Validated spectrophotometric methods for simultaneous determination of Omeprazole, Tinidazole and Doxycycline in their ternary mixture

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A B S T R A C T

A comparative study of smart spectrophotometric techniques for the simultaneous determination of Omeprazole (OMP), Tinidazole (TIN) and Doxycycline (DOX) without prior separation steps is developed. These techniques consist of several consecutive steps utilizing zero/or ratio/or derivative spectra. The proposed techniques adopt nine simple different methods, namely direct spectrophotometry, dual wavelength, first derivative-zero crossing, amplitude factor, spectrum subtraction, ratio subtraction, derivative ratio-zero crossing, constant center, and successive derivative ratio method. The calibration graphs are linear over the concentration range of 1–20 μg/mL, 5–40 μg/mL and 2–30 μg/mL for OMP, TIN and DOX, respectively. These methods are tested by analyzing synthetic mixtures of the above drugs and successfully applied to commercial pharmaceutical preparation. The methods that are validated according to the ICH guidelines, accuracy, precision, and repeatability, were found to be within the acceptable limits.

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1. Introduction

Peptic ulcers are painful sores in the lining of the stomach or the duodenum as a result of imbalance between digestive fluids, caused by infection with a type of bacteria called Helicobacter pylori or excess production of the acid producing cells of the stomach. For bacterial infection, the most effective treatment is a combination of 2 antibiotics (e.g. Tinidazole and Doxycycline) and 1 proton pump inhibitor (e.g. Omeprazole).

The three investigated drugs are officially listed in B.P. [1] and U.S.P. [2]; the chemical structures of the drugs are shown in Fig. 1.

Omeprazole (OMP) is a proton pump inhibitor, used in treatment of peptic ulcer disease and NSAID-associated ulceration, in gastroesophageal reflux disease and the Zollinger–Ellison syndrome [3]. OMP has been estimated as single or in combination with other drugs using several techniques including spectrophotometry [4,5], spectrofluorimetry [6], electrochemical methods [7], thin layer chromatography (TLC) [8,9], high performance liquid chromatography (HPLC) [10,11] and capillary electrophoresis [12].

Tinidazole (TIN) represents a class of drugs, namely anti-parasites that have an activity against anaerobic bacteria and protozoa. It is used in peptic ulcer with other antimicrobials and proton pump inhibitor [3]. TIN was determined as single or in combinations using different techniques including spectrophotometry [13–15], electrochemical methods [16], TLC [17] and HPLC [18–20].

Doxycycline (DOX) is a tetracycline derivative which is bacteriostatic with a broad spectrum of antimicrobial activity including many aerobic and anaerobic Gram-positive and Gram-negative pathogenic bacteria and some protozoa. It is used in triple therapy along with Tinidazole and proton pump inhibitor in the treatment of peptic ulcer [3].

Doxycycline had been estimated in pharmaceutical formulations by UV-spectrophotometry [21,22], spectrofluorimetry [23], electrochemical methods [24], TLC [25] HPLC [26,27] and capillary electrophoresis [28].

OMP was simultaneously determined in the presence of TIN using different techniques including spectrophotometry [5], electrochemical methods [29] TLC [8,9] and HPLC [30], while TIN was simultaneously determined in the presence of DOX using spectrophotometric technique [31].

Literature survey reveals that the three drugs were simultaneously determined using HPLC technique [32–34] while no reported methods have been reported for determination of the three drugs in combination using spectrophotometric methods.

The aim of this work was to develop spectrophotometric methods based on smart original mathematical techniques for resolving the ternary mixture of OMP, TIN and DOX drugs with spectral interfering problems without preliminary separation. Consequently, we conduct a comparative study between the two recently developed methods namely; constant center (CC) [35], spectrum subtraction (SS) [36] and...
amplitude factor (P-Factor) [37–39] and conventional spectrophotometric methods namely; dual wavelength (DW) [40,41], ratio subtraction (RS) [42] first derivative (′D) [43] derivative ratio-zero crossing (′DD) [44] and successive ratio-derivative [45] in terms of specificity and validation and prove their effectiveness compared to the reported methods. The proposed methods are very simple, accurate, precise and do not require any sophisticated apparatus or computer programs.

For applying the proposed methods, only a spectrophotometer with simple software to measure and manipulate the spectra of the studied drugs is needed. At the same time the adopted methods are smart and can be applied for most of the binary and ternary mixtures with no need for any sophisticated conditions or difficult requirements. The only requirement for the dual wavelength method is the selection of two wavelengths for each drug in a way so that the difference in absorbance is zero for another drug. While for ratio subtraction, one of the drugs should be extended than the other one.

2. Experimental

2.1. Apparatus and software

Spectrophotometric measurements were carried out on JASCO V-630 BIO Double-beam UV–vis spectrophotometer (S/N C367961148), using 1.00 cm quartz cells. Scans were carried out in the range from 200 to 400 nm at 0.1 nm intervals. Spectra Manager II software was used.

2.2. Samples and solvents

2.2.1. Pure samples

Omeprazole (OMP), Tinidazole (TIN) and Doxycycline (DOX) were kindly supplied by El-Hikma Pharmaceutical Company, Cairo, Egypt; the purity was certified to be 100.10 ± 1.34, 100.88 ± 1.65 and 100.14 ± 1.12 according to the reported method for OMP and TIN [5] and official method [2] for DOX.

