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Article

DRUG FORMULATIONS AND CLINICAL METHODS

Three Different Approaches Based on Derivative Ratio Spectra for Spectrophotometric Resolution of a Quaternary Mixture in Semisolid Dosage Form

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Abstract

Background: Recent incorporated spectrophotometer software supporting mathematical methods was considered as an optimum key for the resolution of multicomponent mixtures.
Objective: Several spectrophotometric techniques are introduced for the determination of mixtures of tretinoin (TN), hydroquinone (HQ), and fluocinolone acetonide (FA), in the presence of the preservative methyl paraben (MP), without any separation procedure, taking into consideration the presence of two minor components and the severe overlap of their spectra.
Method: Constant multiplication coupled spectrum subtraction resolved the quaternary mixture into the zero-order absorption spectrum of TN alone and a severely overlapped, ternary mixture of HQ, FA, and MP. Three approaches based on the derivative ratio spectra were applied to resolve this ternary, severely overlapped mixture: derivative ratio-zero-crossing point method, factorized derivative ratio method, and double divisor derivative ratio method.
Results: The work was conducted over a concentration range of 1–10, 4–38, and 4–35 μg/mL, for TN, HQ, and FA, respectively. The results obtained were compared statistically to each other and to the official methods, showing no significant difference.
Conclusions: The proposed methods were successfully applied for the simultaneous determination of the three drugs in the presence of MP in synthetic mixtures and in their combined dosage form (Trimelasma® cream) with very good accuracy and precision.
Highlights: This was a comparative study between conventional and new methods for resolving ternary, severely overlapped mixtures. Mathematical manipulation steps and enrichment techniques aided accurate quantification of the minor components in mixtures.

Tretinoin (TN), also named all-trans-retinoic acid (ATRA) (1), is a derivative of vitamin A (retinol) (2). It is known to be important in controlling reproduction, proliferation, and differentiation of cells. It is beneficial in management of acne and photo-damaged skin cases and keratinization disorders such as ichthyosis and keratosis follicularis (3). Its chemical name is (2*E* , 4*E* , 6*E* ,

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8E)-3,7-dimethyl-9–(2,6,6-trimethylcyclohexen-1-yl)nona-2,4,6,8-tetraenoic acid (1). The U.S. pharmacopeia

(USP) official method for determination is non-aqueous acid-base titration (1). The literature also revealed its determination via spectrophotometry (4–6) and chromatography by HPLC (7–11).

Hydroquinone (HQ) is a phenol derivative used as a bleaching agent (12). Its chemical name is benzene-1,4-diol (1). The USP official method for determination is redox titration (1). The literature also revealed its determination via spectrophotometry (13, 14) and chromatography by HPLC (11, 13, 15–17).

Fluocinolone acetonide (FA) is a steroid preparation which is very effective when applied locally in high dilution for treatment of dermatological conditions such as melasma (18) and dermatitis (19). Its chemical name is 6 alpha, 9 alpha-difluoro-16 alpha-hydroxyprednisolone-16, 17-acetonide (20). The British pharmacopeia (BP) official method for determination is a spectrophotometric method (2). The literature also revealed its determination via spectrophotometry (21–23) and chromatography by demonstrating HPLC methods (24, 25).

The literature also revealed the determination of the ternary mixture of TN, HQ in presence of FA as interfering substance without quantitative determination of it due to its very low concentration via HPLC method (10). Determination of TN, HQ, and methyl paraben (MP) spectrophotometrically has been also reported (14).

The aim of this work is to quantitatively determine, via spectrophotometric methods, TN, HQ, and FA in the presence of the preservative MP, in Trimelasma® cream, with acceptable accuracy and precision, and overcome the challenges caused by the presence of two minor components and the severe overlap of their spectra. To the best of our knowledge, no analytical quantitative method has been proposed for TN, HQ, and FA, in the presence of MP.

The chemical structures of the four components under investigation are shown in Figure 1.

METHOD

Theoretical Background

Factorized derivative ratio method coupled with spectrum subtraction (FDRM-SS) (27)

This method is an approach for factorized spectra (28–31) for resolving the severely overlapped spectra of ternary mixtures (X, Y, and Z), where one of the components is chosen as a divisor (Z') and its contribution is eliminated through derivatization using a suitable order where Z/Z' is cancelled by derivatization. The adequate wavelength is then selected, where X/Z' spectrum has maxima or minima at the zero point of the third component Y/Z' (either zero-crossing or zero-contribution point). This factorized derivative ratio of X is prepared, where $P_{X(\lambda \text{ zero point})} = 1$, by dividing the derivative of the ratio spectra (DD^1) of X by its recorded peak amplitude value at this specified wavelength ($\lambda_{\text{zero point}}$).

