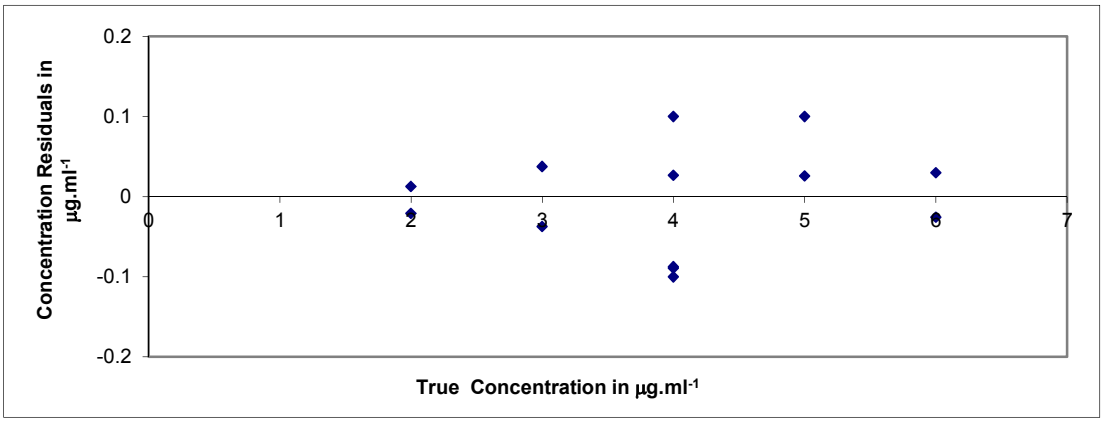


Fig. 31.Concentration residuals versus predicted concentration of Mox.HCl in the validation set using CLS model.

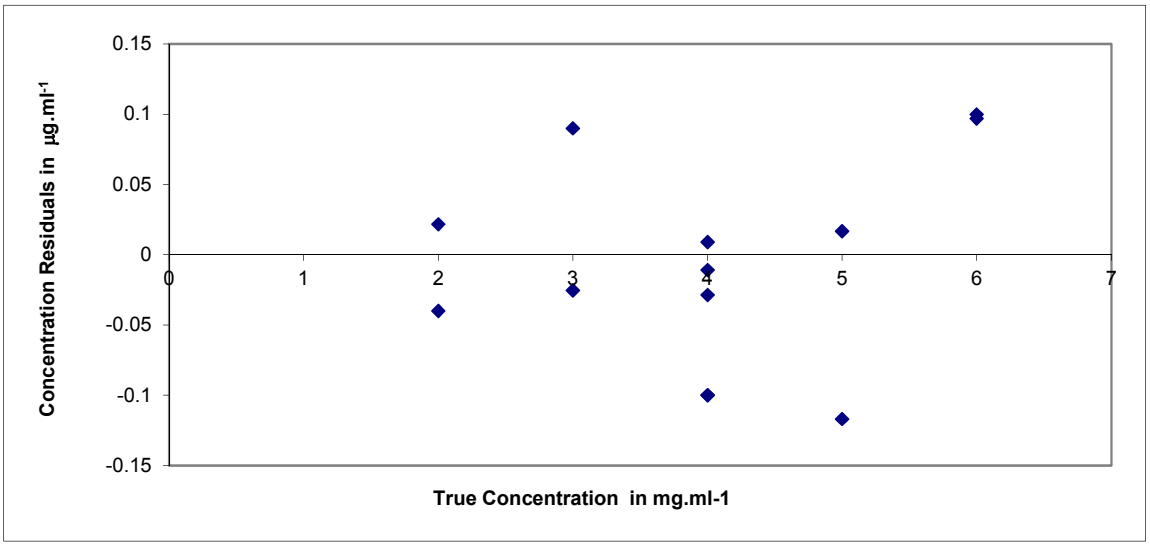


Fig. 32. Concentration residuals versus predicted concentration of HCTZ in the validation set using CLS model.

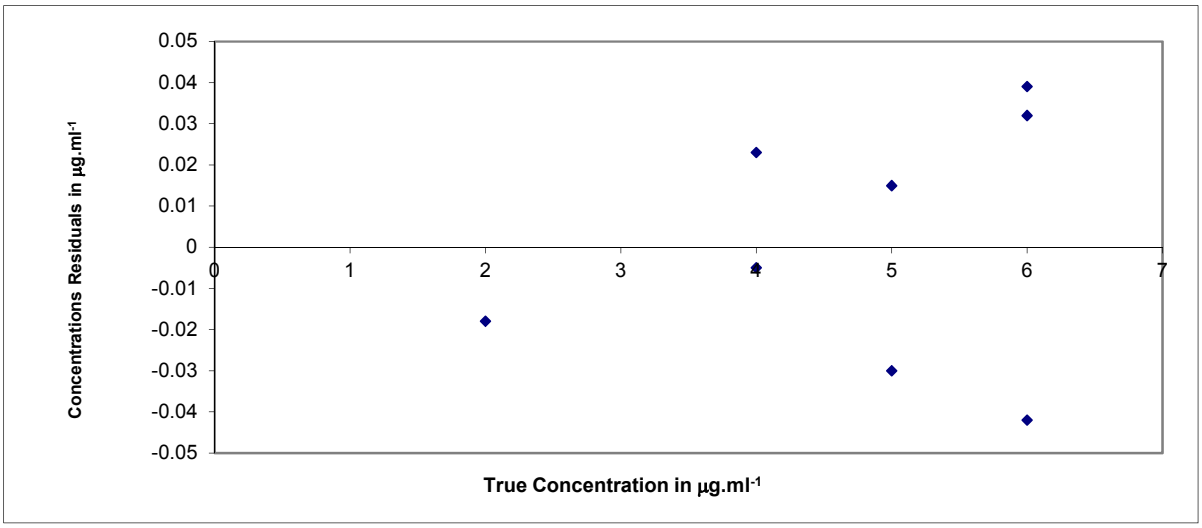


Fig. 33. Concentration residuals versus predicted concentration of Mox.HCl in the validation set using PCR model.

