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Research Article

APPLICATION OF THREE NOVEL SPECTROPHOTOMETRIC METHODS MANIPULATING RATIO SPECTRA FOR RESOLVING A PHARMACEUTICAL MIXTURE OF CHLORPHENOXAMINE HYDROCHLORIDE AND CAFFEINE

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ABSTRACT

Three spectrophotometric methods are presented for the determination of a binary mixture of Chlorphenoxamine hydrochloride (CPX) and Caffeine (CAF) in laboratory prepared mixture and pharmaceutical dosage form without prior separation. Method (I) is an extended ratio subtraction method (EXRSM) coupled with ratio subtraction method (RSM), which depends on subtraction of the plateau values from the ratio spectrum. Method (II)is a ratio difference spectrophotometric method (RDSM), which depends on the difference in value between two different wavelengths of the ratio spectrum. Method (III) is a mean centering of ratio spectra (MCR). Mathematical explanation of the three methods is illustrated. Calibration curves of the three methods are linear over the concentration ranges of 4-24 μgml^{-1} and 2-24 μgml^{-1} for CPX and CAF, respectively. The three methods proved to be simple, specific, accurate and precise. Solvent used is double distilled water. The three methods are validated as per the ICH guidelines where accuracy, precision, repeatability and robustness are found to be within the acceptable limits.

Keywords: Chlorphenoxamine HCl, Caffeine, Extended ratio subtraction method, Ratio subtraction method, Mean centering ratio spectra, Mean centering, Ratio difference, Ratio spectra

INTRODUCTION

Chlorphenoxamine hydrochloride (CPX),2- [1-(4-Chlorophenyl)-1–phenylethoxy] -N, N-dimethylethanamine.HCL, is a histamine H_1-receptor antagonist with antimuscarinic properties [1] and Caffeine (CAF), 3, 7-Dihydro-1, 3, 7-trimethyl-1 H- purine-2, 6-dione, is a xanthine derivative with CNS stimulant activity [1, 2]. Combination of the two drugs is available as Allergex Caffeine tablets produced by the Egyptian International Pharmaceutical Industries Co. (EIPICO). CPX provides symptomatic relief in allergic conditions, and CAF is added to counteract the sedative effects of CPX. The chemical structures of the two investigated drugs are shown in Fig. 1.

The literature in hand describes several articles dealing with the simultaneous determination of the components of this pharmaceutical mixture. It includes colorimetry [3], derivative spectrophotometry [4,5], chemometry [4], TLC spectrodensitometry [6] and HPLC [7]. On the other hand, literature survey reveals that no ratio spectra UV-spectroscopic methods [8-10] were reported for this simultaneous determination of this combination.

Thus, the purpose of this study was to solve the problem of overlapping spectra of both drugs by developing rapid, simple and precise spectrophotometric methods for their simultaneous determination.

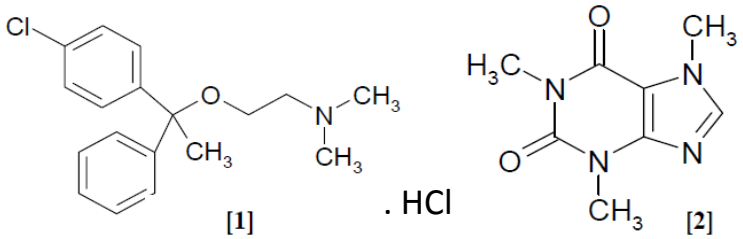

Fig. 1: The Chemical Structure of Chlorphenoxamine Hydrochloride [1] and Caffeine [2].

Theory of the proposed methods

Extended ratio subtraction method (EXRSM)

Extended ratio subtraction method (EXRSM) starts with the ratio subtraction method (RSM) [9] which depends on that, if you have a mixture of two drugs X and Y having overlapped spectra, you can determine X by dividing the spectrum of the mixture (X+Y) by a known concentration of Y as a divisor Y'. The division will give a new curve that represents *X/Y' + constant*. Measure the value of this constant *X/Y'* in the plateau region. If we subtract this constant value, then multiply the obtained curve after subtraction by Y' (the divisor), then we can obtain the zero order absorption spectrum (D^0) of X, (original spectrum of X). This can be summarized in the following equations:

(X+Y)/Y' = X/Y' + Y/Y' = X/Y' + constant

X/Y' + constant – constant = X/Y'

X/Y' x Y' = X

The concentration of X is calculated using the regression equation representing the linear relationship between the absorbance at its λ_{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 D^0 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 D^0 of Y (original spectrum of Y).

Y/X' + X/X' – X/X' = Y/X' x X' = Y

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

Ratio difference spectrophotometric method (RDSM)

This method is based on that the amplitude difference between two points on the ratio spectra of a mixture is directly proportional to the concentration of the component of interest. The independence of the interfering component is the major advantage of this method [10].

This method depends on that, if you have a mixture of two drugs X and Y having overlapped spectra, you can determine X by dividing the spectrum of the mixture by a known concentration of Y as a divisor Y'. The division will give a new curve that represents *(X+Y)/Y'* i.e. *X/Y' + Y/Y'*, where *Y/Y'* is a constant. By selecting two wavelengths λ_1 and λ_2 on the obtained ratio spectrum and subtracting the amplitudes at these two points, the constant *Y/Y'* will be cancelled along with any other instrumental error or any interference from the sample matrix. This can be summarized as the following:

(X+Y)/Y' = X/Y' + Y/Y'= X/Y' + constant

Suppose the amplitudes at the two wavelengths are P1 and P2 at λ_1 and λ_2 respectively; by subtracting the two amplitudes the interfering Y shows no interference; then:

P1 – P2 = (X/Y')1 + constant – {(X/Y')2 + constant}

P1 – P2 = (X/Y')1 – (X/Y')2

where; P1 is the peak amplitudes of the ratio spectrum at λ_1, P2 is the peak amplitudes of the ratio spectrum at λ_2.

