Computation of geometric representation of novel spectrophotometric methods used for the analysis of minor components in pharmaceutical preparations

Hayam M. Lotfy, Sarah S. Saleh, Nagiba Y. Hassan, Hesham Salem

Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, Kasr-El Aini, 11562 Cairo, Egypt
Pharmaceutical Chemistry department, Faculty of Pharmaceutical sciences & Pharmaceutical industries, Future University in Egypt (FUE), 12311 Cairo, Egypt
Analytical Chemistry Department, Faculty of Pharmacy, October University for Modern Sciences and Arts (MSA), 11787 6th October, Egypt
Analytical Chemistry Department, Faculty of Pharmacy, Deraya University, Minia, Egypt

HIGHLIGHTS

- Minor component represents a challenge for spectrophotometric analysis.
- Novel geometrical spectrophotometric solutions for minor components.
- Geometrical relation by standard addition or subtraction GAM, GIAM, RHPSAM, CAUC.
- Minor component tetryzoline with ofloxacin and prednisolone in ratio (1:7.5:5).
- Minor component tetryzoline with sodium cromoglicate in ratio (1:80).

GRAPHICAL ABSTRACT

Abstract

Novel spectrophotometric methods were applied for the determination of the minor component tetryzoline HCl (TZH) in its ternary mixture with ofloxacin (OFX) and prednisolone acetate (PA) in the ratio of (1:5:7.5), and in its binary mixture with sodium cromoglicate (SCG) in the ratio of (1:80). The novel spectrophotometric methods determined the minor component (TZH) successfully in the two selected mixtures by computing the geometrical relationship of either standard addition or subtraction. The novel spectrophotometric methods are: geometrical amplitude modulation (GAM), geometrical induced amplitude modulation (GIAM), ratio H-point standard addition method (RHPSAM) and compensated area under the curve (CAUC). The proposed methods were successfully applied for the determination of the minor component TZH below its concentration range. The methods were validated as per ICH guidelines where accuracy, repeatability, inter-day precision and robustness were found to be within the acceptable limits. The results obtained from the proposed methods were statistically compared with official ones when compared to the reported HPLC method, which proved that the developed methods could be alternative to HPLC techniques in quality control laboratories.
1. Introduction

The minor component is the component of a mixture which is present in very low concentration either in dosage forms or biological fluids. The analysis of minor component always represents a challenge for analytical chemists as it requires sensitive and specific methods for its analysis such as GC and LC–MS etc. The problem arises upon applying spectrophotometric techniques due to the problem of interference of the spectrum of the major ones leading to hindering its accurate determination by conventional spectrophotometric techniques. Another problem with the minor component is the deviation from Beer’s law which occurs in case of low concentrations, where transmittance values are close to 100% and where incident light approaches transmitted light [1]. Therefore, special approaches were developed to eliminate those problems based on either standard addition [2–5] or blank subtraction [6–8].

The selected minor component for this work is tetryzoline HCl (TZH) with a chemical formula of 4,5-dihydro-2-(1,2,3,4-tetrahydro-1-naphthalenyl)-1H-imidazole hydrochloride. It is a sympathomimetic agent with marked alpha adrenergic activity exhibiting a vasoconstrictor effect, so it is used as conjunctive and nasal decongestant. Tetryzoline HCl (TZH) is combined together with antibiotics, corticosteroids and anti-histamines to formulate anti-infective eye preparations to treat acute and sub-acute conjunctivitis, keratitis and corneal ulcers [9]. Different analytical techniques were reported for TZH determination in pharmaceutical preparations and in biological fluids such as: colorimetric determination [10], spectrophotometry [11–14], high performance liquid chromatographic method (HPLC) [16–21] and gas chromatography [22]. Two ophthalmic combinations were selected for the analysis of the minor component TZH; the first one is mixture (A): ofloxacin (OFX), prednisolone acetate (PA) and TZH in the ratio of (7:5:1) respectively, while the second one is mixture (B): sodium cromoglicate (SCG) and TZH in the ratio of (80:1) respectively. The structural formulae of the components of interest are shown in Fig. 1. The UV absorption spectra of both mixtures are shown in Fig. 2.

Different spectroscopic and liquid chromatographic methods have been previously reported for the determination of the cited drugs: OFX [23–27], PRD [28–32], SCG [33–36] and TZH [15,16]. For mixture (A), HPLC methods [37,38] and spectrophotometric methods [39] were reported for its analysis, where the later used standard TZH addition to the dosage form and then the claimed concentration of TZH in the preparation was calculated after subtraction of the added concentration; Meanwhile, no methods have been reported for the analysis of mixture (B).

This work presented four novel spectrophotometric methods for the determination of the minor component (TZH) in the two selected mixtures based on computing the geometrical relationship of either standard addition, such as: geometrical amplitude modulation (GAM), geometrical induced amplitude modulation (GIAM) and ratio H-point standard addition method (RHPSAM); or blank subtraction such as compensated area under the curve (CAUC). The four methods were applied to the synthetic mixtures and pharmaceutical dosage forms of the drugs of interest where the minor component (TZH) was accurately estimated in addition to the major components present. The determination of each component concentration was done with no interference of the added excipients. The obtained results from the spectrophotometric methods were compared to each other to ensure their accuracy and precision.

2. Theory

2.1. Geometrical amplitude modulation method (GAM)

A new approach is introduced to determine the concentration of the minor component X in the presence of the major component Y. It is based on the geometric representation of the effect of standard addition of X on the response of the binary mixture of (X and Y), and the interpretation of this result as a regression equation. The method is a novel ratio spectrum manipulating method using normalized spectrum of the divisor obtained by dividing certain spectrum of Y component by its concentration.

2.1.1. In case of Y is more extended than X

By using the normalized spectrum of Y as a divisor, two points were selected in the overlapped region of the ratio spectra of X and Y (P1 and P2), where the amplitudes were calculated as follow:

\[
P_1 = \frac{[\alpha_{X1} C_{X(added)}]}{[\alpha_{Y} C_{Y}]} + \frac{[\alpha_{X1} C_{X(minor)}]}{[\alpha_{Y} C_{Y}]} + \frac{[\alpha_C X_{C}]}{[\alpha_{Y} C_{Y}]} \]

\[
P_2 = \frac{[\alpha_{X2} C_{X(added)}]}{[\alpha_{Y} C_{Y}]} + \frac{[\alpha_{X2} C_{X(minor)}]}{[\alpha_{Y} C_{Y}]} + \frac{[\alpha_C X_{C}]}{[\alpha_{Y} C_{Y}]} \]

By calculating the difference \(\Delta P = (P1 - P2)\)

\[
\Delta P = \frac{[\alpha_{X1} C_{X(added)}]}{[\alpha_{Y} C_{Y}]} + \frac{[\alpha_{X1} C_{X(minor)}]}{[\alpha_{Y} C_{Y}]} - \frac{[\alpha_{X2} C_{X(added)}]}{[\alpha_{Y} C_{Y}]} + \frac{[\alpha_{X2} C_{X(minor)}]}{[\alpha_{Y} C_{Y}]} \]

This can be rearranged as follows:

![Fig. 1. The structural formulae of (a) ofloxacin (OFX), (b) prednisolone acetate (PA), (c) sodium cromoglicate (SCG) and (d) tetryzoline hydrochloride (TZH).](image-url)
\[ \Delta P = \left[ \frac{a_{X1} \cdot C_{X(added)}}{\lambda} \right] / \frac{a_Y}{\Delta a_X} - \left[ \frac{a_{X2} \cdot C_{X(added)}}{\lambda} \right] / \frac{a_Y}{\Delta a_X} - \left[ \frac{a_{X1} \cdot C_{X(minor)}}{\lambda} \right] / \frac{a_Y}{\Delta a_X} \]

where \( \Delta a_X \) is equivalent to the difference of absorptivities of \( X \) component \( (\Delta a_{X1} = \Delta a_{X2}) \).

