Three New Methods for Resolving Ternary Mixture with Overlapping Spectra: Comparative Study

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Received February 10, 2017; accepted March 9, 2017

Metronidazole (MET) has the chemical name of 2-(2-methyl-5-nitro-1H-imidazol-1-yl)ethanol.1) It is officially reported in British Pharmacopoeia (BP),3) United States Pharmacopoeia (USP),2) and European Pharmacopoeia.3) It is widely used in treatment of protozoal diseases such as trichomoniasis and giardiasis,4,5) as it has antibacterial activities against anaerobic bacteria. Diloxanide furoate (DLX) is 4-(N-methyl-2,2-dichloroacetamido)phenyl 2-furoate,1) and it is reported in both BP and USP.2,2) It is a derivative of dichloroacetamide and is used in the treatment of intestinal amoebiasis.6) Mebeverine HCl (MEB) is reported only in BP,11) and is chemically known as 4-(ethyl(4-methoxy-α-methylphenethyl)-aminobutyl) veratrerate hydrochloride. MEB acts as antispasmodic drug that directly acts on the smooth muscle of the gastrointestinal tract.9) Both MET and DLX were formulated in combined dosage form to exert a synergistic effect which was useful for treatment of intestinal and extra-intestinal amoebic infections.7,8) On the other hand, MEB was added to MET and DLX combination to relief colic during amoebae and giardia infections.5)

After extensive literature review, some methods have been reported for determination of the studied ternary mixture such as spectrophotometric methods including derivative ratio,7) successive derivative ratio spectra,9) mean ratio spectra and multivariate calibration methods.9) Also, different chromatographic methods like TLC-densitometry,7,10) and RP-HPLC methods5,8) have been published for quantitation of the studied mixture.

All the published spectrophotometric methods depended on either measuring the three drugs in several successive steps which is time consuming, using complex algorithms or high cost programs and softwares (like multivariate calibration methods which need Matlab8 and partial least squares (PLS) Toolbox). Furthermore, the reported chromatographic methods need high cost instruments, chemicals and several sample pretreatment steps which is money and time consuming. Due to these drawbacks, it was thought worthy to develop and validate novel spectrophotometric methods to achieve accurate quantitation of the three drugs without preliminary separation and with minimum data manipulation steps. The developed methods are used for simultaneous determination of the three studied components with the aid of mathematical techniques to resolve their overlapped spectra without derivatization steps or complex algorithms. The developed area under the curve ("AUC"), modified absorption factor method (MAFM) and modified amplitude center method (MACM) are first introduced for resolving ternary mixture of overlapping spectra. The newly introduced analytical methods are a modification of the recently developed spectrophotometric methods,11–22) while the same author published a PLS chemometric method for analysis of the same ternary mixture since 2012.9) Ternary mixture of MET, DLX and MEB was used as a sample mixture to test the validity and applicability of the newly established methods.

Theories of the Developed Methods

"AUC" Method "AUC" method is a widely used simple spectrophotometric method for resolving binary mixtures depending on area under the curve in zero order,11–14) or ratio absorption spectra.15,16) Here we test the applicability of using this method for the first time to resolve a ternary mixture of overlapping spectra. Theory of "AUC" method for binary mixture was previously illustrated.11)

On applying the method to a ternary mixture, suppose we...
have a mixture of three components: X, Y and Z. Three absorbance ranges are selected where each of the three components showing absorbance at each region. By calculating the absorptivity value \((a)\) for each component at each region, where \((a)\) is equal to:

\[
a = \frac{\text{"AUC" of the component}}{\text{concentration of the component}} \text{ (\(\mu g/mL\))}
\]

By using the calculated \((a)\) values for each of the three studied components, the following three equations can be obtained:

\[
A_1 = a_1C_1 + a_2C_2 + a_3C_3
\]

\[
A_2 = a_1C_1 + a_2C_2 + a_3C_3
\]

\[
A_3 = a_1C_1 + a_2C_2 + a_3C_3
\]

Where \(A_1, A_2\) and \(A_3\) are the areas under the curves of the ternary mixture at the selected ranges, \(a_1, a_2\) and \(a_3\) are the absorptivity values at the selected ranges and \(C\) is the concentration in \((\mu g/mL)\).

By applying Cramer’s rule and matrices in the previous equations, concentrations of \(X, Y\) and \(Z\) in the ternary mixture can be calculated.

**MAFM** This method is a modification of the previously published absorption factor method,\(^{17}\) that was mainly used for resolving binary mixtures. The modification was carried out in order to apply the previous method to ternary or more complicated mixtures. Moreover, this method has advantage over the amplitude factor method,\(^{17–19}\) in that it does not need any derivatization or division steps, hence signal to noise ratio is enhanced.