2.2.2. Market sample

TRIO capsules dosage form, labeled to contain 20 mg (OMP)/50 mg(TIN)/50 mg(DOX) was kindly supplied by El-Hikma Pharmaceutical Company.

2.2.3. Solvents

Solvents were of spectroscopic analytical grade; ethanol and water (Sigma Aldrich, Germany).

2.3. Standard solutions

Stock standard solution of each of OMP, TIN and DOX (1 mg/mL) in ethanol:water (90:10, v/v) is prepared. The prepared solutions were found to be stable without any degradation when stored in dark glass wares in the refrigerator at 4 °C for 3 days.

Working standard solutions for OMP and DOX (50 μg/mL) and TIN (100 μg/mL) were prepared from stock solutions (1 mg/mL) by appropriate dilutions with ethanol:water (90:10, v/v).

2.4. Procedure

The zero-order absorption spectra (D0) of 10 μg/mL for each of OMP, TIN and DOX were recorded in the range of 200–400 nm against ethanol:water (90:10, v/v) as a blank.

2.4.1. Construction of calibration graphs

Aliquots equivalent to 10–200 μg OMP, 50–400 μg TIN and 20–300 μg DOX were accurately transferred from their working standard solutions into three separate series of 10– mL volumetric flasks then completed to volume with the same solvent. The spectra of the prepared standard solutions were scanned from 200 to 400 nm and stored in the computer.

Calibration graphs were constructed relating the absorbance of zero order spectra (D0) of OMP at 302 nm, TIN at 315 nm while DOX at 276 nm and 351 nm versus the corresponding concentrations.

For dual wavelength method calibration graphs relating the difference between the absorbance at 287.2 nm and 263.2 nm for OMP and that between 292.7 nm and 309.6 nm for DOX versus the corresponding concentrations and the regression equations were computed.

Then the first derivative spectra (D1) (Δλ = 8.00 and scaling factor = 10) were recorded and calibration graphs were constructed relating the amplitude of the obtained (D1) spectra of OMP, TIN, and DOX at 313 nm, 352 nm and 380 nm respectively versus the corresponding concentrations and the regression equations were computed. A factor (PFIN/P352) relating the amplitude of TIN at 380 nm and 352 nm was calculated to be 0.146.

For constant center method the stored absorption spectra of TIN and DOX were divided by the absorption spectra of 5 μg/mL DOX and 5 μg/mL TIN, respectively where the obtained ratio spectra were recorded. Calibration graphs were constructed by plotting the difference between the amplitudes of the obtained ratio spectra at [350 nm and 360 nm] and [361 nm and 374 nm]; versus amplitudes at 350 nm and 374 nm for TIN and DOX respectively and the regression equations were computed.

For derivative ratio-zero crossing method the stored (D0) spectra of OMP were divided by the spectrum of 5 μg/mL DOX and the first derivative of the obtained spectra is recorded. A calibration graph relating the amplitude at 313.9 nm versus the corresponding concentrations of OMP was constructed and the regression equations were computed.

For successive ratio-derivative spectra, the stored zero order absorption spectra (D0) of different concentration of OMP were divided by the spectrum of 5 μg/mL of DOX and the ratio spectra were obtained. First derivatives of the ratio spectra were obtained with Δλ = 8 and scaling factor 1. These vectors (D1 of the ratio spectra) were divided by (d/dλ) (5 μg/mL of TIN/5 μg/mL of DOX) corresponding to the derivative of the ratio of the spectra of TIN and DOX and therefore, second ratio spectra were obtained. First derivative of these vectors was obtained Δλ = 8 and the calibration graph of OMP was constructed by plotting the amplitude of the resulting spectra at 314.5 nm against its corresponding concentration. The same steps were performed for TIN using the spectrum of 5 μg/mL of DOX as the first divisor followed by the spectrum of (d/dλ) (5 μg/mL of OMP/5 μg/mL of DOX) as the second divisor; and for DOX using the spectrum of 5 μg/mL of TIN as
the first divisor followed by the spectrum of \((d/d\lambda)(2 \mu g/mL of OMP / 5 \mu g/mL of TIN)\) as the second divisor. The calibration graphs of TIN and DOX were constructed by plotting the amplitude of the resulting spectra at 355.5 nm and 353 nm, respectively, against their corresponding concentrations \((\Delta \lambda = 8.00)\).

2.4.2. Application to laboratory prepared mixtures

Into a series of 10 mL volumetric flask, accurate aliquots of OMP, TIN and DOX were transferred from their working standard solutions to prepare six mixtures containing different ratios of the cited drugs. The volumes were completed with ethanol:water (90:10, v/v). The spectra of the prepared solutions were recorded at 200–400 nm, stored in the computer and manipulated as mentioned under construction of calibration graphs.

Each drug in the ternary mixture can be determined and analyzed by more than one method using different approaches.