Practically, Eq. (4) can be mathematically obtained from the regression equation representing the geometric linear relationship between the differences of ratio amplitudes \( \Delta P \) versus the added \( X \) concentration \( (C_{X(added)}) \):

\[ \Delta P = M_1 \left[ C_{X(added)} \right] + A \]

where \( M_1 \) is the slope which is equal to \( (\Delta a_{X1}/a_Y) \) and \( A \) is the intercept which is equal to \( (\Delta a_{X2}/a_Y \cdot C_{X(minor)}) \).

So by the extrapolation of the linear geometric representation of \( \Delta P \) versus \( C_{X(added)} \), the concentration of \( C_{X(minor)} \) in mixture can be directly determined, as shown in Fig. 3a. This extrapolation can be computed mathematically using the slope and intercept of the obtained regression equation as follow:

\[ \frac{A}{M_1} = C_{X(minor)} \]

For the determination of major component \( Y \) \( [C_{Y(major)}] \), amplitude modulation method is done through the direct recording of the constant value \( (P_{\text{const}}) \) using ratio spectrum which is a straight line that is parallel to the wavelength axis in the region where \( Y \) is extended.

\[ P_{\text{const}} = \left( \frac{a_Y}{a_Y \cdot C_Y} \right) = C_Y / C_Y \]

... \( C_Y = 1 \)

Then \( P = C_Y \)

...This constant amplitude value directly represents concentration of major component \( [C_Y] \).

The obtained amplitude of the ratio spectrum was modulated to concentration which represented the recorded concentration of \( Y \) \( [C_{Y(major)}] \). (Recorded of \( Y \)).

To eliminate any error, the recorded concentration of \( Y \) could be corrected to the actual concentration by using the following regression equation:

\[ C_{\text{recorded}} = \text{slope} \cdot C_{Y(major)} + \text{intercept} \]
The slope was found to be around unity and the intercept was around zero.

2.1.2. In case of X and Y are showing severely overlapping spectra with an isosbestic point

By using the normalized spectrum of Y as a divisor, two points are selected in the overlapped region of the ratio spectra of X and Y including the isosbestic point ($P_{iso}$ and $P_1$) and the difference was calculated between as shown before in Eq. (4) but by using $P_{iso}$ instead of $P_1$.

$$\Delta P = P_{iso} - P_1.$$  

Where $\Delta P$ is equivalent to the difference of absorptivities of X component ($\alpha_{X1} - \alpha_{X2}$). By plotting the ratio amplitudes at $P_{iso}$ versus the added (X) concentration, A straight line is obtained, where the extrapolation of linear geometric representation of $\Delta P$ versus added $C_X$ ($C_X(minor)$) in mixture can be directly determined as shown before in Eqs. (5) and (6).

By using the normalized spectrum of Y as a divisor, the amplitude $P_{iso}$ can be calculated as follow:

$$P_{iso} = [\alpha_X/\alpha_Y \cdot C_{X(minor)}] + [\alpha_X/\alpha_Y \cdot C_{X(minor)} + C_Y]$$  

Practically, Eq. (9) can be mathematically obtained from the regression equation representing the geometric linear relationship ($P_{iso}$) versus the added (X) concentration ($C_X(minor)$): $P_{iso} = M_2 \cdot C_{X(minor)} + B$  

Where and ($M_2$) is the slope which is equal to ($\alpha_X/\alpha_Y$) and (B) is the intercept which is equal to ($\alpha_X/\alpha_Y \cdot (C_{X(minor)} + C_Y)$). Since the measurements are done at $x_{iso} (\alpha_X = \alpha_Y)$; then ($M_2$) will be equivalent to one, and (B) will be equivalent to ($C_{X(minor)} + C_Y$).

So the extrapolation of linear geometric representation of $P_{iso}$ versus added $C_X$ will directly represent the sum of ($C_{X(minor)} + C_Y$), as shown in Fig. 3a.

$P_{iso}$ extrapolation = Intercept/slope

$$B/M_2 = \frac{[\alpha_X/\alpha_Y \cdot (C_{X(minor)} + C_Y)]}{\alpha_X/\alpha_Y}$$  

$$B/M_2 = (C_{X(minor)} + C_Y)$$  

So $C_Y$ could be determined by difference between two extrapolations of linear geometric representation of $P_{iso}$ and $\Delta P$ ($P_{iso} - P_1$) as summarized in Eqs. (6) and (12):

$$|C_Y| = B/M_2 - A/M_1$$  

$$|C_Y| = (C_X + C_Y) - |C_X|$$

2.2. Geometrical induced amplitude modulation method (GIAM)

The method is an extension of the novel (GAM) using normalized spectrum of the major component Y as a divisor in order to determine minor component X. This method could be applied for a mixture of X and Y with severely overlapping spectra, where X and Y shows large difference in their absorptivities, and hence no isosbistic point exist at the zero spectrum. So the absorptivity factor is calculated for X and Y ($\alpha_X/\alpha_Y = F$) [40,41].

By using the normalized spectrum of Y as a divisor, two wavelengths of the ratio spectrum were selected ($P_1$ and $P_2$) where $P_1$ is the absorptivity factor point. The difference was calculated for ($P_1 - P_2$).

$$P_1 = [\alpha_{X1}/\alpha_Y \cdot (C_{X(minor)} + C_{X(added)})] + |C_Y|$$

$$P_2 = [\alpha_{X2}/\alpha_Y \cdot (C_{X(minor)} + C_{X(added)})] + |C_Y|$$  

By calculating the difference $\Delta P = P_1 - P_2$.

$$\Delta P = (\alpha_{X1}/\alpha_Y \cdot C_{X(minor)}) + (\alpha_{X2}/\alpha_Y \cdot C_{X(minor)})$$

Where $\Delta P$ is equivalent to the difference of absorptivities of X component ($\alpha_{X1} - \alpha_{X2}$). By plotting the difference of the ratio spectra amplitudes $\Delta P$ ($P_1 - P_2$) versus the added concentration of (X), A straight line is obtained where the extrapolation of linear geometric representation of $\Delta P$ versus added $C_X$ ($C_X(minor)$) in mixture can be directly determined as shown in Eqs. (5) and (6), as shown in Fig. 3b. By using the normalized spectrum of Y as a divisor, the amplitude $P_1$ can be calculated as follow:

$$P_1 = [\alpha_X/\alpha_Y \cdot (C_{X(added)})] + [\alpha_X/\alpha_Y \cdot (C_{X(minor)}) + C_Y]$$

Practically, Eq. (18) can be mathematically obtained from the regression equation representing the geometric linear relationship ($P_{iso}$) versus the added (X) concentration ($C_X(added)$):

$$P_1 = M_2 \cdot C_{X(added)} + B$$

Where and ($M_2$) is the slope which is equal to ($\alpha_X/\alpha_Y$) and (B) is the intercept which is equal to ($\alpha_X/\alpha_Y \cdot (C_{X(minor)} + C_Y$). Since the measurements are done at $x_{iso} (\alpha_X/\alpha_Y = F)$, so ($M_2$) will be equivalent to the absorptivity factor ($F$), while (B) will be equivalent to $|F \cdot (C_{X(minor)} + C_{Y(major)})|$, as shown in Fig. 3b.