The concentration of X is calculated by using the regression equation representing the linear relationship between the differences of the ratio spectra amplitudes at the two selected wavelengths versus the corresponding concentration of drug X.

Similarly, Y could be determined by the same procedure using a known X as a divisor X`.

Mean centering of ratio spectra spectrophotometric method (MCR)

This is a well-established spectrophotometric method in which both binary and ternary mixtures could be determined without previous separation. In this method the ratio spectra are obtained after which the constant is removed by mean centering of the ratio spectra [11].

To explain the mean centering expression, consider a three-dimensional vector [12]:

$$y = \begin{bmatrix} 5 \\ 1 \\ 3 \end{bmatrix}$$

We center or mean center (MC) this column by subtracting the mean of three numbers

$$\text{calling: } y' = \begin{bmatrix} 3 \\ 3 \\ 3 \end{bmatrix}$$

$$MC(y) = y - y' = \begin{bmatrix} 5 \\ 1 \\ 3 \end{bmatrix} - \begin{bmatrix} 3 \\ 3 \\ 3 \end{bmatrix} = \begin{bmatrix} +2 \\ -2 \\ 0 \end{bmatrix}$$

It could be proved that if the vector *y* is multiplied by *n* (a constant number), the mean centered vector is also multiplied by *n* and also if a constant number is added to the vector *y*, the mean center of this vector is not changed.

Consider a mixture of two compounds *X* and *Y*. If there is no interaction among the compounds and Beer's law is obeyed for each compound, it can be written:

$A_m = \alpha x Cx + \alpha y Cy$ (1)

where A_m is the vector of the absorbance of the mixture, αx and αy are the molar absorptivity vectors of *X* and *Y* and *Cx* and *Cy* are the concentrations of *X*, *Y* and *Z*, respectively.

If Eq. (1) is divided by αy corresponding to the spectrum of a standard solution of *Y* in binary mixture, the first ratio spectrum is obtained in the form of Eq. (2) (for possibility of dividing operation, the zero values of αy should not be used in the divisor):

$B = Am/\alpha y = \alpha x\ Cx/\alpha y + Cy$ (2)

If Eq. (2) is mean centered (MC), since the mean centering of a constant (*Cy*) is zero, Eq. (3) would be obtained:

$MC(B) = MC\ [\alpha x C\alpha/\alpha y]$ (3)

Eq. (3) is the mathematical foundation of binary mixture analysis that permits the determination of concentration of each of the active compounds in the solution (*X* in these equations) without interfering from the other compound of the binary system (*Y* in these equations). As Eq. (3) shows there is a linear relation between the amount of MC(*B*) and the concentration of *X* in the solution.

A calibration curve could be constructed by plotting MC(*B*) against concentration of *X* in the standard solutions of *X* or in the standard binary mixtures. For more sensitivity the amount of MC(*B*) corresponding to maximum or minimum wavelength should be measured.

Calibration graphs for *Y* could also be constructed as described for *X*.

MATERIALS AND METHODS

Instrumentation

Spectrophotometric analysis was carried out on a Shimadzu 1601 double beam spectrophotometer with a fixed slit width (2 nm) using a pair of 1 cm matched quartz cells. The spectrophotometer is connected to an IBM PC with an HP inkjet printer. The bundle software, UV-Probe spectroscopy software version 2.1 (Shimadzu, Kyoto, Japan), was used to process absorption.

Software

Microsoft Excel was used for handling data and processing calculations.

Materials

Pure samples

Pure drug samples of CPX and CAF were kindly supplied by EIPICO Pharmaceuticals, 10th of Ramadan City, Egypt. Their purity was checked and found to be 100.62 ± 0.61 and 99.20 ± 0.78 % according to the manufacturer method of analysis [3] and the BP [2], for CPX and CAF, respectively.

Solvents

Double distilled water.

Pharmaceutical dosage form

Allergex Caffeine tablets (EIPICO Pharmaceuticals) Batch No. 1106021, labeled to contain 20 mg CPX and 50 mg CAF per tablet were purchased from local pharmacies.

Stock and working standard solutions

Stock standard solutions

CPX and CAF stock standard solutions (both are 1 mg ml^{-1}), prepared by dissolving 100 mg of CPX and CAF, respectively, in a few milliliters of double distilled water into a 100-ml volumetric flasks and then completing to volume with the same solvent.

Working standard solutions

CPX and CAF working standard solution (both 0.1 mg ml^{-1}), prepared by transferring 10 ml of each of CPX and CAF stock solutions, into a 100-ml volumetric flask and complete to volume with the same solvent.

Procedure

Spectral characteristics and wavelengths selection

The absorption spectra of 16 µg ml^{-1} of CPX and 16 µg ml^{-1} of CAF were recorded over the spectral wavelength range 200–320 nm using double distilled water as blank.

Linearity and construction of calibration curves

Accurately measured aliquots equivalent to 50-600 µg CPX and 25-600 µg CAF are transferred from their working standard solution (both are 0.1 mg ml^{-1}) into two series of 25-ml volumetric flasks and complete to volume with double distilled water. The spectra of the prepared standard solutions are scanned from 200 to 320 nm and stored in the computer.

For extended ratio subtraction method (EXRSM) coupled with ratio subtraction method (RSM)

Calibration curves are constructed relating the absorbance of zero order spectra of CPX at 222.4 nm and CAF at 273.0 nm versus the corresponding concentrations and regression equations are computed.

For ratio difference spectrophotometric method (RDSM)

The stored spectra of CPX are divided by the spectrum of 12 µg ml^{-1}CAF while CAF spectra are divided by the spectrum of 12 µg ml^{-1} CPX.

Calibration curves of CPX and CAF are constructed by plotting the difference between the amplitudes of ratio spectra at 226.3 and 235.0 nm for CPX and 209.1 and 226.7 nm for CAF, versus the corresponding concentrations and the regression equations are computed.

