**ABSTRACT**

Three sensitive, times saving, precise and accurate spectrophotometric techniques have been established and confirmed for the simultaneous determination of ternary admixture of rosuvastatin calcium (ROSCa), timolol maleate (TIM) and diclofenac sodium (DICNa). Method A, first derivative of ratio spectra spectrophotometric (1st DD) which is the most simple simplest one. Using the spectrum of an aliquot of 5 μg/mL of DICNa as a divisor, ROSCa could be determined by measuring 1DD amplitudes at 239.5 and 266.5 nm for ROSCa, while TIM could be similarly determined at 307.7 and 331 nm. DICNa could be determined at 269.7 and 292 nm when 5 μg/mL aliquot of ROSCa was used as a divisor without interference. This method showed limit of detection of 0.556, 0.567 & 0.3 μg/mL and correlation coefficients of 0.9997, 0.9998 and 0.9995 for ROSCa,
increase the consumption and decay of LDL, played through affecting the number of hepatic for cholesterol lowering. Its lipid modifying role is selectivity in the liver which is the organ devoted mevalonate, which is a cholesterol forefather. responsible enzyme that converts HMG inhibitor for HMG ingredient. It is a competitive and selective contain 5, 10, 20 and 40 mg of the active It is available in variety of tablet forms that be stored in airtight container to be protected practically insoluble in anhydrous ethanol. It must slightly soluble in water and methanol and powder, freely soluble in methylene chloride, or almost white color and it is moisture absorbing 1 structure of C -dihydroxyhept sulfonyl) amino] pyrimidine (ROSCa) is fluorophenyl) (MCRS). The arithmetical description of this method was explained. The method was performed for the estimation of these drugs together in one run without preseparation in authentic laboratory prepared mixtures, bulk and spiked human plasma. The wave lengths 277.9, 313.1 and 333.12 nm were used for the quantitative estimation of each of the studied drugs with good correlation coefficients (0.9998, 0.997& 0.9999 and LOD of 0.297, 0.150 & 0.24 µg/mL for the studied drugs in the previously mentioned order. Method C, successive-ratio derivative spectra method (SRDS). The mathematical description of the method was explained. By using this method, ROSCa, TIM and DICNa could be determined at 264, 339 and 300 nm, respectively. Correlation coefficients were 0.9998, 0.9999 & 0.9995 and LOD values of 0.374, 0.258 & 0.769 µg/mL in the same order. The three methods were linear in the range 5-25 µg/mL. The obtained results has been statistically compared with those obtained by the published one, showing no significant difference regarding accuracy and precision at p = 0.05. The developed methods do not need sophisticated techniques or instruments, besides being sensitive, selective and eco-friendly.

Keywords: Rosuvastatin calcium; first derivative; ratio spectra; mean centering; successive derivative ratio spectra; ternary mixture.

ABBREVIATIONS

ROSCa : Rosuvastatin calcium; TIM : Timolol maleate; DICNa : Diclofenac sodium; 1stDD : First derivative of ratio spectra; MCRS : Mean centering of ratio spectra; SRDS : Successive-ratio derivative spectra.

1. INTRODUCTION

The chemical form of rosuvastatin calcium (ROSCa) is calcium bis [(3R,5S,6E)-7-{4-(4-fluorophenyl)-6-(1-methylethythyl)-2-{methyl methyl sulfonyl) amino] pyrimidine-5-yI}-3,5-dihydroxyhept-6-enoate] with a chemical structure of C$_{46}$H$_{34}$CaF$_2$N$_{12}$O$_{13}$S$_2$ as shown in Fig. 1-a.

Its molecular weight is 1001 [1,2]. It has a white or almost white color and it is moisture absorbing powder, freely soluble in methylene chloride, slightly soluble in water and methanol and practically insoluble in anhydrous ethanol. It must be stored in airtight container to be protected from light at 2-8°C [1,2].

It is available in variety of tablet forms that contain 5, 10, 20 and 40 mg of the active ingredient. It is a competitive and selective inhibitor for HMG-CoA reductase which is the responsible enzyme that converts HMG-CoA to mevalonate, which is a cholesterol forefather. ROS has proved to have high consumption and selectivity in the liver which is the organ devoted for cholesterol lowering. Its lipid modifying role is played through affecting the number of hepatic LDL receptors on the cell-surface in order to increase the consumption and decay of LDL, thus reducing the hepatic production of VLDL, which, in turn, reduces the quantity of both LDL and VLDL.

Published literature showed that various methods have been developed for the quantitative estimation of ROSCa either individually or simultaneously including spectrophotometric [3-10], capillary electrophoresis [11], liquid chromatography (HPLC, UPLC) with UV detection or mass spectrometry [12-20], complexometric titration [21], thin layer chromatography (TLC) [22-25], electrochemical [26-28], chemometric [29] and spectrofluorimetric methods [30] have been reported for the determination of ROS-Ca in pure, dosage forms or in physiological fluids.