So through the extrapolation of linear geometric representation of $P_1$ versus added $C_X$:

$$P_1$$ extrapulation = Intercept/slope

$$B/M_2 = \frac{|F \cdot (C_{X(minor)} + C_Y)/F|}{C_Y}$$  

$$B/M_2 = \frac{|C_{X(minor)}| + |C_Y|}{F}$$  

And by calculating the difference between two extrapolations of ($P_1 - \Delta P$) which is the difference between Eqs. (21) and (6):

$$|C_Y| = B/M_2 - A/M_1$$  

$$|C_Y| = (C_X + C_Y) - |C_X|$$

2.3. Ratio H-point standard addition method (RHSAM)

This method has the same theoretical background of the conventional H-point standard addition method which is done by plotting the absorbance at the two selected wavelengths versus the added analyte concentration (X), where the interfering substance (Y) exhibits the same absorbance, two straight lines are obtained that have a common point with coordinates $H (-C_{H}, A_{H})$, where $C_{H}$ is the unknown analyte concentration and $A_{H}$ is the analytical signal due to the interferent (Y). The method has been applied to binary $C_Y$ mixtures of drugs with overlapped absorbance spectra [3–5]. By introducing a new modification which is using the normalized spectrum of (Y) as the divisor [42,43], the determination of both components (X) and (Y) is done using the peak amplitudes of the ratio spectra at any two wavelengths along the ratio spectra to be plotted versus the added concentrations of component (X), and the two straight lines obtained will intercept at the so-called...
RH point having (–C RH; P RH), where C RH is the unknown analyte concentration (C x) and P RH is equivalent to the concentration of interfering component (C y). The concentrations of X and Y can be calculated mathematically following the through the following equations:

\[
C_x = (B - A)/(M_1 - M_2) \\
C_y = (M_1B - M_2A)/(M_1 - M_2)
\]

where \( M_1 \) and \( M_2 \) are the slopes of the standard addition calibration lines obtained on applying the RHPSAM at \( \lambda_1 \) and \( \lambda_2 \), respectively; A and B are the intercepts of the two regressions, such that \( A = (X/Y)_1 + (Y/Y)_2 \) and \( B = (X/Y)_2 + (Y/Y) \).

2.4. Compensated area under the curve method (CAUC)

This method is considered to be a modification of the derivative compensation ratio technique [14–16], but instead of using the amplitude of derivative spectra, the area under the curve was used. It could be applied when the absorption spectra of two components (X) and (Y) overlap to a large extent or when analysis of a minor component (X) in the presence of a major component (Y) represents a problem.

This method involves calculating the AUC ratios (range AUC\(_x\)/range AUC\(_y\)) for pure major component (Y) against a blank solution. The mixture solution containing (X + Y) is placed in the sample cell and the area under the curve (AUC) was recorded for the difference absorption spectra of [mixture (X + Y)] against the reference cell. The mixture solution containing (X + Y) is placed in the sample cell and the area under the curve (AUC) was recorded for the difference absorption spectra of [mixture (X + Y)] against the reference cell. The concentration (Cx) could be determined after substitution in it using the average of AUC ratio of pure (Y).

2.4.1. Geometrical approach

It consists of plotting the above mentioned AUC ratios of the mixture against the concentration of the minor component (Cx) in the reference cell where a line with very slight curvature is obtained. The concentration (Cx) is calculated from the graph as it is the concentration corresponding to the AUC ratio of the mixture which is equal to the AUC ratio of the pure component (Y) previously obtained from spectra of pure (Y) at different concentration.

2.4.2. Computed geometrical approach via regression equation

This is a new modification, where the regression equation representing the geometrical approach was computed. Then the concentration (Cx) could be determined after substitution in it using the average of AUC ratio of pure (Y).

3. Experimental

3.1. Apparatus and software

Shimadzu – UV 1800 double beam UV–Visible spectrophotometer (Japan) with matched 1 cm quartz cells at 200–800 nm range was used for all absorbance measurements. Spectra were automatically obtained by Shimadzu UV-Probe 2.32 system software.

3.2. Materials and reagents

3.2.1. Pure samples

Tetryzoline HCl (TZH) reference standard was supplied by Sigma–Aldrich, USA, with a purity of 100.06 ± 0.59. Ofloxacin (OFX) and 80% solution of benzalkonium chloride were kindly supplied by Egyptian International Pharmaceutical Industries Co. (EIPICO), Cairo, Egypt, with a purity of 99.68 ± 1.21 for OFX. Prednisolone acetate (PA) and Sodium cromoglicate (SCG) were kindly supplied by Sigma Pharmaceutical Industries Limited, Al-Monofeya, Egypt, with purities of 100.26 ± 0.83 and 100.80 ± 0.743, respectively. The purities were tested by the official methods [44].

3.2.2. Market samples

Loxtra® eye drops (batch No. PK0033), labeled to contain 3 mg of ofloxacin, 2 mg of prednisolone acetate, 0.4 mg of TZH and 0.05 mg of benzalkonium chloride per one mL. Croma® eye drops (batch No. PD0080), labeled to contain 40 mg of sodium cromoglicate, 0.5 mg of TZH and 0.1 mg of benzalkonium chloride per one mL. Both preparations were manufactured by Jamjoompharma, Kingdom of Saudi Arabia were purchased from the local market.

3.2.3. Solvents

Spectroscopic analytical grade methanol was supplied from (S.d.fine-chem limited – Mumbai) and distilled water.

3.3. Standard solutions

3.3.1. Stock solutions

For mixture A: OFX, PA and TZH were prepared in a solvent mixture of methanol: water (50:50 v/v), of concentration 1 mg/mL. The least amount of methanol was used to economize the use of organic solvent, which is cost effective and eco-friendly. For mixture B: SCG and TZH were prepared in distilled water of concentration 1 mg/mL.

3.3.2. Working solutions

The solutions were freshly prepared by dilution from the stock solutions with the same solvent mixture to obtain a concentration 10 μg/mL for TZH and 200 μg/mL for OFX, PA and SCG.

3.4. Procedure

3.4.1. Spectral characteristics

The zero-order absorption spectra (D 0) of the two mixtures assembling the ratios present in the cited pharmaceutical dosage forms were recorded at (200–400 nm) against solvent mixture as a blank and in presence of 1 μg/mL of benzalkonium chloride. The first mixture consisted of 15 μg/mL of OFX, 20 μg/mL of PA and 2 μg/mL of TZH, while the second one consisted of 40 μg/mL of SCG and 0.5 μg/mL of TZH.

