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Spectrophotometric methods for determination of Amprolium hydrochloride and Ethopabate binary mixture

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ABSTRACT

Five simple, specific, accurate and precise UV- spectrophotometric methods are adopted for the simultaneous determination of Amprolium hydrochloride (AMP) and Ethopabate (ETH), a binary mixture with overlapping spectra without preliminary separation. The first method is first derivative of the ratio spectra (^{1}DD) for determination of AMP and ETH at 234.7 nm and 306.8 nm respectively. The second method is the mean centering of the ratio spectra for determination of AMP and ETH at 238.8 nm and 313 nm respectively. The third method is based on dual wavelength selection for determination of AMP and ETH at 235.3 nm & 308 nm and 244 nm & 268.4 nm respectively. The fourth method is ratio difference method for determination of AMP and ETH at 239 nm & 310 nm and 239 nm & 313 nm respectively. The fifth one is area under the curve (AUC) method where the areas between 235.6-243 nm and 268.3-275 nm are selected for determination of AMP and ETH simultaneously. These methods are tested by analyzing synthetic mixtures of the two drugs and they are applied to their pharmaceutical veterinary preparation. Methods are validated according to the ICH guidelines and accuracy, precision and repeatability are found to be within the acceptable limit.

Highlights

- These methods are sensitive and resolve binary mixture with overlapping spectra.
- The good recovery and accuracy make them applicable in QC laboratories
- The proposed methods can be used in QC without the difficulties of HPLC
- The proposed methods were validated according to ICH guidelines.
- The proposed methods have been applied successfully on the veterinary formulation

KEYWORDS

Spectrophotometry;
Amprolium hydrochloride;
Ethopabate;
Ratio spectra;
Dual wavelength;
Area under curve.

INTRODUCTION

Coccidiosis is a term sometimes applied to infections with protozoa of the order *Eucoccidiorida.* The predominant coccidian infections in man are caused by *Cryptosporidium, Cyclospora cayetanensis, Isospora, Plasmodium, and Toxoplasma*. Coccidian protozoa, primarily *Eimeria,* cause economically important infections in domesticated animals[1]. Amprolium hydrochloride and Ethopabate are widely used to treat and prevent coccidiosis in chickens. Since both are usually used as a combination, it is important to develop simple spectrophotometric methods to determine them simultaneously.

Amprolium Hydrochloride is 1-[(4-amino-2-propyl-5-pyrimidinyl) methyl]-2- methylpyridinium chloride Hydrochloride[2] (Figure 1). It is an antiprotozoal used in veterinary practice alone or with other drugs such as ethopabate, for the control of coccidiosis in pigeons and in poultry[1]. Ethopabate is methyl 4-acetamido-2-ethoxybenzoate[2] (Figure 2). It is an antiprotozoal used in veterinary practice with other drugs, such as amprolium, for the control of coccidiosis in poultry[1].

Literature survey revealed that Amprolium hydrochloride and Ethopabate are official in British Pharmacopoeia[3]. There are many reported methods for the determination of either AMP, ETH, together or in combination with other drugs in different matrices such as pharmaceutical formulation, surface water, eggs, chicken muscles, chicken plasma, chicken liver and chicken feed. These methods include liquid chromatography coupled with ultraviolet (UV)[4,5] or fluorescence[6-9] detection, and liquid chromatography mass spectrometry (LC–MS)[10-16]. Thin layer chromatography[4]. Spectrophotometric methods[17-21] Atomic spectrometry[22]. Capillary electrophoresis[23]. Electrochemical method[24].

Figure 1 : Structural formula for Amprolium hydrochloride

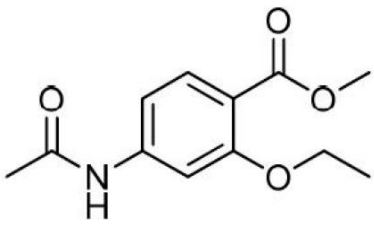

Figure 2 : Structural formula for Ethopabate

The aim of the study is to develop simple, rapid, sensitive and selective spectrophotometric methods for the determination of components with overlapping spectra in their binary mixtures simultaneously, providing the advantage of minimal data processing and without the need of sophisticated instruments, expensive solvents or large number of samples.

Theoretical background

(a) Mean centering method

This method is based on the mean centering of ratio spectra[25,26]. To explain the mean centering expression, let us consider a three dimensional vector[27].

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

$$\text{Calling: } y` = \begin{bmatrix} 3 \\ 3 \\ 3 \end{bmatrix}$$

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

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 as follows:

$$A_m = \alpha_x C_x + \alpha_y C_y \quad (1)$$

where Am is the vector of the absorbance of the mixture, α_x and α_y are the molar absorptivity vectors of x and y; and Cx and Cy are the concentrations of x and y, respectively. If Eq. (1) is divided by ¸y corresponding to the spectrum of a standard solution of y in binary mixture, the ratio spectrum is obtained in the form of Eq. (2) (for possibility of dividing op-

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eration, the zero values of ¸y should not be used as a divisor):

$$B = A_m / \alpha_y = \alpha_x C_x / \alpha_y + C_y \quad (2)$$

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

$$MC(B) = MC \left[\frac{\alpha_x C_x}{\alpha_y} \right] \quad (3)$$

Eq. (3) represents the mathematical foundation of bicomponent analysis that permits the determination of concentration of each of the active compounds in the solution (x in this equation) without interfering from the other compound of the binary system (y in these equations). Eq. (3) shows that 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.

(b) Ratio difference method

Recently, Elzanfaly et al.[28-30] developed a novel simple, rapid and selective method to determine components having overlapping spectra in binary mixtures simultaneously. For any two drugs X and Y with overlapping spectra, when the spectrum of X is divided by a divisor of a certain concentration of Y, a ratio spectrum will result, and a linear relationship between the difference in amplitudes at any two wavelengths and the corresponding concentration of X will result, while the ratio spectrum of Y will be a straight line of constant amplitude parallel to the x-axis and the difference in amplitudes of Y at any two wavelengths will be zero. Mathematically it can be explained as follows: In the ratio spectrum of a lab mixture of X and Y is divided by a divisor Y‘

$$P_1 = P_{1x} + K$$

$$P_2 = P_{2x} + K$$

where P_1 and P_2 are the amplitudes of the mixture spectrum at λ_1 and λ_2, respectively. P_{1X} and P_{2X} are the amplitudes of X at λ_1 and λ_2, respectively. K is the constant resulting from Y/Y‘

$$\Delta P_{(\lambda 1 - \lambda 2)} = P1 - P2 = (P_{1x} + K) - (P_{2x} + K) = P_{1x} - P_{2x}$$

A calibration curve relating the difference in amplitudes in the ratio spectrum at λ_1 and λ_2 $\Delta P_{(\lambda 1 - \lambda 2)}$ using a certain concentration of Y as a divisor to the corresponding concentration of X will be used for the determination of X in the unknown samples of the binary mixture. Similarly component Y can be obtained by using certain concentration of X as a divisor.