The chemical form of timolol maleate (TIM) is (S)-1-(tert-butylamino)-3-[(4-morpholin-4-yl-1,2,5-thiadiazol-3-yl)oxy]propan-2-ol maleate(1:1) as shown in Fig. 1-b. It is white, odourless powder, has melting point of 201.5-202.5°C. Soluble 1 in 15 of water, 1 in 21 of alcohol, and 1 in 40 of chloroform; soluble in methanol; practically insoluble in ether. A 2% solution in water has a pH of 3.8 to 4.3. Ophthalmic preparations have a pH of 6.5 to 7.5. Its molecular weight equals 432.5. It is the maleate salt form of timolol, a propanolamine derivative and a non-selective beta-adrenergic antagonist with antihypertensive property. Timolol competitively binds to beta-1-adrenergic receptors in the heart and vascular smooth muscle and beta-2-receptors in the bronchial and vascular smooth muscle, resulting in a decrease in beta-adrenergic stimulation. Its main use is in the treatment of the elevated intraocular pressure in patients who suffer
open-angle glaucoma or ocular hypertension [31]. TIM as a nonspecific adrenergic blocker was the first one that has been used as an antiglaucoma agent, proving more efficiency than the other newer blockers. TIM is used as antihypertensive agent as well [32]. It was quantitatively determined by HPLC [31-34], spectrophotometric [35,36] in bulk, pharmaceuticals and physiological fluids, UPLC methods [37] and an electrochemical one [38].

Diclofenac sodium (DICNa) is a synthetic, nonsteroidal anti-inflammatory and analgesic compound. It is odorless, white to off-white crystalline, slightly hygroscopic powder. The molecular formula of DICNa is \( C_{14}H_{10}Cl_NaO_2 \) as Fig. 1-c shows [32]. Its molecular weight equals 318.13 [39]. It is used as analgesic, anti-inflammatory and antipyretic agent. It is also used for controlling pain and ocular inflammation. It inhibits the formation of prostaglandins through the reduction of the formation of cyclooxygenase enzyme. Many articles have been reported for the estimation of DICNa such as spectrophotometric [40-43], chromatographic [44-46] and electrochemical methods [47-49].

An HPLC method [32] was proposed for the determination of ROSCa, TIM and DICNa in pharmaceuticals and physiological fluids in one run. Thus, a single method has been reported for the estimation the mentioned drugs in combination. The combination of rosuvastin calcium, timolol maleate and diclofenac sodium is not official in any of the commonly known pharmacopeias. While this combination is fruitful for the treatment of patients with multiple diseases such as hyperlipidemia, lowering the blood cholesterol, anti-hypertensive, anti-inflammatory, analgesic and antipyretic. The selected drugs in this study are usually co-prescribed in clinical practice. So the aim of this work is to develop alternative accurate, applicable and smart first derivative of ratio spectra, mean centering of ratio spectra and successive-ratio derivative spectra methods for the simultaneous determination of ROSCa, TIM and DICNa in ternary mixture and in spiked human plasma.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Used chemical reagents and solvents used throughout the work were of analytical grade, such as methanol and double distilled water.

2.1.1 Pure standards

TIM and DICNa were kindly supplied by EPCI part of HIKMA group, Beni-Suef, Egypt. ROSCa was provided as a gift from SPIMACO Pharmaceutical Company, Alexandria, Egypt with claimed purity of 100.5%. The reported content of ROSCa in BP Pharmacopeia is in the range 97.0 - 102.0%.

2.1.2 Apparatus

All absorbance measurements have been performed on UV-1800 double beam UV-Visible spectrophotometer (Shimadzu-Japan) with highest resolution in which is the spectral bandwidth for the spectral range 190-1100 nm and is matched with 1 cm quartz cells. Data analysis is performed by software (UV-Probe 2.5.2). An m-file running under MATLAB R2015a.
program was used for performing the data analysis of the mean centering of ratio spectra method.

2.2 Procedure

2.2.1 Preparation of standard stock solutions

1000µg/mL stock standard solutions of ROSCa, TIM and DICNa were prepared by dissolving accurately weighed 100 mg of each of them in three 100 mL volumetric flasks, add about 70 mL of methanol. Sonicate for 10 min till complete dissolution then completes to the mark with the same solvent and mix well.

The working standard solutions (100µg/mL) were prepared by appropriate dilution from stock ones. All standards were freshly prepared and stored in the refrigerator.