3.4.2. Construction of calibration graphs

3.4.2.1. Mixture (A). Working solutions equivalent to (1–15 μg/mL), (2–40 μg/mL) and (3–15 μg/mL) of OFX, PA and TZH, respectively, were prepared separately in a solvent mixture of methanol: water (50:50 v/v). The absorption spectra of the prepared solutions were measured at (200–400 nm) and stored in the computer. A calibration graph was constructed by plotting the absorbance of the zero order spectra of OFX at 298.5 nm against the corresponding concentration and the regression equation was computed. The absorption spectra of PA and TZH solutions were divided by the normalized PA spectrum and the amplitudes were plotted against the corresponding concentrations of each drug and the regression parameters were computed. The AUC at the selected wavelength ranges A1 (235–250 nm) and A2 (260–265 nm) were recorded for PA solutions, and then the average of the AUC ratio (A1/A2) was calculated. A calibration graph was constructed by plotting the AUC of PA at A2 (260–265 nm) against the corresponding concentration and the regression equation was computed.
3.4.2. Mixture (B). Working solutions equivalent to (5–40 μg/mL) and (3–15 μg/mL) of SCG and TZH, respectively, were prepared separately in distilled water. The absorption spectra of the prepared solutions were measured at (200–400 nm) and stored in the computer. The absorption spectra were divided by the normalized SCG spectrum and the amplitudes at 221, 223, 250 and 325 nm for SCG and TZH were recorded. These amplitudes were plotted against the corresponding concentrations of each drug and the regression parameters were computed. The AUC at the selected wavelength ranges \( A_1 \) (228–236.5 nm) and \( A_2 \) (320–330 nm) were recorded for SCG solutions, and then the average of the AUC ratio \( (A_1/A_2) \) was calculated. A calibration graph was constructed by plotting the AUC of SCG at \( A_2 \) (320–330 nm) against the corresponding concentration and the regression equation was computed.

3.4.3. Assay of synthetic mixtures
3.4.3.1. For GAM, GIAM and RHPSAM. Mixture (A). Nine synthetic mixtures with three different concentrations of each component: (50, 100, 150 μg/mL of OFX), (100, 200, 300 μg/mL of PA) and (5, 10, 20 μg/mL of TZH) were prepared in solvent mixture. One milliliter of each mixture was transferred to a set of 1–10 mL volumetric flasks and then standard addition of different aliquots of TZH (20–90 μg) was applied and the volumes were completed to the mark with the same solvent. The mixtures spectra were divided by the spectrum of OFX (9 μg/mL) as a divisor where the obtained constant values at the region (320–340 nm) was recorded. This constant values were subtracted from the obtained ratio spectra and then the product spectra was further multiplied by the spectrum of OFX divisor in order to recover the spectra of binary mixtures of (PA + TZH). The recorded constant values were the multiplied by the spectrum.
Table 1
Results of several experiments for the analysis of synthetic mixtures (A) at different concentration ratios of OFX, PA and TZH.

<table>
<thead>
<tr>
<th>Taken</th>
<th>Found</th>
<th>OFX</th>
<th>PA</th>
<th>TZH</th>
<th>Regression equation</th>
<th>r</th>
<th>Sc</th>
<th>Found PA</th>
<th>Found TZH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P_{226} = 1.0037 C + 10.516</td>
<td>0.9996</td>
<td>0.196</td>
<td>9.968</td>
<td>0.509</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AΔ = 0.9744 C + 0.497</td>
<td>0.997</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P_{226} = 1.0157 C + 10.942</td>
<td>0.9998</td>
<td>0.066</td>
<td>9.778</td>
<td>0.995</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AΔ = 0.9699 C + 0.965</td>
<td>0.998</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P_{226} = 1.0152 C + 11.966</td>
<td>0.9998</td>
<td>0.115</td>
<td>9.797</td>
<td>1.990</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AΔ = 0.9707 C + 1.932</td>
<td>0.996</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P_{226} = 0.9957 C + 0.488</td>
<td>0.997</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AΔ = 0.9652 C + 0.982</td>
<td>0.997</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P_{226} = 0.9911 C + 22.044</td>
<td>0.9998</td>
<td>0.156</td>
<td>20.206</td>
<td>2.036</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AΔ = 0.9674 C + 1.970</td>
<td>0.999</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P_{226} = 1.0049 C + 40.476</td>
<td>0.9988</td>
<td>0.183</td>
<td>39.775</td>
<td>0.504</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AΔ = 0.9973 C + 0.503</td>
<td>0.998</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P_{226} = 1.0059 C + 40.951</td>
<td>0.9977</td>
<td>0.083</td>
<td>39.701</td>
<td>1.010</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AΔ = 0.9745 C + 0.984</td>
<td>0.993</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P_{226} = 0.9909 C + 42.026</td>
<td>0.9995</td>
<td>0.043</td>
<td>40.375</td>
<td>2.036</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AΔ = 0.9736 C + 1.981</td>
<td>0.996</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>P_{226} = 0.9815 C + 42.067</td>
<td>0.9994</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AΔ = 0.1819 C + 40.436</td>
<td>0.9853</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

| Mean R² ± SD | 100.03 ± 0.65 | 99.34 ± 1.13 | 101.09 ± 0.98 | 99.87 ± 0.32 | 100.79 ± 1.24 |

a In µg/mL, average of three experiments.
b In regression equations 'C' is the added concentration of TZH and AΔ is difference in amplitudes at (226–250) nm.
c Standard error of standard addition lines.
d Ratio present in Loxtra® dosage form.
of OFX divisor to obtain the $D_0$ spectra of pure OFX from which the concentrations of OFX present in the synthetic mixtures were calculated using its $\lambda_{\text{max}}$ 298.5 nm. The obtained spectra of binary mixtures of (PA + TZH) were divided by the normalized divisor of PA, where the amplitudes at the isosbestic point (226 nm) 240 nm and 250 nm were recorded. The difference between amplitudes $\Delta P$ at (226–250 nm) was calculated. For GAM, two lines were plotted representing the amplitudes at 226 nm and $\Delta P_{(226-250)}$ against the added concentrations of TZH for each mixture where the extrapolations for each line was calculated. For RHPSAM, two lines were plotted representing the amplitudes at 221 and 250 nm against the added concentrations of TZH for each mixture.

3.4.3.2. For CAUC. Mixture (A). The AUC at the selected wavelength ranges $A_1$ (235–250 nm) and $A_2$ (260–265 nm) were recorded for the previously prepared mixture containing (15 OFX + 10 PA + 2 TZH in $\mu$g/mL) using different concentrations of TZH in blank cell (0–3 $\mu$g/mL) after the resolution of OFX using ratio subtraction as under Section 3.4.3.1. The AUC ratio ($A_1/A_2$) was calculated.