In order to illustrate this method, suppose that we have the ternary mixture of \(X, Y\) and \(Z\) where the spectrum of \(X\) is extended over \(Y\) and \(Z\) while the components \(X\) and \(Y\) have extended spectra over component \(Z\). In order to resolve these overlapped spectra, three wavelengths are chosen \((\lambda_1, \lambda_2\) and \(\lambda_3\)) on the basis that \(Y\) and \(Z\) do not interfere with \(X\) at \(\lambda_1\) while at \(\lambda_2\) contribution is only due to \(X\) and \(Y\) (no interference from \(Z\)) and at \(\lambda_3\) all spectra are overlapped.

In order to determine concentration of component \(X\), an absorption factor \((F_{x_{\lambda_1}})\) for pure \(X\) is calculated that is representing the ratio:

\[
F_{x_{\lambda_1}} = \text{absorbance of } X \text{ at } \lambda_2 / \text{absorbance of } X \text{ at } \lambda_1
\]

Then the postulated absorbance of \(X\) in the ternary mixture at \(\lambda_2\) (at which \(Y\) interferes) \([P_{x_{\lambda_2}}]\) can be obtained by recording the mixture absorbance at \(\lambda_1\) \([A_{m_{\lambda_1}}]\) and using the previously calculated \(F_{x_{\lambda_1}}\):

\[
P_{x_{\lambda_2}} = F_{x_{\lambda_1}} \cdot A_{m_{\lambda_1}}
\]

By constructing of a calibration curve for \(X\) at \(\lambda_2\) and using the previously calculated absorbance value at \(\lambda_1\) \([P_{x_{\lambda_2}}]\), concentration of \(X\) can be obtained. Then subtraction of the postulated absorbance of \(X\) from the total mixture absorbance at \(\lambda_2\) \([A_{m_{\lambda_2}}]\), getting the absorbance due to \(Y\) at \(\lambda_2\) \([P_{y_{\lambda_2}}]\) as follow:

\[
P_{y_{\lambda_2}} = A_{m_{\lambda_2}} - P_{x_{\lambda_2}}
\]

By the same way, using the estimated absorbance due to \(Y\) at \(\lambda_2\) and applying on a previously computed regression equation for \(Y\) at \(\lambda_2\), the concentration of \(Y\) can be calculated.

In order to get concentration of \(Z\), factors for pure \(X\) and \(Y\) \([F_{x_{\lambda_2}}, F_{y{\lambda_2}}]\) are calculated as follow:

\[
F_{x_{\lambda_2}} = \text{absorbance of } X \text{ at } \lambda_3 / \text{absorbance of } X \text{ at } \lambda_2
\]

\[
F_{y_{\lambda_2}} = \text{absorbance of } Y \text{ at } \lambda_3 / \text{absorbance of } Y \text{ at } \lambda_2
\]

In order to calculate the contribution of \(X\) and \(Y\) at \(\lambda_3\) \([P_{x_{\lambda_3}}, P_{y_{\lambda_3}}]\) respectively, the previously calculated hypothesized absorbance values of \(X\) and \(Y\) at \(\lambda_2\) \([P_{x_{\lambda_2}}, P_{y_{\lambda_2}}]\) and the estimated factors \([F_{x_{\lambda_2}}, F_{y_{\lambda_2}}]\) are used as follow:

\[
P_{x_{\lambda_3}} = F_{x_{\lambda_2}} \cdot P_{x_{\lambda_2}}
\]

\[
P_{y_{\lambda_3}} = F_{y_{\lambda_2}} \cdot P_{y_{\lambda_2}}
\]

Absorbance due to \(Z\) at \(\lambda_3\) can be obtained by subtraction:

\[
P_{z_{\lambda_3}} = A_{m_{\lambda_3}} - [P_{x_{\lambda_3}} + P_{y_{\lambda_3}}]
\]

Where \(A_{m_{\lambda_3}}\) is the total mixture absorbance at \(\lambda_3\), \(P_{x_{\lambda_3}}\) and \(P_{y_{\lambda_3}}\) are the postulated absorbance values of \(X\) and \(Y\) at \(\lambda_3\), respectively. By substitution in the regression equation representing the relation between the absorbance of \(Z\) at \(\lambda_3\) to its corresponding concentration, concentration of component \(Z\) can be obtained.

