Development of normalized spectra manipulating spectrophotometric methods for simultaneous determination of Dimenhydrinate and Cinnarizine binary mixture
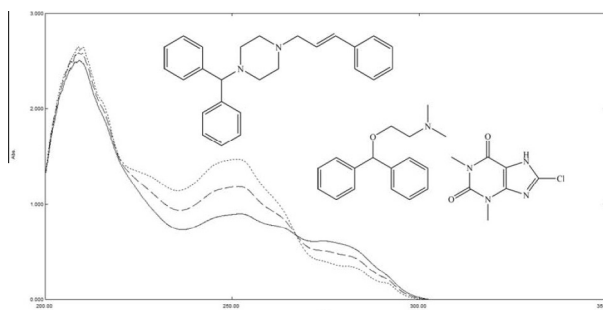
Nesrine T. Lamie, Ali M. Yehia *

Analytical Chemistry Department, Faculty of Pharmacy, Cairo University, El-Kasr El-Aini Street, 11562 Cairo, Egypt

HIGHLIGHTS

- Completely overlapped spectra were smartly resolved using normalized spectra.
- Novel techniques utilizing the division spectra for simultaneous determination of binary mixture.
- Advantages of the proposed methods over the conventional spectrophotometric techniques.

GRAPHICAL ABSTRACT

Normalized spectrum was utilized as a divisor for simultaneous determination of Dimenhydrinate and Cinnarizine binary mixture with minimum manipulation steps. The proposed methods were simultaneous constant center, simultaneous derivative ratio spectrophotometry and ratio H-point standard addition method.

ARTICLE INFO

ABSTRACT

Simultaneous determination of Dimenhydrinate (DIM) and Cinnarizine (CIN) binary mixture with simple procedures were applied. Three ratio manipulating spectrophotometric methods were proposed. Normalized spectrum was utilized as a divisor for simultaneous determination of both drugs with minimum manipulation steps. The proposed methods were simultaneous constant center (SCC), simultaneous derivative ratio spectrophotometry (S^1DD) and ratio H-point standard addition method (RHPSAM). Peak amplitudes at isoabsorptive point in ratio spectra were measured for determination of total concentrations of DIM and CIN. For subsequent determination of DIM concentration, difference between peak amplitudes at 250 nm and 267 nm were used in SCC. While the peak amplitude at 275 nm of the first derivative ratio spectra were used in S^1DD; then subtraction of DIM concentration from the total one provided the CIN concentration. The last RHPSAM was a dual wavelength method in which two calibrations were plotted at 220 nm and 230 nm. The coordinates of intersection point between the two calibration lines were corresponding to DIM and CIN concentrations. The proposed methods were successfully applied for combined dosage form analysis, Moreover statistical comparison between the proposed and reported spectrophotometric methods was applied.

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* Corresponding author. Tel.: +20 2 25353400; fax: +20 2 23628246.
  E-mail addresses: aliyehia00@yahoo.com, ali.yehia@pharma.cu.edu.eg (A.M. Yehia).

Introduction

Dimenhydrinate (DIM); 2-benzhydryloxy-N,N dimethyl-ethanamine; 8-chloro-1,3-dimethyl-7H-purine-2,6-dione [1], Fig. 1a. It is 8-chlorotheophilline salt of diphenhydramine and used to prevent motion sickness [2]. Cinnarizine (CIN); 1-(diphenylme thyl)-4-(3-phenyl-2-propenyl) piperazine [1], Fig. 1b. It is a piperazine derivative with antihistamine, sedative and calcium-channel blocking activities. CIN is used for the symptomatic treatment of nausea and vertigo caused by Ménière's disease and other vestibular disorders, it is also used for prevention and treatment of motion sickness [3]. The combination therapy of both drugs is recommended as it showed the lowest rate of adverse events compared to single Dimenhydrinate or placebo [4].

