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Comparative Study Between Recent Methods Manipulating Ratio Spectra and Classical Methods based on two-wavelength selection for the determination of Binary Mixture of Antazoline hydrochloride and Tetryzoline hydrochloride

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Abstract

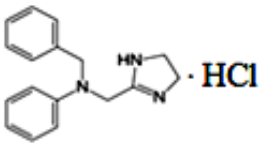
A comparative study was developed between two classical spectrophotometric methods (dual wavelength method and Vierordt's method) and two recent methods manipulating ratio spectra (ratio difference method and first derivative of ratio spectra method) for simultaneous determination of Antazoline hydrochloride (AN) and Tetryzoline hydrochloride (TZ) in their combined pharmaceutical formulation and in the presence of benzalkonium chloride as a preservative without preliminary separation. The dual wavelength method depends on choosing two wavelengths for each drug in a way so that the difference in absorbance at those two wavelength is zero for the other drug. While Vierordt's method, is based upon measuring the absorbance and the absorptivity values of the two drugs at their λ_{max} (248.0 and 219.0 nm for AN and TZ, respectively), followed by substitution in the corresponding Vierordt's equation. Recent methods manipulating ratio spectra depend on either measuring the difference in amplitudes of ratio spectra between 255.5 and 269.5 nm for AN and 220.0 and 273.0 nm for TZ in case of ratio difference method or computing first derivative of the ratio spectra for each drug then measuring the peak amplitude at 250.0 nm for AN and at 224.0 nm for TZ in case of first derivative of ratio spectrophotometry. The specificity of the developed methods was investigated by analyzing different laboratory prepared mixtures of the two drugs. All methods were applied successfully for the determination of the selected drugs in their combined dosage form proving that the classical spectrophotometric methods can still be used successfully in analysis of binary mixture using minimal data manipulation rather than recent methods which require relatively more steps. Furthermore, validation of the proposed methods was performed according to ICH guidelines; accuracy, precision and repeatability are found to be within the acceptable limits. Statistical studies showed that the methods can be competitively applied in quality control laboratories.

Keywords: Antazoline, Tetryzoline**,** Dual wavelength**,** Vierordt's method, Ratio difference, Derivative ratio.

1- Introduction:

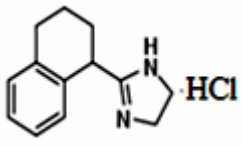
Antazoline HCl is 4,5-dihydro-N-phenyl-N-(phenylmethyl)-1H-imidazole-2-methanamine hydrochloride (Figure 1.a). [1,2]. Antazoline has antihistaminic and anticholinergic properties used to relieve nasal congestion and is used in eye drops to relieve the symptoms of allergic conjunctivitis [3]. Tetryzoline HCl is a 2-[(1*RS*)-

1,2,3,4-Tetrahydronaphthalen-1-yl)-4,5-dihydro-1*H*-imidazole hydrochloride (Figure 1.b).[1,2]. It is a sympathomimetic drug (alpha agonist) that constricts blood vessels and is used as nasal and conjunctival decongestant [4].



a

Antazoline HCl



b

Tetryzoline HCl

Fig.1. Chemical structures of AN and TZ

The combination of the two drugs is available in the market as eye drop (Trillerg®) for treatment of allergic-inflammatory affections of the conjunctiva. Literature survey reveals that there are only four analytical reports for simultaneous determination of the selected drugs in their pharmaceutical preparation: three HPLC methods [5–7] and one HPTLC method [8].

No spectrophotometric methods are reported for the simultaneous determination of the selected drugs in their pharmaceutical preparation, this is probably due to the absence of distinctly measurable peak in the absorption spectrum of TZ especially at low concentrations.

Therefore the aim of this paper is to develop, rapid and sensitive spectrophotometric methods for the simultaneous determination of AN and TZ in their binary mixture in the presence of benzalkonium chloride as a preservative without preliminary separation. A comparative study between classical spectrophotometric methods and recent methods manipulating ratio spectra is also performed to highlight the advantages and disadvantages of such methods.

2- EXPERIMENTAL

2.1. Instruments

Shimadzu UV-2400 PC Series Spectrophotometer (Tokyo – Japan) with two matched 1cm quartz cells using the following spectral parameters; a single fast scan mode and a fixed slit width (2 nm). Connected to an IBM-PC computer loaded with Shimadzu UVPC software and was equipped with HP desk jet printer and used for all the absorbance measurements and treatment of data.

2.2. Materials and Reagents

Pure samples were kindly supplied by Shanghai Yurui Bio-Tech Pharmaceutical Co., Ltd, China. Their purities were found to be 99.78%±1.007 and 99.79%±1.487 for AN and TZ; respectively according to the reported HPTLC method [8]. Benzalkonium chloride was kindly supplied by Orchidia Pharma, (Cairo, Egypt). Pharmaceutical formulation; Trillerg eye drops Batch No.0514102 was kindly supplied by Orchidia Pharma, (Cairo, Egypt). Each ml is claimed to contain 0.5 mg of AN, 0.4 mg of TZ and 0.05 mg of benzalkonium chloride. Sodium hydroxide and methanol were of analytical grade and obtained from ADWIC (Cairo, Egypt).