For TIN

i. **First derivative-zero crossing method**, the amplitude of the first derivative spectra of ternary mixture is recorded at 352 where OMP and DOX are zero-crossing.

ii. **Constant center method**, the absorption spectra of the laboratory prepared mixtures were divided by the absorption spectra of 5 \(\mu g/mL\) standards TIN. The ratio spectra were recorded at 361 nm and 374 nm. The postulated amplitudes at 374 nm were calculated using the corresponding regression equation. The constant value was obtained after subtraction the recorded amplitude of the mixtures and its postulated amplitude at the specified wavelength. The obtained constant value for each mixture is then multiplied by the spectra of 5 \(\mu g/mL\) standard TIN so the original spectra of TIN were obtained and the absorbance is recorded at \(\lambda_{\text{max}}\) of TIN (315 nm). The constant center method is not only used for determination of TIN but also for the resolution of its spectra from the ternary mixture using spectrum subtraction method, and the mixture will act as a binary mixture of OMP and DOX only.

iii. **Successive ratio-derivative spectra methods**, the stored \(\left(D_0\right)\) spectra of the mixtures were divided by the spectrum of 5 \(\mu g/mL\) DOX as first divisor. First derivatives of the ratio spectra were obtained with \(\Delta \lambda = 8\) and scaling factor 1. These vectors \((D_1)\) of the ratio spectra are divided by \((d/d\lambda)(2 \mu g/mL of OMP / 5 \mu g/mL of TIN)\) as the second divisor corresponding to the derivative of the ratio of the spectra of TIN and DOX and therefore, second ratio spectra were obtained. The amplitudes of the resulting spectra were recorded at 353 nm.

The concentrations of the drug were calculated by substitution in the corresponding regression equation after applying the corresponding manipulating steps for each method.

For DOX

DOX can be determined either in the presence of both TIN and OMP or in the presence of OMP alone after the resolution of TIN spectrum using **constant center** followed by **spectrum subtraction** thus obtaining the spectra of OMP and DOX as a resolved binary mixture.

a— Determination of DOX in the presence of both TIN and OMP

i. **First derivative-zero crossing coupled with amplitude factor method**, the amplitude of the first derivative spectra of ternary is recorded at 380 nm where OMP shows no contribution while the contribution of TIN is canceled adopting amplitude factor method.

ii. **Constant center method**, the absorption spectra of the prepared laboratory mixtures were divided by the absorption spectra of 5 \(\mu g/mL\) standard DOX. The ratio spectra were recorded at [350 nm and 360 nm] respectively. The postulated amplitudes at 350 nm were calculated using the corresponding regression equation. The constant value was obtained after subtraction the recorded amplitude of the mixtures and its postulated amplitude at the specified wavelength. Multiply the obtained constant value for each mixture by the spectra of 5 \(\mu g/mL\) standard DOX so the original spectra of DOX were obtained and the absorbance is recorded at \(\lambda_{\text{max}}\) of DOX (276 nm). The constant center method can also be used for resolution of the whole spectrum of DOX if followed by spectrum subtraction.

iii. **Successive ratio-derivative spectra method**, the stored \(\left(D_0\right)\) spectra of each mixture was divided by the spectrum of 5 \(\mu g/mL\) TIN as first divisor. First derivatives of the ratio spectra were obtained with \(\Delta \lambda = 8\) and scaling factor 1. These vectors \((D_1)\) of the ratio spectra are divided by \((d/d\lambda)(2 \mu g/mL of OMP / 5 \mu g/mL of TIN)\) as the second divisor corresponding to the derivative of the ratio of the spectra of TIN and DOX and therefore, second ratio spectra were obtained. The amplitudes of the resulting spectra were recorded at 355.5 nm.

b— Determination of DOX in the presence of OMP only.

i. **Direct spectrophotometry**, the absorbance of the zero order spectra \((D_0)\) of the prepared laboratory mixtures is recorded at 354 nm, where OMP has no contribution. DOX determination is followed by division by 5 \(\mu g/mL\) DOX as a divisor, a constant is obtained as a straight line parallel to the wavelength axis in the region where DOX is more extended than OMP. The constant is determined and subtracted, obtaining the pure spectra of OMP alone as single drug.

ii. **Dual wavelength method**, the difference between the absorbances of the zero order spectra \((D_0)\) of the prepared laboratory mixtures at 292.7 nm and 309.6 nm was calculated.

iii. **First derivative-zero crossing method**, the amplitude of the first derivative spectra of ternary is recorded at 380 nm where OMP shows no contribution.

The concentrations of the drug were calculated by substitution in the corresponding regression equation after applying the corresponding manipulating steps for each method.

For OMP

Omeprazole (OMP) can be determined either in the presence of both TIN and DOX or in presence of DOX alone after the resolution of TIN spectrum using constant center followed by spectrum subtraction thus obtaining the spectra of OMP and DOX as a resolved binary mixture. Omeprazole (OMP) can also be determined as a pure resolved single drug after the resolution of TIN spectrum using constant center followed by spectrum subtraction and then the resolution of DOX adopting either constant center followed by spectrum subtraction or ratio subtraction in the region where DOX is more extended than OMP.
a— Determination of OMP in the presence of both TIN and DOX
  i. First derivative-zero crossing, the amplitude of the first derivative spectra of ternary is recorded at 313 nm where both TIN and DOX show no contribution.
  ii. Derivative ratio-zero crossing, the absorption spectra of the prepared laboratory mixtures were divided by the absorption spectra of 5 μg/mL standard DOX. The first derivative of the obtained ratio spectra were recorded at [313.9 nm].
  iii. Successive ratio-derivative spectra, the stored (D^0) spectra of mixtures were divided by the spectrum of 5 μg/mL DOX as first divisor. First derivatives of the ratio spectra were obtained with Δλ = 8 and scaling factor = 1. These vectors (D^1 of the ratio spectra) are divided by (d/dλ) (5 μg/mL of TIN / 5 μg/mL of DOX) as the second divisor corresponding to the derivative of the ratio of the spectra of TIN and DOX and therefore, second ratio spectra were obtained. The amplitudes of the resulting spectra were recorded at 314.5 nm.