2.2.2 Construction of calibration curves

a) For pure standards of ROSCa, TIM and DICNa

Aliquots (1.25, 2.5, 3.75, 5 and 6.25 mL) of each of ROSCa, TIM and DICNa were transferred separately from their standard working solutions (100µg/mL) to 25 mL volumetric flasks to obtain a series of concentrations (5,10,15,20 and 25µg/mL) for each. Spectra of ROSCa, TIM and DICNa (zero order ones) are scanned in the range of 200-400 nm (Fig. 2) and stored. \( \lambda_{\text{max}} \) is determined for each individual drug solution. Calibration curves are plotted relating the absorbance to the corresponding concentrations of each drug.

b) For laboratory prepared mixtures

Different laboratory mixtures of the three drugs were prepared in the range 5-25 µg/mL by transferring suitable aliquots from each standard working solution to a series of 25 mL volumetric flasks with different ratios as illustrated in the first column of Table 2. Each flask was completed to the mark with methanol. After mixing well, the absorbance was recorded for each mixture against methanol as blank.

c) For spiked human plasma samples

In general, the collection of blood samples should be ethical approved and must be under the umbrella of Human Research Committee in case of human volunteers administrating the drugs, but in our case blood samples were collected from healthy human volunteers who did not administrate any drugs, then plasma samples were spiked with the studied drugs. So different volumes of each of ROSCa, TIM and DICNa were transferred from their corresponding 100µg/mL working standard solutions into three series of 25-mL measuring flasks to obtain concentrations in the range of 5-25 µg /mL of each drug. 1mL of plasma was added to each flask then was completed with methanol to the mark. Methanol in this case acts both as a solvent and a protein precipitating agent. Another series of flasks was prepared containing the three drugs with each other's in different proportions, and then 1mL of plasma was added to each flask and completed to the mark with methanol. The blank was similarly prepared but without adding the drugs.

Samples were vortex mixed well, then poured into centrifuge tubes and were centrifuged for 5 minutes at 3500 rpm, where the precipitated plasma protein separates. The clear solutions were carefully decanted to clean tubes and the spectra of the individual drugs and their mixtures were scanned simultaneously against the blank and the nominal concentration for each drug was estimated by using the corresponding regression equation.

2.3 Methods

From the zero order absorption spectra, it was noticed that the three drugs could not be determined directly due to their overlapped spectra as shown in Fig. 2. In an attempt to resolve this overlapping, 1st, 2nd derivatives and Extended Ratio Subtraction methods are applied on the spectra. But as shown in Fig. 3, the overlapping could not be resolved. Thus the following methods are proposed to overcome this overlapping problem.

2.3.1 First derivative of ratio spectra spectrophotometric method (1DD)

In this method the stored spectrum of one of the pure drug standards is divided by that of a chosen drug divisor. Then the first derivative is obtained using suitable \( \Delta \lambda \) and scaling factor. The amplitudes of the first derivative of the ratio spectra at one maximum and one minimum for each drug are plotted against the respective concentrations and the calibration curves are constructed [50,51]. By applying this method for the determination of ROSCa, DICNa and TIM, DICNa and TIM were used as divisors separately for the determination of ROSCa. Using ROSCa
and DICNa in case of TIM and ROSCa and TIM for DICNa. The optimal Δλ = 8.0 and scaling factor = 1.0. The chosen divisors are 5 μg/mL standard solutions of ROSCa, DICNa and TIM.

2.3.2 Mean Centering of Ratio Spectra Method (MCRS)

(1) Theoretical background

To clarify the expression of mean centering (MC), let considering a five-dimensional vector \( k \):

\[
k = [6 \ 10 \ 2 \ 8 \ 4]^T
\]

The mean vector of the vector \( k \) is

\[
\bar{k} = [6 \ 6 \ 6 \ 6 \ 6]^T
\]

The vector \( k \) can be mean centered by subtracting the mean vector \( \bar{k} \) from it as follows:

\[
MC(k) = k - \bar{k}
\]

\[
MC(k) = [0 \ 4 \ -4 \ 2 \ -2]^T
\]

Where \( MC(k) \) is the mean center of the vector \( k \).

Let \( n \) is a constant vector where

\[
n = [2 \ 2 \ 2 \ 2 \ 2]^T
\]

It is clear that if the vector \( y \) is multiplied by \( n \) (a constant number), the mean centered vector is also multiplied by \( n \) and also if a constant number is added to the vector \( y \), the mean center of this vector is not changed.