Dimenhydrinate and Cinnarizine are official in British Pharmacopeia and each of them was determined by potentiometric titration and liquid chromatographic method [5]. In addition, some spectrophotometric [6,7] chromatographic [8–11] methods have been reported in the literature for the determination of either DIM or CIN in pharmaceutical formulations, biological samples and with other drugs in combination. Few methods have been reported for analysis of the cited drugs in their binary mixture, including classical derivative, derivative ratio, Q absorbance ratio and bivariate spectrophotometric methods [12–14] and other chromatographic [15,16] methods. Although the previously reported spectrophotometric methods could determine DIM and CIN in binary mixtures, they required several manipulation steps. Moreover, repetition of manipulation procedure should be applied in order to determine each drug in the binary mixture as in derivative ratio spectrophotometric method.

The aim of this work was to use the normalized spectra to resolve the sever spectral overlap of DIM and CIN binary mixture. Novel spectrophotometric methods were developed, namely, simultaneous constant center (SCC), simultaneous derivative ratio (S^1DD) and ratio H point standard addition method (RHPSAM). The proposed spectrophotometric methods could be easily used accurately and precisely for simultaneous determination of the studied binary mixture with simple manipulation procedures.
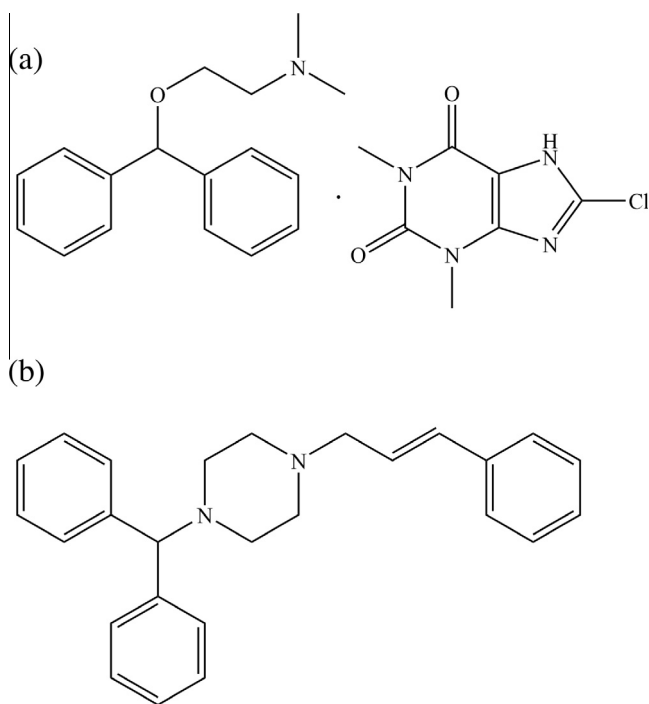

Fig. 1. Chemical structures of (a) Dimenhydrinate and (b) Cinnarizine.

Experimental

Apparatus and software

A dual beam Shimadzu (Kyoto/Japan) UV–vis. spectrophotometer, model 1650 UV-PC. The bundle software, UV PC personal spectroscopy software version 3.7 (SHIMADZU) was used to process absorption and derivative spectra, scans were carried out in the range from 200 nm to 400 nm at 0.1 nm intervals using 1.00 cm quartz cells.

Materials and reagents

Pure standards were kindly supplied by Amoun Pharmaceutical Company, Cairo, Egypt. Their purity were found to be 100.80 ± 1.245 and 100.24 ± 0.961, for DIM and CIN, respectively, according to the reported spectrophotometric method [12].

Amocerebral plus® tablets; Batch No. 124820, were kindly supplied by Amoun Pharmaceutical Company, Cairo, Egypt. Tablets are claimed to contain 40 mg Dimenhydrinate and 20 mg Cinnarizine per tablet.

Methanol; spectrophotometric grade was purchased from E. Merck, Darmstadt, Germany.

Solutions

Stock standard solutions (1.0 mg mL^{-1})

Stock standard solutions were prepared by accurately weighing 50.0 mg of DIM and CIN bulk powder into two separate 50-mL volumetric flasks; 25.0 mL methanol was added in each, shaken for few min and the volumes were made up to the mark with methanol.

Working standard solutions (100.0 µg mL^{-1})

Working standard solutions were prepared by transferring 5.0 mL of the drugs stock standard solutions (1.0 mg mL^{-1}), separately, into two 50-mL measuring flasks and the volumes were made up to the mark with methanol.