EXPERIMENTAL

Apparatus

Spectrophotometer: SHIMADZU UV-1601 PC, dual beam UV-visible spectrophotometer with two matched 1-cm quartz cells, connected to an IBM compatible personal computer (PC) and an HP-600 inkjet printer. Bundled UV–PC personal spectroscopy software version (3.91) is used to process the absorption. The spectral band width is 0.1 nm with wavelength scanning speed of 2800 nm min^{-1}.

Calibrated Dragon lab® micropipettes.

Software

Minitab® Release 14.12.0

Materials

(a) Pure standards

Amprolium hydrochloride and Ethopabate are kindly supplied by Prima Vet pharmaceutical company, Cairo, Egypt. Their purity is found to be 100.35% and 99.5%, respectively, according to the reported spectrophotometric method[17].

(b) Pharmaceutical preparation

Amprolium & Ethopabate PREMIX 25%® (Batch No. 1203322) is a feed additive labelled to contain 250 gm amprolium hydrochloride and 16 gm ethopabate per one kilogram, manufactured by Adwia Co. S.A.E. 10th of Ramadan city, Egypt.

(c) Reagents

Methanol, a spectrophotometric analytical grade is obtained from ADWIC, Egypt.

Procedures

(a) Standard solutions

AMP stock standard solution: 200 μg/mL in methanol

ETH stock standard solution: 200 μg/mL in methanol

(b) Methods

(A) For (^{1}DD) method

Different aliquots equivalent to 10-400 μg/mL of AMP and 10-100 μg/mL of ETH are accurately transferred from their standard stock solutions (200 μg/mL) into two separate series of 10-mL volumetric flasks then completed to volume with methanol. The spectra of the prepared standard solutions are scanned from 200–400 nm using methanol as blank and stored in computer. For the determination of AMP in presence of ETH, the stored spectra of AMP are divided by the spectrum of 2 μg/mL ETH, then the first derivative of the ratio spectra (^{1}DD) with $\Delta\lambda$ = 4 nm is obtained. The amplitude of the first derivative peak of (AMP/ETH) is measured at 234.7 nm. A calibration curve relating the peak amplitude at 234.7 nm to the corresponding concentrations in μg/mL of AMP is constructed. For the determination of ETH in presence of AMP, the stored spectra of ETH are divided by the spectrum of 5 μg/mL AMP, then the first derivative of the ratio spectra (^{1}DD) with $\Delta\lambda$ = 4 nm is obtained. The amplitude of the first derivative peak of (ETH/AMP) is measured at 306.8 nm. A calibration curve relating the peak amplitude at 306.8 nm to the corresponding concentrations in μg/mL of ETH is constructed.

(B) For mean centering method

Different aliquots equivalent to 10-350 μg/mL of AMP and 10-120 μg/mL of ETH are accurately transferred from their standard stock solutions (200 μg/mL) into two separate series of 10-mL volumetric flasks then completed to volume with methanol. The scanned spectra of AMP are divided by the spectrum of 2 μg/mL of ETH and the obtained ratio spectra are mean centered. The same is applied to ETH spectra as they are divided by the spectrum of 5 μg/mL of AMP, the obtained ratio spectra are smoothed with $\Delta\lambda$ = 4 nm and are then mean centered. The calibration curves for both AMP and ETH are constructed by plotting the mean centered values at 238.8 nm and 313 nm for AMP and ETH, respectively, versus the corresponding concentration.

(C) For dual wavelength method

Different aliquots equivalent to 20-320 μg/mL of AMP and 10-140 μg/mL of ETH are accurately transferred from their standard stock solutions (200 μg/mL) into two separate series of 10-mL volumetric flasks then completed to volume with methanol. Using the scanned spectra, absorbance values at both 235.3 and 308 nm (for AMP) and at both 244 and 268.4 nm (for ETH) are measured. AMP is determined by plotting the difference in absorbance at 235.3 and 308 nm (difference is zero for ETH) against its corresponding concentration. Similarly, for determination of ETH, the difference in absorbance at 244 and 268.4 nm (difference is zero for AMP) is plotted against its corresponding concentration. The concentrations of the two drugs are calculated each from the corresponding calibration curve equation.

(D) For ratio difference method

Different aliquots equivalent to 10-400 μg/mL of AMP and 10-140 μg/mL of ETH are accurately transferred from their standard stock solutions (200 μg/mL) into two separate series of 10-mL volumetric flasks then completed to volume with methanol. For the determination of AMP in presence of ETH, the stored spectra of AMP are divided by the spectrum of 2 μg/mL of ETH, then the amplitude difference of the ratio spectra (AMP/ETH) at 239 and 310 nm ($\Delta P_{239-310}$) is plotted against the corresponding concentrations in μg/mL of AMP. For the determination of ETH in presence of AMP, the stored spectra of ETH are divided by the spectrum of 5 μg/mL of AMP, the obtained ratio spectra are smoothed with $\Delta\lambda$ = 4 nm, then the amplitude difference of the ratio spectra (ETH/AMP) at 239 and 313 nm ($\Delta P_{239-313}$) is plotted against the corresponding concentrations in μg/mL of ETH.

(E) For area under the curve method

Different aliquots equivalent to 10-400 μg/mL of AMP and 10-140 μg/mL of ETH are accurately transferred from their standard stock solutions (200 μg/mL) into two separate series of 10-mL volumetric

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flasks then completed to volume with methanol. Area under the curve is calculated for the wavelength ranges selected for determination of AMP and ETH which are 235.6-243 nm (λ_1–λ_2) and 268.3-275 nm (λ_3–λ_4), the absorptivity '*Y*' values of each of the two drugs are determined at the selected wavelength ranges. The absorptivity '*Y*' values are determined as, Y = area under curve of component (from 235.6-243 nm or 268.3-275 nm)/concentration of the component (in µg/mL). Mixed standards are prepared and their area under the curve are measured at the selected wavelength ranges. Concentration of two drugs in mixed standard and the sample solution are calculated using the corresponding equations.