**Fig. 2.** Zero order absorption spectra of 5μg/mL of each of ROSCa, TIM and DICNa mixture using methanol as a blank

(a) (b)
Thus the mean centering of Eq. (3) will be as follows:

\[ MC(X) = MC\left(\frac{a_{a}C_{a}}{a_{c}}\right) + MC\left(\frac{a_{b}C_{b}}{a_{c}}\right) \]  

The mean centering of any constant equals zero, then

\[ MC(C_{a}) = MC(C_{b}) = MC(C_{c}) = 0 \]  

Thus the mean centering of Eq. (3) will be as follows:

\[ MC(Y) = MC\left(\frac{MC(X)}{MC[a_{b}/a_{c}]}\right) = MC\left[\frac{MC[a_{a}C_{a}/a_{c}]}{MC[a_{b}/a_{c}]}ight] \]  

The 2\textsuperscript{nd} ratio spectrum can be attained by dividing Eq. (5) by \( MC[a_{a}/a_{c}] \) corresponding to the mean centering of the ratio of the spectra of the standard solutions of \( b \) and \( c \) as the following equation (the zero values of \( MC[a_{a}/a_{c}] \) must not be used in the divisor for the possibility of the dividing operation):

\[ Y = \frac{MC(X)}{MC[a_{a}/a_{c}]} = MC\left[\frac{MC[a_{a}C_{a}/a_{c}]}{MC[a_{b}/a_{c}]}ight] + C_{b} \]  

Fig. 3. UV spectra of 5 µg/mL of each of ROSCa, TIM and DICNa: (a) 1\textsuperscript{st} derivative spectra, (b) 2\textsuperscript{nd} derivative spectra and (c) Extended ratio subtraction method

\( \text{(2) Application of the MCRS method for a ternary mixture of the three drugs} \)

Considering a mixture of the three drugs \( a, b \) and \( c \), where they are compatible with each other’s and Beer’s law is obeyed for each drug, it can be written:

\[ \text{Abs} \text{(Mix)} = a_{a}C_{a} + a_{b}C_{b} + a_{c}C_{c} \]  

where \( \text{Abs} \text{(Mix)} \) is the absorbance vector of the mixture, \( C_{a}, C_{b} \) and \( C_{c} \) are the concentrations of \( a, b \) and \( c \), and \( a_{a}, a_{b} \) and \( a_{c} \) are the molar absorptivity vectors of \( a, b \) and \( c \), respectively.

By dividing Eq. (2) by \( a_{c} \) corresponding to the spectrum of a standard solution of \( c \) in the combination mixture of the three drugs (the zero values of \( a_{c} \) must not be used in the divisor for the possibility of the dividing operation), the 1\textsuperscript{st} ratio spectrum can be attained as in Eq. (3):

\[ X = \frac{\text{Abs} \text{(Mix)}}{a_{c}} = \frac{a_{a}C_{a}}{a_{c}} + \frac{a_{b}C_{b}}{a_{c}} + C_{c} \]  

The mean centering of any constant equals zero, then

\[ MC(C_{a}) = MC(C_{b}) = MC(C_{c}) = 0 \]  

Eq. (7) extracts one of the active components to be determined \( a \) in this eq.) in the solution based on that there is no interference from the other components of a ternary system \( b \) and \( c \) in this mixture. Also, the concentrations of the other active components can be calculated in the same manner. It is found that there is a linear relationship between \( C_{a} \) in the solution and the quantity of \( MC(Y) \) as Eq. (7) shows. A calibration graph could be established by plotting \( MC(Y) \) versus \( C_{a} \) either in the standard ternary mixtures or in the standard solutions of \( a \). To obtain more sensitive results, the quantity of \( MC(Y) \) should be measured in correspondence with minimum or maximum wavelengths. Also, calibration curves for the other components \( b \) and \( c \) can be constructed in the same manner [51-53]. This method is applied for the determination of the concentrations of ROSCa, DICNa and TIM, respectively by substituting in these equations (eq.2 to eq.7) separately.
2.3.3 Successive Ratio-Derivative Spectra Method (SRDS)

As a step, for applying the successive ratio-derivative spectra method [54], the following equation can be obtained by taking the 1st derivative of Eq. (3) where the 1st derivative of any constant (e.g. C_a, C_b, and C_c) equals to zero.

\[
\frac{d(Y)}{d\lambda} = \frac{d}{d\lambda} \left[ \frac{a_2C_a}{a_c} \right] + \frac{d}{d\lambda} \left[ \frac{a_2C_b}{a_c} \right] + \frac{d}{d\lambda} \left[ \frac{a_2C_c}{a_c} \right] (8)
\]

By dividing Eq. (8) by \((d/d\lambda) [a_2/a_c]\) corresponding to the derivative of the ratio of the spectra of the standard solutions of b and c as the following equation (the zero values of \((d/d\lambda) [a_2/a_c]\) must not be used in the divisor for the possibility of the dividing operation):

\[
Z = \frac{\frac{d(Y)}{d\lambda}}{(d/d\lambda)[a_2/a_c]} = \frac{(d/d\lambda)[a_2C_a/a_c]}{(d/d\lambda)[a_2/a_c]} + C_b (9)
\]