**MACFM** This newly introduced method is a combination between the previously used amplitude center (ACM),\(^{20}\) and ratio difference (RD),\(^{21,22}\) spectrophotometric methods. It depends on simultaneous quantitation of the three studied components using a single divisor [the same as ACM] through two steps; amplitude calculation via ratio difference and amplitude subtraction depending on using the principle of RD method [advantage over ACM]. Additionally, it does not require that any of the three components should have extended spectrum [advantage over ACM] and all the three components can be determined at a single wavelength [difference from RD method].

For a mixture of three components; \(X, Y\) and \(Z\) with severely overlapped spectra. The spectrum of the ternary mixture is divided by standard spectrum of one of the three components (suppose \(Z\)) where a new spectrum for each component was obtained and represented as follow:

\[
A_m = A_x + A_y + A_z
\]

\[
A_m / Z' = A_x / Z' + A_y / Z' + A_z / Z'
\]

\[
A_m = A_x + A_y + A_z = A_x / Z' + A_y / Z' + \text{constant}
\]

By selecting two wavelengths; \(\lambda_1\) and \(\lambda_2\) at which the ratio difference for \((A_x / Z')\) is zero while \((A_y / Z')\) has significant difference. Hence \((A_x / Z')\) is constant, so the ratio difference between \(\lambda_1\) and \(\lambda_2\) is also equal to zero. Then the regression equation representing a relation between the ratio difference values for different concentrations of pure \(X\) versus the corresponding ratio amplitude at any of the selected wavelengths (suppose \(\lambda_2\)) is computed and can be expressed as follow:

\[
(A_x / Z')_{\lambda_1} - (A_x / Z')_{\lambda_2} = (A_y / Z')_{\lambda_2} \cdot \text{slope} \pm \text{intercept}
\]

Where \(((A_x / Z')_{\lambda_1} - (A_x / Z')_{\lambda_2})\) is the ratio difference at the selected wavelengths and \((A_y / Z')_{\lambda_2}\) is the postulated ratio amplitude of \(X\) at \(\lambda_2\). By recording the ratio difference of the mixture at \(\lambda_1\) and \(\lambda_2\) and then substitution in Eq. 5, the hypothesized ratio amplitude of \(X\) at \(\lambda_2\) can be estimated and used for quantitation of \(X\) by applying in a regression equation relating the ratio amplitude of \(X\) at \(\lambda_2\) versus its corresponding concentration.

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By the same way, the assumed ratio amplitude of $Y$ at $\lambda_2$
can be obtained by selecting other two wavelengths (provided
that $\lambda_2$ is one of them), $\lambda_2$ and $\lambda_3$ at which $(A/Z)$ has zero
difference, difference is zero for the constant $(A/Z')$ while
$(A/Z')$ has significant difference. Then the equation relating
the ratio difference of various concentrations of pure $Y$ to the
corresponding ratio amplitude at $\lambda_2$ is computed and repre-
sented as follow:

$$(A/Z)_{\lambda_2} - (A/Z')_{\lambda_2} = (A/Z)_{\lambda_2} \text{ slope} \pm \text{intercept} \quad (6)$$

Where $[(A/Z)_{\lambda_2} - (A/Z')_{\lambda_2}]$ is the ratio difference at the
selected wavelengths and $[(A/Z)_{\lambda_2}]$ is the assumed ratio am-
plitude of $Y$ at $\lambda_2$. By calculating the ratio difference of the
mixture between $\lambda_2$ and $\lambda_3$ and applying in Eq. 6, to get the
postulated ratio amplitude of $Y$ at $(A/Z)_{\lambda_2}$. By substitution in
the regression equation representing the ratio amplitude of $Y$
at $\lambda_2$ versus its corresponding concentration, concentration of
$Y$ can be calculated.

The final step is to calculate the postulated ratio amplitude of
$Z$ at $\lambda_2$ by subtraction of the estimated ratio amplitudes of $X$
and $Y [(A/Z')_{\lambda_2}, (A/Z')_{\lambda_2}]$ from the total ratio amplitude of the
mixture at $\lambda_2 ([(A/Z)_{\lambda_2}])$. 

$$(A/Z)_{\lambda_2} - [(A/Z)_{\lambda_2} + (A/Z')_{\lambda_2}] = (A/Z)_{\lambda_2} \quad (7)$$

Similarly, regression equation for pure $Z$ at $\lambda_2$ representing
the relation between the ratio amplitude at $\lambda_2$ and the cor-
responding concentration is computed and used to determine
concentration of $Z$ in the ternary mixture.