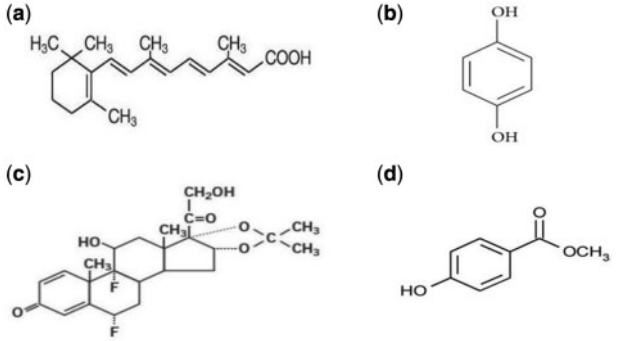

Figure 1. The chemical structure of (a) TN, (b) HQ, (c) FA, and (d) MP.

DD^1 factorized spectrum (FS') = $P_X/P_XV_{(\lambda \text{ zero point})}$

For the analysis of laboratory- prepared mixtures, the component Z' is used as a divisor, then derivatization under specified experimental parameter the DD^1 spectrum of the components X (X/Z') in the mixture could be obtained by multiplying the recorded amplitude at the chosen wavelength ($\lambda_{\text{zero point}}$) by the previously computed DD^1 factorized spectrum for X.

P_X in the mixture = DD^1 factorized spectrum (FS') × $P_XV_{(\lambda \text{ zero point})}$

The concentration of X is calculated via a regression equation representing the linear relationship of the amplitudes of pure X using Z' as a divisor versus the corresponding concentrations using specified graphical representation P_{maxima} to zero ($P_{max–zero}$), P_{minima} to zero ($P_{min–zero}$), or peak to peak ($P_{max-min}$).

The DD^1 spectrum of component Y (Y/Z') is obtained by subtracting it from the DD^1 spectrum of the ternary mixture, then its concentration is calculated using its constructed regression equation via specified graphical representation. Similarly, the third component Z is obtained via the same procedure using X or Y as a divisor.

Double divisor ratio spectra derivative method (DDRD)

DDRD (32) is used for resolving the severely overlapped spectra of ternary mixtures (X, Y, and Z). If the Beer–Lambert Law is obeyed for three compounds at all wavelengths, then the spectra of the ternary mixture at wavelength λi can be given by:

$$A_{m,\lambda i} = \varepsilon_{X,\lambda i}C_X + \varepsilon_{Y,\lambda i}C_Y + \varepsilon_{Z,\lambda i}C_Z \quad \text{(Eq 1)}$$

where, $A_{m,\lambda i}$ = the absorbance of the mixture at wavelength λi; $\varepsilon_{X,\lambda i}$, $\varepsilon_{Y,\lambda i}$, and $\varepsilon_{Z,\lambda i}$ are the molar absorptivities of X, Y, and Z at wavelength λi; and C_X, C_Y, and C_Z are the molar concentration of X, Y, and Z, respectively.

A similar expression of the binary mixture (a double divisor) of two compounds in a ternary mixture can be written:

$$A_{m,\lambda i} = \varepsilon_{X,\lambda i}C_X + \varepsilon_{Y,\lambda i}C_Y \quad \text{(Eq 2)}$$

The ternary mixture is divided by an equimolar binary mixture (a double divisor X + Y) of two compounds from those present in the ternary mixture followed by derivatization.

If Eq. 1 is divided by Eq. 2 (double divisor), the resulting expression for the ratio spectra can be given as:

$$\frac{A_{m,\lambda i}}{\varepsilon_{X,\lambda i}C_X^0 + \varepsilon_{Y,\lambda i}C_Y^0} = \frac{\varepsilon_{X,\lambda i}C_X + \varepsilon_{Y,\lambda i}C_Y}{\varepsilon_{X,\lambda i}C_X^0 + \varepsilon_{Y,\lambda i}C_Y^0} + \frac{\varepsilon_{Z,\lambda i}C_Z}{\varepsilon_{X,\lambda i}C_X^0 + \varepsilon_{Y,\lambda i}C_Y^0} \quad \text{(Eq 3)}$$

The ratio of $(\varepsilon_{X,\lambda i}C_X + \varepsilon_{Y,\lambda i}C_Y/\varepsilon_{X,\lambda i}C_X^0 + \varepsilon_{Y,\lambda i}C_Y^0)$ is equal to constant (k) (or very close to 1) with respect to λi in a certain range of wavelength and if this above constant is replaced in Eq. 3, it can be obtained Eq. 4:

$$\frac{A_{m,\lambda i}}{\varepsilon_{X,\lambda i}C_X^0 + \varepsilon_{Y,\lambda i}C_Y^0} = k + \frac{\varepsilon_{Z,\lambda i}C_Z}{\varepsilon_{X,\lambda i}C_X^0 + \varepsilon_{Y,\lambda i}C_Y^0} \quad \text{(Eq 4)}$$

In the double divisor procedure, the standard concentrations of C_X^0 and C_Y^0in Eq. 2 are equal or very close to each other ($C_X^0 = C_Y^0$) or ($C_X^0 \cong C_Y^0$) which can be expressed as:

$$\varepsilon_{X,\lambda i}C_X^0 + \varepsilon_{Y,\lambda i}C_Y^0 = C_X^0[\varepsilon_{X,\lambda i} + \varepsilon_{Y,\lambda i}] \quad \text{(Eq 5)}$$