2.3. Standard solutions

Stock Standard solutions of AN and TZ (0.1mg/ml) in 0.1 M methanolic NaOH.

2.4. Laboratory prepared mixtures containing different ratios of AN and TZ

Into a series of 10-mL volumetric flasks, aliquots of AN and TZ were transferred from their corresponding stock standard solutions (0.1 mg/mL) of each, and then the volume was completed with methanolic NaOH. That prepares mixtures containing different ratios of the two drugs including the ratio of their commercial product.

2.5. Procedures

2.5.1. Linearity and construction of calibration curves

Aliquots equivalent to (30–300 µg) and (50–450 µg) of AN and TZ, respectively were separately transferred from their stock standard solutions (0.1 mg/ml) into two series of 10-mL volumetric flasks. Then volumes were made-up to the mark with 0.1 M methanolic NaOH. The spectra of the prepared standard solutions were scanned from 200 to 400 nm, stored in the computer and used for the construction of the proposed methods.

2.5.1.1. For Classical Spectrophotometric Methods (dual wavelength method and Vierordt's method)

a) Dual wavelength method:

The calibration curves were constructed relating the difference in absorbance of zero order spectra between 262.0 nm and 273.0 nm for AN and the difference between 230.0 nm and 258.6 nm for TZ versus the corresponding concentrations and the two regression equations were computed.

b) Vierordt's method:

The absorbances of the both drugs were recorded at 248.0 nm and 219.0 nm and the absorptivity values, $E_{(1\%, 1cm)}$ were calculated by using following formula:-

$E_{(1\%, 1cm)} = A/bC$ Where, A = absorbance b = path length of cell (1cm) and C = concentration in gm/100ml

Calibration curves were constructed relating the absorbance of zero order spectra of AN at 248.0 nm (λmax) and TZ at 219.0 nm (λmax) versus the corresponding concentrations and the two corresponding regression equations were computed.

2.5.1.2. For recent methods manipulating ratio spectra (ratio difference method and first derivative of ratio spectrophotometry)

For the determination of AN, the stored spectra of AN were divided by the spectrum of 45 µg/ml TZ, while for the determination of TZ, the stored spectra of TZ were divided by the spectrum of 30 µg/ ml AN**.**

a) For ratio difference spectrophotometric method:

Calibration curves of AN and TZ were constructed by plotting the difference between the peak amplitudes of ratio spectra at 255.5 & 269.5 nm for AN and 220.0 & 273.0 nm for TZ, versus their the corresponding concentrations then the corresponding regression equations were computed.

b) For the first derivative of ratio spectra method:

The first derivative of the ratio spectra was obtained using $\Delta\lambda = 4$ and scaling factor 100 for both AN and TZ. Calibration curves of AN and TZ were constructed by plotting the peak amplitudes of the first derivative of the ratio spectra at 250.0 nm for AN and at 224.0 nm for TZ.

2.5.2. Application of the proposed methods for the determination of AN and TZ in laboratory prepared mixtures

For dual wavelength, ratio difference and first derivative of ratio spectra methods:

For the determination of AN and TZ, the absorption spectra of laboratory prepared mixtures [2.4.], were scanned and stored. Then procedures were performed as described in linearity. The concentration of each drug was calculated using the corresponding regression equation.

For Vierordt's method:

For the determination of AN and TZ, the recorded spectra of the laboratory-prepared mixtures [2.4.], were measured at 248.0 and 219.0 nm. Then concentrations of the two drugs were calculated from the following equations:

$$\mathbf{C}_{x\,(AN)} = (A^2 \times \alpha_{y1}) - (A^1 \times \alpha_{y2}) / (\alpha_{x2} \times \alpha_{y1}) - (\alpha_{x1} \times \alpha_{y2})$$

$$\mathbf{C}_{y(TZ)} = (A^1 \times \alpha_{x2}) - (A^2 \times \alpha_{x1}) / (\alpha_{y1} \times \alpha_{x2}) - (\alpha_{y2} \times \alpha_{x1})$$

Where, C_X and C_Y are the concentrations of AN and TZ in sample solution respectively. A^1 and A^2 are absorbance of sample at 248.0 nm and 219.0 nm, where as α_{x1} and α_{x2} are absorptivity of AN at 248.0 nm and 219.0 nm and α_{y1} and α_{y2} are absorptivity of TZ at 248.0 nm and 219.0 nm, respectively

2.5.3. Application to pharmaceutical preparation

To determine the content of AN and TZ in Trillerg eye drops (each 1 ml labeled to contain 0.5 mg AN and 0.4 mg TZ), Ten eye drops solutions were mixed carefully. Then an accurately volume equivalent to 10 mg AN and 8 mg TZ was transferred to a 100-mL volumetric flask, then the volume was completed to the mark with 0.1 M methanolic NaOH. Further dilutions were made using the same solvent to obtain solutions in the linearity range.