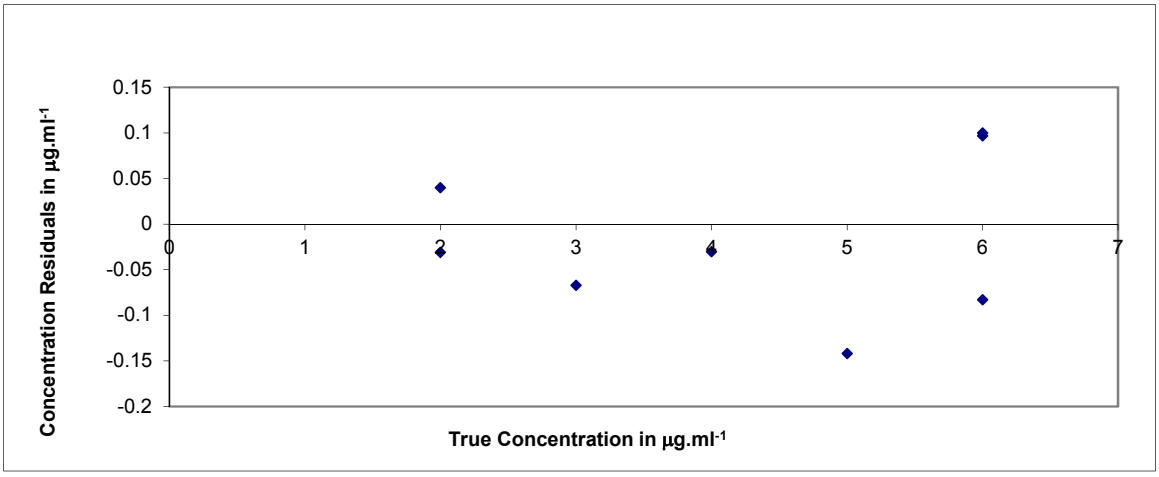


Fig. 34. Concentration residuals versus predicted concentration of HCTZ in the validation set using PCR model.

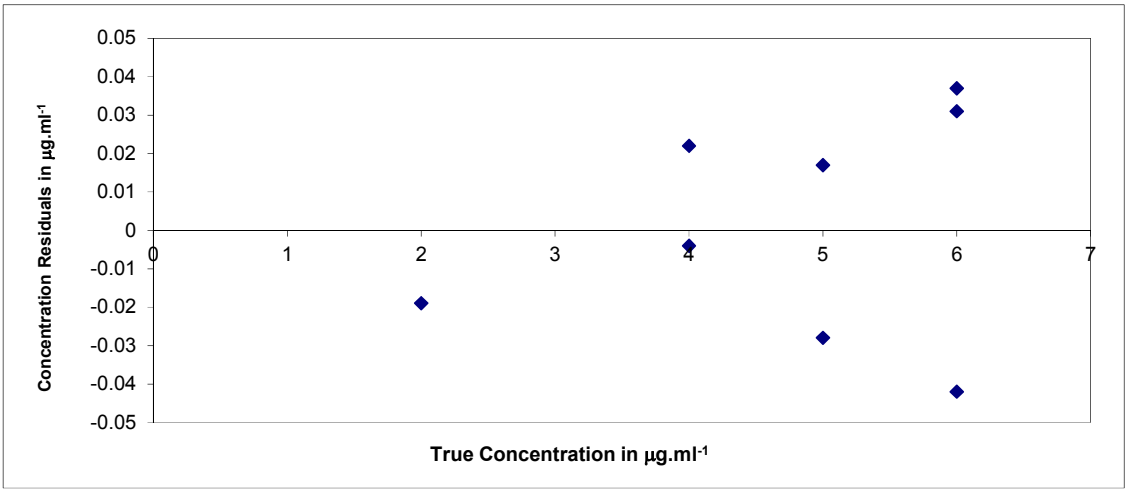


Fig. 35. Concentration residuals versus predicted concentration of Mox.HCl in the validation set using PLS model.

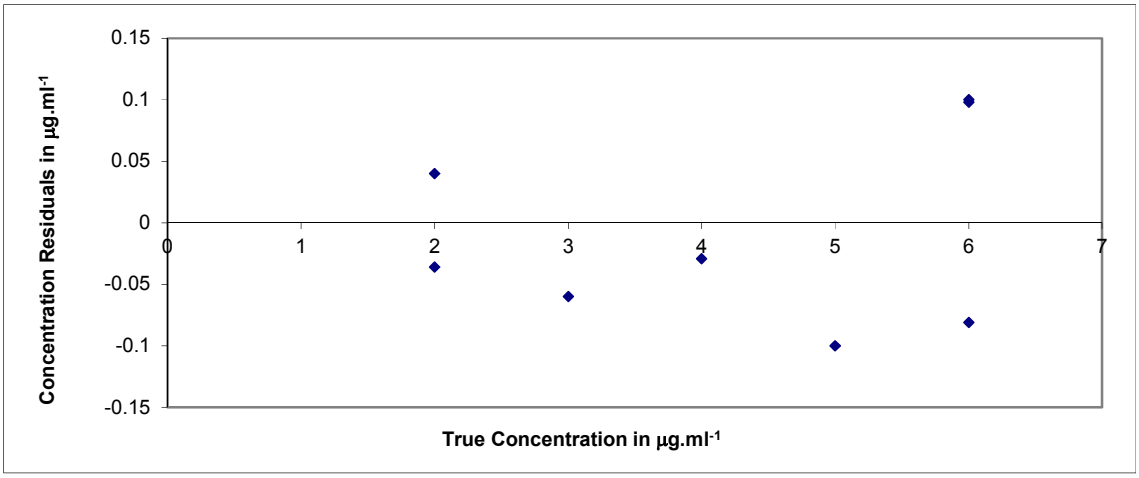


Fig. 36. Concentration residuals versus predicted concentration of HCTZ in the validation set using PLS model.