When Eq. 5 is substituted with Eq. 3 the following equation can be obtained:

$$\frac{A_{m,\lambda i}}{C_X^0[\varepsilon_{X,\lambda i} + \varepsilon_{Y,\lambda i}]} = k + \frac{\varepsilon_{Z,\lambda i}C_Z}{C_X^0[\varepsilon_{X,\lambda i} + \varepsilon_{Y,\lambda i}]} \quad \text{(Eq 6)}$$

The first (or high order) derivation of Eq. 6 can be taken with respect to λi in the selected region of wavelength, in this case, the derivation of a constant (k) is zero and we can obtain the following Eq. 7:

$$\frac{d}{d\lambda}\left[\frac{A_{m,\lambda i}}{C_X^0[\varepsilon_{X,\lambda i} + \varepsilon_{Y,\lambda i}]}\right] = \frac{d}{d\lambda}\left[\frac{\varepsilon_{Z,\lambda i}C_Z}{C_X^0[\varepsilon_{X,\lambda i} + \varepsilon_{Y,\lambda i}]}\right]\frac{C_Z}{C_X^0} \quad \text{(Eq 7)}$$

In this method, the concentration C_Z is proportional to the derivative signals in the coincidence points corresponding to the maximum and minimum of wavelength for pure Z and its ternary mixture with X and Y.

As explained above, the analogous procedures can be used for the estimation of C_X and C_Y in a ternary mixture.

Derivative ratio spectrum zero-crossing method (DRSZ)

DRSZ is method for resolving ternary mixtures (32), by simultaneous use of the zero-crossing method and Salinas's method (33). For a mixture of three compounds, X, Y, and Z, having severely overlapped spectra and Beer's Law is obeyed for all compounds over the whole wavelength range.

The absorption spectrum of the mixture is defined by the Eq. (1). If divided by the standard solution of one of the components (e.g., X of concentrationC_X^0) and the first derivative of the result is obtained, the following equation can be written:

$$\frac{d}{d\lambda}\left[\frac{A_{m,\lambda i}}{\varepsilon_{X,\lambda i}C_X^0}\right] = \frac{C_Y}{C_X^0}\frac{d}{d\lambda}\left[\frac{\varepsilon_{Y,\lambda i}}{\varepsilon_{X,\lambda i}}\right] + \frac{C_Z}{C_X^0}\frac{d}{d\lambda}\left[\frac{\varepsilon_{Z,\lambda i}}{\varepsilon_{X,\lambda i}}\right] \quad \text{(Eq 8)}$$

The divisor component is eliminated via derivatization. The concentration of one component is determined by substituting the peak amplitude at the zero-crossing point of the other in the corresponding regression equation.

Spectrum addition (SA)

SA is an enrichment technique that is adopted in the dosage form (34). The sum of the stored spectra can be performed with the help of Spectra manager® version 2 software. The application of a sample enrichment technique is essential for the analysis of mixtures where one or more component is present in low concentration to facilitate the determination of one or more minor components since the choice of the optimum concentration range depends on the spectral characteristics of the compound, its absorptivity, and its ratio in the mixture without a need to increase its concentration in the binary or ternary mixtures, which might lead to a deviation from Beer's law due to the electrostatic attraction between ions (35) when transmittance values are close to 100% where incident light approaches transmitted light. The sample enrichment technique involves adding known amounts of standard to one or more aliquots of the processed sample solution, enhancing the analyte signal.

Constant multiplication (CM)

In a mixture of two or more components showing an extended spectrum of one component, CM (36, 37) allows getting the zero-order absorption spectrum of the extended component, and when coupled to spectrum subtraction (SS) (38) the less extended components are obtained.

Instrumentation

Spectrophotometric measurements are made using a double-beam UV-Vis spectrophotometer model V-760, connected to a compatible computer with software (Microsoft excel 2010, Spectra manager). The absorption spectra of the solutions are measured in a 1.00 cm quartz cell. Scans are carried out in the range of 200–400 nm at room temperature.

Chemicals and Reagents

(a) *Pure samples*.—TN, HQ, FA, and MP supplied by Marcyrl Pharma with purity 100.22 ± 0.78, 100.33 ± 0.53, 99.99 ± 0.53, and 100.14 ± 0.55, respectively, according to the USP and BP official methods.

(b) *Pharmaceutical formulation*.—Trimelasma cream, each 1.00 g claiming to contain 0.50 mg TN, 40.00 mg HQ, 0.10 mg FA, and 1.80 mg MP, is manufactured by Marcyrl Pharmaceutical Company, and was purchased from the Egyptian market.

(c) *Solvents*.—Analytical grade methanol was supplied by Carlo Erba .

Standard Solution

A standard stock solution (500.00 μg/mL) was prepared by dissolving the required amount of each drug in methanol, then the volume was completed to 100 mL using methanol.

Working standard solutions (50.00 μg/mL) were prepared by appropriate dilution from the previously mentioned stock solution using methanol.

Procedure

TN, HQ, and FA spectra

Zero-order absorption spectrum of 10.0 μg/mL of TN, HQ, and FA were obtained by scanning over the range 200–400 nm against methanol as a blank.