(F) Application of the proposed methods for the determination of AMP and ETH in laboratory prepared mixtures

Solutions containing different ratios of AMP and ETH are prepared. Zero order absorption spectra of these mixtures are recorded using methanol as a blank. By applying the proposed methods, the concentrations of AMP and ETH in the prepared mixtures are calculated from the corresponding equations.

(G) Application of the proposed methods for the determination of AMP and ETH in pharmaceutical formulation (Amprolium & Ethopabate PREMIX 25%®)

A portion of the premix equivalent to 25 mg of AMP and 1.6 mg of ETH is accurately weighed, sonicated in 25 mL methanol for 15 min and filtered into 100- mL volumetric flask. The residue is washed three times each with 5 mL methanol and then completed to volume with methanol. The procedures of each method are followed and the concentrations of AMP and ETH are calculated from the corresponding equations. The validity of the methods is assessed by applying the standard addition technique.

RESULTS AND DISCUSSION

Molecular absorption spectroscopy has been widely studied to develop analytical methods for the determination of drugs in pharmaceutical preparations for quality control purposes. The main challenge is that most active drugs absorb in the UV region and exhibit overlapping spectra that hinder their simultaneous determination. The zero-order absorption spectra (D_0) of AMP and ETH show severe overlapping, (Figure 3), which prevents the analysis of one drug in presence of the other. By applying the proposed techniques to the spectral data of the mixture, this overlapping can be resolved, allowing the determination of each of AMP and ETH in presence of the other.

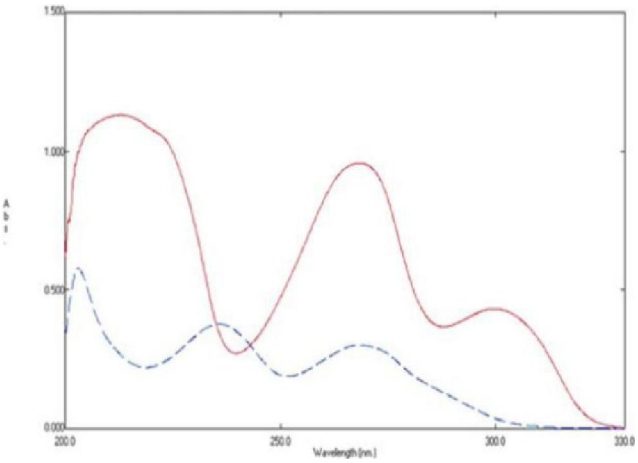

Figure 3 : Zero order absorption spectra of 10 µg/mL AMP (- - - -),10 µg/mL ETH (-), using methanol as blank

METHOD DEVELOPMENT AND OPTIMIZATION

^{1}DD method

Salinas et al.[31] developed a spectrophotometric method, which is based on the derivation of the ratio-spectra for resolving binary mixtures.

In this method the absorption spectrum of the mixture is divided by the absorption spectrum of a standard solution of the interferent giving a ratio spectrum, then the first derivative of this ratio spectrum is obtained. The concentration of the analyte is then determined from a calibration curve.

The main advantage of this method is that the whole spectrum of the interferent is cancelled. Accordingly, the choice of the wavelength selected to construct the calibration curve is not critical.

In order to optimize ^{1}DD method for determination of AMP and ETH, it is necessary to test the influence of many variables such as scanning speed, the concentration of the standard solution used as a divisor, the wavelength increment over which the deriva-

tive is obtained ($\Delta\lambda$) and the smoothing function. It is found that fast scanning speed, $\Delta\lambda$=4 nm and scaling factor 1 gave best compromise in terms of signal to noise ratio, peak resolution and sensitivity throughout the determination. Effect of divisor concentration is also tested, for determination of AMP, different concentrations of ETH are used (1, 2, 4 µg/mL) and for determination of ETH, different concentrations of AMP are used (5, 8, 10 µg/mL) and the divisor concentrations 2 and 5 µg/mL of ETH and AMP, respectively, are found the best regarding average recovery percent when they are used for the prediction of AMP and ETH concentrations, respectively, in bulk powder as well as in laboratory prepared mixtures.

The absorption spectra of AMP are divided by the absorption spectrum of 2 µg/mL ETH and the absorption spectra of ETH are divided by the absorption spectrum of 5 µg/mL AMP, for determination of AMP and ETH, respectively, giving ratio spectra shown in (Figure 4 and 5). These gave the best compromise in terms of sensitivity, repeatability and signal to noise ratio. Us-

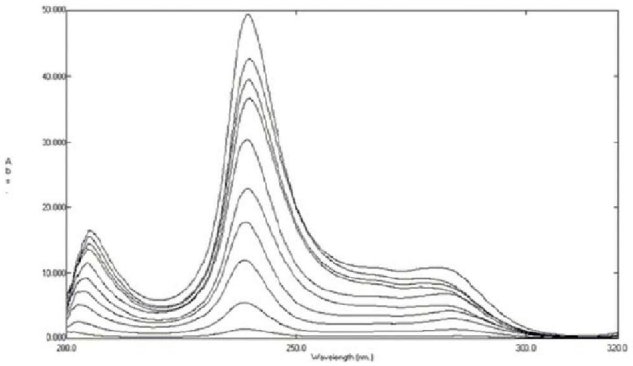

Figure 4 : Ratio spectra of AMP (1–40 µg/mL) using 2 µg/mL of ETH as a divisor and methanol as blank

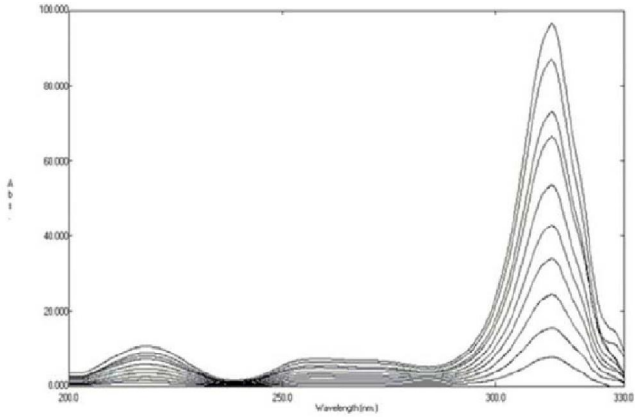

Figure 5: Smoothed ratio spectra of ETH (1–10 µg/mL) using 5 µg/mL of AMP as a divisor and methanol as blank