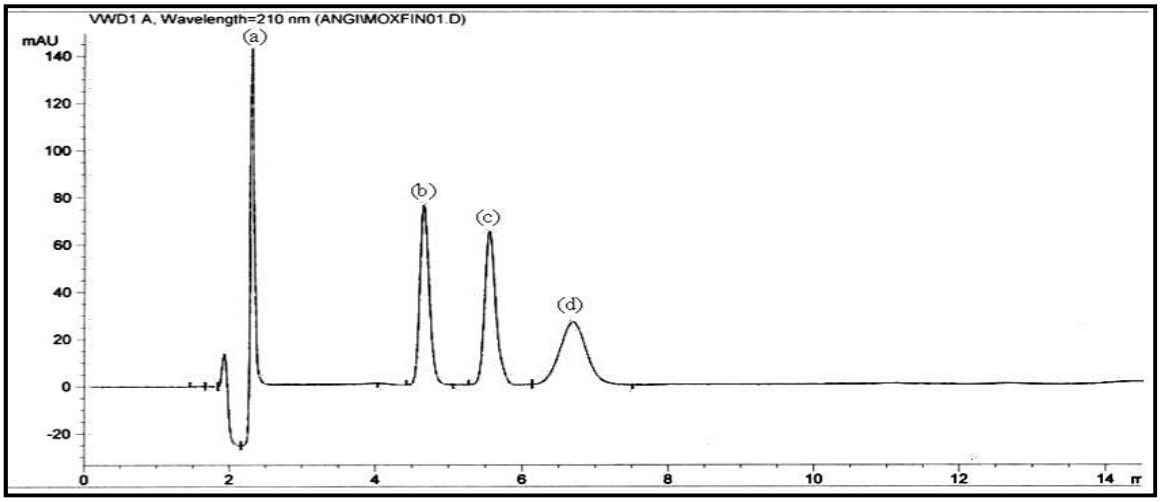


Fig. 37. Scanning profile of Hplc chromatogram of Mox.HCl (6.66 min), HCTZ (5.44 min.), DSA (4.63 min.) and Mox-at (2.33 min.), each of (10 µg.ml^{-1}).

The factor based methods [PCR and PLS] are suggested to be preferred than the CLS method as they do not require that the concentration values for all of the components present have to be provided in the concentration matrix. Only Knowledge of the constituents is required, therefore CLS requires knowing the complete composition [concentration of every constituent] of the calibration matrix (Aguilar *et al*, 2005).For application of these multivariate methods, a training set of 25 mixtures with different concentrations of Mox , HCTZ, DSA and Mox-at in the range of (2.0-6.0 µgml^{-1}) as shown in table (6).With regard to CLS, both the absorbance and the concentration matrices were used to construct the CLS model and subsequently obtain the K matrix "absorbitivity matrix" displayed in figure (11). The mean percentage recovery and the standard deviation values the studied drugs in the samples of the validation set is demonstrated in table (7).

In factor based methods [PCR and PLS], appropriate selection of the number of factors to be used to construct the model is the key to achieving correct quantitation 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. In this method test set validation method was used, it was clear from the loadings plots (figure 12), scores plots (figures 13-16), plots of explained Y variance (figures 17-20), residual validation variance (figures 21-24) versus the number of factors or latent variables, that the model selected 4 latent variables. The proposed PLS-2 and PCR calibration models were evaluated by a test set of 8 samples of different concentrations of the components, picked at random.Table (8, 9) represents the mean percentage recovery and the standard deviation values for the test set used in validation of

the proposed PCR & PLS methods. To validate the predictive ability of the three proposed models, several diagnostic tools were applied, the first method was carried out by plotting the known concentrations against the predicted concentrations as shown in figures (25-30). The points lay on a straight line in all plots, the regression analysis for all these linear relationships has been carried out and the results showed that all the plots had a slope of nearly one and an intercept close to zero.

Also the concentration residuals were plotted versus the predicted concentrations as shown in figures (31-36), as can be seen the plots show that the residual values very randomly around zero for all samples indicating that these residuals are the noise associated with the measurement. The plots shows no features for any sample indicating good predictive ability of the models.The third method depends on calculation of the validation diagnostics including SEP, MSEP, RMSEP and S^2, where their numerical values are indicated in table (8). The small values of the calculated validation diagnostics indicate the negligible error of prediction and the high predictive ability of the proposed methods. The proposed chemometric methods were successfully applied to the determination of Mox and HCTZ in commercial Vigoran® tablets. Satisfactory results were obtained in good agreement with the label claim as shown in table (9). These results were statistically compared to those of the published HPLC method with regard to accuracy and precision using student's t-test and F-ratio test at 95% confidence level as demonstrated in table (10). The calculated values did not exceed the theoretical ones, indicating that there is no significant difference between the developed methods and the published HPLC method.