Factorized spectrum of HQ

The DD^1 spectrum of HQ using 2 μg/mL MP as a divisor was divided by the recorded amplitudes at 226.5 nm and stored on the computer.

Linearity and calibration graphs

Portions equivalent to 1.00–10.00 μg/mL TN, 4.00–38.00 μg/mL HQ, and 4.00–35.00 μg/mL FA were transferred from working solutions (50.00 μg/mL) for TN, HQ, and FA, into three series of 10 mL volumetric flasks. These were diluted to volume using methanol. The spectra of the prepared standards were scanned against methanol as blank in the range 200–400 nm and saved on the computer.

Calibration graph of TN. Zero-order absorption spectra (D^0) for TN were recorded directly and saved on the computer. The calibration graph was made by relating the λ_{max} of the scanned spectrum at 338 nm to the corresponding concentration.

Calibration graphs of HQ.

DDRD. The absorption spectra of the solutions (prepared at different concentrations of HQ) and the ternary mixture were recorded and divided by the sum of the absorption spectra of solutions of MP and FA (10 μg/mL each in methanol) as "double divisor" to get the ratio spectra. Their derivatives were then plotted at the coincidence point (peak amplitude = 226.7 nm) against the concentrations of the HQ and stored on the computer in order to compute the regression equation.

DD^1. The first derivative spectra of the ratio spectrum resulted from dividing the zero-order absorption spectra (D^0) of HQ by 2.00 μg/mL MP as a divisor. Two regression equations were constructed either by plotting the peak amplitudes at $P_{max\text{-}min}$ ($P_{229.2–221.3}$) or by plotting the amplitude at peak minima 284.4 nm versus the corresponding concentrations of HQ, and the regression equations were computed.

Calibration graphs of FA.

DDRD. The absorption spectra of the solutions (prepared at different concentrations of FA) and the ternary mixture were recorded and divided by the sum of the absorption spectra of solutions of MP and HQ (10.00 μg/mL each in methanol) as "double divisor" to get the ratio spectra. Their first derivatives were then plotted at the coincidence point (peak amplitude = 252.6 nm) against the concentrations of the FA and stored on the computer in order to compute the regression equation.

DD^1. The first derivative of ratio spectra resulted from dividing the zero-order absorption spectra (D^0) of FA by 2.00 μg/mL MP as a divisor and then the peak amplitudes at peak minima 221.4 nm are plotted against the concentrations of FA conducting the regression equation.

Applying the spectrophotometric methods for determining TN, HQ, and FA, in the presence of MP in laboratory-prepared mixtures

The laboratory-prepared mixtures containing different ratios of TN, HQ, and FA in the presence of 2.00 μg/mL MP, were divided by normalized D^0 TN, and then the constant from the extended region 375–395 nm was multiplied by the normalized TN spectrum to get the D^0 spectrum. TN concentration was obtained by substituting the λ_{max} 338 nm in the corresponding regression equation.

The recovered TN spectrum was subtracted from its corresponding mixture to get resolved ternary mixtures of HQ, FA, and MP.

Approach 1. The first derivative of the ratio spectra of the ternary using 2.00 μg/mL MP as a divisor was obtained and the P_{min} 284.4 nm was substituted in the corresponding regression equation to get the HQ concentration.

Approach 2. Dividing the resolved mixture by equimolar (10.00 μg/mL) double divisors (MP + FA) or (MP + HQ), followed by derivatization of the ratio spectra. The concentration of HQ or FA was obtained by substituting the P_{max} 226.7 nm for HQ or P_{max} 252.6 nm for FA in the corresponding regression equations.

Approach 3. The peak amplitude at 226.5 nm of the first derivative of the ratio spectra of the ternary mixture using MP as a divisor, was multiplied by the prepared factorized HQ spectrum to resolve the DD^1 HQ in mixture alone, then substituting $P_{229.2–221.3}$ of the resulted spectrum in the corresponding regression equation to get HQ concentration. Then, by SS of the resolved DD^1 HQ from the first derivative of the ratio spectrum of the resolved ternary mixture (HQ+FA+MP) results in the DD^1 FA, where the P_{min} at 221.4 nm is substituted in the corresponding regression equation to obtain the FA concentration in mixture.

Application to the pharmaceutical formulation

Dosage form extraction. One gram of cream was accurately weighed in a 100 mL beaker and then 40 mL methanol spiked with 1.00 mg standard TN was added with continuous stirring. This was placed in a sonicator in a dark room for 15 min, then the contents of the beaker were transferred to a 100 mL volumetric flask and diluted to 70 mL with methanol. The volumetric flask was then placed in the sonicator in a dark room for 5 min. Methanol was used to dilute to volume. The 100 mL volumetric flask was placed in the refrigerator till the base of the cream agglutinated and precipitated. This was centrifuged at 4°C at a speed of 3500 rpm. The clear supernatant and filter was then removed.