b— Determination of OMP in the presence of DOX only
  i. Dual wavelength method, the difference between the absorbances of the zero order spectra (D^0) of the prepared laboratory mixtures at 287.2 nm and 263.2 nm was calculated.
  ii. First derivative-zero crossing method, the amplitude of the first derivative spectra of ternary is recorded at 313 nm where DOX shows no contribution.

b— Determination of OMP as single resolved drug
  OMP can be determined as a single drug using direct spectrophotometric method measuring the absorbance at its λ_{max} (301.5 nm) after the resolution of TIN and DOX. The concentrations of the drug were calculated by substitution in the corresponding regression equation after applying the corresponding manipulating steps for each method.

2.4.3. Application to pharmaceutical dosage form

The contents of 10 capsules were emptied, weighed, finely powdered and homogenously mixed. A portion equivalent to 10 mg OMP, 250 mg TIN and 25 mg DOX was accurately weighed and dissolved in ethanol:water (90:10) by shaking in ultrasonic bath for about 30 minutes. The solution was filtered into a 100 mL measuring flask and the volume was completed with the same solvent. 1 mL of the previously prepared solution is transferred to 10 mL volumetric flask and the volume was completed with the same solvent. Proceed as under laboratory prepared mixture for each drug. The concentration of each drug was calculated using the specified regression equation.

3. Results and discussion

Resolving the overlapped spectra of multi-component mixtures without prior separation of the constituent analytes was rather a difficult task. In the last few years, the development of methods for the resolution of such mixtures has grown dramatically. Spectrophotometric methods were found to be very easy to apply, very rapid, sensitive and yet very cheap for analysis of mixture. Several methods were published on the analysis of TIN, OMP and DOX each in its single form, while, only three articles adopting chromatographic methods have been published for their analysis in mixture form [32–34]. No spectrophotometric methods have been reported for the mixture analysis. Spectrophotometric methods are preferable over other hypeninated analytical instrumentalations or techniques such as LC-MS, GC-MS, LC-NMR, etc.; which always require optimization of conditions such as pH, temperature, flow rate, etc.

Fig. 2. Zero order absorption spectra of 1 μg/mL OMP (—–), 25 μg/mL TIN (–) and 2.5 μg/mL DOX (—–).

After several trials, it was found that mixture of ethanol:water 90:10 was a best solvent for the cited drugs in their mixture since Doxycycline is sparingly soluble in ethanol.

The zero order spectra of pure drugs were severely overlapped which hinders their determination as shown in Fig. 2. The choice of the optimum concentration range depends on the spectral characteristics of the compound, its absorptivity and its ratio in the mixture. The contribution of TIN in the mixture is greater than that of OMP and DOX because it represents the major component in the pharmaceutical dosage form (1 OMP: 25 TIN: 2.5 DOX). OMP did not show any absorption and its contribution to the absorption of the mixture above 340 nm was considered to be negligible.

a— Simultaneous determination of TIN, DOX and OMP
  The three drugs can be simultaneously determined in the presence of each other either progressively using first derivative-zero crossing-coupled with amplitude factor method, or successively using constant center coupled with spectrum subtraction method and successive ratio-derivative spectra.

3.1. First derivative-zero crossing-coupled with amplitude factor method

The first order absorption spectrum [Fig. 3] was used for determination of the studied drugs progressively. The D^1 spectra showed that OMP has a peak at 313 nm where both TIN and DOX are zero crossing i.e. both drugs have no contribution. Also TIN has a significant amplitude 352 nm at which both OMP and DOX are zero crossing. Thus two calibration graphs could be constructed relating the peak amplitudes at 313 nm and 352 nm for standard solutions OMP and TIN, respectively and the corresponding concentrations for determination of both drugs, respectively.

On the other hand, DOX shows a peak at which OMP is zero crossing while TIN shows contribution, i.e. TIN has some interference at amplitude of derivative peak (λ_{1} = 380 nm) of DOX, the contribution of TIN can be canceled via the amplitude factor method by subtracting

Fig. 3. First derivative order absorption spectra of 1 μg/mL OMP (—–), 25 μg/mL TIN (–) and 2.5 μg/mL DOX (—–).

b— Determination of DOX in the presence of TIN only
  i. Dual wavelength method, the difference between the absorbances of the zero order spectra (D^0) of the prepared laboratory mixtures at 287.2 nm and 263.2 nm was calculated.
  ii. First derivative-zero crossing method, the amplitude of the first derivative spectra of ternary is recorded at 313 nm where TIN shows contribution, i.e. TIN has some interference at amplitude 352 nm while DOX shows no contribution.
  iii. Successive ratio-derivative spectra, the stored (D^0) spectra of ternary is recorded at 313 nm where TIN and DOX show no contribution.
the amplitude due to TIN at peak amplitude of DOX using experimentally calculated amplitude factor. For application of the amplitude factor method a second wavelength (\(\lambda_2 = 352\) nm) where DOX doesn’t show any contribution should be selected as summarized in the following equations:

\[
P_{\text{380}}(\text{DOX} + \text{TIN}) = P_{\text{352}}(\text{TIN})
\]

Peak amplitude of DOX at \(\lambda_1\) = \(P_{\text{1}}(\text{DOX} + \text{TIN}) - P_{\text{1}}/P_{\text{2}} \times P_{\text{2}}(\text{DOX} + \text{TIN})
\]

where: \(P_{\text{1}}\) and \(P_{\text{2}}\) is the amplitude of TIN at \(380\) nm and \(352\) nm, respectively. \(P_{\text{1}}/P_{\text{2}}\) is called the amplitude factor [ratio of the peak amplitude at the two wavelengths \(P_{\text{380}}/P_{\text{352}}\)], it is constant for pure TIN and calculated to be 0.146. \(P_{\text{380}}(X + Y)\) and \(P_{\text{352}}(\text{DOX} + \text{TIN})\) are the amplitudes of the mixture at 380 nm and 352 nm, respectively.