For Chromatographic method:

The effectiveness of employing stationary phases of different selectivities, as opposed to the more common optimization protocol of manipulating the mobile phase for separation of complex mixtures is an approach that was under estimated in the past. Resolution of multicomponent mixtures especially of actives, inactives and their degradates is considered challenging especially when composed of components of high structural similarity which lead to similar chromatographic behaviour and in the sometime compounds with opposite chromatographic behaviour. In the present work, a complex mixture of Mox and HCTZ and their alkaline degradates was presented and several trials have been done in order to resolve and quantitatively determine the active ingredients in presence of their degradates.First, different chromatographic columns were tried including C_{18} and C_8 with different lengths (15 cm and 25 cm), it was found impossible to separate the mixture under isocratic conditions using those columns due to two main problems:

1. Almost similar chromatographic behaviour of DSA and HCTZ which causes almost complete overlapping of their peaks.
2. Under the chromatographic conditions that resolves HCTZ from DSA, Mox which is a more hydrophobic pseudopeptide with a strongly retentive behaviours was strongly retained and was not eluted from the columns used.
3. The problem of non retention of Mox at which leads to its very early elution [causing it to be coeluted with solvent front at a retention time less than 1 minute].

All these reasons led to a conclusion that a polar RP must be used. Cyanopropyl columns presented on excellent solution, where they are classified as normal phase materials with intermediate polarities. The dual character phases have applications in normal and reversed phase chromatography. In the latter technique, chromatographers use them as short alkyl chain phase which minimizes retention of strongly hydrophobic compounds causing them to elute more quickly and with a much better peak shape. In the normal phase mode, the cyanofunctionality provides a different, more gentle interaction with polar compounds than does base silica by different retention mechanisms including pi-pi, dipole dipole and hydrogen bonding when used with non polar mobile phase. All this provides better discriminating power of the cyanobonded phase which certainly provides increased optimization potential. CN columns were also of choice for separation of certain mixtures of peptide and small organic molecules which couldn't be resolved on C_8 or C_{18} column (Majors, 2001; Waksmundzka-Hajnas *et al*, 2001).

Coming to the mobile phase, preliminary trials were conducted using a mixture of methanol-water or acetonitrile water in different ratio and at different PH values but there arised the problem of multiple and irregular shaped chromatographic peaks of peptides. Large sized peptides which are too large to partition into the hydrophobic phase, adsorb to the hydrophobic surface after they enter the column until the mobile phase strength reaches the critical point necessary to cause desorption, they then desorb and interact only slightly with the surface as they elute down the column, that causes the problem of multiple peaks (Waksmundzka-Hajnas *et al*, 2001; Carr, 2002) and because Mox is a large sized pseudopeptide the same problem is faced during its analysis and this necessitates the use of buffers as ammonium acetate and potassium dihydrogen phosphate to control PH. The best buffer was aqeous potassium dihydrogen phosphate in a concentration of 10 mM.

Regarding the PH values, at highly acidic PHs, Mox at showed a split peak due to its cis trans isomerization, while Mox was strongly retained and eluted as a very broad peak that was not well defined. At pH [6.0 - 7.0], all of the compounds were eluted as single, symmetric and good shaped peaks.

The effect of organic modifier on the retention behaviour and peak shapes of the investigated compounds was also studied. It could be arranged in an ascending order Mox-at < DSA < HCTZ <<<<<<< Mox ranging from almost no effect on retentive behaviour of Mox at to critical effect on retention of Mox where becuae of the pseudo-peptide nature of Mox which controls its rentetion behaviour on RP columns by adsorption desorption mechanisms, a practical consequence of this mechanism of interaction is that it is very sensitive to the organic modifier concentration [large changes occur in the retention time with relatively small changes in the organic modifier concentration].

In other words, the number of organic modifier molecules required to desorb Mox is very precise and therefore desorption occurs over a very narrow window of organic modifier concentration. This results in complete retention until the critical organic modifier concentration is reached and sudden desorption taken place (Carr, 2002; Zhou *et al*, 1991).

Using this critical concentration of either acetonitrile or methanol causes either overlapping of HCTZ and DSA or HCTZ and Mox respectively therefore arises the need for a mixture of both modifiers to produce optimum resolution of these compounds and triethyl amine was added in a concentration of 0.1% as a suitable modifier to suppress peak tailing of Mox.

Finally a ratio of 70% buffer: 20% ACN: 10% methanol was found optimum for separation of the mixture with good retention times with the whole run lasting less than 10 minutes as demonstrated in figure (37).

Linear calibration graphs were constructed for Mox and HCTZ by plotting the detector response in terms of peak area against the corresponding concentrations of the studied drugs and the proposed methods were found to be linear over the range of (2.0-12.0 $\mu g.ml^{-1}$) and (1.0-11.0 $\mu g.ml^{-1}$) for Mox and HCTZ respectively. The linear regression equations were computed and found to be:

P.A = 79.427 C + 2.1468 r = 0.9999 for Mox.

P.A = 56.380 C + 0.2734 r = 0.9999 for HCTZ.

Where P.A is the mean peak area of five determinations and C is the corresponding concentration in $\mu g.ml^{-1}$ and r is the correlation coefficient.

The mean percentage recoveries ± SD of pure samples were found to be 100.19±0.193 and 100.02±0.172 for Mox and HCTZ respectively which confirms that the proposed method can be successfully applied for the analysis of the studied drugs in pure powder form. The results are summarized in table (11).

The proposed method could be stability indicating for the determination of the studied drugs in laboratory prepared mixtures of their degradates with mean percentage recoveries ±SD of 100.79±1.034 and 99.10±0.816 for Mox and HCTZ respectively.

Since the robustness of an analytical procedure is a measure of its capacity to remain unaffected after slight but deliberate variation in the analytical conditions. Seperation of the investigated compounds in combination was performed under these conditions. There was a slight decrease or increase in the the t_R of values of all peaks however, it didn't significantly affect the chromatographic performance [K' & R_s] and the calculated R_s were always less than 2 ensuring complete separation.