Mixture (B). Nine synthetic mixtures with three different concentrations of each component: (100, 200, 400 $\mu$g/mL of SCG) and (5, 10, 20 $\mu$g/mL of TZH) were prepared in distilled water. One milliliter of each mixture was transferred to a set of 10-mL volumetric flasks and then standard addition of different aliquots of TZH (20–90 $\mu$g) was applied and the volumes were completed to the mark with distilled water. The mixtures spectra were divided by the normalized divisor of SCG, where the amplitudes at the absorptivity factor point (223 nm) and 250 nm were recorded. The difference between amplitudes $\Delta P$ at (223–250 nm) was calculated. For GIAM, two lines were plotted representing the amplitudes 223 nm and $\Delta P_{(223-250)}$ against the added concentrations of TZH for each mixture where the extrapolations for each line was calculated. For RHPSAM, two lines were plotted representing the amplitudes at 221 and 250 nm against the added concentrations of TZH for each mixture.

Fig. 6. Plots of geometrical induced amplitude modulation (GIAM) between amplitudes at absorptivity factor point ($P_{223}$) and amplitude differences ($\Delta P_{223-250}$) against the added TZH concentrations for different concentrations of synthetic mixture (B) [SCG + TZH].

Fig. 7. Using SCG normalized divisor for different concentrations of SCG: (a) 40 $\mu$g/mL, (b) 20 $\mu$g/mL, (c) 10 $\mu$g/mL; where SCG concentration is equivalent to the constant amplitude at the extended region at (305–325 nm).
Table 2
Results of several experiments for the analysis of synthetic mixtures (B) at different concentration ratios of SCG and TZH.

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Taken</th>
<th>Found</th>
<th>SCG (305–325)</th>
<th>GIAM</th>
<th>RHP-SAM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SCG</td>
<td>TZH</td>
<td>Regression</td>
<td>equation(^c)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>0.5</td>
<td>10.011</td>
<td>P(_{223} = 0.4981 , C + 10.25)</td>
<td>P(_{221} = 0.641 , C + 10.042)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(\Delta P = 0.5582 , C + 0.274)</td>
<td>0.9994</td>
<td>0.9996</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>1</td>
<td>10.034</td>
<td>P(_{223} = 0.5087 , C + 10.585)</td>
<td>P(_{221} = 0.656 , C + 10.361)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(\Delta P = 0.3624 , C + 0.568)</td>
<td>0.9997</td>
<td>0.9999</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>2</td>
<td>9.998</td>
<td>P(_{223} = 0.5079 , C + 11.084)</td>
<td>P(_{221} = 0.6679 , C + 10.984)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(\Delta P = 0.5770 , C + 1.164)</td>
<td>0.9991</td>
<td>0.9995</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>0.5</td>
<td>20.246</td>
<td>P(_{223} = 0.4967 , C + 20.249)</td>
<td>P(_{221} = 0.6667 , C + 20.005)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(\Delta P = 0.5725 , C + 0.2909)</td>
<td>0.9992</td>
<td>0.9991</td>
</tr>
<tr>
<td>5</td>
<td>20</td>
<td>2</td>
<td>19.986</td>
<td>P(_{223} = 0.4968 , C + 20.504)</td>
<td>P(_{221} = 0.6745 , C + 20.261)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(\Delta P = 0.5651 , C + 0.566)</td>
<td>0.9994</td>
<td>0.9996</td>
</tr>
<tr>
<td>6</td>
<td>20</td>
<td>1</td>
<td>20.090</td>
<td>P(_{223} = 0.5028 , C + 20.98)</td>
<td>P(_{221} = 0.6761 , C + 19.978)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(\Delta P = 0.5609 , C + 1.125)</td>
<td>0.9994</td>
<td>0.9997</td>
</tr>
<tr>
<td>7(^e)</td>
<td>40</td>
<td>0.5</td>
<td>40.392</td>
<td>P(_{223} = 0.4997 , C + 40.242)</td>
<td>P(_{221} = 0.6434 , C + 40.054)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(\Delta P = 0.5733 , C + 0.281)</td>
<td>0.9998</td>
<td>0.9996</td>
</tr>
<tr>
<td>8</td>
<td>40</td>
<td>1</td>
<td>40.528</td>
<td>P(_{223} = 0.4971 , C + 40.102)</td>
<td>P(_{221} = 0.6445 , C + 40.398)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(\Delta P = 0.5701 , C + 0.570)</td>
<td>0.9999</td>
<td>0.9997</td>
</tr>
<tr>
<td>9</td>
<td>40</td>
<td>2</td>
<td>39.780</td>
<td>P(_{223} = 0.4957 , C + 41.022)</td>
<td>P(_{221} = 0.6564 , C + 40.966)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(\Delta P = 0.5658 , C + 1.144)</td>
<td>0.9999</td>
<td>0.9999</td>
</tr>
</tbody>
</table>

Mean R\(^2\) ± SD    100.42 ± 0.64

\(^a\) In µg/mL, average of three experiments.
\(^b\) SCG is determined via GAM method through extended amplitude constant at (305–325 nm).
\(^c\) In regression equations ‘C’ is the added concentration of TZH and \(\Delta P\) is difference in amplitudes at (223–250) nm.
\(^d\) Standard error of standard addition lines.
\(^e\) Ratio present in Croma\(^a\) dosage form.
3.4.4. Application to pharmaceutical preparation

Mixture (A). One milliliter was accurately transferred from the pharmaceutical dosage form (Loxtra® eye drops) to a 10-mL volumetric flask and diluted to the mark with solvent mixture. 500 μL of the prepared solution was transferred into a set of 10-mL volumetric flasks and then standard addition of different aliquots of TZH (20–90 μg) was applied and the volumes were completed to the mark with the same solvent.

Mixture (B). One milliliter was accurately transferred from the pharmaceutical dosage form (Croma® eye drops) to a 100-mL volumetric flask and diluted to the mark with distilled water. One milliliter of the prepared solution was transferred into a set of 10-mL volumetric flasks and then standard addition of different aliquots of TZH (20–90 μg) was applied and the volumes were completed to the mark with distilled water.

For both mixtures solutions, proceed as detailed under Section 3.4.3. When carrying out the standard addition technique, different dilutions were prepared in order to obtain a final concentration of 12 μg/mL of OFX, 8 μg/mL of PA for mixture A, and a final concentration of 20 μg/mL of SCG; where different known concentrations of pure standard of each drug were added to the pharmaceutical dosage form before proceeding in the previously mentioned methods.

4. Results and discussion

Analysis of complex mixtures, especially those containing minor components, always required the use of hyphenated analytical instruments such as LC–MS or GC–MS, which brought high cost and time consumption. Recently, spectrophotometric techniques attracted the attention of analysts for different applications. Besides, the existing spectrophotometric methods were found to be very easy to apply, rapid, sensitive and yet very cheap for analysis of any mixture.
This work presented the application of four novel spectrophotometric methods applied for the analysis of mixtures containing minor component. The proposed methods were based on computing the geometrical relationship of either standard addition, such as: geometrical amplitude modulation (GAM), geometrical induced amplitude modulation (GIAM) and ratio H-point standard addition method (RHPSAM); or blank subtraction such as compensated area under the curve (CAUC). The methods were applied for the determination of the minor component tetryzoline HCl (TZH) in its ternary mixture (A) with ofloxacin (OFX) and prednisolone acetate (PA) in the ratio of (7.5:5:1), and in its binary mixture (B) with sodium cromoglicate (SCG) in the ratio of (80:1) with no interference of benzalkonium chloride (added preservative), where it exhibits UV absorption at (200–220 nm) only.