Laboratory prepared mixture solutions

Laboratory mixtures of DIM and CIN were prepared by transferring different aliquots from their respective stock standard solutions (1.0 mg mL^{-1}) equivalent to 1.0, 1.5 and 2.0 mg of DIM and 0.8, 1.0 and 1.2 mg of CIN into nine separate 10-mL volumetric flasks. The prepared mixtures were containing different ratios of both drugs surrounding the dosage form.

Pharmaceutical formulation solutions

Ten tablets of Amocerebral plus® were weighted and finely powdered. Amounts of powdered tablets equivalent to 40.0 mg of DIM and 20.0 mg of CIN were accurately weighted and transferred into separate 100-mL beakers; sonicated in 50 mL methanol for 10 min and filtered into 100-mL volumetric flasks. Every residue was washed three times each using 10 mL methanol and the solutions were completed to the mark with the same solvent.

Procedure

Spectral characteristics of DIM and CIN normalized spectra

Normalized spectra for DIM and CIN were computed from the scanned zero order absorption spectra (^0D) of several concentrations for each drug using methanol as a blank. The scanning range was 200–350 nm. Then the average of each component's normalized spectra were obtained.
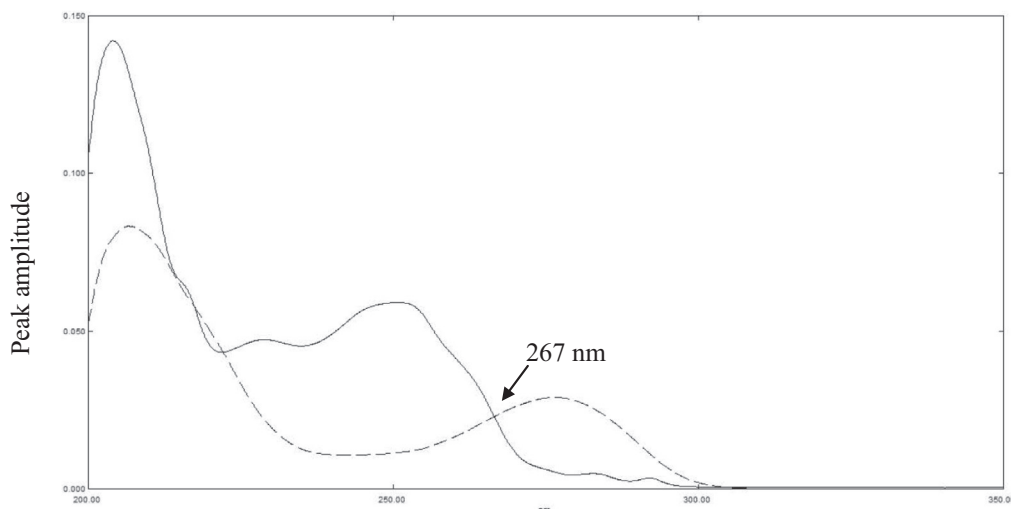
Fig. 2. Normalized spectra of DIM (—) and CIN (-----) in methanol, showing an isoabsorptive point at 267 nm.

Construction of calibration curves and assay of blind pure samples

Aliquots equivalent to 100.0–450.0 μg of DIM and 40.0–200.0 μg of CIN were accurately transferred from their corresponding working standard solutions (100.0 μg mL^{-1}), into two separate series of 10-mL volumetric flasks and the volumes were completed with methanol. The zero order absorption spectra (^{0}D) of the two drugs were recorded against methanol as a blank. Ratio spectra of both components were obtained by dividing their recorded spectra by the average normalized CIN spectrum.

Simultaneous constant center "SCC". The peak amplitudes at 267 nm (isoabsorptive point) were measured for DIM and CIN ratio spectra. In addition, the peak amplitudes of ratio spectra at 250 nm were measured for only DIM. Calibration plots were constructed for DIM and CIN using peak amplitudes at 267 nm; while the difference between the two amplitudes at 250 nm and 267 nm were plotted against the corresponding peak amplitude at 267 nm for only DIM. The regression parameters were computed for each calibration plot.