With a micropipette, 0.95 mL were accurately transferred into a 10 mL volumetric flask. Methanol was then used to dilute to volume to obtain a working solution, containing 1.425 μg/mL TN, 38.00 μg/mL HQ, 0.095 μg/mL FA, and 1.71 μg/mL MP. The scanned spectrum was then enriched with 4.00 μg/mL FA by spectrum addition using the Spectra manager software.

Results and Discussion

The four components TN, HQ, FA, and MP are combined in Trimelasma cream. The first challenge arises from the ratio of the components present in the dosage form, where TN: HQ: FA: MP were in the ratio 0.475:38:0.095:1.71. Taking into consideration the linearity range of each drug, it is impossible to determine the two minor components TN and FA simultaneously with the other components unless enrichment of both TN and FA is applied. Since TN is extended in the region 375–400 nm as shown in Figure 2, it is enriched by a standard addition technique, while FA is enriched by a spectrum addition technique as it is severely overlapped with the other components present in the mixture. The accurate addition of 4 μg/mL by the spectrum addition technique revealed more accurate, precise results when applied on the dosage form portion under spectrophotometric determination. Enrichment of the minor component TN by standard addition is applied by adding an exactly known concentration of TN on the portion of cream dosage form to be extracted, and then proceeding in the extraction and dilution steps to get a final concentration of TN within the proposed linearity range of the drug (amount of TN in the cream portion + standard added TN), so the total response can be substituted in the proposed regression equation to get the concentration. To get the actual concentration of TN present in the dosage form portion, the amount of standard added drug is subtracted from the total concentration of the drug found in the analyzed sample. Spectrum addition is an enrichment technique used when one or more components are present in low concentrations in the mixtures and require enrichment. Spectrum addition differs from standard addition techniques in that the standard added drug is in the form of a spectrum that is mathematically added to the dosage form spectrum with the aid of the Spectramanager software. Figure 3 shows the spectrum of the dosage form portion to be analyzed. The advantage of using the SA technique for the enrichment of FA is that it inhibits the interference which varies as the ratio of analyte concentration to sample matrix changes, and can be successfully applied during the manipulation steps of the analysis of ternary mixtures after

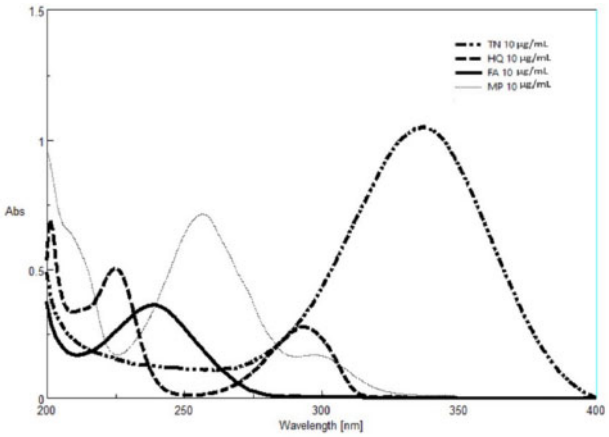

Figure 2. Zero order absorption spectra of TN, HQ, FA, and MP, each 10 µg/mL in methanol.

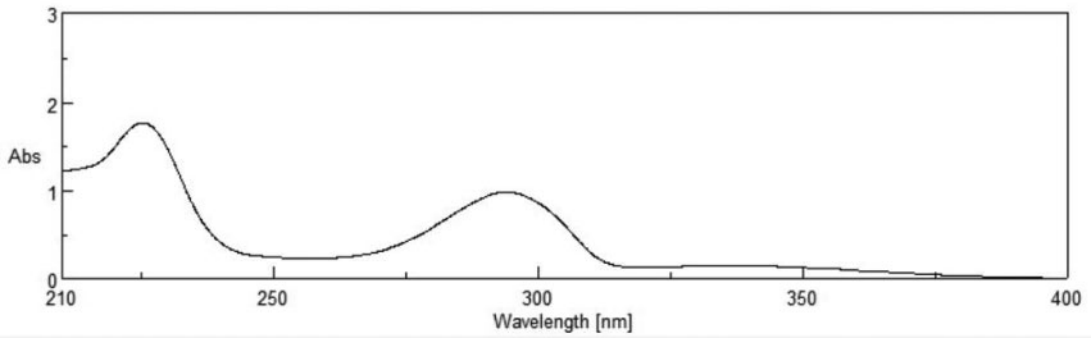

Figure 3. Dosage form extraction solution spectrum after standard addition of 0.95 µg/mL TN and spectrum addition (4 µg/mL) of FA.

resolution in order to enrich the drugs in the resolved binary mixture. It also showed significant advantages over the standard addition technique regarding simplicity, robustness, and little sample preparation.

The second challenge arises from the complex overlap of the four components. As shown in Figure 2 the zero-order absorption spectrum of TN is more extended than HQ in the region 375–400 nm, while the other three components' spectra are severely overlapped in the region 200–350 nm. Constant multiplication (CM) is applied to obtain the D^0 TN, by division of the D° absorption spectrum of the laboratory mixture by the D° spectrum of normalized 1 µg/mL TN, the constant is obtained from the plateau region at 375–400 nm. This constant is then multiplied by the D° absorption spectrum of normalized 1 µg/mL TN to get the D° absorption spectrum of TN present in the mixture. Absorbance at λ_{max} 338 nm is substituted in the regression equation to get TN concentration.