4.1. Geometrical amplitude modulation method (GAM)

This method could be applied to a mixture where the major component is extended over the minor one or when both components exhibit severely overlapping spectra with an isosbestic point. To apply this method to mixture (A), firstly OFX should be resolved via ratio subtraction method [45,46] using OFX divisor in order to recover the zero order absorption spectrum of binary mixture of (PA + TZH). By applying constant multiplication method, the zero order absorption spectrum of OFX present in the mixture could be obtained and its concentration was calculated using its $\lambda_{\text{max}}$, as shown in Fig. 4. The spectrum of binary mixture of (PA + TZH) was then divided by the PA normalized divisor; and then the amplitudes at isosbestic point (P226) and the amplitude difference $\Delta P_{226-250}$ were plotted against the added concentrations of TZH for each mixture where the extrapolations for the two line were calculated, as shown in Fig. 5. To analyze the mixtures geometrically, it was found that the extrapolation for line (P226) represented the total concentrations of (PA + TZH) present in each mixture; while the extrapolation for line ($\Delta P_{226-250}$) eliminated the amplitude of PA, which is a constant along the whole spectrum, represented the concentrations of TZH only present in each mixture. Then concentration of PA was calculated by
difference. In order to compute those geometrical extrapolations, the concentrations of PA and TZH can be directly calculated through the previous Eqs. (6) and (10) as follow:

\[ C_{TZH} = \frac{A}{M_1} \]

where \( A \) and \( B \) are the intercepts; \( M_1 \) and \( M_2 \) are the slopes of the standard addition calibration lines obtained at \( \Delta P_{226-250} \) and \( \Delta P_{226} \), respectively. The calculations for each synthetic mixture were listed in Table 1.

To apply this method to mixture (B) where SCG spectrum is extended than that of TZH, the mixture spectra were divided by the SCG normalized divisor; and then the difference between amplitudes \( \Delta P_{223-250} \) were plotted against the added concentrations of TZH for each mixture where the extrapolations were calculated, as shown in Fig. 6. To analyze the mixtures geometrically, it was found that the extrapolation for line \( \Delta P_{223-250} \) eliminated the amplitude of SCG, which is a constant along the whole spectrum, represented the concentrations of TZH only present in each mixture. The concentration of SCG was equivalent to the constant amplitude at the extended region at \( 305-325 \text{ nm} \) as shown in Fig. 7. In order to compute the geometrical extrapolation, concentration of TZH can be directly calculated through Eq. (6) as follow:

\[ C_{TZH} = \frac{A}{M_1} \]
Table 4
Assay parameters and validation sheet obtained by applying the proposed spectrophotometric methods for mixture A.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>OFX</th>
<th>PA</th>
<th>TZH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength (nm)</td>
<td>D₀</td>
<td>P 224</td>
<td>P 226</td>
</tr>
<tr>
<td>Slope</td>
<td>0.1059</td>
<td>1.0908</td>
<td>1.0779</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.0061</td>
<td>–0.8246</td>
<td>–0.8347</td>
</tr>
<tr>
<td>Correlation coeff. (r)</td>
<td>0.9998</td>
<td>0.9998</td>
<td>0.9997</td>
</tr>
<tr>
<td>Mean</td>
<td>99.74</td>
<td>100.00</td>
<td>99.81</td>
</tr>
<tr>
<td>RSD%</td>
<td>1.025</td>
<td>0.522</td>
<td>1.752</td>
</tr>
<tr>
<td>Accuracy</td>
<td>100.93 ± 0.67</td>
<td>101.23 ± 0.99</td>
<td>99.34 ± 1.12</td>
</tr>
<tr>
<td>Repeatability</td>
<td>0.943</td>
<td>1.102</td>
<td>0.549</td>
</tr>
<tr>
<td>Inter-day precision</td>
<td>1.102</td>
<td>1.231</td>
<td>0.881</td>
</tr>
<tr>
<td>Robustness</td>
<td>1.532</td>
<td>0.779</td>
<td>1.002</td>
</tr>
</tbody>
</table>

a Average of three experiment.
b Mean ± standard deviation of 3 concentrations of each drug (4, 6 and 8 µg/mL).
c Relative standard deviation of 3 concentrations of each drug (4, 6 and 8 µg/mL).
d Robustness were checked by testing the effect of solvent (45%, 55%, 60% methanol).

Table 5
Assay parameters and validation sheet obtained by applying the proposed spectrophotometric methods for mixture B.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>SCG</th>
<th>P 221</th>
<th>P 223</th>
<th>P 250</th>
<th>P 325</th>
<th>A₁ (228–236.5)</th>
<th>A₂ (120–330)</th>
<th>TZH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength (nm)</td>
<td>D₀</td>
<td>P 221</td>
<td>P 223</td>
<td>P 250</td>
<td>P 325</td>
<td>A₁ (228–236.5)</td>
<td>A₂ (120–330)</td>
<td>P 221</td>
</tr>
<tr>
<td>Range (µg/mL)</td>
<td>2.5–40</td>
<td>2.5–40</td>
<td>5–40</td>
<td>2.5–40</td>
<td>2.5–40</td>
<td>3–15</td>
<td>3–15</td>
<td>3–15</td>
</tr>
<tr>
<td>Slope</td>
<td>0.9718</td>
<td>0.9722</td>
<td>0.9874</td>
<td>0.9957</td>
<td>0.4083</td>
<td>0.1639</td>
<td>0.6638</td>
<td>0.5084</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.2686</td>
<td>0.2378</td>
<td>0.0860</td>
<td>0.0163</td>
<td>–0.1196</td>
<td>–0.0317</td>
<td>–0.2742</td>
<td>–0.1046</td>
</tr>
<tr>
<td>Correlation coeff. (r)</td>
<td>0.9999</td>
<td>0.9999</td>
<td>1.000</td>
<td>1.000</td>
<td>0.9999</td>
<td>0.9999</td>
<td>0.9999</td>
<td>0.9999</td>
</tr>
<tr>
<td>Mean</td>
<td>99.76</td>
<td>99.77</td>
<td>99.37</td>
<td>99.79</td>
<td>99.88</td>
<td>100.37</td>
<td>100.06</td>
<td>100.07</td>
</tr>
<tr>
<td>RSD%</td>
<td>0.887</td>
<td>0.884</td>
<td>0.721</td>
<td>0.232</td>
<td>1.095</td>
<td>1.624</td>
<td>0.451</td>
<td>1.240</td>
</tr>
<tr>
<td>Accuracy</td>
<td>99.43 ± 0.91</td>
<td>100.14 ± 1.08</td>
<td>100.11 ± 0.65</td>
<td>101.03 ± 0.97</td>
<td>100.11 ± 1.12</td>
<td>101.22 ± 0.99</td>
<td>99.50 ± 0.60</td>
<td>100.79 ± 1.67</td>
</tr>
<tr>
<td>Repeatability</td>
<td>0.742</td>
<td>0.699</td>
<td>0.422</td>
<td>0.421</td>
<td>0.932</td>
<td>0.681</td>
<td>0.669</td>
<td>0.865</td>
</tr>
<tr>
<td>Inter-day precision</td>
<td>0.866</td>
<td>0.989</td>
<td>0.588</td>
<td>0.661</td>
<td>1.100</td>
<td>0.832</td>
<td>0.789</td>
<td>0.899</td>
</tr>
<tr>
<td>Robustness</td>
<td>0.438</td>
<td>1.155</td>
<td>0.789</td>
<td>1.008</td>
<td>0.459</td>
<td>0.789</td>
<td>0.611</td>
<td>1.120</td>
</tr>
</tbody>
</table>