For dual wavelength, Vierordt's, ratio difference and first derivative of ratio spectra methods:

The procedures were completed as described under linearity or laboratory prepared mixtures. The concentrations of AN and TZ were calculated by substituting in the corresponding regression equations.

The analysis was done in triplicates. Standard addition technique was applied by mixing the solution content of the eye drop with different increments of pure AN and TZ standards before proceeding in the above mentioned procedures.

3- Result and discussion

The analytical problem of spectrophotometric multicomponent analysis is that the analyte of interest is often accompanied by other co-formulated compounds absorbing in the same spectral region. In this case, spectral overlapping requires resolution by special procedures. Resolution of overlapped spectra can be done by several spectrophotometric methods.

This paper describes the development and validation of spectrophotometric methods for the determination of AN and TZ in their combined dosage form without any interference from benzalkonium chloride (added as a preservative in the dosage form). This is, followed by a comparison between classical and recent methods to determine their applicability in the resolution of such spectra.

This mixture is a challenging mixture and literature survey reveals that, no spectrophotometric methods are reported for the simultaneous determination of these drugs, because the abosrptivities of TZ at 265.0 nm and 273.0 nm are very small, and no absorption maxima in the range of 200-250 nm in distilled water, alcohol or in 0.1 M HCl (Figure 2). In 0.1 M NaOH, TZ shows a peak maxima at 219.0 nm, but unfortunately AN is insoluble in this solvent. While 0.1 M methanolic NaOH is found to be a good solvent for the determination of AN and TZ, as they are freely soluble in this solvent, and the spectrum of TZ shows a λ_{max} at 219.0 nm that can

be used for its determination by classical spectrophotometric techniques. Furthermore the spectrum of TZ is completely overlapped by the spectrum of AN (Figure 3). Moreover, the presence of benzlkoniumm chloride as a preservative in the dosage form for adds another challenge, since it is a UV absorbing compound.

Different laboratory prepared mixtures are tried, contained the same concentration of benzalkonium chloride in the dosage form and different concentrations of it up to 10 times its concentration in the dosage form. Benzalkonium chloride does not show any absorbance, (the absorbance of 10 µg is 0.0049 and 0.0058 at 256.0 nm and 262.0 nm, respectively).

The chosen methods are dual wavelength method and Vierordt's method as examples of classical spectrophotometric methods and ratio difference and first derivative of ratio spectra methods as examples of recently developed methods manipulating ratio spectra. These methods are chosen because they are very simple and require no sophisticated calculation.

3.1. For Classical Spectrophotometric Methods:-

- **Dual wavelength method:**

This method [9,10] offers an efficient way for analyzing a component in presence of an interfering component. For elimination of interferences, dual analytical wavelengths were selected in a way to make the absorbance difference zero for one drug in order to analyze the other drug.

For the determination of AN two wavelengths (262.0 and 273.0 nm) were selected where the absorbance difference between the two wavelengths is directly proportional to the concentration of AN and the absorbance difference of TZ at these wavelengths is zero, while we choose (230.0 and 258.6 nm) were selected for the determination of TZ, (Figure **3)**.

Linear relationships were obtained between the absorbance difference (262.0 & 273.0 nm for AN and 230 & 258.6 nm for TZ) and the corresponding drug concentrations in the range of 3 – 30 µg/mL and 5 – 45 µg/mL for AN and TZ respectively. The regression equations were computed and found to be:

$A_{AN} = 0.0142C-0.001$ r = 0.9998

$A_{TZ} = 0.0169C+0.0011$ r = 0.9998

where A is the difference in absorbance, C is concentration in µg/mL and r is the correlation coefficient. The mean percentage recoveries were 100.26 ± 0.715 and 100.37 ± 0.863, for AN and TZ, respectively, Table 1.

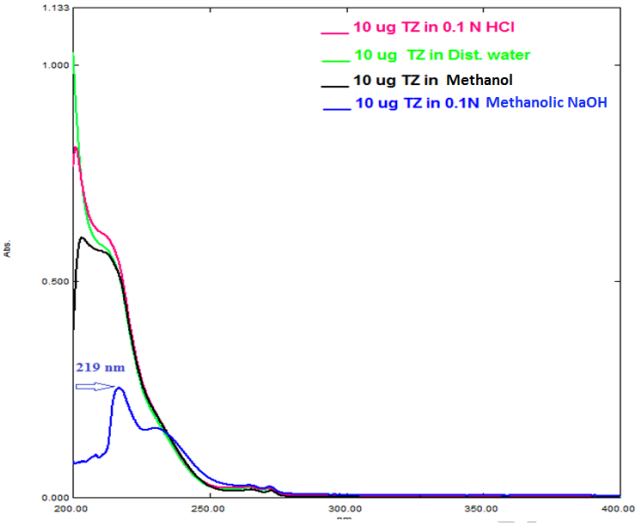


Figure (2): Zero–order absorption spectra of TZ (10 µg/mL) using different solvents.