Table 1. Accuracy of the proposed double divisor ratio spectra derivative spectrophotometry (DDRS-DS) for the analysis of pure samples of Mox.HCl and HCTZ.

Mox.HCl			HCTZ		
Taken (µg.ml-1)	Found* (µg.ml-1)	Recovery (%)	Taken (µg.ml-1)	Found* (µg.ml-1)	Recovery (%)
3	2.98	99.33	3	3.01	100.33
5	4.94	98.80	5	4.99	99.80
7	7.13	101.86	7	7.01	100.14
9	8.93	99.33	9	9.02	100.22
11	11.05	100.46	11	11.04	100.36
12	12.01	100.08	13	13.00	100.00
Mean±SD		99.98±1.097			100.14±0.213

** Average of three determinations.*

Table 2. Results obtained for the analysis of laboratory prepared mixtures containing different ratios of Mox.HCl and HCTZ by the proposed double divisor ratio spectra derivative spectrophotometry in presence of their alkaline degradation

Mox.HCl			HCTZ		
Taken ($\mu g.ml^{-1}$)	Found* ($\mu g.ml^{-1}$)	Recovery (%)	Taken ($\mu g.ml^{-1}$)	Found* ($\mu g.ml^{-1}$)	Recovery (%)
2	2.01	100.50	10	9.92	99.20
4	3.96	99.00	8	8.11	101.38
6	5.91	98.50	6	5.94	99.00
8	8.15	101.88	4	3.93	98.25
10	9.97	99.69	2	1.96	98.00
Mean±SD		99.91±1.332			99.17±1.335

** Average of three determinations.*

Table 3. Quantitative determination of Mox.HCl and HCTZ in pharmaceutical preparation and application of standard addition technique by the proposed double divisor ratio spectra derivative spectrophotometry (DDRS-DS)

Pharmaceutical formulation		Taken (µg.ml-1)	Found* (µg.ml-1)	Standard addition technique		
				Pure added (µg.ml-1)	Pure found** (µg.ml-1)	Recovery (%)
FEmpress® plus tablets claimed to contain 15 mg/25 mg Mox.HCl/ HCTZ tablet (Batch No. 003599)	Moexipril hydrochloride	3.00	99.00±0.995	2	2.00	100.00%
				4	4.08	102.00
				6	6.12	102.00
				8	8.13	101.63
	Mean±SD					101.41 ±0.954
	Hydrochlorothiazide			2	1.99	99.50
		5.00	100.33±0.352	4	3.99	99.75
				6	6.01	100.17
				8	7.98	99.75
	Mean±SD					99.79 ±0.278

* *Average of ten determinations.* ** *Average of five determinations.*

Table 4. Results of assay validation parameters obtained by applying the proposed ratio subtraction method, for determination of Mox.HCl and HCTZ.

Parameters	Fos	HCTZ
Linearity	2-12µg.ml-1	2.14 µg.ml-1
Correlation coefficient (r)	0.999	0.9999
Slope	0.0794	0.08822
Intercept	0.0902	0.004253
Standard error of the slope	0.000302216	0.000353894
Confidence limit of the slope	0.078596-0.080275	0.087310555-0.089129981
Confidence limit of the intercept	0.002353927	0.003165322
Confidence limit of the intercept	0.083647-0.096718	-0.003883862-0.012389576
Standard error of estimation	0.002528523	0.003745259
Accuracy (mean±SD)	99.98±1.097	100.14±0.213
Selectivity	99.91±1.332	99.17±1.335
Precision (RSD%)		
Repeatability*	1.302	0.290
Intermediate precision*	1.400	0.437

* *The intra-day and inter-day relative standard deviations of the average of concentrations 2.0, 8.0, 12.0 µgml^{-1} for Mox.*
* *The intra-day and inter-day relative standard deviations of the average of concentrations 4.0, 8.0, 14.0 µgml^{-1} for HCTZ.*

Table 5. Statistical comparison between the results obtained by applying the proposed double divisor ratio spectra derivative spectrophotometry and the reported method for determination of Mox.HCl and HCTZ.

Items	Ratio subtraction		Reported Method*	
	Mox.HCl	HCTZ	Mox.HCl	HCTZ
Mean	99.00	100.33	99.37	100.83
SD	0.995	0.352	0.951	0.556
RSD%	1.005	0.351	0.957	0.551
N	10	10	5	5
Variance	0.990	0.124	0.904	0.309
Student's t-test (2.306)	0.694	2.151		
F-value (6.388)	1.095	2.495		

* *The numbers between parenthesis are the theoretical values.*

Table 6. The concentration of mixtures of Mox.HCl and HCTZ and their degradates in the training set.

Sample No.	Mox.HCl	Mox-at	HCTZ	DSA
1	4	4	4	4
2	4	2	2	6
3	2	2	6	3
4	2	6	3	6
5	6	3	6	4
6	3	6	4	3
7	6	4	3	3
8	4	3	3	5
9	3	3	5	6
10	3	5	6	5
11	5	6	5	4
12	6	5	4	6
13	5	4	6	6

14	4	6	6	2
15	6	6	2	5
16	6	2	5	2
17	2	5	2	4
18	5	2	4	5
19	2	4	5	5
20	4	5	5	3
21	5	5	3	2
22	5	3	2	3
23	3	2	3	4
24	2	3	4	2
25	3	4	2	2

Table 7. Results obtained for the determination of Mox.HCl and HCTZ in the validation set by classical least squares method (CLS)