Blind samples of DIM and CIN were prepared and the zero order absorption spectrum of each sample was recorded against methanol as a blank, then divided by the normalized CIN spectrum. The peak amplitudes at 267 nm were measured for the determination of both drugs' concentrations, while difference between peak amplitudes at 250 nm and 267 nm were calculated in order to determine DIM concentrations.

Simultaneous derivative ratio "S^{1}DD". The first derivative of DIM ratio spectra were obtained, using scaling factor = 1 and $\Delta\lambda = 4$ nm then the peak amplitudes at 275 nm were plotted against its corresponding concentrations and the regression parameters were computed.

Blind samples of DIM were prepared and the zero order absorption spectra were recorded against methanol as a blank, then divided by the normalized CIN spectrum to obtain ratio spectra. The first derivative of these ratio spectra were calculated using the previously described parameters. The peak amplitudes at 275 nm were measured for the determination of DIM concentrations.

Ratio H-point standard addition method "RHPSAM". The peak amplitudes of ratio spectra were measured at 220 nm and 230 nm for both DIM and CIN division spectra using normalized CIN as a devisor. The peak amplitudes were plotted against the corresponding concentrations and the regression parameters were computed.

Assay of DIM and CIN pure samples were performed after standard addition of different aliquots of DIM working standard solutions (100.0 μg mL^{-1}) on each sample, separately. The volumes were completed to the mark with methanol and the zero order absorption spectra of standard addition series for each sample were recorded against methanol as a blank. The recorded spectra were divided by the normalized CIN spectrum and peak amplitudes at 220 nm and 230 nm were measured then plotted against the corresponding added DIM concentrations. Two regression equations were computed at the two specified wavelengths. Then these regression parameters were used for the determination of DIM and CIN concentrations in their pure samples.

Assay of laboratory prepared mixtures

For SCC and S^{1}DD methods, tenfold dilutions were applied to the synthetic mixtures and the zero order absorption spectra were recorded against methanol as a blank. After obtaining the ratio spectra using CIN normalized spectrum as a divisor, peak amplitudes at 267 nm were measured for the determination of total DIM and CIN concentrations. For the determination of DIM concentrations; difference between peak amplitudes at 250 nm and 267 nm of the ratio spectra were used in SCC. While, peak amplitudes at 275 nm of the obtained derivative ratio spectra were used in S^{1}DD. Concentrations of CIN in mixtures were calculated by subtraction.

On the other hand, 1.0 mL aliquots were transferred from the previously prepared synthetic mixtures into separate sets of 10-mL volumetric flasks and then standard additions of DIM working standard solution were applied to those sets. The volumes were completed to the mark with methanol. The procedure of RHPSAM, previously stated under the calibration was followed in order to determine the concentrations of DIM and CIN.