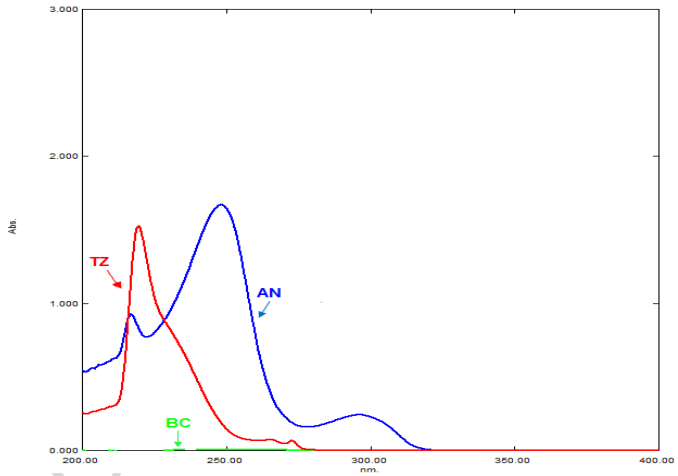


Figure (3): Zero–order absorption spectra of AN (30 µg/mL), TZ (45 µg/mL) and benzalkonium chloride (10 µg/mL) in 0.1 M methanolic NaOH.

- **Vierordt's method:**

  Since AN and TZ have overlapping spectra between 200-280 nm, they cannot be determined in binary combinations by direct UV measurements (Figure 3). But the proposed Vierordt's method [11,12] enable the calculation of the concentration of the drugs using their UV absorptions. For calculations in this method, it is necessary to select two points on the wavelength scale where the absorptivities are at a maximum. The wavelengths so chosen for either of the substances should not coincide with a sharply sloping part of the spectral curve of the other compound. The suitable pair of wavelengths for the mixture of AN and TZ were 248.0 nm (λ_{max} of AN) and 219.0 nm (λ_{max} of TZ). The absorptivities of AN and TZ at these

wavelengths, on which calculations of Vierordt's method are based, were given in Table 2.

Linear relationships were obtained between the absorbance and the corresponding drug concentrations in the range of 3 – 30 µg/mL and 5 – 45 µg/mL for AN at 248.0 nm and TZ at 219.0 nm, respectively. The regression equations were computed and found to be:

A_{248AN} = 551.8C+0.0047 r = 0.9998 A_{219AN} = 312.59C-0.0941 r = 0.9991

A_{219TZ} = 337.57C+0.0706 r = 0.9992 A_{248TZ} = 42.733C+0.0002 r = 0.9995

Where A is the absorbance, C is concentration in µg/mL and r is the correlation coefficient. The mean percentage recoveries were 100.59 ± 0.749 and 100.66 ± 0.723, for AN and TZ, respectively, Table 1

3.2. For Recent Methods Manipulating Ratio Spectra:-

- **Ratio difference spectrophotometric method (RDM)**

This is a newly developed method [13-15] having the ability for solving severely overlapped spectra without prior separation; meanwhile it does not 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 sample matrix containing an interfering component. It uses the analytical data of the ratio spectrum at two accurately selected wavelengths λ_1 and λ_2 to nullify the interferent contribution. So the following requirements were applied: at the selected wavelength pair, the difference in analyte ratio spectrum have to be linear while the difference in interferent ratio spectrum is remaining zero with changing the concentration. Also, the difference in amplitude due to the analyte ratio spectrum at the two selected wavelengths should be as large as possible to reach good accuracy and sensitivity. Similarly, another two wavelengths are selected for the estimation of the second component (interferent). Thus, the overlapped spectra of the cited drugs suggested that a ratio difference method is a suitable method for the determination of AN and TZ in their combined dosage form. For method optimization, some important decisions were carefully taken. Different divisor concentrations of AN and TZ were tried and different wavelength pairs were investigated to meet the method requirements. Ratio difference method starts by scanning the zero order absorption spectra of the laboratory-prepared mixtures (AN and TZ).