Recovery of the severely overlapped ternary mixture (HQ + FA + MP) is achieved by spectrum subtraction, where the recovered TN spectrum is subtracted from the original spectrum of the whole mixture with the aid of the Spectramanager software. Three approaches based on derivative ratio spectra are applied to resolve the ternary mixture which are DRSZ, DDRD, and FDRM-SS.

Approach 1

DRSZ succeeded in obtaining only the concentration of HQ. As the first derivative of the ratio spectra of the ternary mixture using MP as a divisor show a peak amplitude at 284.4 nm corresponding to HQ where FA has no contribution. HQ concentration is obtained by substitution of the peak amplitude at 284.4 nm in corresponding regression equations. Trials for applying DRSZ to obtain FA concentration were conducted by using the HQ spectrum as a divisor, but they failed to obtain a spectrum where there is a peak corresponding to FA while MP shows a zero-crossing point. So, approaches 2 and 3 were applied.

Approach 2

The double divisor derivative ratio method is applied twice to obtain the concentration of both HA and FA. Dividing the D^0 spectrum of the resolved ternary mixture (FA + HQ + MP) by the equimolar double divisor mixture (MP + HQ; 10 µg/mL each) followed by derivatization, where absorbance at the coincidence point 252.6 nm as shown in Figure 4a is substituted in the regression equation to get FA concentration. Dividing the D^0 spectrum of the resolved ternary mixture (FA + HQ + MP) by the equimolar double divisor mixture (MP + FA) 10 µg/mL each followed by derivatization where absorbance at the coincidence point 226.7 nm as shown in Figure 4b is substituted in the regression equation to get HQ concentration.

Approach 3

FDRM-SS successfully resolved both HQ and FA in their DD^1 forms, allowing each one to be measured separately at its $P_{max\text{-}min}$, giving spectra identical to the DD^1 spectra of the pure drugs, eliminating the contribution of other components in the mixture. It is used to obtain the concentrations of HQ and FA using 2 µg/mL MP as a divisor. Where multiplying the peak amplitude at 226.5 nm of

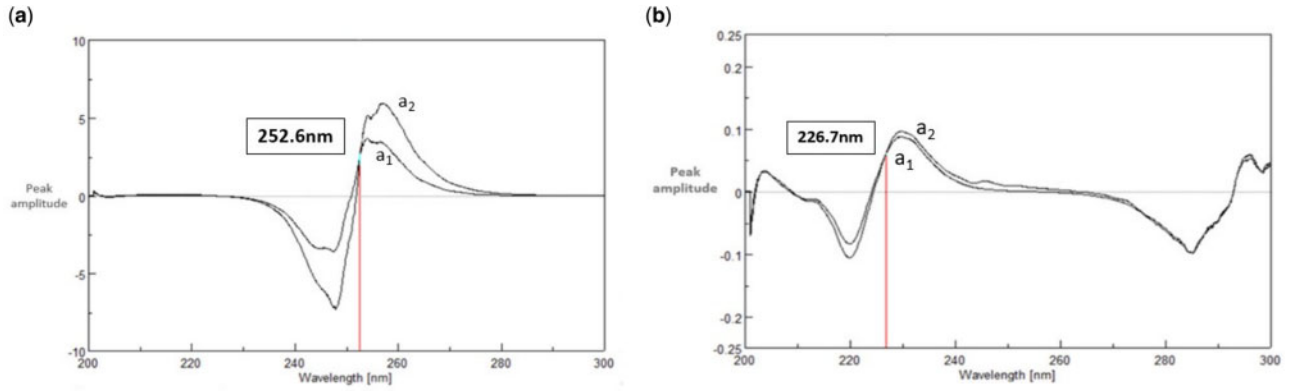

Figure 4. (a) The coincident spectra of the first derivative of the ratio spectra of (a_1) 7 μg/mL pure FA and (a_2) ternary mixture of HQ, FA, and MP each 7 μg/mL, using a mixture of HQ and MP, each 10 μg/mL, as divisor. (b) The coincident spectra of the first derivative of the ratio spectra of (a_1) 7 μg/mL pure HQ and (a_2) ternary mixture of HQ, FA, and MP each 7 μg/mL, using a mixture of FA and MP, each 10 μg/mL, as divisor.