Experimental

Equipment The used equipment was a double beam UV-
Visible spectrophotometer model UV-1601 PC (SHIMADZU,
Japan) that was connected to IBM compatible computer. The
software was UVPC personal spectroscopy version 3.7 and a
quartz cell with 1 cm path length was used.

Samples

Pure Samples

Powdered samples of MET, DLX and MEB were obtained
as a gift from EVA PHARMA for Pharmaceuticals and Medi-
cal Appliances S.A.E, Egypt with purity of 100.5, 98.9 and
100.4%, respectively according to manufacturer certificates of
analysis.

Pharmaceutical Dosage Form Sample

Dimetrol® film coated tablets with a batch No. of 909537
were manufactured by EVA PHARMA for Pharmaceuticals
and Medical Appliances S.A.E, Egypt. They were labeled to
contain 375 mg MET, 250 mg DLX and 50 mg MEB per tablet.

Solvents Methanol was the solvent of choice throughout
this work and it was of HPLC grade (Sigma-Aldrich® Chemie
GmbH, Germany).

Solutions Stock solutions of MET, DLX and MEB in the
concentration of 1 mg/mL were prepared by weighing accur-
ately 0.1 g of each in three separate 100 mL volumetric flasks
using methanol as a solvent.

Working solutions of MET, DLX and MEB (0.1 mg/mL) were
obtained by diluting 10 mL of their respective stock solutions
(1 mg/mL) in three 100 mL volumetric flasks and using metha-
ol for dilution.

Solutions of pharmaceutical dosage form: Ten Dimetrol®
coated tablets were weighed, grinded and then the obtained
powder was mixed well. An accurate weight equal to 150 mg
MET, 100 mg DLX and 20 mg MEB was transferred to 100 mL
volumetric flask, 75 mL methanol was used for dissolution and
then the sample solution was ultra sonicated for 30 min. After
that the solution was left to cool, the volume was adjusted
with methanol to obtain a stock solution containing 1.5 mg/mL
MET, 1 mg/mL DLX and 0.2 mg/mL MEB, and the solution
was then filtered. Suitable dilution of the prepared sample sol-
solution was made to get sample working solution of 0.15 mg/mL
MET, 0.1 mg/mL DLX and 0.02 mg/mL MEB. Then concentra-
tions within the linearity ranges of the developed methods
were prepared by further dilutions of the prepared sample
working solution.

Synthetic Mixtures Solutions

Several mixtures containing different concentrations of
MET, DLX and MEB were laboratory prepared from their
stock solutions (1 mg/mL) in a set of 10 mL volumetric flasks
and using methanol as a solvent.

Procedure

Linearity and Ranges

Different concentrations of pure MET, DLX and MEB in the
ranges of 1–38, 1–25 and 1–25 µg/mL, respectively were
prepared in methanol using their respective working solutions
(0.1 mg/mL). UV scanning of the prepared samples was car-
rried out in the range of 200–400 nm and then the stored spec-
tra were used for construction of calibration curves.

Method (I): “AUC” Method

The stored spectra of the prepared MET, DLX and MEB
samples were used for calculating “AUC” in the ranges of
225–235, 240–250 and 280–290 nm. The absorbptivity values
‘a’ for each component at the selected regions were calculated
and the concentrations of each component were obtained by
applying Cramer’s rule and matrices (1–3).

Method (II): MAFM

Two calibration curves were constructed at 300 nm, one for
pure MET and the other for MEB in the concentration ranges of
1–38 and 2–25 µg/mL, respectively relating the absorbance
values at the selected wavelength to their corresponding
concentrations and the regression equations were calculated.
While for DLX the equation was computed using the concentra-
tions in the range of 1–25 µg/mL and the absorbance values
at 246 nm. Additionally, absorbance factors for pure MET and
MEB were calculated and represented as follow:

$$F_1(\text{MET}) = \frac{ABS_{\text{MET}}(300 \text{nm})}{ABS_{\text{MET}}(319 \text{nm})}$$

$$F_2(\text{MET}) = \frac{ABS_{\text{MET}}(246 \text{nm})}{ABS_{\text{MET}}(300 \text{nm})}$$

$$F_2(\text{MEB}) = \frac{ABS_{\text{MEB}}(246 \text{nm})}{ABS_{\text{MEB}}(300 \text{nm})}$$

Where $ABS_{\text{MET}}(300 \text{nm})$, $ABS_{\text{MET}}(319 \text{nm})$ and $ABS_{\text{MET}}(246 \text{nm})$
are the absorbance values of pure MET at 300, 319 and 246 nm,
respectively. $ABS_{\text{MEB}}(246 \text{nm})$ and $ABS_{\text{MEB}}(300 \text{nm})$ are the absorbance
of pure MEB at 246 and 300 nm, respectively.