Quantitative estimation of DOX was carried out using the following equation.

Amplitude of DOX at 380 nm

\[
= P_{\text{380}}(\text{DOX} + \text{TIN}) - (0.146) \times P_{\text{352}}(\text{DOX} + \text{TIN})
\]

The main advantage of the first derivative-zero crossing is the chance of doing easy measurements in correspondence of peaks so it permits the use of the wavelength of highest value of analytical signals (a maximum or a minimum), and 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 the presence of other compounds and ingredients which possibly interfere in the assay, while its main disadvantage of this measuring technique is a not too great precision of measurements. Additionally the advantage of the amplitude factor method is the determination of the active compounds with minimum manipulation steps.

3.2. Constant center coupled with spectrum subtraction method

The zero order spectra of the studied drugs revealed that OMP didn’t show any absorption above 350 nm while DOX and TIN are extended. Constant center method was adopted for determination of both DOX and TIN with satisfactory precision. By applying the proposed method the original spectra of DOX and TIN will be obtained. If their spectra are subtracted from the total spectra of the ternary mixture successively, the result will be the spectrum of pure OMP which in turn can be determined.

In the constant center method, the absorption spectrum of the mixture was scanned and divided by the absorption spectrum of a known concentration of both TIN and DOX divisors, respectively and the ratio spectra are obtained representing:

\[
\left(\frac{\text{OMP}}{\text{TIN}} + \frac{\text{DOX}}{\text{TIN}} + \text{constant}\right)\text{ and } \left(\frac{\text{OMP}}{\text{DOX}} + \frac{\text{TIN}}{\text{DOX}} + \text{constant}\right).
\]

The selected divisors should compromise between minimal noise and maximum sensitivity. The divisor concentrations 5 \(\mu\)g/mL TIN and 5 \(\mu\)g/mL DOX gave the best results regarding average recovery percent when used for the analysis of TIN and DOX concentrations in mixtures, respectively.

Ratio difference at two selected wavelength was applied to the ratio spectra of the cited drugs. The requirements for the selection of these two wavelengths is the contribution of both TIN and DOX only with no contribution of OMP in addition the ratio spectrum of one drug showed the same value (constant) whereas the other drug showed significant difference in these two ratio values at these two selected wavelengths \(\lambda_1\& \lambda_2\) with concentrations. The two selected wavelengths are 374 nm and 361 nm and 350 nm and 360 nm for TIN and DOX respectively as shown in Figs. 4 and 5.

Ratio difference at two selected wavelength was applied to the ratio spectra of the cited drugs

\[
\left(\frac{\text{OMP}}{\text{TIN}} + \frac{\text{DOX}}{\text{TIN}} + \text{constant}\right)_1 - \left(\frac{\text{OMP}}{\text{TIN}} + \frac{\text{DOX}}{\text{TIN}} + \text{constant}\right)_2
\]

(1)

\[
\left(\frac{\text{OMP}}{\text{DOX}} + \frac{\text{TIN}}{\text{DOX}} + \text{constant}\right)_1 - \left(\frac{\text{OMP}}{\text{DOX}} + \frac{\text{TIN}}{\text{DOX}} + \text{constant}\right)_2
\]

(2)
Since OMP has no contribution at the selected wavelengths i.e. OMP/TIN and OMP/DOX are zero and Eqs. (1) and (2) can be written as Eqs. (3) and (4), respectively.

\[
\left( \frac{\text{DOX}}{\text{TIN}} \right)_{1} - \left( \frac{\text{DOX}}{\text{TIN}} \right)_{2} 
\]

(3)

\[
\left( \frac{\text{TIN}}{\text{DOX}} \right)_{1} - \left( \frac{\text{TIN}}{\text{DOX}} \right)_{2} 
\]

(4)

For the determination of TIN in the mixture, the zero order spectrum of TIN at 374 nm was obtained after multiplication of the calculated constant value by the interfering drug (DOX was canceled) versus the corresponding ratio amplitudes at 374 nm.

\[ P_{1} - P_{2} = 0.508 \left( \frac{\text{DOX}}{\text{TIN}} \right) - 0.00004 \]

where; \( P_{1} \) and \( P_{2} \) are the ratio amplitudes at 374 nm and 361 nm of the ratio spectra of different concentrations of TIN (2–30 μg/mL) using 5 μg/mL TIN as a divisor and \( \left( \frac{\text{DOX}}{\text{TIN}} \right) \) is the corresponding ratio amplitudes of the ratio spectra at 374 nm.