For mean centering of ratio spectra method (MCR)

The scanned spectra are exported to Microsoft Excel for subsequent calculations, and then the spectra of CPX are divided by the normalized spectrum of CAF, the obtained ratio spectrum is mean centered. The same procedure is applied to CAF.

The Calibration curves for CPX and CAF are constructed by plotting the mean centered values at 226.8 and 274.0 nm, respectively, versus the corresponding concentrations and the regression equations are computed.

Application of extended ratio subtraction, ratio difference and mean centering of ratio spectra for the determination of CPX and CAF in laboratory prepared mixtures

Solutions containing different ratios of CPX and CAF were prepared by transferring accurately measured aliquots from their standard working solutions into a series of 25-ml volumetric flasks and the volume was completed to the mark with double distilled water. The final concentration ranges were 4 - 24 µg ml^{-1} for CPX and 2 - 24 µg ml^{-1} for CAF. Zero order absorption spectra of these different laboratory prepared mixtures were recorded from 200 to 320 nm using double distilled water as blank and the procedure under linearity for each method was then followed. Concentrations of CPX and CAF in the prepared samples were calculated from the corresponding computed regression equations.

Application to pharmaceutical preparation

To determine the content of CPX and CAF in commercial tablets (each tablet labeled to contain 20 mg CPX and 50 mg CAF), 20 tablets were weighed and finely powdered. A portion of powder equivalent to one tablet was weighed accurately and transferred to a 100-ml beaker. 50 ml of double distilled water was added, stirred using a magnetic stirrer for 15 min and filtered through 0.5µm Whatman filter paper into a 100-ml volumetric flask. The residue was washed three times each with 10 ml of double distilled water and the solution was completed to the mark with the same solvent. From the above prepared solution, further dilutions were prepared in the obtained linearity ranges using the same solvent. The general procedure described above under each method was followed to determine the concentration of both drugs in the prepared dosage form solution. The analysis was done in triplicates. Concentrations of CPX and CAF in the prepared samples were calculated from the corresponding computed regression equations.

RESULTS AND DISCUSSION

This paper describes the application of three recently developed spectrophotometric ratio-spectra methods for the simultaneous determination of CPX and CAF. The zero order absorption spectra of pure drugs show overlapping which hinders their direct determination as shown in Fig. 2.

Extended ratio subtraction method (EXRSM) coupled with ratio subtraction method (RSM)

Extended ratio subtraction method (EXRSM) starts after the application of the ratio subtraction method (RSM) [9]. The RSM depends on that, when a mixture of CPX and CAF, where the spectrum of CAF is more extended (Fig. 2), the determination of CPX in the mixture could be done by scanning the zero order absorption spectra of the laboratory-prepared mixtures (CPX and CAF), dividing them by a carefully chosen concentration of standard CAF` (12 µg ml^{-1}) as a divisor. This chosen concentration of the divisor gives the best regression over the proposed concentration range. This will produce new ratio spectra which represent *CPX/CAF' + constant* as shown in Fig. 3a and 3b. Next, subtraction of the values of these constants *CAF/CAF'* in the plateau region (280-290 nm) is done, as shown in Fig. 4a and 4b. This is followed by multiplication of the obtained spectra by the divisor CAF` (12 µgml^{-1}) as shown in Fig. 5a and 5b, which corresponds to the original spectra of CPX. These obtained spectra are used for the direct determination of CPX at 222.4 nm and calculation of the concentration from the corresponding regression equation (obtained by plotting the absorbance values of the zero order curves of CPX at 222.4 nm against the corresponding concentrations).

The determination of CAF could be done by the extended ratio subtraction by dividing these obtained spectra of CPX by a carefully chosen concentration of standard CPX` (12µg$ml^{-1}$) producing ratio spectra that represent the constants *CPX/CPX '*in plateau (210-240 nm) as shown in Fig. 6. The previously scanned zero order absorption spectra of the laboratory-prepared mixtures (CPX and CAF) were divided by standard CPX` (12 µg ml^{-1}) as a divisor producing new ratio spectra which represent *CAF/CPX' + constant* as shown in Fig. 7. Then subtraction of these obtained constants *CPX/CPX'* as shown in Fig. 8, which is followed by multiplication of the obtained spectra by the divisor CPX` (12 µg ml^{-1}) as shown in Fig. 9. Finally, the original spectra of CAF (Fig. 9) could be obtained which are used for direct determination of CAF at 273.0 nm and calculation of the concentration from the corresponding regression equation (obtained by plotting the absorbance values of the zero order curves of CAF at 273.0 nm against the corresponding concentrations).

The extended ratio subtraction method has an advantage that the extended drug in the mixture could be determined at its λ_{max} which could not be achieved by the previously established ratio subtraction method [9] which had determined unextended drug only. Therefore, the two methods are considered to be complementary to each other since the two components of interest in the mixture could be determined.

Ratio difference spectrophotometric method (RDSM)

The most striking feature of the ratio difference method is its simplicity, rapidity and accuracy [10]. This is a newly developed method having the ability for solving severely overlapped spectra without prior separation; meanwhile it doesn't require any sophisticated apparatus or expensive computer programs.

The utilization of ratio difference method is to calculate the unknown concentration of a component of interest present in a mixture containing both the component and an interfering component.

The only requirement in the ratio difference method is the contribution of the two overlapped spectra at the two selected wavelengths λ_1 and λ_2 where the ratio spectrum of the

interfering component shows the same amplitude (constant) whereas the component of interest shows significant difference in these two amplitude values at these two selected wavelengths with concentration. Similarly, another two wavelengths are selected for the estimation of the second component. Thus, the overlapped spectra of the cited drugs suggested that a ratio difference method was a suitable method for simultaneous determination of CPX and CAF.