Then, the 1st derivative of Eq. (9) will be as follows:

\[
\frac{d(Z)}{d\lambda} = \frac{d}{d\lambda} \left[ \frac{(d(Y)/d\lambda)[a_2/a_c]}{(d/d\lambda)[a_2/a_c]} \right] = \frac{d}{d\lambda} \left[ \frac{(d/d\lambda)[a_2C_a/a_c]}{(d/d\lambda)[a_2/a_c]} \right] (10)
\]

Eq. (10) extracts mathematically one of the active components to be determined \(a\) in this equation in the solution with no interference from the other components of a ternary system \(b\) and \(c\) in this mixture. Also, the concentrations of the other active components can be calculated in the same manner. It is found that there is a linear relationship between \(C_a\) in the solution and the quantity of \(d(Z)/d\lambda\) as Eq. (10) shows. A calibration graph could be established by plotting \(d(Z)/d\lambda\) versus \(C_a\) either in the standard ternary mixtures or in the standard solutions of \(a\). To obtain more sensitive results, the quantity of \(d(Z)/d\lambda\) should be measured in correspondence with minimum or maximum wavelengths. Also, calibration curves for the other components \(b\) and \(c\) can be constructed in the same manner.

In the present work, this method is used for the determination of ROSCa, DICNa and TIM respectively by substituting in these equations (eq.8 to eq.10) separately.

3. METHODS DEVELOPMENT AND OPTIMIZATION

Selection of the solvent, wavelengths and divisors is one of the key factors to adjust and develop the method, so different solvents were tried such as water, ethanol and methanol. The solvent which led to develop the most resolved spectra with maximum sensitivity and minimum noise should be chosen and the use of methanol satisfies these requirements. Also, selection of the divisor at concentrations of 5 µg/mL of ROSCa, TIM and DICNa in 1st DD showed good recovery and selectivity.

4. RESULTS AND DISCUSSION

The main aim of this work is to develop novel, accurate, sensitive and precise spectrophotometric methods for the determination of a mixture of the three drugs viz., ROSCa, DICNa and TIM in their pure form, laboratory prepared mixtures and spiked human plasma. As mentioned previously all of the zero order, 1st and 2nd derivative spectrophotometric methods failed to achieve simultaneous determination of the three drugs due to their overlapped spectra. To overcome this problem, the 1stDD, MCRS and SRDS methods are used. For convenience, each of them will be discussed separately.

4.1 Applying the 1St DD Method for the Ternary Mixture

The proposed method proved its ability to solve the overlapped spectra of the three drugs as shown in Figs. 4, 5 and 6, in which the stored zero spectra of the standards of ROSCa and TIM are divided by the spectrum of 5 µg/mL of DICNa and their first derivatives are developed using \(\Delta\lambda=8\) and scaling factor =1.0. The amplitudes of the 1st derivative of the ratio spectra at one maximum 239.5nm, one minimum at 266.5 nm for ROSCa and one maximum at 307.7nm, one minimum at 331 nm for TIM are plotted against the corresponding concentrations of ROSCa and TIM, hence the calibration curves are constructed. Also, the spectra of the DICNa and TIM pure standards are divided by the standard spectrum of 5 µg/mL of ROSCa, then the first derivative is achieved using scaling factor = 1.0 and \(\Delta\lambda=8\). The amplitudes of the first derivative of the ratio spectra at one maximum 269.7 nm, one minimum at 292 nm for DICNa and at one maximum at 278.7 nm, one minimum at 317 nm for TIM are plotted against the mixed concentrations of DICNa and TIM, hence calibration curves are constructed. The ratio spectra of the pure standards ROSCa and DICNa are obtained by dividing their spectra by the spectrum of 5 µg/mL of TIM and the first derivative is created using scaling factor= 1.0 and \(\Delta\lambda=8\). Similarly, the peak amplitudes of the first derivative of the ratio spectra at one minimum 284.4 nm, one maximum at 264.7 nm for DICNa and at one maximum at 239.5nm, one minimum at 269.5 for
ROSCa are plotted versus the corresponding concentrations of DICNa and ROSCa, and then the calibration curves are constructed.

Fig. 4. Ratio spectra and first derivative of the ratio spectra of different concentrations of solution of: (a) ROSCa and (b) TIM in methanol when 5 μg/mL solution of DICNa in methanol is used as a divisor (Δλ = 8 nm)
Fig. 5. Ratio spectra and first derivative of the ratio spectra of different concentrations of solution of: (a) DICNa and (b) TIM in methanol when 5 μg/mL solution of ROSCa in methanol is used as a divisor (Δλ = 8 nm)

Fig. 6. Ratio spectra and first derivative of the ratio spectra of different concentrations of solution of: (a) ROSCa and (b) DICNa in methanol when 5 μg/mL solution of TIM in methanol is used as a divisor (Δλ = 8 nm)
4.2 Applying the MCRS Method

The developed MCRS method is based on getting the MC of ratio spectra. MCRS method is performed for the determination of ROSCa, TIM and DICNa drugs in a mixture in one run without preseparation. To optimize the proposed method, different divisor concentrations were tested to develop high selective method. 5μg/mL is the most suitable concentration. The spectra of the drugs were created in the range 222-349 nm as shown in Fig. 7.