a Average of three experiment.
b Mean ± standard deviation of 3 concentrations of each drug (5, 10 and 15 µg/mL).
c Relative standard deviation of 3 concentrations of each drug (5, 10 and 15 µg/mL).
d Robustness were checked by testing the effect of solvent (5%, 7%, 10% methanol).
where A and M$_1$ are the intercept and slope of the standard addition calibration line obtained at ($\Delta$P$_{223–250}$). The calculations for each synthetic mixture were listed in Table 2.

This method applies minimum manipulation steps to determine minor and major components using only one divisor (normalized divisor). The requirement of this method is the existence of isoabsorptive point of both components or the extension of the spectrum of the major one. The advantage of this method over the ratio H-point standard addition method (RHSAM) is the simplicity of the geometric computation using the simple Eqs. (6) and (10). Several wavelengths were tested and the chosen wavelengths showed the best recoveries for the synthetic mixtures.

4.2. Geometrical induced amplitude modulation method (GIAM)

This proposed method is an extension of the novel (GAM) which could be applied for a mixture of two components with severely overlapping spectra and a large difference in their absorptivities, and hence no isoabsorptive point exists. To apply this method to mixture (B), absorptivity factor point ($\lambda_p$) should be chosen; which is the crossing point obtained between different concentrations of the two drugs where the absorptivities of the two drugs are not equal and subsequently their ratio is not unity but they represents by the absorptivity factor ($F$) which is equal to the inverse of the ratio of the used concentrations. Several crossing points were checked for the best linearity correlation and the best recoveries when applied to synthetic mixtures. Therefore, $\lambda_p$ 223 nm was chosen to calculate the absorptivity factor ($F$) where the absorptivity of SCG is double that of TZH ($\alpha$TZH/$\alpha$SCG) and so, $F = 1/2$, as shown in Fig. 3b.

The mixtures spectra mixture were divided by the SCG normalized divisor; and then the amplitudes at absorptivity factor point ($P_f$) at 223 nm and difference between amplitudes $\Delta P_{223–250}$ were plotted against the added concentrations of TZH for each mixture where the extrapolation for each line was calculated, as shown in Fig. 4. To analyze the mixtures geometrically, it was found that the extrapolation for $\lambda_p$ line (223 nm) represented the concentrations of $[2 \; C_{SCG} + C_{TZH}]$ present in each mixture; while the extrapolation for line ($\Delta P_{223–250}$) eliminated the amplitude of SCG, which is a constant along the whole spectrum, represented the concentrations of TZH only present in each mixture. In order to compute those geometrical extrapolations, the concentrations of SCG and TZH can be directly calculated through the previous Eqs. (6) and (20) as follow:

$$C_{TZH} = \frac{A}{M_1}$$

$$C_{SCG} = [B/M_2] - \frac{|A/M_1| * F}{1 - F}$$

where A, B are the intercepts; M$_1$ and M$_2$ are the slopes of the standard addition calibration lines obtained at ($\Delta$P$_{223–250}$) and ($P_f$), respectively. The calculations for each synthetic mixture were listed in Table 2.

This method has the same advantages of the geometrical amplitude modulation (GAM) of minimum manipulation and simplicity in addition to the possibility of its application in more complex mixtures where large difference in their absorptivities occurs and no isoabsorptive point exists.

### Table 6
Application of the proposed methods for the analysis of the selected pharmaceutical formulations of mixtures (A) and (B).

| Methods   | Mixture (A) | | | | |
|-----------|-------------|-----|-----|-----|
|           | OFX | PA | TZH | |
| D$_0$ (298.5 nm) | 101.16 ± 0.63 | – | – | |
| GAM       | – | 100.55 ± 0.66 | 100.25 ± 1.04 | |
| RHSAM     | – | 98.80 ± 1.33 | 100.38 ± 0.49 | |
| CAUC      | – | 99.10 ± 0.95 | 100.25 ± 0.38 | |

| Methods   | Mixture (B) | | | | |
|-----------|-------------|-----|-----|-----|
|           | P (305 – 325 nm) | SCG | TZH | |
|           | 98.96 ± 0.76 | – | – | |
| GIAM      | 101.11 ± 0.79 | 100.80 ± 0.78 | – | |
| RHSAM     | 100.72 ± 1.05 | 101.39 ± 0.49 | – | |
| CAUC      | 99.30 ± 0.85 | 100.25 ± 0.80 | – | |

### Table 7
Results of one-way ANOVA for comparison of the proposed methods for the determination of PA, SCG and TZH in pure form.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degree of freedom</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>F value</th>
<th>P value</th>
<th>F critical</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA</td>
<td>Between columns</td>
<td>6</td>
<td>0.655</td>
<td>0.109</td>
<td>0.107</td>
<td>2.342</td>
</tr>
<tr>
<td></td>
<td>Within columns</td>
<td>39</td>
<td>39.968</td>
<td>1.025</td>
<td>0.095</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>45</td>
<td>40.623</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCG</td>
<td>Between columns</td>
<td>6</td>
<td>6.590</td>
<td>1.098</td>
<td>1.699</td>
<td>2.342</td>
</tr>
<tr>
<td></td>
<td>Within columns</td>
<td>39</td>
<td>25.206</td>
<td>0.646</td>
<td>0.147</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>45</td>
<td>31.796</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TZH</td>
<td>Between columns</td>
<td>9</td>
<td>1.313</td>
<td>0.146</td>
<td>0.114</td>
<td>2.086</td>
</tr>
<tr>
<td></td>
<td>Within columns</td>
<td>47</td>
<td>60.209</td>
<td>1.281</td>
<td>0.999</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>56</td>
<td>61.521</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* There was no significance difference between the methods using one-way ANOVA at P < 0.05.