Method (III): MACM

The stored spectra pure MET, DLX and were divided by
standard spectrum of 12 µg/mL MEB. The resulted ratio
spectra were then used for construction of their calibration
curves using their corresponding ratio amplitudes at 235 nm
from which the regression equations were computed. More-
over, a calibration plot for pure MET representing the relation
between the ratio difference at (235–253.8 nm) to the cor-
responding ratio amplitude at 235 nm was constructed and
the equation was then computed. Similarly, a calibration plot
for DLX was constructed but using the difference between (235–242 nm) and the ratio amplitude at 235 nm.

Analysis of Laboratory Prepared Mixtures

The prepared mixtures were UV scanned in the range of 200–400 nm and their spectra were used for measuring concentrations of the studied drugs by applying in the developed methods.

Method (I)

Steps under linearity were followed and then by applying in the following equations, concentrations of MET, DLX and MEB in the ternary mixture could be obtained.

\[
\begin{align*}
A_{225-235} &= 0.1918C_{MET} + 0.3672C_{DLX} + 0.3475C_{MEB} \\
A_{240-250} &= 0.1514C_{MET} + 0.5708C_{DLX} + 0.1352C_{MEB} \\
A_{280-290} &= 0.2730C_{MET} + 0.0550C_{DLX} + 0.1421C_{MEB}
\end{align*}
\]

Method (II)

The absorbance value at 319 nm was recorded for each mixture and then multiplied by the previously calculated factor \(F_{1(MET)}\) to calculate the corresponding absorbance of MET at 300 nm. Subtracting the resulted postulated absorbance value of MET from the total absorbance at 300 nm getting the absorbance due to MEB. While the factors \(F_{2(MET)}\) and \(F_{1(MEB)}\) were used to get the corresponding absorbance values of MET and MEB at 246 nm using their previously calculated absorbance values at 300 nm. By subtraction form the total absorbance of the mixture at 246 nm, contribution of DLX at 246 nm could be calculated. By using the calculated absorbance values of MET, MEB (at 300 nm) and DLX (at 246 nm) and the previously computed regression equations at 300 nm (for MET and MEB) and 246 nm (for DLX), concentrations of MET, MEB and DLX in the ternary mixture could be obtained.

Method (III)

The stored spectra of the laboratory prepared mixtures were divided by standard spectrum of 12 µg/mL MEB. The ratio difference between 235–253.8 and 235–242 nm were recorded and then used for calculating the consequent ratio amplitudes of MET and DLX \(P_{MET}\) and \(P_{DLX}\) using the following equations:

\[
\begin{align*}
\Delta A_{235-253.8} &= 0.6724 P_{MET(235nm)} + 0.0075 \\
\Delta A_{235-242} &= 0.4647 P_{DLX(235nm)} + 0.0033
\end{align*}
\]

By recording the total ratio amplitude at 235 nm, the postulated ratio amplitude of MEB at 235 nm could be calculated by subtraction. Then the previously computed regression equations at 235 nm to were used to get the concentrations of MET, DLX and MEB in their mixture.

Analysis of Pharmaceutical Dosage Form

The recorded spectra of the prepared samples were used instead of the spectra of the synthetic mixtures and then instructions detailed under analysis of laboratory prepared mixtures were followed. Concentrations of MET, DLX and MEB were estimated using the corresponding regression equations. Additionally, standard addition technique was performed in order to evaluate accuracy of the developed methods and it was carried out on three different levels.

Results and Discussion

In this work, our aim was to resolve the overlapped spectra of MET, DLX and MEB (Fig. 1), using accurate and simple methods without using complex algorithms, derivatization, or preliminary separation steps. Nowadays, spectrophotometric methods become the analytical method of choice in most pharmaceutical factories especially in rush working for analysis of several batches in short analysis time and low cost.

In all the developed methods, univariate approach was utilized in order to find specific wavelengths for accurate measurements of the three studied components without interference from each other. The introduced methods consisted of consecutive steps depending on either zero order spectra or ratio spectra with the aid of some mathematical techniques which was advantageous over the previously published spectrophotometric methods that depended mainly on derivatization steps, \(^7,8\) or on using complex softwares. \(^9\) The developed methods were “AUC,” MAFM and MACM.

In the initial step and during methods optimization, different solvents were tried like methanol, ethanol, water, 0.1 N HCl and 0.1 N NaOH. Regarding selectivity methanol was chosen.