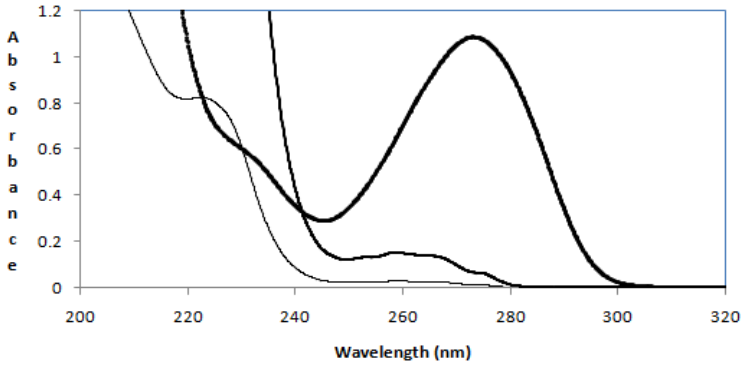

Fig. 2: Zero order absorption spectra of 20 µg ml^{-1} (- - - -), 100 µg ml^{-1} (— . . — .) of Chlorphenoxamine hydrochloride and 20 µg ml^{-1} of Caffeine (·····).

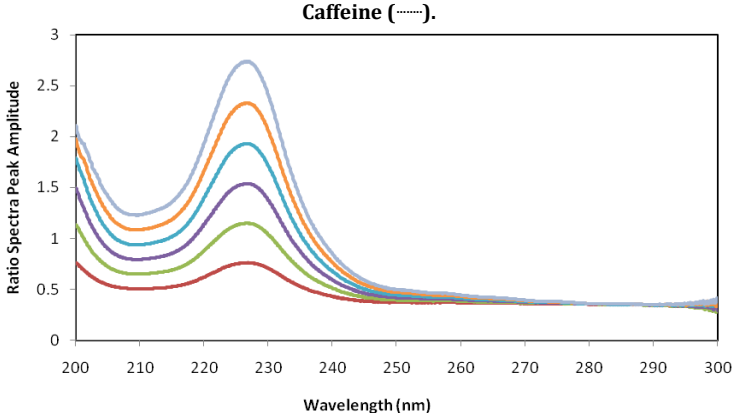

Fig. 3a: Ratio Spectra of some laboratory prepared mixture of CPX (4-24 µg ml^{-1}) and CAF (4 µg ml^{-1}) using 12 µg ml^{-1} of CAF as a divisor.

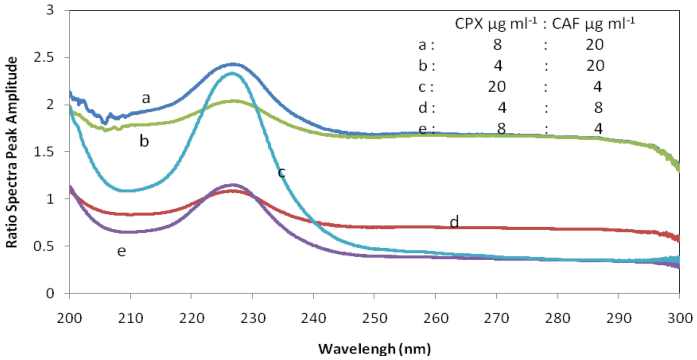

Fig. 3b: Ratio Spectra of some laboratory prepared mixtures of CPX and CAF using 12 µg ml^{-1} of CAF as a divisor.

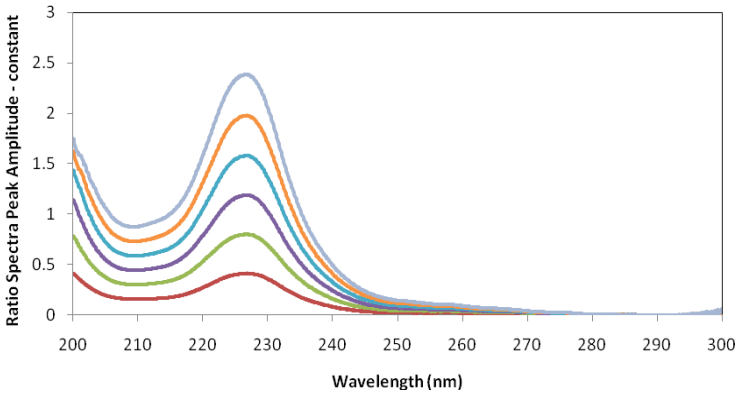

Fig. 4a: Ratio spectra of some laboratory prepared mixtures of CPX (4-24 µg ml^{-1}) and CAF (4 µg ml^{-1}) using 12 µg ml^{-1} of CAF as a divisor after subtraction of the constant.

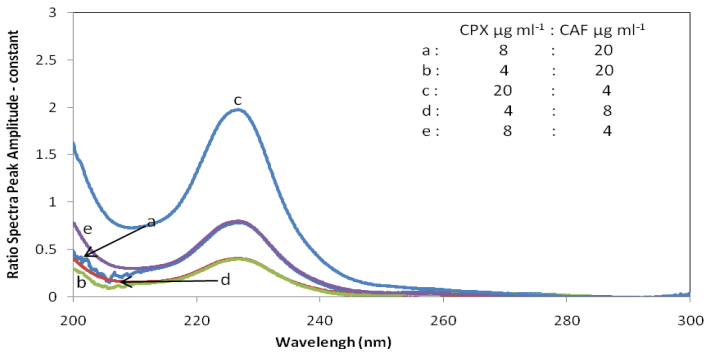

Fig. 4b: Ratio spectra of some laboratory prepared mixtures of CPX and CAF using 12 µg ml^{-1} of CAF as a divisor after subtraction of the constant.