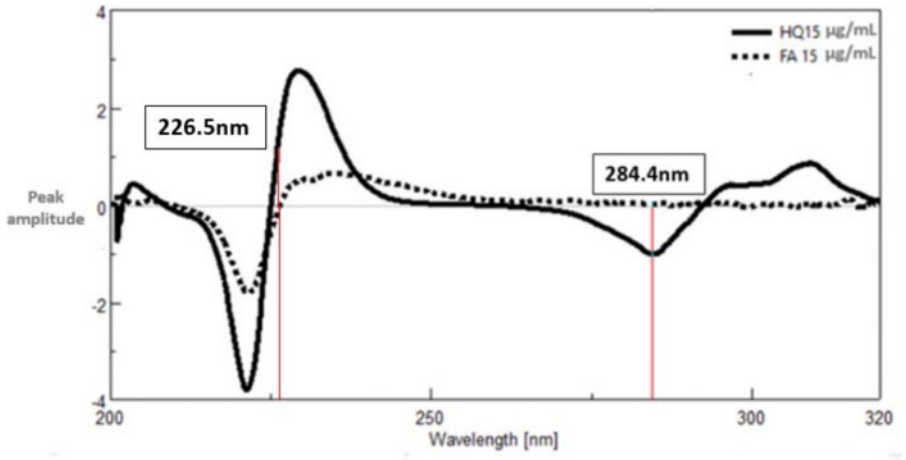

Figure 5. The first derivative of the ratio spectra of HQ and FA (each 15 μg/mL) using MP (2 μg/mL) as a divisor.

the derivative ratio of the resolved mixture of HQ, FA, and MP using 2 μg/mL MP as a divisor (HQ/MP) (at zero crossing of FA) shown in Figure 5 by the factorized spectrum of HQ/MP to get the first derivative of the ratio spectrum of HQ/MP present in mixture, where the $P_{229.2–221.3}$ as shown in Figure 5 is substituted in the corresponding regression equation to obtain HQ concentration. Coupling derivatization with factorized spectra and spectrum subtraction in FDRM-SS, is the best approach for resolving the ternary, severely overlapped mixture of HQ, FA, and MP, as the MP contribution is eliminated by derivatization, DD^1 HQ is obtained by FDRM, and DD^1 FA is obtained by SS.

Validation

Validation of the methods were carried out according to International Conference on Harmonization (ICH) (39) guidelines.

Linearity Range

Linearity was computed by making several calibration curves on three consecutive days. The calibration curves were made within the range of concentrations that were confirmed as having been applied on the dosage form. Every data point was assayed in triplicate. The regression equation parameters and the concentration ranges for the proposed methods are shown in Table 1.

LOD and LOQ

Both LOD and LOQ for the suggested methods were computed as per ICH guidelines (39) and the values are shown in Table 1. LOD and LOQ were computed utilizing the slope of the calibration curve and standard deviations of the regression line residuals. The sensitivity of the nominated methods is affirmed by low values of LOD and LOQ.

Accuracy

It is the measure of exactness of the proposed method or the closeness of agreement between the measured value and the claimed one expressed by recovery % with an accepted range of (98–102%).

The results were found to be accurate by implementing the suggested methods for assessment of five blind samples (three replicates each) of TN, HQ, and FA within linearity ranges. Concentrations were achieved and the favorable percentage recoveries imply good recovery within the accepted range as listed in Table 1.

Table 1. Assay parameters and method validation obtained by applying the proposed spectrophotometric methods

Parameter	TN	HQ			FA	
	D^0 338 nm	DDRD $P_{226.7\,nm}$	DD^1 $P_{229.2–221.3\,nm}$	DD^1 $P_{284.4\,nm}$	DDRD $P_{252.6\,nm}$	DD^1 $P_{221.4\,nm}$
Range	1–10 μg/mL		4–38 μg/mL		4–35 μg/mL	
Slope	0.1031	0.0082	0.4321	0.0678	0.3559	0.1175
Intercept	0.0125	0.0011	0.0386	0.0037	0.121	0.0399
Correlation coefficient (r)	0.9999	0.9999	1.0000	1.0000	0.9999	0.9999
Accuracy, mean ±SD	99.70 ± 0.83	100.08 ± 1.09	100.19 ± 0.84	99.93 ± 0.74	99.98 ± 0.48	100.00 ± 0.48
Intra-day precision (RSD)[a]	0.600	0.569	0.697	0.641	0.198	0.373
Inter-day precision (RSD)[a]	0.717	0.915	1.338	1.312	0.696	1.036
LOD	0.08	0.37	0.18	0.30	0.48	0.48
LOQ	0.24	1.13	0.54	0.90	1.45	1.45

[a] Both intra-day ($n = 3$) and inter-day relative standard deviations were held of concentrations 4, 5, 10 μg/mL for TN, 4, 5, 12 μg/mL for HQ, and 4, 10, 15 μg/mL for FA.

Table 2. Determination of TN, HQ, and FA in laboratory-prepared mixtures using the proposed spectrophotometric methods