For determination of AN, several divisor concentrations 10, 15, 25, 35 and 45 µg/mL of TZ were tested, the best results were obtained when using 45 µg/mL of TZ as a divisor which yields the minimal noise and gave the highest accuracy and recoveries for determination of AN. Practically, the previously scanned ratio spectra were divided by TZ' (45 µg/ mL) as a divisor to produce new ratio spectra which represent AN/TZ' + constant as shown in (Figure 4). The amplitudes at 255.5 & 269.5 nm were selected and subtracted, so the constant TZ/TZ' was be cancelled. Similarly, the difference of amplitudes at the two selected wavelengths (220.0 & 273.0 nm) using standard AN' (30 µg/mL) as a divisor were recorded for the estimation of TZ as shown in (Figure 5).The concentration of AN and TZ were calculated using their corresponding regression

equations. The regression equations for amplitude difference (255.5 & 269.5 nm for AN and 220.0 & 273.0 nm for TZ) were computed and found to be:

$AD_{AN} = 0.3539C + 0.0031$ r = 0.9998

$AD_{TZ} = 0.0327\ C + 0.0697$ r = 0.9991

Where AD is the amplitude difference, C is concentration (µg/mL) and r is the correlation coefficient. The mean percentage recoveries were 99.99 ± 1.03 and 100.18 ± 1.23, for AN and TZ, respectively, Table 1.

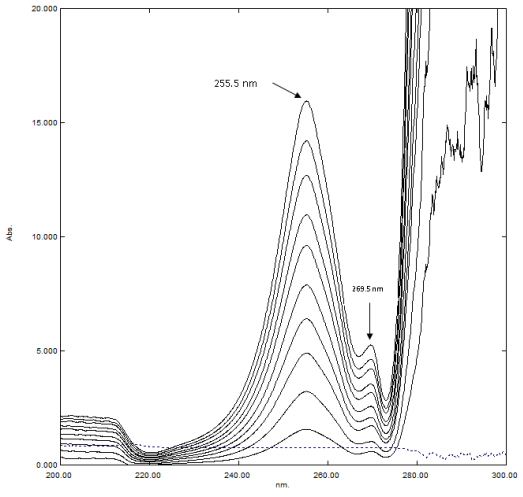


Figure (4): Ratio spectra of AN (3–30 µg/ mL) (—) and TZ 40 µg/ mL (- - -) using 45 µg/ mL of TZ' as a divisor and 0.1 M methanolic NaOH as a blank.

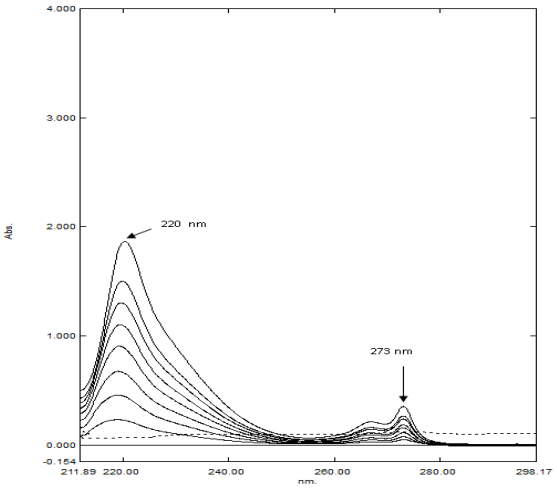


Figure (5): Ratio spectra of TZ (5–45 µg/ mL) (—) and AN 27 µg/ mL (- - -) using 30 µg/ mL of AN' as a divisor and 0.1 M methanolic NaOH as a blank.

- **The first derivative of ratio spectra:**

The main advantage of this method is the chance of easy measurements in correspondence to peaks so it permits the use of the wavelength of highest value of analytical signals (maximum or minimum) [16].

Moreover, the presence of a lot of maxima and minima is another advantage by the fact that these wavelengths give an opportunity for the determination of active compounds in presence of other active compounds or excipients which possibly interfere with the analysis. The main parameters that affect the shape of the derivative ratio spectra such as wavelength, scanning speed and the wavelength increment over which the derivative is obtained (Δλ) were studied and it was found that fast scanning speed, Δλ=4 and scaling factor 100 gave best compromise in terms of signals to noise ratio, peak resolution and sensitivity throughout the determination.

The first derivative of the developed ratio spectra obtained in the ratio difference method were calculated with Δλ = 4 nm and scaling factor 100. The regression equations for the peak amplitude at 250.0 nm for AN (Figure 6) and at 224.0 nm for TZ (Figure 7) were computed and found to be:

$$^{1}DD_{250AN} = 4.0079C + 0.3367 \qquad r = 0.9998$$

$$^{1}DD_{224TZ} = 0.2845C + 0.3387 \qquad r = 0.9991$$

Where ^{1}DD is the peak amplitude, C is concentration (µg/mL) and r is the correlation coefficient. The mean percentage recovery was 100.06 ± 0.68 at 250 nm for AN. While the mean percentage recovery for TZ was 100.55±1.00 at 224 nm, Table 1.