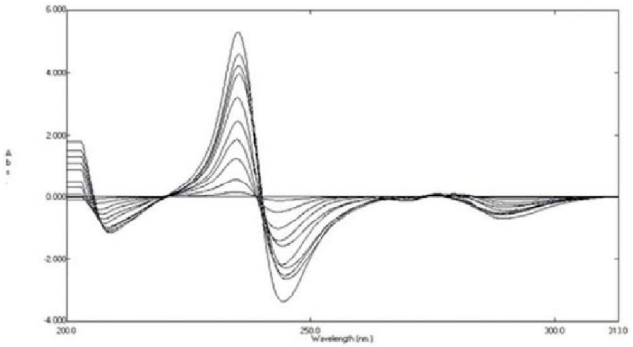

Figure 6: First derivative of ratio spectra of AMP (1–40 µg/mL) using 2 µg/mL of ETH as a divisor and methanol as blank

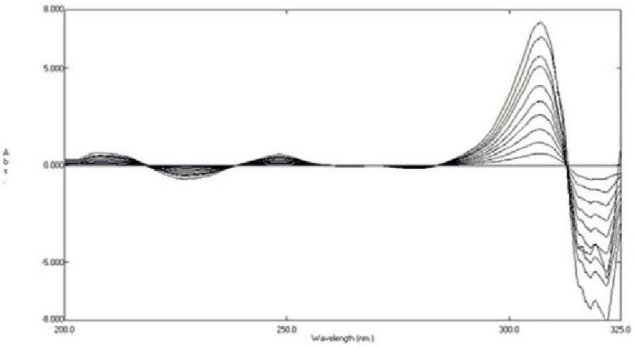

Figure 7: First derivative of smoothed ratio spectra of ETH (1–10 µg/mL) using 5 µg/mL of AMP as a divisor and methanol as blank

ing the first derivative of the ratio spectra presented in (Figure 6 and 7), linearities are studied and calibration curves are constructed relating the peak amplitude at 234.7 nm and at 306.8 nm to the corresponding concentration of AMP and ETH, respectively. The linear regression equations are found to be:

$$P_{AMP} = 0.1299C + 0.0063 \quad r = 0.9997$$

$$P_{ETH} = 0.7604C - 0.2957 \quad r = 0.9992$$

where C is the concentration of AMP and ETH in µg/mL, P is the peak amplitude of the first derivative of the ratio spectrum curve and r is the correlation coefficient.

Mean centering method

For further improvement of the selectivity to resolve the overlap present between AMP and ETH, a method based on the mean centering of ratio spectra is applied; this method eliminates the derivative step and therefore the signal-to-noise ratio is enhanced[25,26]. The absorption spectra of AMP are divided by the absorption spectrum of 2 µg/mL ETH and the absorption spectra of ETH are divided by the ab-

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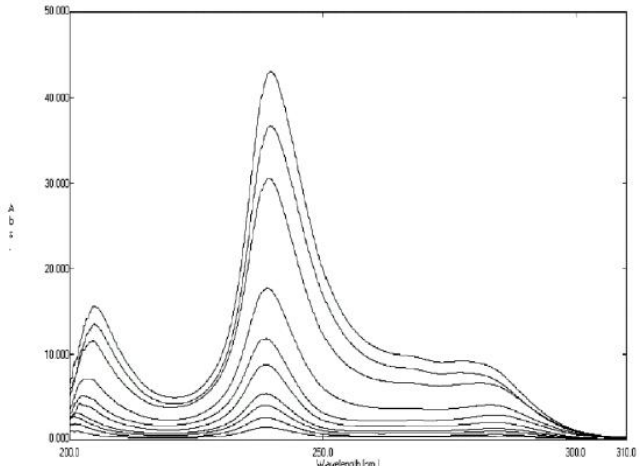

Figure 8: Ratio spectra of AMP (1–35 µg/mL) using 2 µg/mL of ETH as a divisor and methanol as blank

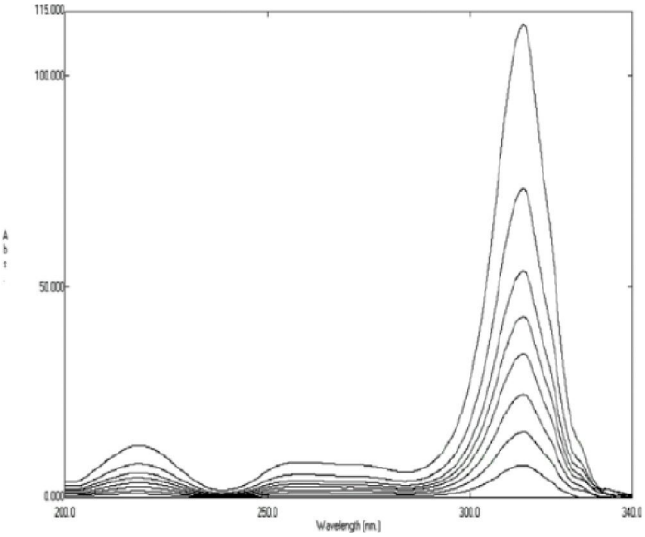

Figure 9: Smoothed ratio spectra of ETH (1–12 µg/mL) using 5 µg/mL of AMP as a divisor and methanol as blank

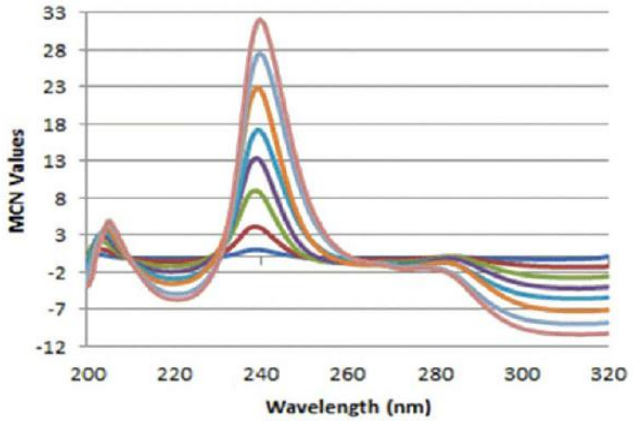

Figure 10: Mean centered ratio spectra of AMP (1-35 µg/mL) using 2 µg/mL of ETH as a divisor and methanol as blank

sorption spectrum of 5 µg/mL AMP for determination of AMP and ETH, respectively (Figure 8 and 9). The