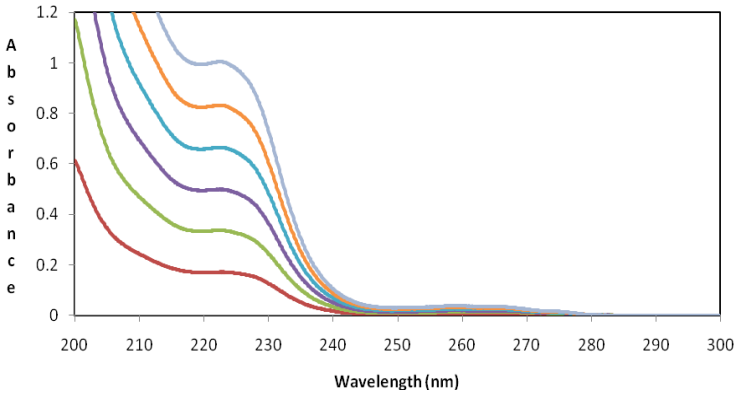

Fig. 5a: The zero order absorption spectra of CPX (4-24 µg ml^{-1}) (X) obtained by the proposed ratio subtraction method for the analysis of laboratory prepared mixtures after multiplication by the divisor CAF` (Y`).

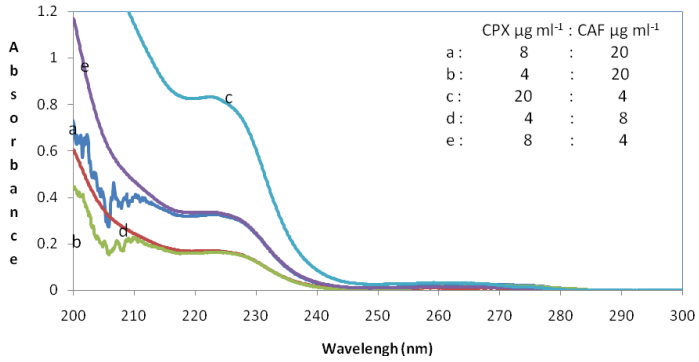

Fig. 5b: The zero order absorption spectra of different concentrations of CPX (X) obtained by the proposed ratio subtraction method for the analysis of laboratory prepared mixtures after multiplication by the divisor CAF` (Y`).

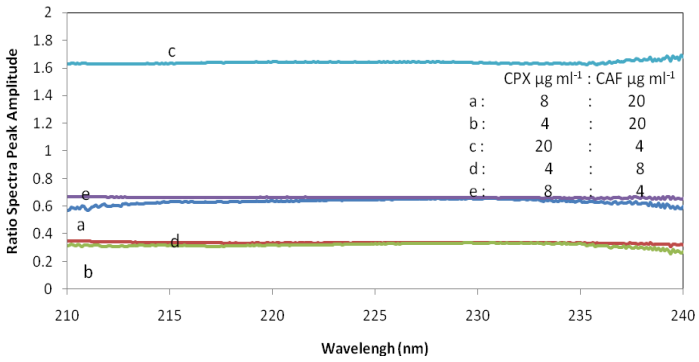

Fig. 6: Ratio spectra of obtained spectra of CPX (X) using 12 µg ml^{-1} of CPX` (X`) as a divisor.

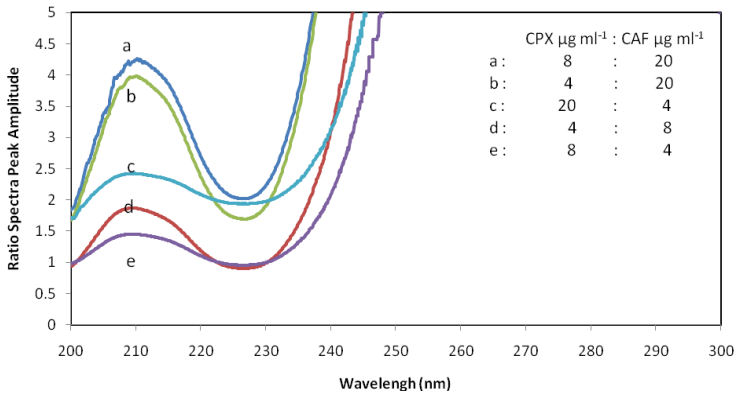

Fig. 7: Ratio spectra of laboratory prepared mixtures of CPX (X) and CAF (Y) using 12 µg ml^{-1} of CPX` (X`) as a divisor.

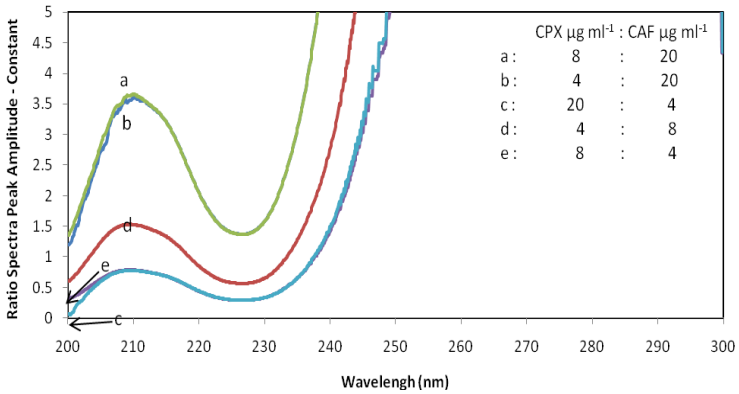

Fig. 8: Ratio spectra of laboratory prepared mixtures of CPX (X) and CAF (Y) using 12 µg ml^{-1} of CPX` (X`) as a divisor after subtraction of the constant.