Methods (I)

The proposed method is used for resolving ternary mixture of severely overlapped spectra and it represents a simple way for simultaneous estimation of MET, DLX and MEB ternary mixture by using simple mathematical calculations applying Cramer’s rule. During optimization of the method different wavelength ranges were tested in order to
obtain the highest possible selectivity where the wavelength ranges of 225–235, 240–250 and 280–290 nm were the best ranges regarding method selectivity (Fig. 2).

On applying this method, concentrations of the studied drugs in the synthetic mixtures could be obtained by using their calculated absorptivity values and plying Cramer’s rule and matrices given in Table 1.

Method (II) This method can be successfully used for simultaneous determination of the ternary mixtures or more complex ones. Ternary mixture of MET, DLX and MEB was used as an example to test the validity of the newly introduced method.

The method depended on that MET has extended spectrum over MEB and DLX (in the region above 320 nm) while MET and MEB spectra extended over DLX (in the region above 295 nm) (Fig. 2). On applying the method, three wavelengths were selected, 319 nm (λ1) (at which no interference from MEB and DLX), 300 nm (λ2) (at which contribution is due to MET and MEB) and 246 nm (λ3) (at which the three components interfered with each other). Besides, three absorbance factors for pure MET and MEB were calculated $F_{1\text{MET}}=0.9450$, $F_{2\text{MET}}=0.3186$ and $F_{\text{MEB}}=1.2833$.

Since the absorbance of the ternary mixture at 319 nm was due to MET only, the contribution due to MET and MEB separately in the mixture at 300 nm could be calculated as previously mentioned under “Theory of the Method.”

Concentrations of MET and MEB were then calculated from the corresponding regression equations previously computed at 300 nm. The equations parameters are given in Table 1.

On the other hand, absorbance of the mixture at 246 nm was due to MET, MEB and DLX. By using the previously calculated factors ($F_{2\text{MET}}=0.3186$, $F_{\text{MEB}}=1.2833$ for pure MET and MEB, respectively) and the absorbance of the mixture at 246 nm, postulated absorbance of DLX at 246 nm could be obtained, as previously mentioned under “Theory of the Method.”

The calculated absorbance of DLX at 246 nm was used to

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Table 1. Assay and Validation Parameters of the Proposed Spectrophotometric Method

| Parameters                              | Method I | Method II | Method III |
|----------------------------------------|----------|-----------|------------|
| Wavelength (nm)                        | MET      | DLX       | MEB        |
| Calibration range µg/mL                | 1–38     | 1–25      | 1–25       |
| Calibration range µg/mL                | 300 nm   | 246 nm    | 300 nm     |
| Calibration range µg/mL                | 235 nm   |            |            |
| Wavelength range (1): 225–235 nm       |          |           |            |
| Wavelength range (2): 240–250 nm       |          |           |            |
| Wavelength range (3): 280–290 nm       |          |           |            |
| Slope                                  | $A_{225-235} = 0.1918C_{\text{MET}} + 0.3672C_{\text{DLX}} + 0.3475C_{\text{MEB}}$ | 0.0461     | 0.0586     | 0.0114     |
| Intercept                              | $A_{240-250} = 0.1514C_{\text{MET}} + 0.5708C_{\text{DLX}} + 0.1421C_{\text{MEB}}$ | -0.0008    | -0.0036    | -0.0055    |
| Slope                                  | $A_{280-290} = 0.2730C_{\text{MET}} + 0.0550C_{\text{DLX}} + 0.1421C_{\text{MEB}}$ | 0.9999     | 0.9999     | 0.9999     |
| Correlation coefficient (r)            |          |           |            |
| Accuracy (% Found ± S.D.)              | 99.56±1.79 | 101.00±1.97 | 98.72±2.57 |
| Precision Repeatability (%RSD)         | 1.58     | 1.04      | 1.39       |
| Intermediate precision (%RSD)          | 2.01     | 0.58      | 1.43       |

a) Average of three experiments. b) Mean of 9 concentrations of each drug. c) Standard deviation of 3 concentrations of each drug (5, 15 and 20µg/mL) on the same day. d) Standard deviation of 3 concentrations of each drug (5, 15 and 20µg/mL) on three successive days.
measure its concentration in the ternary mixture using the regression equation, presented in Table 1, previously computed for pure DLX at 246 nm.

Method (III): This method is used to resolve the ternary mixture and simultaneously determine the studied components progressively using a single divisor and a single detection wavelength.