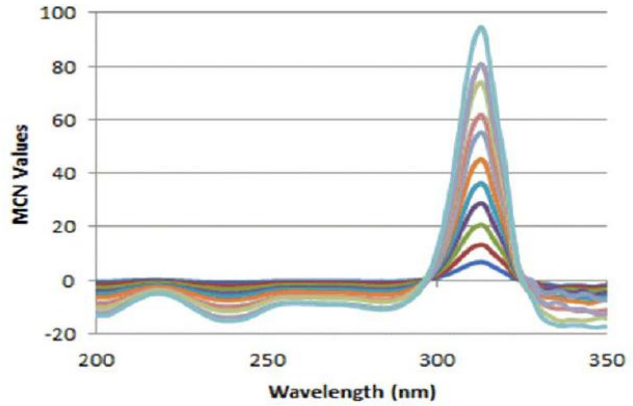

Figure 11: Mean centered ratio spectra of ETH (1-12 µg/mL) using 5 µg/mL of AMP as a divisor and methanol as blank

obtained ratio spectra are mean centered and the concentrations of AMP and ETH are determined by measuring the amplitude at 238.8 and 313 nm, respectively (Figure 10 and 11). The linear regression equations are found to be:

$$MCN_{AMP} = 0.887C + 0.3348 \quad r = 0.9998$$

$$MCN_{ETH} = 8.0927C - 3.0243 \quad r = 0.9995$$

where C is the concentration of AMP and ETH in µg/mL, MCN is the peak amplitude of the mean centered ratio spectrum curve and r is the correlation coefficient.

Dual wavelength method

The principle of dual wavelength method is that the absorbance difference at two points on the spectra is directly proportional to the component of interest, independent of the interfering component. The requirement for dual wavelength method is the selection of two wavelengths where the interfering component shows the same absorbance while the component of interest shows significant difference in absorbance. Different sets of wavelengths are tried, (Figure 12) shows that absorbance values of AMP are the same at 244 and 268.4 nm therefore these two wavelengths are selected for determination of ETH. The same for the two wavelengths 235.3 and 308 nm at which the absorbance values of ETH are the same, hence these two wavelengths are selected for determination of AMP. Difference in absorbances of AMP at 235.3 and 308 nm are plotted against its concentration in the range of 2-32 µg/mL, also for ETH, difference in absorbances at 244 and 268.4 nm are plotted against its concentration in the range of 1-14 µg/mL. The concentration of AMP

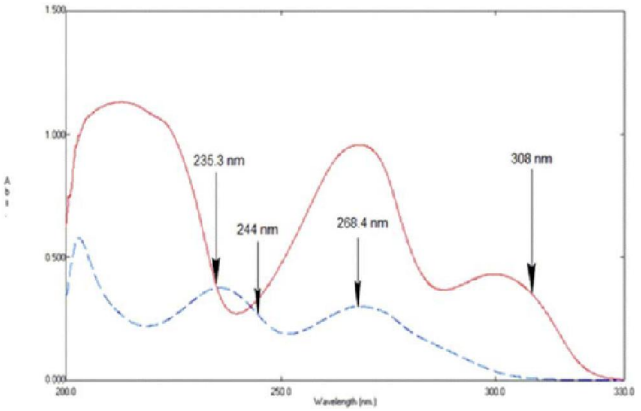

Figure 12: Zero order absorption spectra of 10 µg/mL of AMP (- - - -) and 10 µg/mL of ETH (—) showing the selected wavelengths for dual wavelength method using methanol as a blank

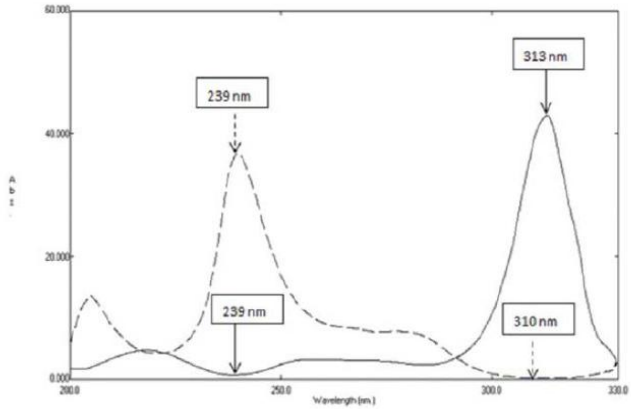

Figure 14: Ratio spectra of 30 µg/mL of AMP (- - - -) and 5 µg/mL of ETH (—) showing the selected wavelengths for dual wavelength method using methanol as a blank

and ETH can be calculated from the following regression equations:

ΔA_{AMP} = 0.0409C – 0.0029 r = 0.9999

ΔA_{ETH} = 0.0637C – 0.0141 r = 0.9999

where ΔA_{AMP} is the absorbance difference at 235.3 and 308 nm. ΔA_{ETH} is the absorbance difference at 244 and 268.4 nm. C is the concentration of AMP and ETH in µg/mL. r is the correlation coefficient.

Ratio difference method

The method comprises two critical steps[28]

The first is the choice of the divisors, the selected divisors should compromise between minimal noise and maximum sensitivity. Different concentrations of divisor are used (1, 2 and 4 µg/mL) of ETH and (5, 8 and 10 µg/mL) of AMP and the divisor concentrations 2 and 5 µg/mL of ETH and AMP, respectively, are found to be