Assay of pharmaceutical formulation

Aliquots equivalent to 0.5 mL were transferred from pharmaceutical dosage form solution into 10-mL volumetric flasks, the volumes were completed to the mark with methanol and measured directly to determine the DIM and CIN concentrations using SCC and S^{1}DD method. While to apply RHPSAM, standard additions of DIM working solution were applied to the transferred aliquots, and the volumes were completed to the mark with methanol.
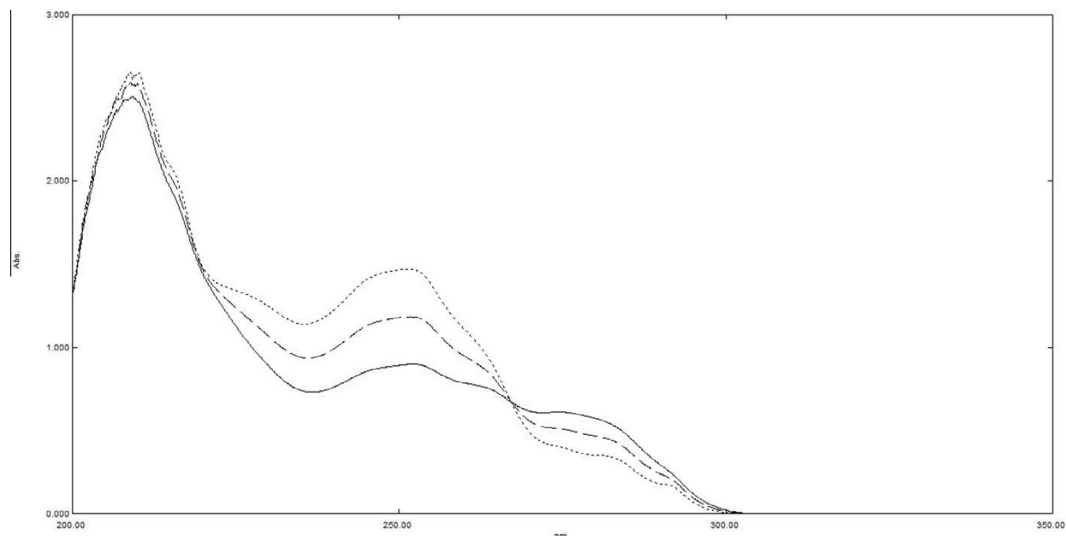
Fig. 3. Scanned spectra of three binary mixtures of DIM and CIN having a concentration of DIM 10 µg mL^{-1} + CIN 20 µg mL^{-1} (—), DIM 15 µg mL^{-1} + CIN 15 µg mL^{-1} (– – – –) and DIM 20 µg mL^{-1} + CIN 10 µg mL^{-1} (………).
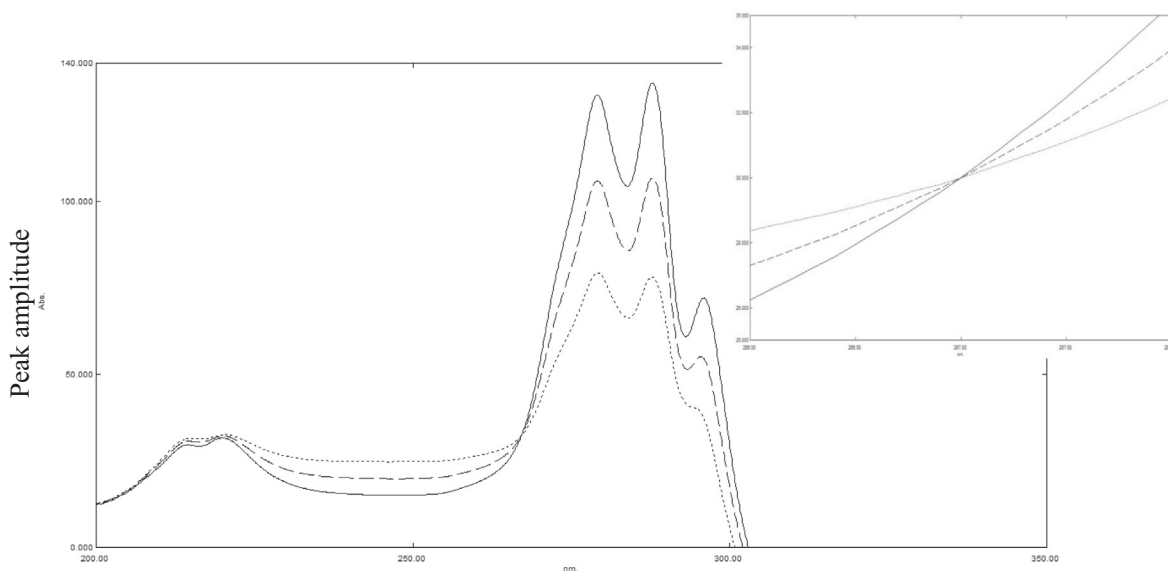
Fig. 4. Division spectra of three binary mixtures of DIM and CIN having a concentration of DIM 10 µg mL^{-1} + CIN 20 µg mL^{-1} (—), DIM 15 µg mL^{-1} + CIN 15 µg mL^{-1} (– – – –) and DIM 20 µg mL^{-1} + CIN 10 µg mL^{-1} (………), using the normalized CIN spectrum as a divisor.

Then the parameters of the two regression equations were computed and used to determine the concentrations of each drug.