The constant value was calculated by monitoring the effect on the amplitude of the ratio spectrum of TIN at 374 nm \( \left( \Delta P_{\text{recorded}} - \Delta P_{\text{postulated}} \right) \), so the constant value was calculated by measuring the difference between the recorded amplitude and postulated amplitude at this wavelength.

\[
\frac{\text{TIN}}{\text{DOX}} = \frac{\text{DOX}}{\text{TIN}} + \frac{\text{TIN}}{\text{TIN}} - \frac{\text{DOX}}{\text{TIN}} \\
\text{Constant} = \left[ \frac{\text{P}_{\text{recorded}}}{\text{P}_{\text{postulated}}^{'}} \right] 
\]

\( P_{\text{recorded}} \) is the recorded amplitude of the ratio spectrum of the laboratory prepared mixture using 5 μg/mL TIN as a divisor and \( P_{\text{postulated}}^{'}) \) is the calculated amplitude using the previously specified regression equation.

The original spectra of TIN in the mixture could be obtained by multiplying the obtained constant of \( \frac{\text{TIN}}{\text{DOX}} \) by TIN (the divisor) \( \left( \text{Fig. 6} \right) \) which is used for direct determination of TIN at \( \lambda_{\text{max}} \) (315 nm) and calculation of the concentration from the corresponding regression equation as shown in Table 1 (obtained by plotting the absorbance values of the zero order curves of TIN at 315 nm against the corresponding concentrations).

Similarly DOX can be determined using 5 μg/mL DOX as a divisor at 350 nm and 360 nm versus 350 nm to calculate the constant value of DOX via amplitude difference step using the following regression equation:

\[ P_{1} - P_{2} = 0.4863 \left( \frac{\text{TIN}}{\text{DOX}} \right) - 0.0013 \]

where; \( P_{1} \) and \( P_{2} \) are the ratio amplitudes at 350 nm and 360 nm respectively of the ratio spectra of different concentrations of TIN (3–40 μg/mL) using 5 μg/mL DOX as a divisor and \( \left( \frac{\text{TIN}}{\text{DOX}} \right) \) is the corresponding ratio amplitudes at 350 nm. The original spectrum of DOX was obtained after multiplication of the calculated constant value by the 5 μg/mL DOX as a divisor as shown in Fig. 7.

Finally the DOX concentrations in the mixtures are calculated from the corresponding regression equation as shown in Table 1 (obtained by plotting the absorbance values of the zero order curves of DOX at \( \lambda_{\text{max}} \) 267 nm against the corresponding concentrations).
The proposed method was successfully applied to the analysis of TIN and DOX in their laboratory prepared mixtures and in tablet dosage forms (Table 2).

Subtracting the obtained zero order spectra of TIN and DOX from the total spectrum of the ternary mixture, the zero order spectra of OMP would be the result enabling the direct determination of its concentrations in the mixtures from the corresponding regression equation (obtained by plotting the absorbance values of the zero order curves of OMP at λ_{max} 301.5 nm against the corresponding concentrations).

The main advantage of constant center method coupled with the spectrum subtraction over the other spectrophotometric methods is that, the first one was able to determine both components in the binary mixture at their λ_{max} without any limitation of the extension of one of the overlapped spectra using only one divisor with minimum manipulation steps. It is also has advantage over derivative technique since it avoids the critical measurement in this technique either at zero crossing or zero contribution of the interfering substance.

3.3. Successive ratio-derivative spectra

In this work, the successive ratio-derivative spectra method is applied for simultaneous determination of OMP, TIN, and DOX in their ternary mixtures successfully with no need for any preliminary separation step. The method depends on successive derivative of the ratio spectra in two successive steps. The proposed method is simpler and provides reproducible results.

Different factors affecting the determinations including divisor concentrations, Δλ, smoothing factors, and type of solvent used were studied and optimized to provide accurate, precise, and reproducible results.

The spectra of different concentrations in the calibration range of OMP, TIN, and DOX were tried as divisors; the best concentrations were 5 μg/mL of DOX as first divisor and 5 μg/mL of each of TIN and DOX as second divisors for OMP determination, 5 μg/mL of DOX as first divisor and 2 μg/mL and 5 μg/mL of each of OMP and DOX, respectively as second divisors for TIN determination and 5 μg/mL of TIN as first divisor and 2 μg/mL and 5 μg/mL of each of OMP and TIN, respectively as second divisors for DOX determination.

Different Δλ (2, 4, 8) were tried and the best results were obtained upon using Δλ = 8 for the three drugs using scaling factor = 10

Different solvents were tried including, 0.1 N NaOH, and 0.1 N HCl, ethanol, water and mixtures of ethanol and water in different proportions. The best solvent giving the best sensitivity was found to be ethanol/water (90:10 v/v).

For OMP determination, the absorption spectra of different concentrations of pure OMP in the range of 1–20 μg/mL were divided by the spectrum of 5 μg/mL of DOX (first divisor) and the first ratio spectra were obtained (Fig. 8a). First derivatives of these ratio spectra were obtained using Δλ = 8 nm (Fig. 8b). After that, these vectors (first derivative of the ratio spectra) were divided by (d/λ)_{TIN/DOX} corresponding to the zero contribution of the interfering substance.

![Graph](image_url)
First derivatives of the ratio spectra of TIN and DOX (second divisor) and the second ratio spectra were obtained (Fig. 8c). First derivatives of these ratio spectra were obtained using $\Delta \lambda = 8$ nm and scaling factor = 1 (Fig. 8d).