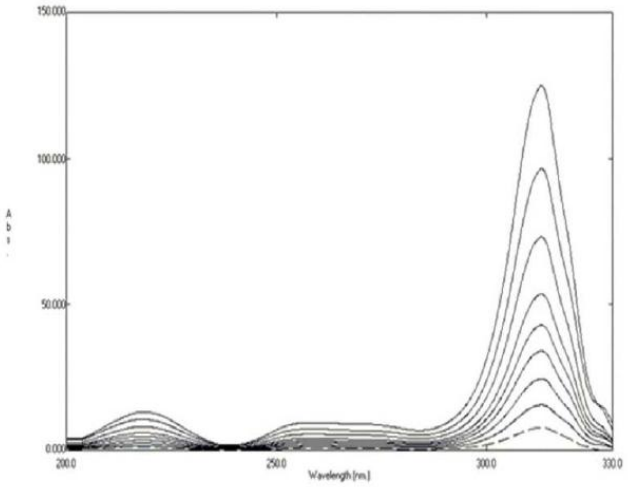

Figure 13: Smoothed ratio spectra of ETH (1–14 µg/mL) using 5 µg/mL of AMP as a divisor and methanol as blank

the best regarding sensitivity, repeatability, signal to noise ratio and average recovery percent when used for the prediction of AMP and ETH concentrations, respectively, in bulk powder as well as in laboratory prepared mixtures, (Figure 4 and 13)

The second critical step is the choice of the wavelengths at which measurements are recorded. Any two wavelengths can be chosen provided that they exhibit different amplitudes in the ratio spectrum and a good linearity is present at each wavelength individually, (Figure 14).

Linear correlation is obtained between the differences in amplitude at 239 & 310 nm for AMP and at 239 & 313 nm for ETH, against the corresponding concentration of AMP and ETH, respectively. The linear regression equations are found to be:

ΔP_{AMP} = 1.1911C + 0.5583 r = 0.9996

ΔP_{ETH} = 9.0073C – 2.0501 r = 0.9996

where ΔP_{AMP} is the amplitude difference at 239 and 310 nm. ΔP_{ETH} is the amplitude difference at 239 and 313 nm. C is the concentration of AMP and ETH in µg/mL. r is the correlation coefficient.

Area under curve method

Selection of the wavelength region to construct AUC method has a great effect on the analytical parameters such as slope, intercept and correlation coefficient. Different wavelength regions are tested where the wavelength ranges 235.6-243 nm and 268.3–275 nm are selected which show good selectivity and percentage recovery, (Figure 15)

Area under curve of the absorption spectra in

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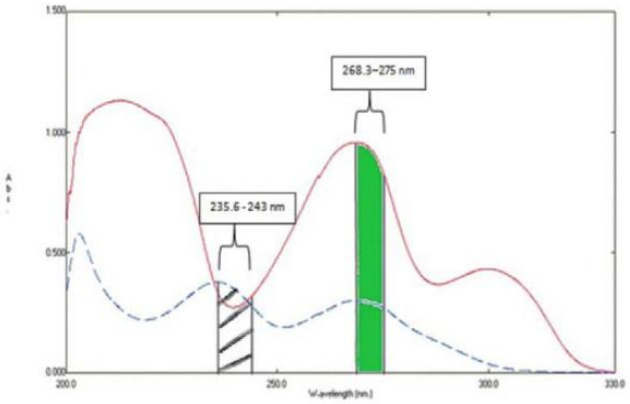

Figure 15: Zero order absorption spectra of 10 µg/mL of AMP (- - -) and 10 µg/mL of ETH (-) showing wavelength ranges for area under curve method using methanol as a blank

the wavelength ranges 235.6-243 nm (λ_1 - λ_2) and 268.3–275 nm (λ_3 – λ_4) of AMP in the concentration range of 1-40 µg/mL is calculated. For ETH, area under curve of the absorption spectra in the wavelength ranges 235.6-243 nm (λ_1 - λ_2) and 268.3-275 nm (λ_3 – λ_4) in the concentration range of 1-14 µg/mL is also calculated. The absorptivity 'Y' values of AMP and ETH are calculated at each wavelength range. The concentrations of AMP and ETH can be obtained by applying Cramer's rule and matrices in Eqs. (1) and (2). Concentration of two the drugs in mixed standard and the sample solution are calculated according to the following equations:

$$A_1 = 0.277C_{AMP} + 0.185C_{ETH}$$
at 235.6-243 nm (λ_1 - λ_2)

$$A_2 = 0.217C_{AMP} + 0.574C_{ETH}$$
at 268.3-275 nm (λ_3 – λ_4) **(2)**

where C_{AMP} and C_{ETH} are the concentrations of AMP and ETH in µg/mL, respectively. 0.277 and 0.217 are the absorptivity (Y value) of AMP at (λ_1 - λ_2) and (λ_3 – λ_4), respectively. 0.185 and 0.574 are absorptivity (Y value) of ETH at (λ_1 - λ_2) and (λ_3 – λ_4), respectively. A_1 and A_2 are the area under curve of sample solutions at the wavelength range (λ_1 - λ_2) and (λ_3 – λ_4), respectively.

To check the ability of the proposed methods for determination of AMP and ETH in their binary mixture, they are applied for determination of the two drugs in their laboratory prepared mixtures with mean percentage recoveries as given in TABLE 1.

The proposed methods are found to be valid and applicable for the analysis of AMP and ETH in their pharmaceutical formulation (Amprolium & Ethopabate PREMIX 25%®) with mean percentage recoveries as shown in TABLE 2. Furthermore, the standard addition technique is performed to assess the accuracy of the proposed methods. The obtained results reveal that there is no interference from excipients in the premix as shown in TABLE 2.

METHOD VALIDATION

Methods validation has been performed according to ICH guidelines[32]:

TABLE 1: Determination of Amprolium hydrochloride and Ethopabate in laboratory prepared mixtures by the proposed methods

No. Of mixtures	Claimed concentration taken (µg/ml)		[1]DDmethod		Mean centering method		Dual Wavelength method		Ratio Difference method		AUC method	
			% Recovery[a]		% Recovery[a]		% Recovery[a]		% Recovery[a]		% Recovery[a]	
	AMP	ETH	AMP	ETH	AMP	ETH	AMP	ETH	AMP	ETH	AMP	ETH
1	32	2	100.01	99.26	99.85	100.79	99.31	99.92	100.49	101.79	98.64	101.33
2	8	4	101.69	100.21	98.28	98.50	99.05	100.33	101.31	100.12	100.25	99.47
3	8	8	99.67	100.69	100.95	98.45	100.96	100.71	98.54	100.69	99.79	101.62
4	16	6	99.62	100.35	99.14	101.59	100.54	99.66	99.68	100.13	98.91	100.02
5	16	10	101.79	99.66	99.57	98.55	100.58	98.82	101.73	98.09	99.22	99.19
6	5	2	100.89	98.68	101.06	99.72	99.27	100.16	99.21	99.29	100.92	100.10
	Mean		100.61	99.81	99.81	99.60	99.95	99.93	100.16	100.02	99.62	100.29
	SD		0.986	0.752	1.069	1.344	0.830	0.652	1.237	1.254	0.865	0.984
	RSD%		0.980	0.753	1.071	1.349	0.830	0.652	1.235	1.254	0.868	0.981