Results and discussion

Spectrophotometric analysis of pharmaceutical combinations with severely overlapped spectra are always a challenging task. Although the simplicity of spectroscopic application, sometimes it fails to resolve spectral overlap of two components; especially when there is no extended spectrum of one component over the other. Most of the cited ratio manipulating spectrophotometric methods were repeating special procedure for the determination of one component using the other component's spectrum as a divisor. Obviously, these were laborious procedures for the determination of binary mixtures. In contrast, the proposed methods offered short time analysis with moderate manipulation steps for the simultaneous determination of DIM and CIN binary mixtures.

A representative approach to test the applicability of spectrophotometric methods is the normalized spectra, which are curves representing the absorptivity of components over the whole range of wavelengths, rather that their corresponding absorbance. Fig. 2, represents the overlaid average normalized spectra of DIM and CIN, the figure showed that both DIM and CIN normalized spectra were intersected at certain wavelength (267 nm); at which both drugs were having the same absorptivity (isoabsorptive point). Importance of normalized spectrum was emphasized when used as a divisor, since it generated a constant line in the ratio spectra. Numerically, this constant was the concentration of the component used as a divisor. For the studied DIM "component X" and CIN "component Y" binary mixture, the Absorbance (A_λ) at a given wavelength (λ) was represented as follow
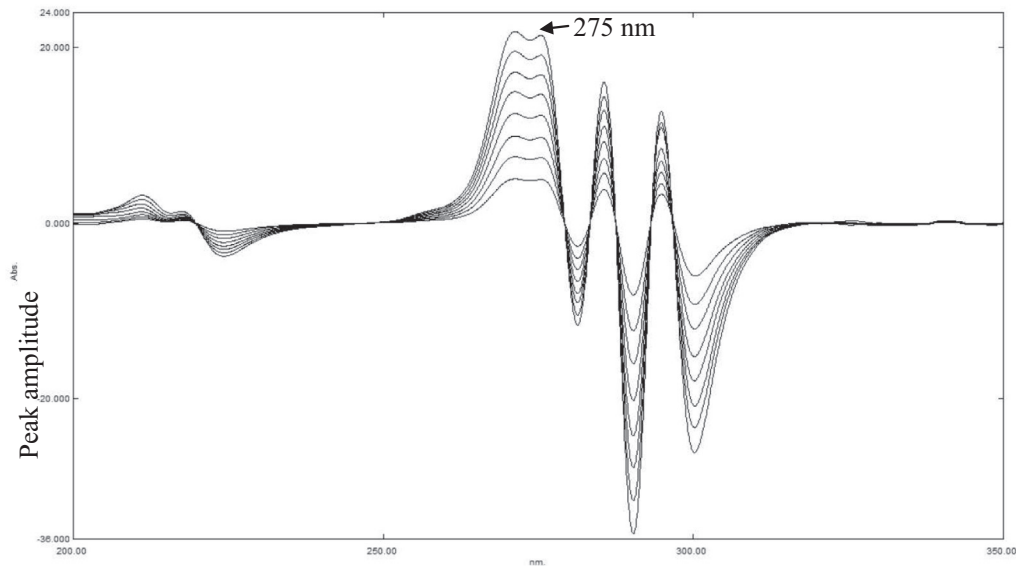
Fig. 5. First derivative of ratio spectra of DIM (10–45 µg mL^{-1}) using normalized CIN spectrum as a divisor.
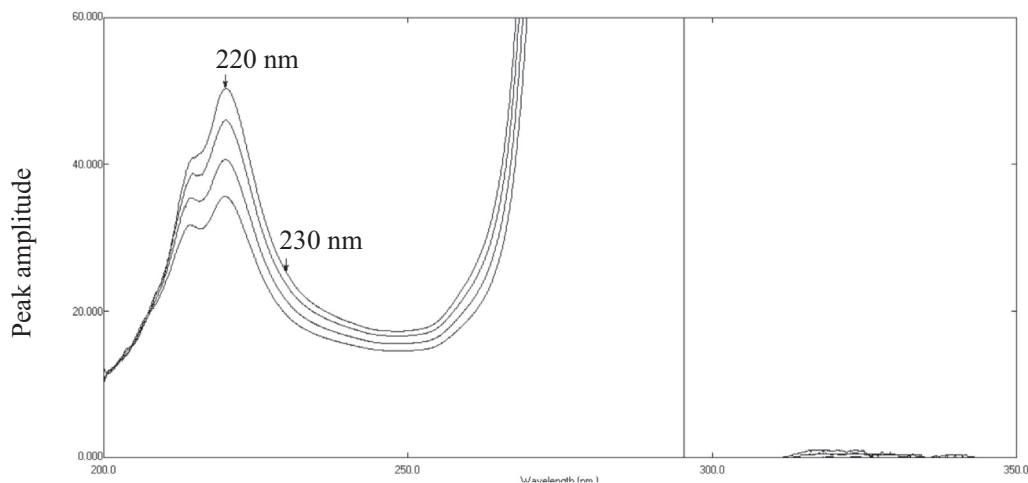