	Mox.HCl			HCTZ		
Sample No.	Taken (□g.ml-1)	Found (□g.ml-1)	Recovery (%)	Taken (□g.ml-1)	Found (□g.ml-1)	Recovery (%)
1	4.00	4.10	102.50	4.00	4.03	100.75
3	2.00	2.02	101.00	3.00	3.03	101.00
5	3.00	2.96	98.67	4.00	4.01	100.25
7	4.00	4.09	102.25	3.00	2.91	97.00
9	3.00	3.04	101.33	6.00	5.90	98.33
11	6.00	6.03	100.50	4.00	3.99	99.75
13	4.00	3.90	97.50	6.00	5.90	98.33
15	6.00	5.97	99.50	5.00	5.12	102.40
17	5.00	4.97	99.40	4.00	4.10	102.50
19	4.00	4.09	102.25	5.00	4.98	99.60
21	5.00	4.90	98.00	2.00	2.04	102.00
23	2.00	1.99	99.50	4.00	.10	102.50
25	4.00	3.97	99.25	2.00	1.98	98.91
Mean±SD			100.13±1.649			100.26±1.803

Table 8. Results obtained for the determination of Mox.HCl and HCTZ in the validation set by the principal component regression (PCR) model in presence of their degradates

	Mox.HCl			HCTZ		
Sample No.	Taken (□g.ml-1)	Found (□g.ml-1)	Recovery (%)	Taken (□g.ml-1)	Found (□g.ml-1)	Recovery (%)
3	2.00	2.02	101.00	6.00	5.90	98.33
5	6.00	5.96	99.33	6.00	5.90	98.33
8	4.00	3.98	99.50	3.00	3.07	102.33
12	6.00	6.04	100.67	4.00	4.03	100.75
13	5.00	4.99	99.80	6.00	6.08	101.33
15	6.00	5.97	99.50	2.00	1.96	98.00
20	4.00	4.01	100.25	5.00	5.14	102.80
22	5.00	5.03	100.60	2.00	2.03	101.50
Mean±SD			100.08±0.633			100.42±1.928

Table 9. Results obtained for the determination of Mox.HCl and HCTZ in the validation set by the partial least squares (PLS) model in presence of their degradates.

	Mox.HCl			HCTZ		
Sample No.	Taken (□g.ml-1)	Found (□g.ml-1)	Recovery (%)	Taken (□g.ml-1)	Found (□g.ml-1)	Recovery (%)
3	2.00	2.02	101.00	6.00	5.90	98.33
5	6.00	5.96	99.33	6.00	5.90	98.33
8	4.00	3.98	99.50	3.00	3.06	102.00
12	6.00	6.04	100.67	4.00	4.03	100.75
13	5.00	4.98	99.60	6.00	6.08	101.33
15	6.00	5.97	99.50	2.00	1.96	98.00
20	4.00	4.00	100.00	5.00	5.10	102.00
22	5.00	5.03	100.60	2.00	2.04	102.00
Mean±SD			100.03±0.645			100.34±1.812

Table 10. Statistical parameters of the validation mixtures of Mox.HCl, HCTZ and their degradates for CLS, PCR and PLS-2 models

	Mox.HCl			HCTZ		
	CLS	PCR	PLS	CLS	PCR	PLS
SEP	0.065775	0.0030028558	0.029345236	0.073608	0.088165429	0.078832553
MSEP	0.003994	0.000789	0.0007535	0.005001	0.0068015	0.00543775
RMSEP	0.063195	0.028089144	0.027449954	0.07072	0.082471207	0.073741101

Such results encouraged the use of the proposed method for the assay of the studied drugs in commercial tablets. The results of which showed no sign for interference of excipients used in tablet formation as demonstrated in table (12).

The accuracy of the method was further confirmed and validated by applying the standard addition technique. The mean percentage recoveries of the authentic added are summarized in table (13). The results of the method validation parameters are listed in table (14). Table (15) shows the system suitability criteria including the retention time [min], capacity factor [K'], selectivity [α], number of theoretical plates [N], resolution [R_s] and symmetry, the results were found in agreement with the USP requirements. The results obtained by the proposed method were statistically compared with those obtained by applying the reported method. Table (16) shows that the values of calculated t- and F were found less than the tabulated ones indicating that there was no significant differences between the two methods.

Table 11. Statistical comparison of the results obtained by the proposed chemometric methods and the reported method for the determination of Mox.HCl and HCTZ .

Items	CLS		PCR		PLS		Reported Method*	
	Mox.HCL	HCTZ	Mox.HCl	HCTZ	Mox.HCL	HCTZ	Mox.HCL	HCTZ
Mean	100.27	100.40	100.16	100.90	100.05	100.75	99.37	100.83
SD	0.997	1.115	0.415	1.321	0.420	1.250	0.951	0.556
RSD%	0.994	1.111	0.414	1.309	0.420	1.241	0.957	0.551
n	5	5	5	5	5	5	5	5
Variance	0.995	1.244	0.215	1.745	0.540	1.563	0.904	0.309
Student's t-test (2.306)	1.457	0.775	1.657	0.103	1.446	0.127		
F-value (6.388)	1.101	4.025	4.205	5.649	5.129	5.060		

* The numbers between parenthesis are the theoretical values.

Table 12. Accuracy of the proposed HPLC method for the analysis of pure samples of Mox.HCL and HCTZ.