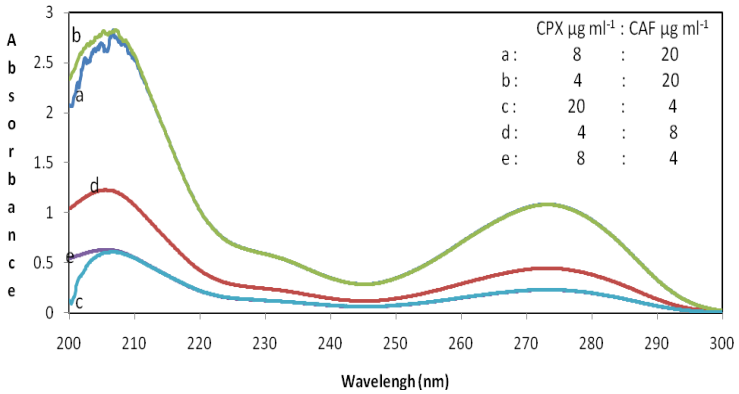

Fig. 9: The zero order absorption spectra of CAF (Y) obtained after multiplication by the divisor using the proposed extended ratio subtraction method for the analysis of laboratory prepared mixtures after multiplication by the divisor CPX` (X`).

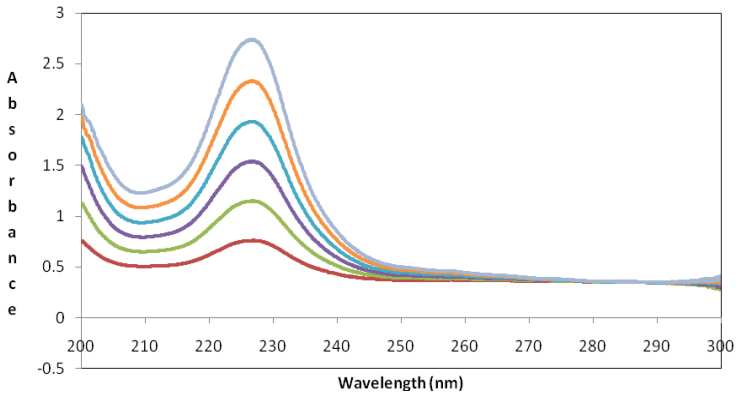

Fig. 10: Ratio Spectra of some laboratory prepared mixture of CPX (4-24 µg ml^{-1}) and CAF 4 µg ml^{-1} using 12 µg ml^{-1} of CAF as a divisor.

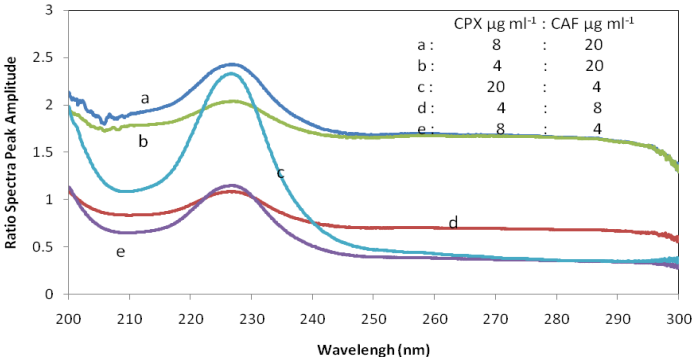

Fig. 11: Ratio Spectra of some mixtures of CPX and CAF using 12 µg ml^{-1} of CAF as a divisor.

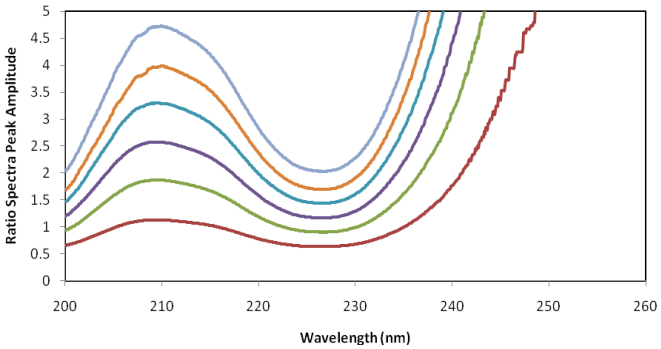

Fig. 12: Ratio Spectra of a mixture of CAF (4-24 µg ml^{-1}) and CPX (4 µg ml^{-1}) using (12 µg ml^{-1}) of CPX as a divisor.

Ratio difference method starts by scanning the zero order absorption spectra of the laboratory-prepared mixtures (CPX and CAF). For determination of CPX, divide the previously scanned ratio spectra by a carefully chosen concentration of standard CAF' (12 µg ml^{-1}) as a divisor to produce new ratio spectra which represent *CPX/CAF' + constant* as shown in Fig. 10 and 11. The amplitudes at 226.3 nm and 235 nm were selected. The amplitudes at these two wavelengths were subtracted, so the constant *CAF/CAF'* will be cancelled. The concentration of CPX was calculated using the corresponding regression equation (obtained by plotting the difference in the amplitude at 226.3 nm and 235.0 nm of the ratio spectra of *CPX/CAF'* against the corresponding concentrations). Similarly, the two selected wavelengths for the estimation of CAF using standard CPX` (12 µg ml^{-1}) as a divisor were 209.1 nm and 226.7 nm as shown in Fig. 12.

Mean centering of the ratio spectra method (MCR)

The developed MCR method depends on the mean centering of ratio spectra, it eliminates the derivative steps and therefore signal-to-noise ratio is enhanced [11].

In order to optimize the developed MCR method, different parameters were tested. Since the wavelength range taken has a great effect on the obtained mean centered ratio spectra, different wavelength ranges were tested and the best results were obtained when using the wavelength range from 200 to 300 nm and 200 to 280 nm for CPX and CAF, respectively. Effect of divisor concentration on the selectivity of the method has been tested. Different concentrations each of CPX and CAF were tested. It was found that the divisor concentration had no significant effect on the specificity of CPX and CAF determination, therefore, normalized spectrum each of CPX and CAF was used as a divisor.

As shown in Fig. 2, the absorption spectra of CPX and CAF are overlapped. So, the absorption spectra of the standard solutions of the CPX with different concentrations were recorded in the wavelength range of 200-300 nm and divided by the normalized spectrum of the CAF and the obtained ratio spectra were mean centered. (Fig. 13) The concentration of CPX was determined by measuring the amplitude at 226.8 nm corresponding to a maximum wavelength as shown in the same figure.