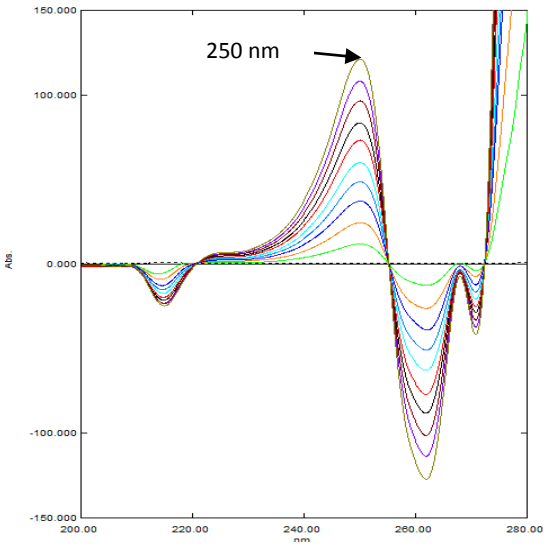


Figure (6): First derivative ratio spectra of AN (3.0 – 30.0 µg/mL) using the spectrum of (45.0 µg/mL) of TZ as a divisor.

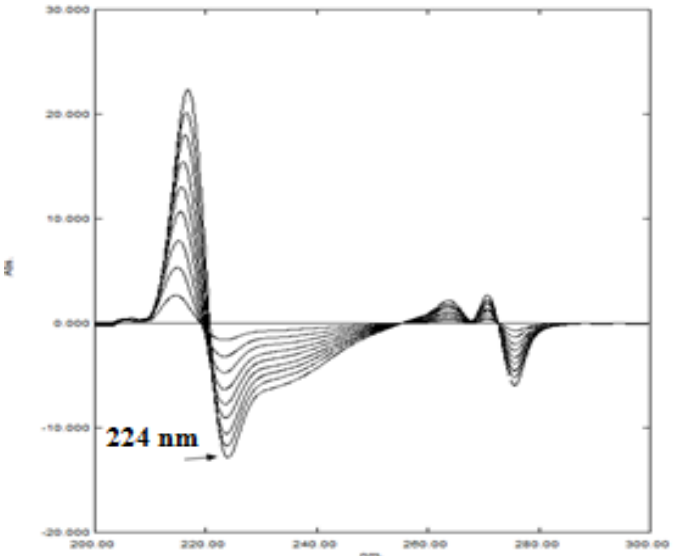


Figure (7): First derivative ratio spectra of TZ (5.0 – 45.0 µg/mL) using the spectrum of (30.0 µg/mL) of AN as a divisor.

The validity of the suggested methods were checked in terms of accuracy by five determinations between 80% and 120% concentration levels and precision (repeatability and intermediate precision) at three concentration levels, Table 1. In order to demonstrate the selectivity and applicability of the proposed methods,

recovery studies were performed by analyzing laboratory prepared mixtures of the two drugs in different ratios including the commercial product ratio, Table 3. The proposed spectrophotometric ratio-spectra methods were successfully applied for the determination of AN and TZ in their combined pharmaceutical formulation (Trillerg eye drop). Furthermore, 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 coformulated dosage form without interference of the pharmaceutical excipients.

Results of the suggested methods for determination of AN and TZ were statistically compared with those obtained by applying the reported HPTLC method [8]. The calculated t-and F-values [17] were found to be less than the corresponding theoretical ones, confirming good accuracy and excellent precision Table 5.

4- Conclusion

From the previous discussion, it could be concluded that all methods were applied successfully for the determination of the selected drugs in their combined dosage form. The proposed classical spectroscopic methods (dual wavelength and Vierordt's method) are simple, accurate and are suitable for simple devices without software or computer programs lacking access to division and derivatization steps. Their main advantage is using minimal data manipulation without the need of the division or any derivative calculations. On the other hand, the recently developed methods (RDM method and ^{1}DD) have the advantages of being more selective than the classical spectrophotometric ones as they don't need critical measurement at fixed wavelengths. The main advantage of the ^{1}DD method is that the whole spectrum of interfering substance is cancelled. Accordingly, the choice of the wavelength used for calibration is not critical. While for the RDM, by calculating the difference between two wavelengths, the noise will be cancelled, and hence signal to noise ratio is enhanced. Generally, all the developed methods do not need sophisticated instruments or any prior separation steps and so they can be used as alternative methods to LC methods in laboratories lacking the required facilities for these techniques for the analysis of any binary mixture without any limitation. They could be used for routine analysis of TZ and AN in their available dosage form without any preliminary separation steps.