Fig. 6. Ratio spectra of synthetic mixture of DIM 15.0 µg mL^{-1} and CIN 10.0 µg mL^{-1}, after addition of different DIM concentrations (10.0–25.0 µg mL^{-1}), using normalized CIN spectrum as a divisor.

$$A_{\lambda} = a_{X\lambda}C_X + a_{Y\lambda}C_Y \tag{1}$$

where $a_{X\lambda}$ and $a_{Y\lambda}$ were the absorptivities of DIM and CIN, respectively. While C_X and C_Y were the concentrations of DIM and CIN, respectively, in the specified mixture.

As previously implied, the ratio spectra obtained using the normalized spectrum of one component as a divisor generated a constant value of its concentration, therefore, dividing Eq. (1) by the normalized spectra of CIN "$a_{Y\lambda}$", Eq. (2) was obtained

$$P_{\lambda} = (a_{X\lambda}C_X/a_{Y\lambda}) + (a_{Y\lambda}C_Y/a_{Y\lambda}) \tag{2}$$

$$P_{\lambda} = (a_{X\lambda}C_X/a_{Y\lambda}) + C_Y \tag{3}$$

Unfortunately in case of DIM and CIN binary mixture, there was no extension of CIN spectrum which prevented the direct determination of its concentration "C_Y". Eq. (3) provided that CIN concentration was a constant value prevailing the whole spectrum, and the upcoming methods were aiming to extract both concentrations of DIM "C_X" and CIN "C_Y".

Simultaneous constant center "SCC"

Theory of SCC based on modification of constant center (CC) method [17,18] in which contribution of component X in the ratio spectrum was obtained from a calibration plot of the difference between peak amplitudes at two wavelength against postulated amplitudes at one of these two wavelengths. The proposed modification was the use of normalized spectrum of component Y as a divisor, therefore the amplitude at isoabsorptive point (267 nm) was actually the concentration of DIM and/or CIN. Direct modulation of the mixture absorbance at isoabsorptive point into its total components' concentration was the core of amplitude modulation method [19,20]. Applying Eq. (3) at the isoabsorptive point (λ = 267 nm)

$$P_{267} = (a_{X267}C_X/a_{Y267}) + C_Y \tag{4}$$

as a_{X267} and a_{Y267} were equal at isoabsorptive point, therefore the following equation was concluded
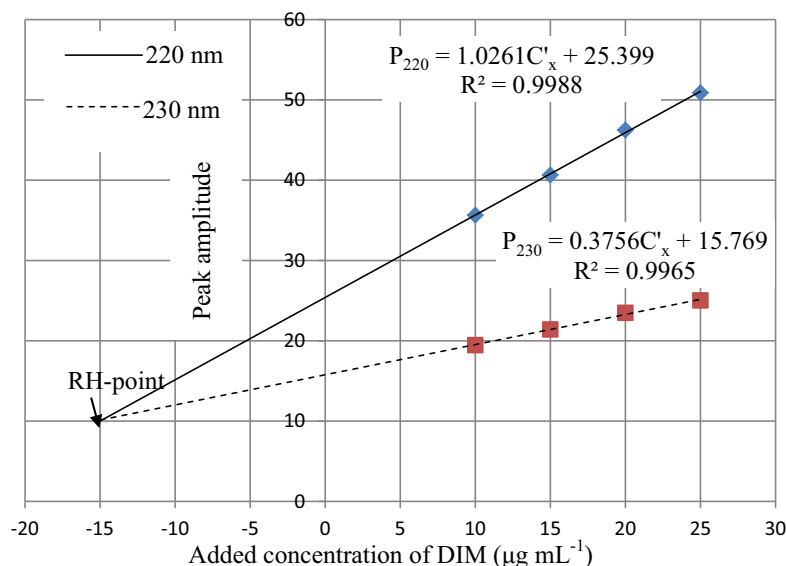
$$P_{267} = C_X + C_Y \tag{5}$$