Similarly, for determination of CAF, the absorption spectra of the standard solution of CAF with different concentrations were recorded in the wavelength range of 200-280 nm and divided by the normalized spectrum of the CPX and the obtained ratio spectra were mean centered. (Fig. 14) The concentration of CAF was determined by measuring the amplitude at 274.0 nm corresponding to a maximum wavelength as shown in the same figure.

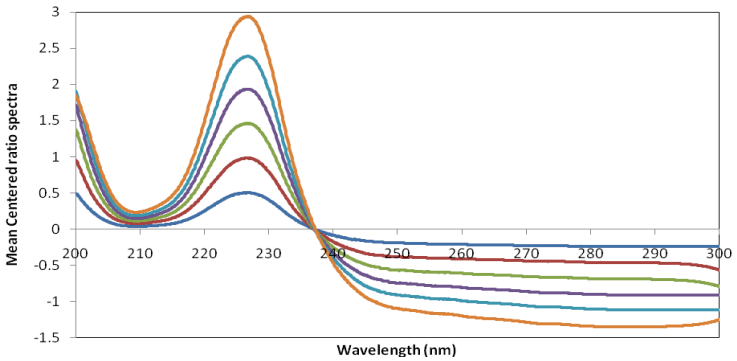

Fig. 13: Mean centered ratio spectra of CPX (4-24 µg ml^{-1}).

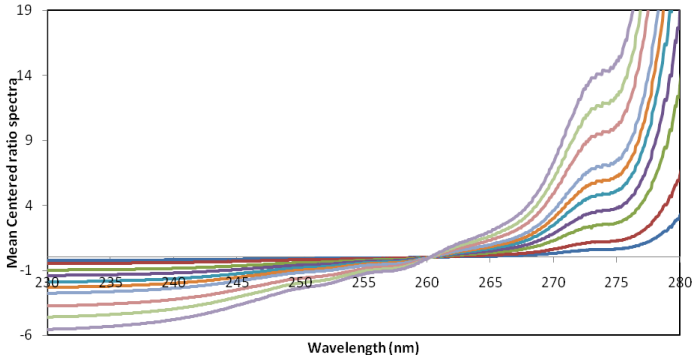

Fig. 14: Mean centered ratio spectra of CAF (2-24 µg ml^{-1}).

Method Validation

Validation of the proposed methods was assessed according to ICH guidelines [13] as shown in tables 1-3.

Linearity

The linearity of the methods was evaluated by analyzing six concentrations of CPX and seven concentrations of CAF ranging among 4-24 µg ml^{-1} and 2-24 µg ml^{-1}, respectively. Each concentration was repeated three times. The assay was performed according to the experimental conditions previously mentioned. The linear regression equations are summarized in Table 1.

Range

The calibration range was established through consideration of the practical range necessary according to adherence to Beer's law and the concentration of CPX and CAF present in the pharmaceutical preparations to give accurate, precise and linear results. Assay parameters are declared in Table 2.

Accuracy

The accuracy of the results was checked by applying the proposed methods for determination of different blind samples of CPX and CAF. The concentrations were obtained from the corresponding regression equations, from which the percentage recoveries suggested good accuracy of the proposed methods. Results are shown in Table 2.

Selectivity

Selectivity of the methods was achieved by the analysis of different laboratory prepared mixtures of CPX and CAF within the linearity range, including the ratio present in the pharmaceutical dosage form. Satisfactory results were obtained as shown in Table 3.

Precision

Repeatability

Three concentrations of CPX (8, 12 and 20 µg ml^{-1}) and CAF (8, 12 and 20 µg ml^{-1}) were analyzed three times intra-daily using the proposed methods. The relative standard deviations were calculated (Table 2).

Reproducibility (Intermediate precision)

The previous procedures were repeated inter-daily on three different days for the analysis of the three chosen concentrations. The relative standard deviations were calculated (Table 2).

Table 1: Linearity studies and regression equations of the proposed methods

Drug	Method	Regression equation	Correlation coefficient (r)
Chlorphenoxamine HCl	RSM	Ya = 0.0416x + 0.0027	0.9999
	RDSM	Yb = 0.0552x + 0.0049	0.9999
	MCR	Yc = 0.1315x + 0.0203	0.9997
Caffeine	EXRSM	Ya = 0.0538x + 0.0132	0.9999
	RDSM	Yb = 0.1092x + 0.0862	0.9997
	MCR	Yc = 0.5950x + 0.0450	0.9998

Where RSM is the ratio subtraction method, EXRSM is the extended ratio subtraction method, RDSM is the ratio difference spectrophotometric method and MCR is the mean centering of ratio spectra method.

Ya: Absorbance of the drug at its λ_{max}; Yb: difference between the amplitudes of the ratio spectra at the two selected wavelengths; Yc: the mean centered values at the specified wavelength and x is the corresponding Concentration

Table 2: Assay Parameters and method validation obtained by applying the proposed methods

Parameter	Chlorphenoxamine HCl			Caffeine		
	RSM	RDSM	MCR	EXRSM	RDSM	MCR
Range µg ml^{-1}	4-24	4-24	4-24	2-24	2-24	2-24
Slope	0.0416	0.0552	0.1315	0.0538	0.1092	0.5950
Intercept	0.0027	0.0049	0.0203	0.0132	0.0862	0.045
Corr. Coef. (r)	0.9999	0.9999	0.9997	0.9999	0.9997	0.9998
Accuracy	99.91 ± 1.073	99.41± 0.726	99.88 ± 1.245	99.05 ± 0.440	100.07 ± 1.250	99.62 ± 1.527
Repeatability[a]	99.80 ± 0.699	99.58 ± 0.813	99.85 ± 0.450	100.09 ± 0.565	99.71 ± 0.561	100.24± 0.458
RSD% [a]	0.700	0.816	0.451	0.565	0.562	0.457
Intermediate Precision [b]	100.14 ± 0.639	99.23 ± 0.843	99.94 ± 0.340	99.98 ± 0.520	99.94 ± 0.494	100.16 ± 0.361
RSD% [b]	0.637	0.849	0.34	0.521	0.494	0.36

a: Intra-day (n=3), average of three concentrations of CPX (8, 12& 20 µg ml^{-1}) repeated 3 times within the same day.

b: Inter-day (n=3), average of three concentrations of CPX (8, 12& 20 µg ml^{-1}) repeated 3 times in three consecutive days.