To resolve the overlapped spectra of MET, DLX and MEB using the newly introduced MACM, the spectrum of the mixture was divided by standard spectrum of 12 µg/mL MEB (Fig. 3). Two wavelength pairs were then chosen on the basis that at the first wavelength pair, the ratio difference was zero for DLX and MEB separately while the difference was significant for MET. At the second pair MET and MEB separately had zero difference while DLX had significant ratio difference. Moreover, the selected pairs should have a common wavelength at which the postulated ratio amplitude corresponding to each of MET/MEB, DLX/MEB and MEB/MEB was obtained and used for its quantitation by applying in calibration curves constructed at this wavelength using different concentrations of pure MET, DLX and MEB. Different wavelength pairs were tested and the wavelength pairs of 235–253.8 and 235–242 nm were selected, respectively.

On applying this method, the postulated ratio amplitude corresponding to (MET/MEB), (DLX/MEB) at 235 nm could be obtained, as previously illustrated under “Theory of the Method.” The assumed ratio amplitude equivalent to (MEB/MEB) was then calculated by subtraction.

Using the calculated ratio amplitudes of MET, DLX and MEB and the regression equations presented in Table 1, concentrations of MET, DLX and MEB in their laboratory-prepared mixtures could be calculated.

After testing the developed methods using the synthetic mixtures, they were further applied to available dosage form containing MET, DLX and MEB where the results obtained were in agreement with the accepted limits (Table 2). In addition, standard addition technique has been carried out on three levels and good percentage recoveries were obtained (Table 2).

When the results obtained by the developed spectrophotometric methods have been compared statistically to those obtained by the reported HPLC method and our previously developed PLS method using One Way ANOVA test [by the aid of Microsoft Excel], the calculated F values were less than the tabulated ones (at $p=0.95$) indicating no significant difference between the newly developed methods and the reported ones (Table 3). The newly introduced methods are novel ones and can be used as alternative to chromatographic methods or multivariate calibration methods.

Methods Validation

Validation of the proposed methods was carried out using USP guidelines, as follow:

Linearity was tested by using different samples of pure MET, DLX and MEB. Beer’s Lambert’s law was obeyed in different ranges presented in Table 1. All the obtained correlation coefficients were near to one and low intercept values were obtained as shown from the data in Table 1.

Table 2. Analysis of Dimetrol® Tablets by the Proposed Methods an Application of Standard Addition Technique

| Parameters | Method I | Method II | Method III |
|------------|----------|-----------|------------|
| Dimetrol® tablets<sup>a)</sup> labeled to contain 375 mg MET, 250 mg DLX and 50 mg MEB/tablet | 102.13±1.680 | 96.91±0.318 | 104.75±0.371 |
| Standard addition<sup>b)</sup> (mean±S.D.) | 99.99±0.87 | 99.99±2.61 | 99.27±1.55 |
| MET (µg/mL) | 99.44±0.498 | 96.50±0.283 | 103.33±1.099 |
| DLX (µg/mL) | 98.75±0.155 | 96.50±0.283 | 103.33±1.099 |
| MEB (µg/mL) | 102.13±1.680 | 96.91±0.318 | 104.75±0.371 |

<sup>a</sup>) Average of five experiments. <sup>b</sup>) Average of three experiments: (pure added equivalent to 4, 5 and 7 µg/mL MET, 3, 4, 6 µg/mL DLX and 10, 12, 13 µg/mL MEB).
Accuracy of the methods was checked by analysis of different samples of pure components following the instructions of each method. The obtained mean percentage recoveries (Table 1) were found to be within the acceptable limits. Additionally, precision was evaluated by testing method repeatability and intermediate precision by triplicate analysis of three different concentrations (10, 15 and 20 µg/mL) of each component three times intraday and seven times interday, respectively. Low values of S.D. were obtained and given in Table 1 confirming that the developed methods are precise. To test selectivity of the newly developed methods, they were applied to analyze different synthetic mixtures of different ratios of MET, DLX and MEB including the ratio in dimetrol® tablets, good percentage recoveries and low S.D. values were observed, Table 4 confirming the ability of the methods to resolve the overlapped spectra each of MET, DLX and MEB.