Table 1: Results of validation parameters of the responses and the regression equations obtained by the proposed methods

Parameters	*Classical Spectrophotometric Methods*				*Recent methods manipulating ratio spectra*			
	Dual Wavelength method		**Vierordt's method**		**Ratio difference spectrophotometric method**		**The first derivative of ratio spectra (^{1}DD)**	
	AN	**TZ**	**AN**	**TZ**	**AN**	**TZ**	**AN**	**TZ**
Slope	0.0142	0.0169	551.798	337.57	0.3539	0.0327	4.0079	0.2845
S.E. of slope	0.000089	0.00011	3.2465	4.9506	0.002152	0.000531	0.023475	0.004657
Intercept	-0.001	0.0011	0.0047	0.0706	0.0031	0.0697	0.3367	0.3387
S.E. of intercept	0.001650	0.000308	0.006043	0.013929	0.040005	0.014944	0.436984	0.131038
Correlation coefficient	0.9998	0.9998	0.9998	0.9992	0.9998	0.9991	0.9998	0.9991
Concentration range µg/ml	3 - 30	5 – 45	3 – 30	5 – 45	3 – 30	5 – 45	3 – 30	5 – 45
Average accuracy (%)	100.26	100.37	100.59	100.66	99.99	100.18	100.06	100.55
S.D.	0.716	0.867	0.754	0.728	1.033	1.231	0.683	1.026
R.S.D. %	0.715	0.863	0.749	0.723	1.033	1.229	0.683	1.010
Repeatability [a] % ± R.S.D.	0.389	0.287	0.503	0.421	0.803	0.621	0.739	0.617
Intermediate precision [b] % ± R.S.D.	0.239	0.133	0.492	0.333	0.492	0.633	0.928	0.419

[a] n = 3×3
[b] n = 3×3

Table 2: Absorptivities (E 1%, 1cm) of AN and TZ in Vierordt's method

AN (X)		TZ (Y)	
	Absorptivity		**Absorptivity**
A^1 at 248 nm	α_{x1}= 551.8	**A^1 at 248 nm**	α_{y1}= 42.733
A^2 at 219 nm			
	α_{x2}= 312.59	**A^2 at 219 nm**	α_{y2}= 337.57

Table 3: Results of analysis of AN and TZ in laboratory prepared mixtures containing different ratios of both drugs in pure powder form by the proposed methods

Ratios	AN (recovery% ± RSD)				TZ (recovery% ± RSD)			
	Dual Wavelength	**Vierordt's method**	**Ratio difference method**	**The first derivative of ratio spectra**	**Dual Wavelength**	**Vierordt's method**	**Ratio difference method**	**The first derivative of ratio spectra**
2:1	101.23±0.319	100.61±0.145	98.66±0.827	102±0.783	101.46±0.909	101.96±0.745	100.06±0.982	100.36±0.348
1:1	101.79±0.865	100.87±0.361	99.23±0.398	100.69±0.211	100.79±0.817	100.72±0.154	101.72±0.129	101.75±0.532
***1.25:1**	100.83±0.421	99.99±0.452	99.9±0.182	101.22±0.982	100.43±0.051	101.32±0.386	100.43±0.673	101.13±0.564
1:2	100.78±0.089	100.18±0.178	101.48±0.733	100.03±0.242	100.36±0.243	101.24±0.067	101.19±0.121	100.61±0.902

*The ratio in Trillerg Eye drop.

Table 4: Determination of AN and TZ in pharmaceutical dosage form by the proposed methods and the application of standard addition technique

Item	AN (recovery% ± RSD)				TZ (recovery% ± RSD)			
	Dual Wavelength	Vierordt's method	Ratio difference method	The first derivative of ratio spectra	Dual Wavelength	Vierordt's method	Ratio difference method	The first derivative of ratio spectra
Trillerg Eye drop B.N.:0514102	100.24±0.179	99.98±0.446	100.71±1.216	100.90±1.169	100.23±0.995	101.89±0.717	101.19±0.997	100.86±1.050
Standard addition	101.53±0.549	100.51±0.345	101.12±0.309	101.52±0.429	99.57±0.208	101.56±0.433	99.65±1.097	99.68±0.496

The added concentrations used in standard addition technique were (5, 10, 15) µg for both AN and TZ.

Table 5: Statistical analysis between the results obtained for the determination of AN and TZ in pure samples by the proposed methods and those obtained by the reported method [8]

Parameters	*Classical Spectroscopic Methods*				*Recent methods manipulating ratio spectra*				Reported HPTLC Method**	
	Dual Wavelength method		Vierordt's method		Ratio difference spectrophotometric method		The first derivative of ratio spectra			
	AN	TZ	AN	TZ	AN	TZ	AN	TZ	AN	TZ
Mean	100.26	100.37	100.59	100.66	99.99	100.18	100.06	100.55	99.78	99.79
S.D	0.716	0.867	0.754	0.728	1.033	1.231	0.683	1.026	1.008	1.487
R.S.D.%	0.715	0.863	0.749	0.723	1.033	1.229	0.683	1.010	1.007	1.487
Variance	0.5112	0.745	0.5625	0.523	1.0671	1.5104	0.4665	1.020	1.0140	2.2111
n	6	6	6	6	6	6	6	6	6	6
Student's t (2.228)*	0.9509	0.8251	1.5761	1.2867	0.3564	0.4947	0.5633	1.0301		
F test (5.05)*	1.9836	2.9679	1.8027	4.2277	1.0524	1.3976	2.1736	2.1677		

*The values between parenthesis are the theoretical values of t and F at (p = 0.05).