[a] **Average of three determinations**

TABLE 2: Determination of Amprolium hydrochloride and Ethopabate in their pharmaceutical formulation by the proposed methods and application of standard addition technique

A

Dosage form	Drug	[1]DD method				Mean centering method			
		Found[a] (%) ± S.D.	Added (µg/ml)	Found[b] (µg/ml)	% recovery	Found[a] (%) ± S.D.	Added (µg/ml)	Found[b] (µg/ml)	% recovery
Amprolium & Ethopabate PREMIX 25%® (250 gm AMP and 16 gm ETH/ Kg)	AMP	99.35 ± 1.186	5	4.998	99.97	99.47 ± 0.257	5	4.975	99.50
			10	9.961	99.61		10	9.963	99.63
			15	15.063	100.42		15	14.974	99.83
		Mean ± S.D.			100 ± 0.406	Mean ± S.D.			99.65 ± 0.166
	ETH	101.74 ± 0.092	2	1.982	99.10	100.17 ± 1.226	2	2.010	100.52
			4	4.029	100.73		4	3.961	99.03
			6	6.053	100.88		6	6.080	101.33
		Mean ± S.D.			100.24 ± 0.987	Mean ± S.D.			100.29 ± 1.167

B

Dosage form	Drug	Dual wavelength method				Ratio difference method			
		Found[a] (%)±S.D.	Added (µg/ml)	Found[b] (µg/ml)	% recovery	Found[a] (%)±S.D.	Added (µg/ml)	Found[b] (µg/ml)	% recovery
Amprolium & Ethopabate PREMIX 25%® (250gm AMP And 16 gm ETH/ Kg)	AMP	98.98 ± 0.286	5	5.019	100.38	99.80 ± 0.451	5	4.957	99.15
			10	10.086	100.86		10	9.843	98.43
			15	15.061	100.41		15	14.980	99.86
		Mean ± S.D.			100.55 ± 0.269	Mean ± S.D.			99.15 ± 0.715
	ETH	100.35 ± 0.4	2	1.973	98.65	101.44 ± 0.945	2	1.992	99.60
			4	3.922	98.05		4	4.033	100.82
			6	5.890	98.17		6	5.963	99.39
		Mean ± S.D.			98.29 ± 0.317	Mean ± S.D.			99.94 ± 0.772

C

Dosage form	Drug	AUC method			
		Found[a] (%) ± S.D.	Added (µg/ml)	Found[b] (µg/ml)	% recovery
Amprolium & Ethopabate PREMIX 25%® (250 gm AMP and 16 gm ETH/ Kg)	AMP	98.52 ± 0.358	5	4.998	99.96
			10	9.903	99.03
			15	14.859	99.06
		Mean ± S.D.			99.35 ± 0.534
	ETH	99.58 ± 0.295	2	2.011	100.54
			4	4.056	101.41
			6	5.948	99.14
		Mean ± S.D.			100.36 ± 1.145

[a] Average of three determinations; [b] Average of four determinations

Linearity

The linearity of the proposed methods is evaluated by analyzing different concentrations of standard solutions of AMP and ETH in triplicates. The values of correlation coefficients are close to unity indicating good linearity, the characteristic parameters for the constructed equations are summarized in TABLE 3.

Range

The calibration range is established through considerations of the practical range necessary according to adherence to Beer's law and the concentration of AMP and ETH present in their pharmaceutical preparation to give accurate, precise and linear re-

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TABLE 3: Assay parameters and method validation for the determination of pure sample of Amprolium hydrochloride and Ethopabate by the proposed methods

Parameters	[1]DD method		Mean Centering method		Dual wavelength method		Ratio difference method		AUC method	
	AMP	ETH	AMP	ETH	AMP	ETH	AMP	ETH	AMP	ETH
λ (nm)	234.7	306.8	238.8	313	difference at 235.3 and 308	difference at 244 and 268.4	difference at 239 and 310	difference at 239 and 313	area between 235.6-243 and 268.3-275	
Concentration range (µg/mL)	1-40	1-10	1-35	1-12	2-32	1-14	1-40	1-14	1-40	1-14
Linearity										
Slope	0.1299	0.7604	0.887	8.0927	0.0409	0.0637	1.1911	9.0073	-	-
Intercept	0.0063	0.2957	0.3348	3.0243	0.0029	0.0141	0.5583	2.0501	-	-
Correlation coefficient (r)	0.9997	0.9992	0.9998	0.9995	0.9999	0.9999	0.9996	0.9996	-	-
Accuracy (mean ± S.D.)	99.76 ± 0.907	100.29 ± 0.842	100.26 ± 1.018	99.94 ± 1.286	99.30 ± 1.097	100.03 ± 1.065	99.27 ± 0.892	100.40 ± 1.814	100.35 ± 1.031	100.39 ± 0.956
Specificity	100.61 ± 0.980	99.81 ± 0.753	99.81 ± 1.071	99.60 ± 1.349	99.95 ± 0.830	99.93 ±0.652	100.16 ±1.235	100.02 ±1.254	99.62 ±0.868	100.29 ±0.981
Precision(%RSD)										
Repeatability[a]	0.437	0.354	0.449	0.259	0.504	0.229	0.507	0.196	0.794	0.363
Intermediate precision[b]	1.027	0.651	0.567	0.941	1.204	0.602	0.836	0.384	1.249	0.925
LOD[c] (µg/ml)	0.062	0.088	0.154	0.116	0.049	0.08	0.174	0.127	0.121	0.103
LOQ[c] (µg/ml)	0.189	0.266	0.465	0.35	0.149	0.244	0.527	0.386	0.366	0.312

[a] The intraday (n = 3), average of three different concentrations repeated three times within day, [b] The interday (n = 3), average of three different concentrations repeated three times in three successive days, [c] Limit of detection and limit of quantitation

sults. TABLE 3.

Specificity

The specificity of the proposed methods is assessed by their application to the analysis of laboratory prepared mixtures containing different ratios of AMP and ETH. Satisfactory results are obtained and presented in TABLE 1, confirming that each of the cited drugs could be successfully determined without interference from the other.