Mox.Hcl			HCTZ		
Taken (µg.ml^{-1})	Found* (µg.ml^{-1})	Recovery (%)	Taken (µg.ml^{-1})	Found* (µg.ml^{-1})	Recovery (%)
3	3.01	100.33	2	2.00	100.00
5	5.01	100.20	4	3.99	99.75
7	7.02	100.29	6	6.01	100.17
9	9.03	100.33	8	8.01	100.13
11	10.98	99.82	10	9.99	99.90
12	12.02	100.17	11	11.02	100.18
Mean±SD		100.19±0.193			100.02±0.172

** Average of three determinations.*

Table 13. Quantitative determination of Mox.HCl and HCTZ in pharmaceutical preparation and application of standard addition technique by the proposed HPLC method:

Pharmaceutical formulation		Taken (µg.ml^{-1})	Found* (µg.ml^{-1})	Standard addition technique		
				Pure added (µg.ml-1)	Pure found* (µg.ml-1)	Recovery (%)
Fempress® plus tablets claimed to contain 15 mg/25 mg Mox.Hcl/HCTZ tablet (Batch No. 003599)	Moexipril hydrochloride	3.00	99.25±0.406	2.00	2.02	101.00
				4.00	4.05	101.25
				6.00	6.05	100.83
				8.00	8.06	100.75
	Mean ±SD					100.96±0.221
	Hydrochlorothiazide	5.00	100.90±0.340	1.00	1.00	100.00
				3.00	3.00	100.00
				5.00	4.99	99.80
				7.00	5.98	99.67
	Mean±SD					99.87±0.162

* *Average of five determinations.*

Table 14. Results of assay validation parameters obtained by applying the proposed HPLC method for determination of Mox.Hcl and HCTZ.

Parameters	Mox.HCl	HCTZ
Linearity	2-12 µg.ml-1	1-11 µg.ml-1
Correlation coefficient (r)	0.9999	0.9999
Slope	79.42678071	56.38032771
Intercept	-2.146818333	0.273422048
Standard error of the slope	0.348016362	0.229748172
Confidence limit of the slope	78.46053239-80.39302904	55.74244453-57.0182109
Confidence limit of the intercept	2.710658017	1.586205519
Confidence limit of the intercept	-9.672811515-5.379174848	-4.130590501-4.677434596
Standard error of estimation	2.911714	1.922211119
Accuracy (mean±SD)	100.19±0.193	100.02±0.172
Selectivity	100.79±1.034	99.10±0.816
Precision (RSD%)		
Repeatability*	0.240	0.129
Intermediate precision*	0.326	0.237

* *The intra-day and inter-day relative standard deviations of the average of concentrations 2.0, 8.0, 12.0 µgml^{-1} for Mox.*

* *The intra-day and inter-day relative standard deviations of the average of concentrations 1.0, 5.0, 11.0 µgml^{-1} for HCTZ.*

Table 15. The system suitability test results of the developed high performance liquid chromatographic method for determination of Mox. HCL and HCTZ in presence of their degradates.

Parameters	System suitability test results	
	Mox.HCL	HCTZ
Retention time (min)	6.67	5.44
Capacity factor (K)	2.42	1.79
Selectivity	1.25	1.16
Number of theoretical plates (N)	2555	8170
Resolution (Rs)	3.53	3.40
Symmetry	0.93	0.89

Table 16. Statistical comparison between the results obtained by applying the HPLC method and the reported method for the determination of Mox.HCl and HCTZ .

Items	HPLC method		Reported method*	
	Mox.HCL	HCTZ	Mox.HCL	HCTZ
Mean	99.25	100.90	99.37	100.83
SD	0.406	0.340	0.951	0.556
RSD%	0.409	0.337	0.957	0.551
N	5	5	5	5
Variance	0.165	0.115	0.904	0.309
Student's t-test (2.306)	0.262	0.220		
F-value (6.388)	5.493	2.680		

* *The numbers between parenthesis are the theoretical values.*

Conclusion

All the presented methods are not only an advance in the analysis of complex mixtures but also considered the first to determine Mox and HCTZ in presence of their degradates. The presented methods were also found suitable for application on pharmaceutical formulations and bulk samples with acceptable results.

References:

Aguilar, M.I., 2005. HPLC of Peptides and Protein Methods and Protocols. Vol. 251.

Banker, G.S. and C.T. Rhades, 2002. "Modern Pharmaceutics", 4th ed., Marcel Dekker, Inc.

Bebawy, L.I., and N. El Koussy, 1997. Stability-Indicating Method for the Determination of Hydrochlorothiazide, Benzydamine Hydrochloride and Clonazepam in the Presence of Their Degradation products. Analytical Letters, 30: 1379-1397.

Belal, F., F.H. Metwally, K.M. Younes and S.M. Amer, 2009. Development of Membrane Electrodes for the Specific Determination of Moexipril Hydrochloride in Dosage Forms and Biological Fluids. Portugaliae Electrochimica Acta, 27: 463-475.

Carr, D., 2002. The Handbook of Analysis and Purification of Peptides and Proteins by RP-HPLC", 3rd ed., Grace Vydac Technical Support Group.

Dinç, E., and F. Onur, 1998. Application of a new spectrophotometric method for the analysis of a ternary mixture containing metamizol, paracetamol and caffeine in tablets. Anal.Chim.Acta, 359: 93-106.

El Shanawane, A.A., S.M. Mostafa and M.S. El Gawish, 2008. Application of a validated, stability indicating LC method to stress degradation studies of ramipril and moexipril. HCl. Chromatographia, 67: 567-573.

El –Yazbi, F.A., H.H. Abdine and R.A.Shaalan, 1999. Spectrophotometric methods for the determination of benazepril hydrochloride in its single and multi-component dosage forms. J.Pharm. Biomed. Anal., 20: 343-350.

Erk, N., 1999. Determination of active ingredients in the pharmaceutical formulations containing hydrochlorothiazide and its binary mixtures with benazepril hydrochloride, triamterene and cilazapril by ratio spectra derivative spectrophotometry and vierordt's method. J.Pharm. Biol. Anal., 20: 155-167.