**** HPTLC method;** The method employed HPTLC aluminium plates precoated with silica gel 60 F254 as the stationary phase. The solvent system consisted of ethylacetate: methanol: ammonia (10:10:1, v/v/v). Densitometric analysis of drugs was carried out in the absorbance mode at 216 nm.

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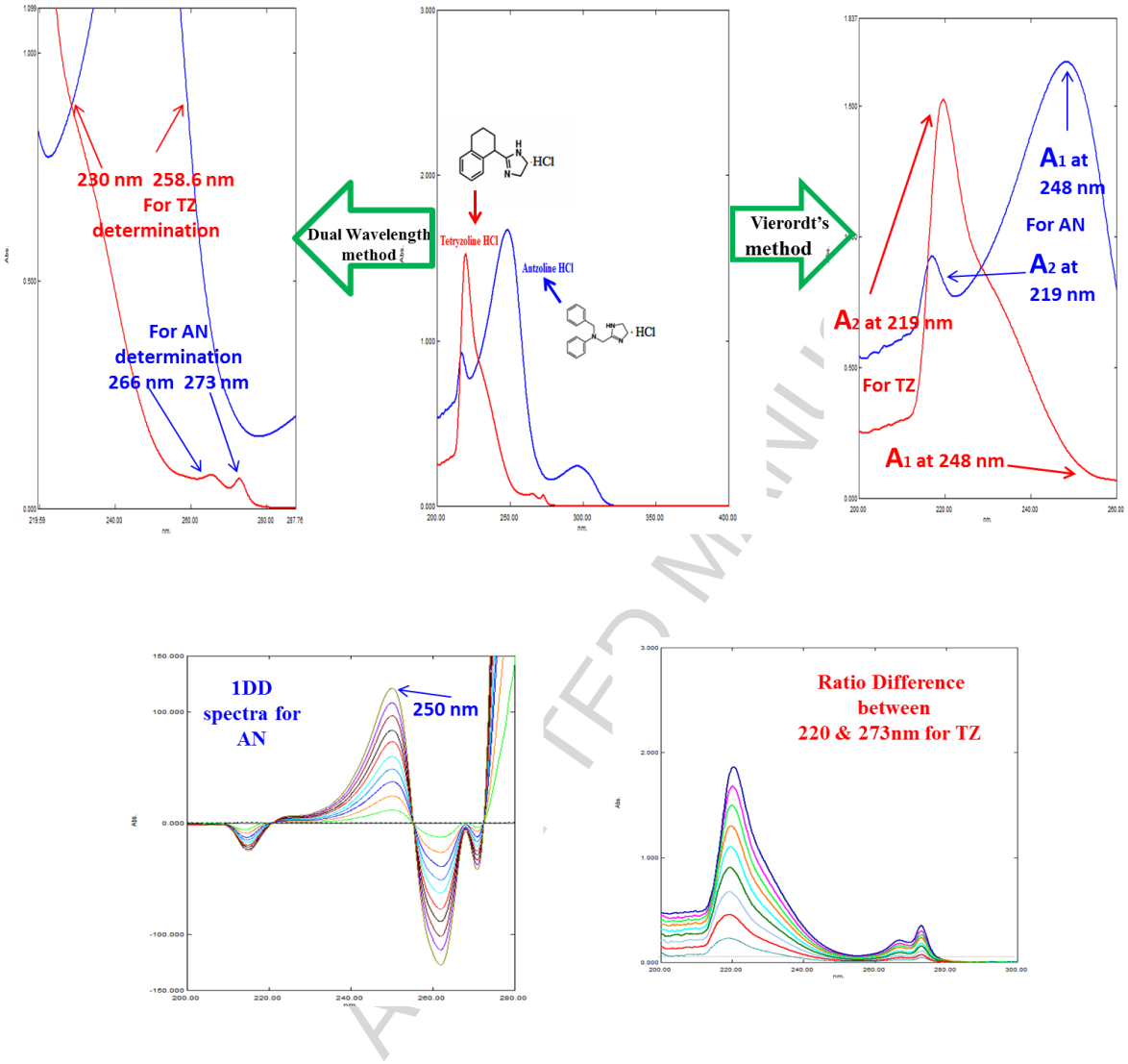
Graphical Abstract



Highlights

- Application of four different spectrophotometric methods.
- The applied methods could be used for simultaneous analysis of complex binary mixtures.
- The applied methods don’t need a special program and could be easily applied in quality control laboratories as they are having equal accuracy and precision compared to HPLC methods; in contrast they are of lower cost.
- Green, safe, economic, highly accurate and reproducible methods.