The stored spectra of different concentrations of the drugs are exported to the MATLAB program. ROSCa, TIM and DICNa concentrations in the authentic ternary mixture and in real samples could be determined by applying the equations from 2 to 7 and assuming that ROSCa is (a), TIM is (b) and DICNa is (c). From the resulted graphs of MC of second ratio spectra for the drugs as in (Fig. 8), it is found that 277.9, 313.1 and 333.1 nm are the suitable wave lengths for the determination of ROSCa, TIM and DICNa, respectively. The calibration curves are constructed by plotting the measured amplitudes versus their corresponding concentrations and r^2 values are computed as in Table 1.

4.3 Applying the SRDS Method

The developed SRDS method is based on getting the derivative of ratio spectra. SRDS method is applied for the determination of ROSCa, TIM and DICNa in the ternary mixtures containing these drugs. To optimize the proposed method, different divisor concentrations were tested to develop high selectivity. 5μg/mL proved as the most suitable concentration. The spectra of the drugs were created in the range 222-349 nm.

The stored spectra of different concentrations of the drugs and their mixtures are exported to the MATLAB program. ROSCa, TIM and DICNa concentration in the ternary mixture and real samples could be determined by applying the equations from 8 to 10 and assuming that ROSCa is (a), TIM is (b) and DICNa is (c). From the resulted graphs of SRDS for the drugs as in (Fig. 10), it is found that 264, 339 and 300 nm are the suitable wave lengths for the determination of ROSCa, TIM and DICNa respectively. The calibration curves are constructed by plotting the measured amplitudes versus their corresponding concentrations and r^2 values are computed as in Table 1.
Fig. 7. The absorption spectra of different concentrations of ROSCa, TIM and DICNa in methanol
Fig. 8. The first ratio spectra, the MC of first ratio spectra (eq.5), the second ratio spectra (eq.6) and the MC of second ratio spectra (eq.7) of ROSCa
Fig. 9. (a) MC of second ratio spectra of TIM (b) MC of second ratio spectra of DICNa

(a)

(b)
4.4 Validation of the Analytical Method

The effectiveness of the method has been proved, according the ICH guidelines (ICH Q2R1), considering linearity, accuracy, selectivity, LOQ and LOD [55].

(1) Linearity and ranges

The linearity of the developed methods is in the concentration range (5.0-25.0μg/mL) for the three drugs. The calibration graphs are obtained by plotting the absorbance versus the corresponding known concentration. The results of the methods have good linearity as indicated by the obtained values of correlation coefficients, slope and intercept, Table 1.

(2) Quantitation (LOQ) and Detection limits (LOD)

LOQ and LOD are based on the Slope and the Standard Deviation of the blank. The analyte concentrations of the samples used for construction of a specific calibration graph should be in LOQ and LOD range. The LOQ =10 × σ /slope and LOD=3.3 × σ /slope, where σ = the standard deviation of the blank and the slope of the calibration line as clarified in Table 1.

(3) Precision

(a) Repeatability:

The intraday RSD % (n = 3), average of three concentrations (5, 10 and 15μg/mL) for each of ROSCa, DICNa and TIM, respectively were repeated three times within the day. The good obtained results are presented in Table 1.

(b) Intermediate precision:

The Intraday RSD% (n=3), the measurements of the average of three concentrations (5, 10 and 15μg/mL) for each of ROSCa, DICNa and TIM respectively are repeated through three days successively. The obtained results are presented in Table 1.

(4) Accuracy and recovery

Accuracy of the developed methods was calculated as recoveries % of pure samples of the target drugs. Accuracy is evaluated by using three concentrations (5 & 10 & 15 μg/mL) for ROSCa, TIM and DICNa, respectively within the linear range by repeating the measurements three times. The corresponding regression equations were used for calculating concentrations. The values of the mean recoveries percent for ROSCa, TIM and DICNa are in between 98% and 102%. The data of accuracy are presented in Table 1.