The concentration of OMP was obtained by substitution in the regression equations representing the linear relationship between the peak amplitude at 314.5 nm of different concentrations of OMP standard solution versus the corresponding concentrations.

Fig. 8. The ratio spectra obtained by dividing the spectra of different OMP concentrations (1–20 μg/mL) by the spectrum of DOX of 5 μg/mL (first divisor) (a). First derivative of the ratio spectra obtained for OMP concentrations with $\Delta \lambda = 8$ nm (b). Second ratio spectra obtained by dividing the first derivative spectra of the first ratio spectra of OMP by the spectrum of $(d/d\lambda) (5 \mu g/mL \text{TIN}/5 \mu g/mL \text{DOX})$ (c). The final spectrum showing OMP determination at peak minimum at 314.5 nm after successive-ratio derivative method with $\Delta \lambda = 8$ nm (d).

Fig. 9. First derivative of the ratio spectra obtained for TIN concentrations with $\Delta \lambda = 8$ nm (a). Second ratio spectra obtained by dividing the first derivative spectra of the first ratio spectra of TIN by the spectrum of $(d/d\lambda) (2 \mu g/mL \text{OMP}/5 \mu g/mL \text{DOX})$ (b). The final spectrum showing TIN determination at peak minimum at 355.5 nm after successive-ratio derivative method with $\Delta \lambda = 8$ nm (c).
Calibration graphs for TIN could be also constructed as described for OMP using standard solutions of DOX of a concentration of 5 μg/mL as first divisor (Figs. 4 and 9a) and using the vector of \( (\frac{d}{d\lambda})_{\text{ONP}}/C_{14}\) \( (\text{DOX})\) corresponding to the derivative of the ratio of the standard spectra each of 2 μg/mL and 5 μg/mL concentrations of OMP and DOX, respectively as second divisor (Fig. 9b and c). The concentration of TIN was obtained by substitution in the regression equations representing the linear relationship between the amplitude at 355.5 nm of different concentrations of TIN standard solution versus the corresponding concentrations.

The same procedures were applied for determination of DOX using standard solutions of TIN of a concentration of 5 μg/mL as first divisor (Figs. 5 and 10a) and using the vector of \( (\frac{d}{d\lambda})_{\text{ONP}}/C_{14}\) \( (\text{TIN})\) corresponding to the derivative of the ratio of the standard spectra each of 2 μg/mL and 5 μg/mL concentrations of OMP and DOX, respectively as second divisor (Fig. 10b and c). The concentration of DOX was obtained by substitution in the regression equations representing the linear relationship between the amplitude at 353 nm of different concentrations of DOX standard solution versus the corresponding concentrations.

The advantage of this method is that it can be applied for resolving ternary mixtures with no limitations, but the disadvantages of this method is the application of several derivatization steps using two divisors for the determination of each component.

### 3.4. Derivative ratio-zero crossing method

Derivative ratio spectra-zero crossing procedure was based on the simultaneous use of the first derivative of ratio spectra and measurements of derivative ratio analytical signals corresponding to the zero crossing points of wavelengths.

This method was adopted for determination of OMP only in the presence of TIN and DOX in the ternary mixtures. In this method, the absorption spectra of the prepared mixtures were recorded and divided by the spectrum of the standard solution of 5 μg/mL of DOX as a divisor. The resulting ratio spectra were smoothed at \( \Delta \lambda = 8 \) nm and their first derivative were plotted with intervals of \( \Delta \lambda = 8 \). The concentrations of OMP in the ternary mixtures were determined by measuring the signals of first derivative spectra of the ratio spectra at 313.9 nm (zero-crossing of TIN) using the corresponding regression equation as depicted in Fig. 11. The main instrumental parameter conditions were optimized to obtain the most distinct curve of first derivative of the ratio spectra.

For selecting a divisor of the appropriate concentration, the standard solutions of 5 μg/mL of DOX were found to be the most suitable. The smoothing factor for the ratio spectra and the influence of the \( \Delta \lambda \) for the first derivative of the ratio spectra were tested and found very

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**Fig. 10.** First derivative of the ratio spectra obtained for DOX concentrations with \( \Delta \lambda = 8 \) nm (a). Second ratio spectra obtained by dividing the first derivative spectra of the first ratio spectra of DOX by the spectrum of \( (\frac{d}{d\lambda})_{2 \mu g/mL \text{ OMP} / 5 \mu g/mL \text{ TIN}} \) (b). The final spectrum showing DOX determination at peak maximum at 353 nm after successive-ratio derivative method with \( \Delta \lambda = 8 \) nm (c).

**Fig. 11.** First derivative of the ratio spectra of OMP (---) and TIN (---) (each 10 μg/mL) after division by the 5 μg/mL DOX.

**Fig. 12.** Zero order absorption spectra of 10 μg/mL OMP (---) and 10 μg/mL DOX (---).
appropriate to use the values of $\Delta \lambda = 8$ and of $\Delta \lambda = 4$, respectively, in the determination of OMP.

The advantage of the first derivative ratio-zero crossing spectrophotometry in this paper has given the good results for the simultaneous determination of three compounds in the ternary mixture, without requiring a separation procedure but its main disadvantage was the critical measurements at the selected wavelengths.

b— Simultaneous determination of OMP and DOX as resolved binary mixture.