### Table 8
Statistical comparison between the results of the reported HPLC method and the proposed methods applied for the pharmaceutical formulation of for mixture (A).

| Items | OFX | | | | | | | | |
|-------|-----|-----|-----|-----|-----|-----|-----|-----|-----| |
|       | D$_0$ (298.5 nm) | Reported HPLC | PA | GAM | RHSAM | CAUC | Reported HPLC | TZH | GAM | RHSAM | CAUC | Reported HPLC | |
| Mean % | 101.16 | 100.29 | 100.55 | 98.80 | 99.1 | 100.25 | 100.25 | 100.38 | 100.25 | 99.56 |
| ±SD   | 0.63 | 0.96 | 0.66 | 1.33 | 0.95 | 0.75 | 1.04 | 0.49 | 0.80 | 0.99 |
| Variance | 0.3969 | 0.9216 | 0.4356 | 1.7689 | 0.9025 | 0.5625 | 2.0816 | 0.2401 | 0.6400 | 0.9801 |
| n     | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | 6 | |
| F-Value (5.050)$^*$ | 2.346 | 1.308 | 3.153 | 1.618 | 1.101 | 4.059 | 1.530 | 0.653 | 0.039 | 0.735 |
| Student’s t-test (2.228)$^*$ | 0.525 | 0.043 | 1.690 | 1.749 | 0.653 | 0.039 | 0.735 | 0.693 | 0.031 | 0.735 |

* Figures between parentheses are the corresponding tabulated t values of at P = 0.05.
4.3. Ratio H-point standard addition method (RHPSAM)

This method is a type of dual wavelength spectrophotometry combining the principles of ratio spectrophotometry and HPSAM. The constants generated in the ratio spectra can be revealed and determined using the fact that H-point standard addition method could determine the proportionality constants, calculated through division by normalized divisor, present in mixtures. For mixture (A), the amplitudes at 223 and 240 nm were selected; while for mixture (B), the amplitudes at 221 and 250 were selected. For each mixture the amplitudes were plotted against the added concentrations of TZH for each mixture, where the two lines intercept at the RH point (–CRH). As shown in Figs. 8 and 9, where the X-coordinate (–CRH) is equivalent to TZH concentration in both mixtures and Y coordinate (Pzh) is the concentration of major component PA and SCG in mixtures (A) and (B), respectively. Accordingly, at this point the concentrations of both components are simultaneously determined using the Eqs. (21) and (22) as follow:

\[ C_{TZH} = \frac{(B - A)}{(M_1 - M_2)} \]

\[ C_{PA \ or \ SCG} = \frac{(M_1 - B - M_2 A)}{(M_1 - M_2)} \]

where M1 and M2 are the slopes of the standard addition calibration lines obtained on applying the RHPSAM at \( \lambda_1 \) and \( \lambda_2 \), respectively; A and B are the intercepts of the two regressions. For mixture (A), \( \lambda_1 \) and \( \lambda_2 \) are 223 and 240; for mixture (B), \( \lambda_1 \) and \( \lambda_2 \) are 221 and 250, respectively. The calculations for each synthetic mixture were listed in Tables 1 and 2.

The advantage of RHPSAM is the absence of any constraints in choosing the two specific wavelengths, as the constant values generated in the ratio curves are extended along the ratio spectra, where the recorded amplitudes were directly modulated prevailing over instrumental noise, as it depends on measuring wavelengths where the major component has constant absorbance value; and this hindered its application on mixture (B).

By using the normalized divisor in the previous three methods: GAM, GIAM and RHPSAM, the results were not affected by the choice of divisor and it minimized the noise in the obtained ratio spectra, contrary from conventional HPSAM in which the choice of the two wavelengths is a critical step and limited to two critical wavelengths where the major component has constant absorbance value; and this hindered its application on mixture (B).

4.4. Compensated area under the curve method (CAUC)

This method is based on standard subtraction of the minor component (TZH) placed in reference cell from the mixture placed in sample cell. It is considered a modification of derivative compensation ratio technique. The mean ratios of AUC of PA and SCG (A1/A2) were selected so that TZH interfere in A1 region only. The AUC ratio was calculated to be 4.646 and 2.955 for pure PA and SCG, respectively, as shown in Figs. 10 and 11. Two mixture solutions were prepared assembling the concentration of both mixtures (A) and (B) in their pharmaceutical dosage forms. These solutions were placed in the sample cell. Different concentrations of pure TZH (0–3 µg/mL) were placed in the reference cell successively. The spectra of the mixture solution against each reference cell were recorded and the AUC ratio was calculated for each mixture. The straight lines obtained, Figs. 10 and 11 and their regression equations were found to be:

\[ R = -0.1039C + 4.8564 \quad r = 0.9994 \quad \text{for mixture(A)} \]

\[ R = -0.0162C + 2.9636 \quad r = 0.9997 \quad \text{for mixture(B)} \]

where R is the recorded AUC ratio of the mixture (A1/A2) against different reference cells, C is the concentration of pure TZH in reference cell; r is the correlation coefficient.

The concentration of TZH present in the mixtures was calculated by substituting in the regression equation with the previously determined mean ratio of AUC of pure PA and SCG for mixture (A) and (B), respectively. The concentrations of PA and SCG were determined using their corresponding AUC at (260–265 nm) and (320–330 nm), respectively, using a blank of the solvent used (zero concentration of TZH). The results were listed in Table 3.

The advantage of this method is providing better sensitivity and prevailing over instrumental noise, as it depends on measuring area of component in a wide range of wavelengths and not at a single wavelength, where the minor component can be determined with blank subtraction rather than standard addition.

The corresponding concentration ranges and calibration equations for the proposed methods were listed in Tables 4 and 5. The proposed procedures were successfully applied for the determination of both pharmaceutical formulations as shown in Table 6.

5. Method validation

The proposed spectrophotometric methods were validated in compliance with the ICH guideline [47] in terms of linearity and range. Accuracy was checked by analysis of five blind samples of each drug. Repeatability and inter-day precision were checked by three replicate analyses of three pure samples of each drug on a single day and on three consecutive days, and the results were expressed as RSD. The precision of standard additions for GAM and GIAM were estimated by calculating standard error (Sx) [48]; and the selectivity for all methods was ascertained by analyzing different mixtures as shown in Tables 1–3. Robustness was checked by minor changes in solvent compositions. The data shown in Tables 4 and 5 proved that the methods were accurate, precise and robust over the mentioned linearity range. Tables 1–3.
and 2 proved the specificity of the proposed methods for the determination of mixtures (A) & (B).

6. Statistical analysis

Table 7 showed one-way ANOVA statistical comparison of the results obtained by the proposed methods and official methods when applied to pure powders of all components. Table 8 showed the calculated t and F values for the statistical comparison of the results obtained by the proposed methods and reported HPLC methods [38] applied for the analysis of Loxtara®, the dosage form of mixture (A); while for mixture (B), no methods were reported for its analysis, so placebo experiments resembling the constituents of the dosage form Croma® were carried on; and the obtained results were statistically compared to the obtained results of the analysis of the dosage form using t and F values, as shown in Table 9. The results from both tables showed that there was no significant difference between the proposed methods with respect to accuracy and precision.

7. Conclusion

This work presented four novel spectrophotometric methods for the determination of the minor component (TZH) in two selected mixtures by computing the geometrical relationship of either standard addition through geometrical amplitude modulation (GAM), geometrical induced amplitude modulation (GIAM) and ratio H-point standard addition method (RHPSAM); or blank subtraction through compensated area under the curve method (CAUC). The advantages of the proposed methods were the simplicity of their geometric computation using simple equations. They were successfully applied for the analysis of the selected mixtures with minimum manipulating steps where satisfactory results were obtained. As a final conclusion, the proposed methods could be successfully applied for the routine analysis of mixtures containing minor component in quality control laboratories without any preliminary separation step.

References