(5) Selectivity

The selectivity of the developed methods for the three drugs in laboratory prepared mixtures containing them in different ratios is obtained within the linear range. The developed methods were evaluated by the analysis of laboratory
Table 1. Validation and regression parameters of the developed methods for determination of ROSCa, TIM and DICNa

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ROSCa</th>
<th>DICNa</th>
<th>TIM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st DD</td>
<td>MCR</td>
<td>SRDS</td>
</tr>
<tr>
<td>Range (µg/mL)</td>
<td>5.25</td>
<td>5.25</td>
<td>5.25</td>
</tr>
<tr>
<td>Slope</td>
<td>0.018</td>
<td>0.021</td>
<td>0.025</td>
</tr>
<tr>
<td>Intercept</td>
<td>0.003</td>
<td>0.001</td>
<td>0.0024</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9997</td>
<td>0.9998</td>
<td>0.9998</td>
</tr>
<tr>
<td>Accuracy (Mean ± RSD)</td>
<td>100.28±1.157</td>
<td>100.65±1.2</td>
<td>100.72±1.16</td>
</tr>
<tr>
<td>LOD(^a) (µg/mL)</td>
<td>0.556</td>
<td>0.297</td>
<td>0.374</td>
</tr>
<tr>
<td>LOQ(^a) (µg/mL)</td>
<td>1.68</td>
<td>0.90</td>
<td>1.135</td>
</tr>
<tr>
<td>Intra-day RSD%</td>
<td>0.121</td>
<td>0.159</td>
<td>0.173</td>
</tr>
<tr>
<td>Inter-day RSD%</td>
<td>0.151</td>
<td>0.245</td>
<td>0.186</td>
</tr>
<tr>
<td>Wave lengths nm</td>
<td>239.5</td>
<td>277.9</td>
<td>264</td>
</tr>
</tbody>
</table>

\(^a\) Limit of detection (3.3× σ/Slope) and limit of quantization (10× σ/Slope)

Table 2. Determination of ROSCa, TIM and DICNa in laboratory prepared mixture by applying the developed methods

<table>
<thead>
<tr>
<th>Ratio of ROSCa: TIM: DICNa</th>
<th>1st DD</th>
<th>MCRS</th>
<th>SRDS</th>
<th>1st DD</th>
<th>MCRS</th>
<th>SRDS</th>
<th>1st DD</th>
<th>MCRS</th>
<th>SRDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1:1</td>
<td>101.5</td>
<td>101.7</td>
<td>98.0</td>
<td>99.1</td>
<td>99.03</td>
<td>98.22</td>
<td>98.7</td>
<td>98.4</td>
<td>98.59</td>
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<td>2:1:1</td>
<td>99.1</td>
<td>98.8</td>
<td>100.1</td>
<td>99.3</td>
<td>103.1</td>
<td>100.25</td>
<td>99.7</td>
<td>101.3</td>
<td>101.1</td>
</tr>
<tr>
<td>3:2:1</td>
<td>101.8</td>
<td>100.7</td>
<td>100.8</td>
<td>99.4</td>
<td>93.3</td>
<td>100.1</td>
<td>100.1</td>
<td>99.9</td>
<td>102.4</td>
</tr>
<tr>
<td>4:3:2</td>
<td>100.5</td>
<td>99.3</td>
<td>99.18</td>
<td>99.5</td>
<td>102.4</td>
<td>99.71</td>
<td>100.3</td>
<td>100.1</td>
<td>99.34</td>
</tr>
<tr>
<td>5:4:3</td>
<td>99.7</td>
<td>100.3</td>
<td>99.79</td>
<td>99.5</td>
<td>100.6</td>
<td>99.73</td>
<td>99.3</td>
<td>100.3</td>
<td>100.5</td>
</tr>
<tr>
<td>Mean ± RSD</td>
<td>100.5±1.16</td>
<td>100.2±1.13</td>
<td>99.57±1.05</td>
<td>99.4±0.18</td>
<td>101.1±1.64</td>
<td>99.60±0.81</td>
<td>99.6±0.61</td>
<td>100.0±1.04</td>
<td>100.39±1.149</td>
</tr>
</tbody>
</table>

\(^*\)Average of three replications
prepared mixtures which contain dissimilar ratios of ROSCa, TIM and DICNa, besides applying the standard addition method to dosage forms. The obtained accuracy proved that the presence of excipients (microcrystalline cellulose NF, lactose monohydrate NF, tribasic calcium phosphate NF, crospovidone NF, magnesium stearate NF, hypromellose NF, triacetin NF, titanium dioxide USP, yellow ferric oxide, and red ferric oxide NF) in the pharmaceutical preparations did not interfere in their analysis of the three drugs as presented in Table 2.

(6) Statistical examination

Statistical comparison between the results obtained for investigation of ROSCa, TIM and DICNa by the suggested methods and those results achieved by the reported one for the simultaneous determination of the three drugs in a mixture [32] where the target compounds were analyzed on Hypersil BDS C(18) column (250 mm × 4.6 mm, 5 μm), applying 0.2% triethylamine) and acetonitrile (40:60, v/v), in isocratic mode as mobile phase, pH 2.75

Fig. 11. Interval plot of the proposed method and the reported method

Table 3. Statistical comparison between the obtained results of the developed methods and those gained by the reported ones (Inside the Fig.: RM [13] has changed to [32])