OMP and DOX can be determined simultaneously either progressively using dual wavelength method and first derivative-zero crossing method or successively using direct spectrophotometry coupled with ratio subtraction, where the ternary mixture acts as binary mixture of both OMP and DOX after the resolution of TIN using constant center method coupled with spectrum subtraction as mentioned in Section 3.2.

3.5. Dual wavelength method

Dual wavelength was used for simultaneous determination of both OMP and DOX employing four wavelengths. From the overlain spectra of OMP and DOX as shown in Fig. 12, two specific wavelengths were selected for each drug. The absorbances at 287.2 nm ($\lambda_1$) and 263.2 nm ($\lambda_2$) were found to be the same for DOX so the difference in absorbance at these two wavelengths ($\Delta A_{287.2-263.2}$) canceled out the contribution of DOX. These two selected wavelengths were employed to determine the concentration of OMP in the binary mixture of OMP and DOX. Similarly, the absorbance at 292.7 nm ($\lambda_3$) and 309.6 nm was selected for the determination DOX, where the difference in absorbance at these two specific wavelengths ($\Delta A_{309.6-292.7}$) canceled out the contribution of OMP. The concentration of OMP and DOX were determined from the corresponding regression equations obtained by plotting the difference in absorbance between ($\lambda_1$) and ($\lambda_2$) and that between ($\lambda_3$) and ($\lambda_4$) against corresponding concentrations of OMP and DOX, respectively.

The main disadvantage of this method is the restriction in the choice of the selected wavelengths which are restricted to those wavelengths with constant absorbance of the interfering substance. This leads to the necessity of critical measurement of the absorbance of the component of interest, as any minor change in the selected wavelengths will affect the results and subsequently show poor reproducibility and robustness.

3.6. First derivative-zero crossing method

Determination of OMP and DOX in the mixtures progressively in one step adopts the first derivative-zero order method (after resolution of TIN using constant center and spectrum subtraction), where the peak amplitudes of first derivative spectra were recorded at 313 nm and 380 nm (zero-crossing of DOX and OMP, respectively) and substituted in the corresponding regression equations for determination of OMP and DOX, respectively as represented in Fig. 13. The main disadvantage of this method is the critical measurement at zero crossing or zero contribution of the interfering substance.

3.7. Direct spectrophotometry coupled with ratio subtraction

The zero-order spectra of OMP and DOX (Fig. 12) showed that DOX is more extended than OMP showing a significant peak at 367 nm which can be utilized for the direct determination of DOX in the presence of OMP with no need for any other step. The concentration of DOX is determined using the regression equation obtained by plotting the absorbance of zero order spectra of different standard solutions of DOX (2–30 $\mu$g/mL) at 367 nm against the corresponding concentrations. The determination of OMP was successively achieved after calculating and subtracting DOX contribution adopting ratio subtraction method using 5 $\mu$g/mL of standard solution of DOX as a divisor (Figs. 14, 15), where the concentration of OMP was determined using the regression equation obtained by plotting the absorbance of different standard solutions of OMP (1–20 nm) at its $\lambda_{max}$ 301.5 nm against the corresponding concentrations. This was summarized in the following equations

\[ \text{OMP} + \text{DOX} \div \text{DOX}' = \left( \frac{\text{OMP}}{\text{DOX}} \right)' = \left( \frac{\text{OMP}}{\text{DOX}} + \text{constant} \right) \]

\[ \left( \frac{\text{OMP}}{\text{DOX}} + \text{constant} \right) - \text{constant} = \left( \frac{\text{OMP}}{\text{DOX}} \right)_{\text{OMP}} \]

\[ \left( \frac{\text{OMP}}{\text{DOX}} \right) \times \text{DOX}' \]

4. Method validation

The proposed spectrophotometric methods were validated in compliance with the ICH guidelines [46] as shown in Table 1.
The specificity of the proposed methods was assessed by the analysis of laboratory prepared mixtures containing different ratios of the drugs, where satisfactory results were obtained over the calibration range as shown in Table 2. The proposed method was also applied for the determination of the drugs in Triu tablets and the validity of the proposed methods was further assessed by applying the standard addition technique as presented in Table 2.

5. Statistical analysis

Validation of the proposed methods was performed according to ICH guidelines [46,47]. Table 3 showed statistical comparisons of the results obtained by the proposed methods and reported method for OMP and TIN [5] and official method for DOX [2]. The calculated t and F values were less than the theoretical ones indicating that there was no significant difference between them with respect to accuracy and precision.

6. Conclusion

From the previous discussion, it could be concluded that the proposed procedures are simple, do not require sophisticated techniques or instruments. They are also sensitive and selective and could be used for routine analysis of OMP, TIN and DOX in their available dosage form without prior separation. The methods are also suitable and valid for application in laboratories lacking liquid chromatographic instruments. High values of correlation coefficients and small values of intercepts validated the linearity of the calibration graphs and the obedience to Beer’s law. The R.S.D. values, the slopes and the intercepts of the calibration graphs indicated the high reproducibility of the proposed methods. As a final conclusion constant center and ratio subtraction method have advantage over those based on derivative technique either first derivative-zero crossing, derivative ratio-zero crossing and successive derivative ratio due to lack of derivative technique so enhance signal to noise ratio. For the methods based on derivatization, first derivative-zero crossing is superior over the two other methods since it needs no divisor. In addition dual wavelength method and those based on zero crossing have a disadvantage which is a critical measurement at the selected wavelengths so have poor robustness.

References