Concn, μg/mL	TN	HQ			FA	
	CM D^0 338nm	DDRD $P_{226.7nm}$	FDRM-SS $P_{229.2–221.3nm}$	DRSZ $P_{284.4nm}$	DDRD $P_{252.6nm}$	FDRM-SS $P_{221.4nm}$
Lab mixture concentration (TN:HQ:FA)			Recovery % ± SD			
6:5:10	100.06 ± 0.17	101.77 ± 0.26	101.86 ± 0.14	100.36 ± 0.25	98.26 ± 0.48	98.81 ± 0.14
5:7:7	99.80 ± 0.16	100.39 ± 0.29	100.29 ± 0.20	98.34 ± 0.20	98.30 ± 0.29	98.81 ± 0.21
4:15:7	101.12 ± 0.28	101.58 ± 0.30	100.55 ± 0.36	98.18 ± 0.47	98.33 ± 0.31	100.88 ± 0.38
7:7:7	100.05 ± 0.11	100.40 ± 0.29	100.50 ± 0.20	98.54 ± 0.20	98.41 ± 0.30	99.02 ± 0.21
1.425:38:4.095[a]	101.96 ± 0.15	100.73 ± 0.16	98.92 ± 0.05	99.81 ± 0.05	99.51 ± 0.17	100.12 ± 0.22
Dosage form batch No.[b] Dosage form			Found % ± SD			
ratio (0.475:38:0.095)	98.94 ± 0.71	98.64 ± 0.01	100.39 ± 0.00	101.29 ± 0.00	101.47 ± 0.34	99.32 ± 0.14

[a] Dosage form ratio mixture after standard addition of 0.95 μg/mL TN and spectrum addition of 4 μg/mL FA.
[b] After subtraction of the 0.95 μg/mL TN and 4 μg/mL FA.

Precision

Intra-day and inter-day precision for the methods were tested by assessing three different concentrations per drug, repeated on the same day or on three consecutive days, within the linearity range. Results are listed in Table 1.

Specificity

Specificity was ascertained by analyzing different laboratory-prepared mixtures of TN, HQ, FA, in different ratios within the linearity range in the presence of MP. The mean ± SD demonstrated good percentage recovery with the lowest standard deviation among the other methods. Satisfactory results are shown in Table 2.

Results for analyzing the four components in Trimelasma cream are shown in Table 2, in addition to the results obtained from the standard and spectrum addition techniques for both TN and FA, respectively. The results showed that the proposed methods are adequately accurate and there is no interference of pharmaceutical excipients.

Statistical Comparison

Statistical results achieved by evaluating the suggested spectrophotometric methods versus the USP and BP official methods for the three drugs under investigation are shown in Table 3. There is no significant difference found in the suggested methods versus the official methods through computing both *F* and *t*-tests. One-way analysis of variance (ANOVA) was implemented as shown in Table 4 and all the methods showed no significant difference upon testing the proposed and the official methods for assessing TN, HQ, and FA.

Conclusions

Enrichment of minor components was successfully achieved by standard and spectrum addition techniques. Mathematical filtration steps on the spectrophotometric spectra successfully resolved the partial overlap of the extended component (TN). A comparative study for the simultaneous determination of the components of the ternary severely overlapped mixture was achieved by DRSZ, DDRD, and FDRM-SS. FDRM-SS succeeded in obtaining both FA and HQ and eliminated any contribution from the preservative. It also allows each one to be measured separately at its $P_{max-min}$, showing maximum accuracy and precision. It also resulted in spectra identical to the DD^1 spectra of the pure drugs, eliminating the contribution of other components in the mixture. Accordingly, the suggested methods can be successfully applied for routine analysis of the studied drugs, either in their pure bulk powders or pharmaceutical formulations, in quality control laboratories.

Table 3. Statistical comparison between results obtained by the proposed methods and the USP (1) official methods for determination of both TN and HQ, and BP (2) official method for FA in pure powder form

	TN		HQ				FA		
Parameters	D^0 338 nm	USP official method[a]	DDRD $P_{226.7\,nm}$	DD^1 $P_{229.2–221.3\,nm}$	DD^1 $P_{284.4\,nm}$	USP official method	DDRD $P_{252.6\,nm}$	DD^1 $P_{221.4\,nm}$	BP official method[b]
Mean	100.12	100.22	100.31	99.88	99.70	100.33	99.90	99.92	99.99
SD	1.05	0.78	0.73	0.46	0.88	0.53	0.53	0.53	0.53
N[d]	6	6	6	6	6	6	6	6	6
Variance	1.1025	0.6084	0.5329	0.2116	0.7744	0.2809	0.2809	0.2809	0.2809
F test (5.05)[c]	1.81	—	1.90	1.33	2.76	—	1.00	1.00	—
Student's *t*-test (2.23)[c]	0.03	—	0.07	1.57	1.51	—	0.31	0.23	—

[a] Volumetric analysis by titration.
[b] Direct spectrophotometric determination: measuring the maximum absorbance at 238 nm.
[c] The figures in parentheses are the corresponding theoretical values at $P = 0.05$.
[d] N = Number of samples.

Table 4. One-way ANOVA testing for the different proposed methods and the USP (1) official methods used for the determination of both TN and HQ, and BP (2) official method for FA in pure powder form

	Source of variation	Sum of squares	Degrees of freedom	Mean square	*F*	*F* critical
TN	Between groups	0.001	1.00	0.001	0.001	4.96
	Within groups	8.55	10.00	0.86		
	Total	8.56	11.00			
HQ	Between groups	1.77	3.00	0.59	1.31	3.10
	Within groups	9.01	20.00	0.45		
	Total	10.79	23.00			
FA	Between groups	0.03	2.00	0.01		
	Within groups	4.22	15.00	0.28	0.05	3.68
	Total	4.25	17.00			

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