Florence, A.T., and D. Attwood, 1998. "Physicochemical Principles of Pharmacy", 3rd ed., London: Mcmillan Press.

Ghasemi, J., B. Abassi, A. Niazi, E. Nadof, and A. Mordai, 2004. Simultaneous Spectrophotometric Multicomponent Determination of Folic Acid, Thiamine, Riboflavin, and Pyridoxal by Using Double Divisor--Ratio Spectra Derivative--Zero Crossing Method. Anal. Lett., 37: 2609-2623.

Hammes, W., B. Hammes, U. Büchsler, F. Paar and H. Bokens, 1995. Simultaneous determination of moexipril and moexiprilat, its active metabolite, in human plasma by gas chromatography-negative-ion chemical ionization mass spectrometry. J. Chromatogr. B Biomed. Appl., 670: 81-89.

Henry, M.B., O.L. Charles and R.L. Wait, 2001. "Physical and Technical Pharmacy", New York, Toronto, London: Mc Graw-Hill Book Co., Inc.

James, I.W., 1998. Pharmaceutical Preformulation: The Physicochemical Properties of Drug Substances, Ellis Horwood, Ltd.

Kalász, H., G. Petroianu, K. Tekes, I. Klebovich, Z. Ludányi and K. Gulyás, 2007. Metabolism of moexipril to moexiprilat: determination of in vitro metabolism using HPLC-ES-MS. Medicinal Chemistry, 3: 101-106.

Kartal, M., and N. Erk, 1999. Simultaneous determination of hydrochlorothiazide and amiloride hydrochloride by ratio spectra derivative spectrophotometry and high-performance liquid chromatography. J. Pharm. Biomed. Anal., 19: 477-485.

Kenneth, R.B., J.P. Randy and M.B. Seasholtz, 1998. Chemometrics Practical Guide, John Willey and Sons Inc., New York.

Kóti, J., V. Háda, G. Petroianu, M.Y. Hasan, K. Tekes, Z. Szücs and H. Kalász., 2006. Monitoring the metabolism of moexipril to moexiprilat using high performance liquid chromatography-electrospray ionization mass spectrometry. J. Chromatogr. Sci., 44: 214-218.

Kramer, R., 1998.Chemometric Techniques for Quantitative Analysis, Marcel Dekker Inc., New York.

Luis, M.L., Fraga J.M.G., and Jimenez A.I., 2001. Simultaneous spectrophotometric determination of diuretics by using multivariate calibration methods. Talanta, 53: 761-770.

Majors, R.E., 2001. New Chromatography Columns and Accessories at the 2001 Pittsburgh Conference, Part I, LC.GC Europe.

Markopoulou, C.K., E.T. Malliou, and J.E. Koundourellis, 2005. Content uniformity and dissolution tests of triplicate mixtures by a double divisor-ratio spectra derivative method. Il Farmaco, 60: 755-762.

Martin, E.M., O. Henandez and J.J.Arias, 1997. Microchem. J., 56: 207-215.

Martin, E., O. Hernandez, A.I. Jimenez, 1998. A partial least-squares multivariate calibration method for the simultaneous spectrophotometric determination of spironolactone, canrenone and hydrochlorothiazide. Anal. Lett., 31: 1857-1877.

Ni, Y., and X. Gong, 1997. Simultaneous spectrophotometric determination of mixtures of food colorants. Anal. Chim. Acta, 354: 163-171.

Panderi, I.E., 1999. Simultaneous determination of benazepril hydrochloride and hydrochlorothiazide in tablets by second-order derivative spectrophotometry. J.Pharm. Biomed. Anal., 21: 257-265.

Prasad, C.V.N., V. Bharadwaj and V. Narsimhan, 1997. Simultaneous determination of metoprolol-hydrochlothiazide and propranolol-hydrochlorthiazide by derivative spectroscopy. J.AOAC Int., 80: 325-330.

Prasad, C.V.N., C. Parihar and K.Sunil, 1998. Simultaneous determination of amiloride HCl, hydrochlorothiazide and atenolol in combined formulations by derivative spectroscopy. J.Pharm. Biomed. Anal., 17: 877-884.

Sidika, E., C.S. Müge and A. Sedef, 2003. Simultaneous determination of moexipril hydrochloride and hydrochlorothiazide in tablets by derivative spectrophotometric and high-performance liquid chromatographic methods. J. Pharm. Biomed Anal., 33: 505-511.

Thomas, E.V., and D.M. Haaland, 1990. Comparison of multivariate calibration methods for quantitative spectral analysis. Anal. Chem., 62: 1091-1099.

Ulvi, V., 1998. Determination of Hydrochlorothiazide and Triamterene in Combined Tablets by Derivative Spectrophotometry. Pharm. Pharmacol. Commun., 4: 193-195.

Waksmundzka-Hajnas, M., A. Petruczynik and A. Hawry, 2001. Comparison of chromatographic properties of cyanopropyl-, diol- and aminopropyl- polar-bonded stationary phases by the retention of model compounds in normal-phase liquid chromatography systems. J. Chromatogr. A, 919: 39-50.

Youssef, R.M. and H.M. Maher, 2008. A new hybrid double divisor ratio spectra method for the analysis of ternary mixtures. Spectrochimica Acta, Part A: Molecular and Biomolecular Spectroscopy, 70: 1152-1166.

Zhou, N.E., C.T. Mant, J.J. Kirkl and R.S. Hodges, 1991. Comparison of silica-based cyanopropyl and octyl reversed-phase packings for the separation of peptides and proteins. J.Chromatogr., 548: 179-193.