Table 3. Results of One Way ANOVA Test for Analysis of Pure Metronidazole, Diloxanide and Mebevirine

| Source of variation | SS     | df | MS  | F     | p-Value | F Crit |
|---------------------|--------|----|-----|-------|---------|--------|
| MET:                |        |    |     |       |         |        |
| Between Groups      | 3.99   | 4  | 1   | 0.22  | 0.93    | 2.7    |
| Within Groups       | 132.83 | 29 | 4.58|       |         |        |
| Total               | 136.82 | 33 |     |       |         |        |
| DLX:                |        |    |     |       |         |        |
| Between Groups      | 18.47  | 4  | 4.62| 2.01  | 0.12    | 2.7    |
| Within Groups       | 66.5   | 29 | 2.29|       |         |        |
| Total               | 84.96  | 33 |     |       |         |        |
| MEB:                |        |    |     |       |         |        |
| Between Groups      | 20.09  | 4  | 5.02| 1.19  | 0.34    | 2.7    |
| Within Groups       | 122.63 | 29 | 4.23|       |         |        |
| Total               | 142.72 | 33 |     |       |         |        |

*Results were statistically compared with those obtained by reported HPLC method on ODS column with a mobile phase consisting of water–methanol–triethylamine (25:75:0.5, by volume, orthophosphoric acid to pH=4), FR=0.7 mL/min with UV at 230 nm and PLS method."

Table 4. Results of Analysis of Laboratory Prepared Mixtures by the Proposed Spectrophotometric Methods

| Concentrations MET : DLX : MEB | Method I | Method II | Method III |
|--------------------------------|----------|-----------|------------|
|                                | MET      | DLX       | MEB        | MET      | DLX       | MEB        | MET      | DLX       | MEB        |
| 22.5 : 15 : 3                  | 102.09   | 101.53    | 103.33     | 103.64   | 103.47    | 99.67      | 98.31    | 98.87     | 103        |
| 4 : 03 : 2                     | 103.35   | 100.87    | 100.5      | 102.9    | 102.47    | 97.6       | 102.55   | 97.2      | 97.3       |
| 9 : 08 : 04                    | 101.33   | 100.13    | 98         | 100.89   | 101.13    | 96.75      | 100.56   | 96.88     | 98.5       |
| 1 : 01 : 1                     | 97.43    | 101.57    | 100.43     | 102.6    | 101.33    | 103.93     | 102.93   | 98.73     | 102.4      |
| 1 : 02 : 01                    | 100.5    | 99.25     | 97.25      | 103.25   | 99.94     | 97.88      | 100      | 100.25    | 97.63      |
| Mean ± S.D.                    | 100.94 ± 2.23 | 100.67 ± 0.99 | 99.90 ± 2.4 | 102.66 ± 1.06 | 101.67 ± 1.35 | 99.17 ± 2.87 | 100.87 ± 1.9 | 98.39 ± 1.37 | 99.77 ± 2.72 |

a) Average of three determinations. b) The ratio in dimetrol® tablets.

Conclusion

In this work three new spectrophotometric methods have been developed and validated for the first time and a ternary mixture of MET, DLX and MEB has been used as a test mixture. The methods had advantages of using either zero order spectra or ratio spectra and they did not need any derivatization steps or complex algorithms, they need only some simple mathematical calculations. They have the advantage of simultaneous determination of the studied drug. They are more money saving than the reported chromatographic methods. Hence, they can be easily applied in quality control laboratories especially those lacking the facilities to chromatographic methods of analysis.

Conflict of Interest

The authors declare no conflict of interest.

References

1) “The British Pharmacopoeia,” Her Majesty’s, The Stationary Office, London, 2007.
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| Total               | 142.72 | 33 |     |       |         |        |

% Recovery

Mean ± S.D.

Accuracy of the methods was checked by analysis of different samples of pure components following the instructions of each method. The obtained mean percentage recoveries (Table 1) were found to be within the acceptable limits. Additionally, precision was evaluated by testing method repeatability and intermediate precision by triplicate analysis of three different concentrations (10, 15 and 20 µg/mL) of each component three times intraday and seven times interday, respectively. Low values of S.D. were obtained and given in Table 1 confirming that the developed methods are precise. To test selectivity of the newly developed methods, they were applied to analyze different synthetic mixtures of different ratios of MET, DLX and MEB including the ratio in dimetrol® tablets, good percentage recoveries and low S.D. values were observed, Table 4 confirming the ability of the methods to resolve the overlapped spectra each of MET, DLX and MEB.

Moreover, the acceptable results obtained on applying the methods to available dosage form, Table 2 proving that tablets additives did not interfere. Results of applying the standard addition technique, Table 2 accessed the accuracy of the methods.

Conclusion

In this work three new spectrophotometric methods have been developed and validated for the first time and a ternary mixture of MET, DLX and MEB has been used as a test mixture. The methods had advantages of using either zero order spectra or ratio spectra and they did not need any derivatization steps or complex algorithms, they need only some simple mathematical calculations. They have the advantage of simultaneous determination of the studied drug. They are more money saving than the reported chromatographic methods. Hence, they can be easily applied in quality control laboratories especially those lacking the facilities to chromatographic methods of analysis.

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