<table>
<thead>
<tr>
<th>Summary of data</th>
<th>Treatments</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>5</td>
</tr>
<tr>
<td>ΣX</td>
<td>504</td>
</tr>
<tr>
<td>Mean</td>
<td>100.8</td>
</tr>
<tr>
<td>ΣX²</td>
<td>50806</td>
</tr>
<tr>
<td>Std.Dev.</td>
<td>0.8367</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Result details</th>
<th>SS</th>
<th>df</th>
<th>MS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Between-treatments</td>
<td>15</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>Within-treatments</td>
<td>11.2</td>
<td>16</td>
<td>0.7</td>
</tr>
<tr>
<td>Total</td>
<td>26.2</td>
<td>19</td>
<td></td>
</tr>
</tbody>
</table>

The f-ratio value is 7.14286. The p-value is .00293. The result is significant at p < .05
Table 4. Recovery results of the developed methods in spiked plasma samples

<table>
<thead>
<tr>
<th>Spiked</th>
<th>ROSCa</th>
<th>Recovery</th>
<th>Spiked</th>
<th>DICNa</th>
<th>Recovery</th>
<th>Spiked</th>
<th>TIM</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>9.90</td>
<td>99</td>
<td>10</td>
<td>10.01</td>
<td>100.1</td>
<td>10</td>
<td>9.89</td>
<td>98.9</td>
</tr>
<tr>
<td>10</td>
<td>9.95</td>
<td>99.5</td>
<td>10</td>
<td>10.12</td>
<td>101.2</td>
<td>10</td>
<td>9.81</td>
<td>98.1</td>
</tr>
<tr>
<td>10</td>
<td>10.04</td>
<td>100.4</td>
<td>10</td>
<td>9.93</td>
<td>99.3</td>
<td>10</td>
<td>9.94</td>
<td>99.4</td>
</tr>
<tr>
<td>10</td>
<td>10.11</td>
<td>101.1</td>
<td>10</td>
<td>9.98</td>
<td>99.8</td>
<td>10</td>
<td>10.06</td>
<td>100.6</td>
</tr>
<tr>
<td>10</td>
<td>10.16</td>
<td>101.6</td>
<td>10</td>
<td>9.85</td>
<td>98.5</td>
<td>10</td>
<td>10.13</td>
<td>101.3</td>
</tr>
<tr>
<td>Mean</td>
<td>10.032</td>
<td>100.32</td>
<td>10.978</td>
<td>99.78</td>
<td>99.78</td>
<td>10.966</td>
<td>99.66</td>
<td></td>
</tr>
<tr>
<td>RSD</td>
<td>-</td>
<td>0.32%</td>
<td>-</td>
<td>1.0%</td>
<td>-</td>
<td>-</td>
<td>1.28%</td>
<td></td>
</tr>
<tr>
<td>%Error</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-0.22%</td>
<td>-</td>
<td>-</td>
<td>-0.34%</td>
<td></td>
</tr>
</tbody>
</table>

adjusted with 85% phosphoric acid at a flow rate of 1mL/min. The column oven temperature was kept at 45°C and the peak response was monitored at 284 nm after injecting a 50 μL sample into HPLC system. The results showed that there are no considerable differences between the suggested spectrophotometric methods and the reported one as Table 3 and Fig. 11 illustrates. One way ANOVA test was used to compare the capability of the developed methods for the simultaneous estimation of ROSCa, TIM and DICNa, by subjecting their results versus the obtained ones by using the reported method [32].

4.5 Application to Spiked Human Plasma

The developed and validated methods have been applied for the analysis of ROSCa, TIM and DICNa in spiked plasma samples. The recovery results were in the range 98-102% and RSD was less than 2.0% as displayed in Table 4.

5. CONCLUSION

Three novel, times saving, accurate and precise spectrophotometric techniques viz., smart first derivative of ratio spectra, mean centering of ratio spectra and successive-ratio derivative spectra methods are developed for the determination of ROSCa, TIM and DICNa simultaneously in ternary mixture in pure laboratory prepared and spiked human plasma samples. These innovative spectrophotometric methods were successfully applied for solving the overlapping spectra of the components of the ternary mixture. Good statistical results were obtained upon comparing those achieved by the developed methods with that of the reported one. Among the advantages of the developed techniques are that they neither need sophisticated instrumentation nor distinctive software; also, they could be applied for the routine examination of these drugs either in their pure bulk standards, spiked plasma samples, in laboratory prepared mixtures in quality control units and in laboratories having no HPLC instruments.

DISCLAIMER

The products used for this research are commonly and predominantly used products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

As per international standard or university standard written patient consent has been collected and preserved by the author(s).

ETHICAL APPROVAL

The collection of blood samples should be ethical approved and must be under the umbrella of Human Research Committee in case of human volunteers administering the drugs, but in our case blood samples were collected from healthy human volunteers who did not administrate any drugs, then plasma samples were spiked with the studied drugs.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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