Accuracy

The accuracy of the results is checked by applying the proposed methods for determination of different concentrations for each of pure AMP and ETH within their linearity ranges, respectively. The concentrations are obtained from the corresponding regression equations then the percentage recoveries are calculated, TABLE 3. To ascertain the accuracy of the proposed methods, recovery studies are carried out by standard addition technique at three different levels, TABLE 2 and 3.

Precision

The precision of the results is checked by applying the proposed methods for determination of three concentrations for each of pure AMP and ETH within their linearity ranges, respectively; where the concentrations are analysed three times each, intra-day, (for repeatability) and on three successive days, (for intermediate precision). The concentrations are obtained from the corresponding regression equations then the percentage recoveries and %RSD values are calculated, TABLE 3.

(a) [1]DD method

Three concentrations of pure AMP (5, 10 and 20 µg/mL) and pure ETH (4, 5 and 6 µg/mL) are analysed.

(b) Mean centering method

Three concentrations of pure AMP (5, 8 and 20 µg/mL) and pure ETH (7, 9 and 10 µg/mL) are analysed.

(c) Dual wavelength method

TABLE 4: Statistical comparison of the results obtained by applying the proposed methods and the reported method for the analysis of pure Amprolium hydrochloride and Ethopabate

Value	[1]DD method		Mean centering method		Dual wavelength method		Ratio difference method		AUC method		Reported method[b][17]	
	AMP	ETH	AMP	ETH	AMP	ETH	AMP	ETH	AMP	ETH	AMP	ETH
Mean	99.76	100.29	100.26	99.94	99.30	100.03	99.27	100.40	100.35	100.39	100.35	99.50
SD	0.907	0.842	1.018	1.286	1.097	1.065	0.892	1.814	1.031	0.956	1.560	1.543
RSD%	0.909	0.840	1.015	1.287	1.105	1.065	0.899	1.807	1.027	0.952	1.555	1.551
N	6	6	6	6	6	6	6	6	6	6	6	6
Variance	0.823	0.709	1.036	1.654	1.203	1.134	0.796	3.291	1.063	0.914	2.434	2.381
Student`s t-test[a]	0.803 (2.228)	1.099 (2.228)	0.118 (2.228)	0.528 (2.228)	1.351 (2.228)	0.648 (2.228)	1.479 (2.228)	0.928 (2.228)	0.002 (2.228)	1.194 (2.228)	–	–
F value[a]	2.959 (5.050)	3.358 (5.050)	2.349 (5.050)	1.438 (5.050)	2.021 (5.050)	2.098 (5.050)	3.054 (5.050)	1.383 (5.050)	2.288 (5.050)	2.602 (5.050)	–	–

[a] The values in parenthesis are the corresponding theoretical values of t and F at (P = 0.05); [b] First derivative spectrophotometry at 288.8 and 320.6 nm for AMP and ETH, respectively

Three concentrations of pure AMP (20, 25 and 30 μg/mL) and pure ETH (5, 6 and 10 μg/mL) are analysed.

(d) Ratio difference method

Three concentrations of pure AMP (5, 10 and 20 μg/mL) and pure ETH (7, 9 and 10 μg/mL) are analysed.

(e) Area under the curve method

Three concentrations of pure AMP (5, 10 and 20 μg/mL) and pure ETH (2, 4 and 6 μg/mL) are analysed.

Detection and quantitation limits

They are calculated from the standard deviation (σ) of the response and the slope of the calibration curve (S) in accordance to the following equations: LOD = 3.3 (σ/S) and LOQ = 10 (σ/S). Results presented in TABLE 3, indicated that the method is sensitive for determination of the studied drugs.

Statistical analysis

Results obtained by the proposed methods for determination of pure AMP and ETH are statistically compared with those obtained by applying the reported spectrophotometric method[17]. The calculated t- and F-values are found to be less than the theoretical ones, confirming accuracy and precision at 95% confidence level, as shown in TABLE 4.

- This manuscript highlights several points which are
- The main advantage of the ratio-spectra derivative spectrophotometry compared to the reported first derivative spectrophotometric methods[17,20] is the ability to do easy measurements in correspondence of peaks i.e. it permits the use of the wavelength of highest value of analytical signals (a maximum or a minimum). Also, the presence of many maxima and minima is another advantage because these wavelengths give an opportunity for the determination of active compounds in the presence of other compounds and excipients which possibly interfere the assay.
- On the other hand, it is found practically that to increase selectivity and to resolve the severe overlap between the zero order absorption spectra of AMP and ETH, mean centering of ratio spectra method is applied which eliminated the derivative step and therefore the signal-to-noise ratio is enhanced.
- The new ratio difference method allows the selection of any two wavelengths for determination of the drugs, such as wavelengths of maximum absorbance that can increase the sensitivity of the method. The Ratio Difference method also has advantages over methods manipulating ratio spectra such as ratio derivative and mean centering in being very simple (one step method). The Ratio Difference method also has advantages over the dual wavelength spectrophotometry in which the selected wavelengths often meet at peak shoulders,

where $\Delta A/\Delta\lambda$ is said to be maximum, therefore selectivity and robustness towards the measured component are extremely affected, while in Ratio Difference method measurements can be done at any two wavelengths throughout the whole ratio spectrum including minima or maxima.

- Area under curve method is a newly established spectrophotometric method which provides a simple way to determine concentration of the component of interest depending on area of its absorption spectrum. This method has the advantage of being simple, sensitive and selective for determination of components in presence of other interfering substances; it is widely applied for determination of different drugs in their binary mixtures.
- All the proposed methods are simple and depend on manipulating the absorption spectra of the studied drugs and don't require any reactions or adjustment of conditions as compared to the tedious, time consuming colorimetric methods[19,21]. Also, the proposed methods are easy to apply for the determination of the studied drugs in their pharmaceutical formulation (for quality control purposes) without the need of sophisticated instruments, expensive solvents or large number of samples as compared to liquid chromatographic techniques[4].

CONCLUSION

From the previous discussion, it can be concluded that the proposed methods are simple, rapid, accurate, precise, sensitive and specific and can be used for the routine analysis of Amprolium hydrochloride and Ethopabate in their available pharmaceutical formulation. The methods are also suitable and valid for application in laboratories lacking liquid chromatographic instruments.

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