Table 3: Determination of Chlorphenoxamine HCl and Caffeine in their binary laboratory mixtures by the proposed methods

Ratios		Chlorphenoxamine HCl (Recovery % ± SD)			Caffeine (Recovery % ± SD)		
CPX : CAF	Conc. µg ml^{-1}	RSM at 222.4 nm	RDSM at 226.3 - 235 nm	MCR at 226.8 nm	EXRSM at 273.0 nm	RDSM at 209.1 - 226.7 nm	MCR at 274.0 nm
2 : 1	8 : 4	100.56 ± 0.762	99.27 ± 0.514	99.17 ± 0.614	99.58 ± 0.415	100.51 ± 0.465	98.93 ± 0.521
5 : 1	20 : 4	99.58 ± 0.527	100.68 ± 0.814	99.87 ± 0.479	99.62 ± 0.361	99.87 ± 0.642	99.62 ± 0.432
2 : 5*	4 : 20	100.19 ± 0.375	100.46 ± 0.671	100.62 ± 0.378	99.87 ± 0.396	99.53 ± 0.613	99.39 ± 0.512
1 : 2	4 : 8	99.28 ± 0.625	99.80 ± 0.527	99.93 ± 0.510	100.24 ± 0.484	100.23 ± 0.341	99.81 ± 0.316
1 : 5	4 : 20	99.81 ± 0.719	100.37 ± 0.351	99.83 ± 0.476	99.68 ± 0.591	100.19 ± 0.354	99.28 ± 0.418

*: The Ratio in Allergex Caffeine tablets.

All calculations were done in triplicates.

Table 4: Determination of Chlorphenoxamine HCl and Caffeine in Allergex Caffeine tablets by the proposed methods and application of the standard addition technique

Product	Claimed (µg ml^{-1})	Standard addition			Recovery (Mean ± SD%)	
Allergex Caffeine (B.N.: 1106021)		Added (µg ml^{-1})	Found (µg ml^{-1})	Recovery %	Proposed method	Standard addition
RSM & EXRSM						
CPX	4	4	3.94	98.50	100.72± 1.218	100.13± 1.410
		8	8.07	100.88		
		12	12.12	101.00		
CAF	10	4	4.01	100.25	99.14 ± 0.40	99.53 ± 0.632
		8	7.94	99.25		
		12	11.89	99.08		
RDSM						
CPX	4	4	3.97	99.25	100.16± 0.54	99.07 ± 0.387
		8	7.89	98.63		
		12	11.92	99.33		
CAF	10	4	3.99	99.75	99.72 ± 0.814	99.49 ± 0.357
		8	7.97	99.63		
		12	11.89	99.08		
MCR						
CPX	4	4	4.06	101.50	99.76 ± 1.051	100.65± 1.161
		8	8.09	101.13		
		12	11.92	99.33		
CAF	10	4	3.98	99.50	98.91 ± 1.342	99.99 ± 1.462
		8	8.13	101.63		
		12	11.86	98.83		

Table 5: Statistical comparison for the results obtained by the proposed methods and the reference methods for the determination of Chlorphenoxamine HCl and Caffeine in pure powder form

Parameter	CPX				CAF			
	Manufacturer Method*	RSM	RDSM	MCR	BP** Method	EXRSM	RDSM	MCR
Mean	100.62	99.91	99.41	99.88	99.20	99.05	100.07	99.62
S.D.	0.605	1.073	0.726	1.245	0.783	0.440	1.250	1.527
N	5	5	5	5	5	5	5	5
Variance	0.366	1.151	0.528	1.550	0.613	0.194	1.563	2.332
student t (2.306)		0.24	0.022	0.276		0.732	0.229	0.602
F (6.388)		3.145	1.442	4.237		3.159	2.550	3.804

*: Manufacturer method is a non-aqueous potentiometric titration method, obtained by personal communication with EIPICO [14].

**: BP method is a non-aqueous potentiometric titration method.

Figures in parenthesis are the corresponding tabulated values at P = 0.05.

Stability

CPX and CAF working solutions in double distilled water showed no spectrophotometric changes up to 2 weeks when stored at room temperature.

Application of the method in Assay of tablets

The proposed spectrophotometric ratio-spectra methods were applied for the determination of CPX and CAF in their combined pharmaceutical formulation (Allergex Caffeine tablets). The validity of the methods was assessed by applying the standard addition technique (Table 4). It shows that the developed methods are accurate and specific for determination of the cited drugs in presence of dosage form excipients.

Statistical Analysis

Results obtained by the proposed methods for the determination of pure samples of CPX and CAF are statistically compared to those obtained by the official methods. The calculated t and F values were found to be less than their corresponding theoretical ones confirming good accuracy and excellent precision (Table 5).

CONCLUSION

From the previous discussion, it could be concluded that the proposed procedures are simple and do not require sophisticated techniques or instruments. They are also sensitive and selective and could be used for routine analysis of CPX and CAF in their available dosage form without prior separation. It is noteworthy to mention that using double distilled water as a solvent, besides being cheap; it is extremely safe to the environment. The methods are also suitable and valid for application in laboratories lacking liquid chromatographic instruments. Moreover, using Microsoft Excel, for manipulation of the spectral data during handling the three proposed methods, eliminates the need for using specific expensive software. Excel, can be downloaded for free or comes pre-installed on any new laptop or desktop as a part of the *Microsoft® Office Starter: reduced-functionality Word &Excel*.

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