Fig. 7. Plot of ratio H-point standard addition method between the peak amplitudes and the added DIM concentrations to synthetic mixture containing 15.0 µg mL^{-1} DIM and 10.0 µg mL^{-1} CIN.

Table 1
Regression and validation parameters for determination of DIM and CIN in bulk powder and results of application analysis by the proposed methods.

Parameter	DIM				CIN			
	SCC	S^{1}DD	RHPSAM		SCC[a]	S^{1}DD[b]	RHPSAM	
	250–267 nm	275 nm	220 nm	230 nm	267 nm		220 nm	230 nm
Linearity Range (µg mL^{-1})	10–45				4–20			
Slope	0.8159	0.4678	1.0432	0.3925	0.9406		0.9808	0.9820
SE of Slope	0.0021	0.0035	0.0106	0.0031	0.0091		0.0087	0.0086
Intercept	−0.2709	0.4920	1.6354	0.5478	0.5666		0.1778	0.1657
SE of intercept	0.0632	0.1038	0.3152	0.0920	0.1183		0.1138	0.1127
Correlation coefficient (r)	0.9999	0.9998	0.9997	0.9998	0.9997		0.9997	0.9997
LOD (µg mL^{-1})	0.26	0.73	0.99	0.77	0.42		0.38	0.38
LOQ (µg mL^{-1})	0.77	2.22	3.02	2.34	1.26		1.16	1.15
Accuracy (Mean ± RSD)	100.07 ± 0.469	99.87 ± 0.930	99.90 ± 1.160		99.96 ± 1.068		99.92 ± 1.290	
Selectivity (Mean ± RSD)	99.92 ± 1.156	100.02 ± 1.139	99.52 ± 0.911		100.08 ± 1.748	100.14 ± 1.815	100.03 ± 1.079	
Precision (RSD) Repeatability Reproducibility	±0.831 ±0.942	±0.509 ±0.517	±0.365 ±0.422		±0.407 ±0.490		±0.481 ±0.523	
Application Analysis (Mean ± RSD)	100.80 ± 1.788	99.98 ± 0.988	100.38 ± 0.546		100.07 ± 1.000	101.70 ± 1.561	100.47 ± 0.903	
Application of Standard addition (Mean ± RSD)	99.33 ± 1.753	99.48 ± 1.511	–		100.54 ± 1.564	100.17 ± 2.078	–	

[a] Results obtained by subtracting total mixture concentration at 267 nm from DIM concentration obtained by SCC method.
[b] Results obtained by subtracting total mixture concentration at 267 nm from DIM concentration obtained by S^{1}DD method.

Eq. (5) justified that the measured peak amplitude at 267 nm "P_{267}" provided the total concentration of DIM and CIN in the mixture. (Fig. 3) showed an isoabsorptive point at 267 nm, where intersection of the scanned zero order spectra of three mixtures having the same total concentration of DIM and CIN was observed. In order to justify Eq. (5) the ratio spectra of those mixtures using the normalized CIN spectrum were calculated, (Fig. 4). The figure showed that peak amplitudes were corresponding to the total concentration of DIM and CIN present in mixtures (30 µg mL^{-1}) at 267 nm. Correlation between peak amplitudes at 267 nm and the corresponding concentrations of CIN were plotted. Total concentration of drugs was calculated using these regression parameters.

Back to SCC, when the amplitude of the ratio spectra at the isoabsorptive point was used as the postulated one, then apparently this calibration plot represented the relation between amplitude difference at two wavelengths and DIM concentrations "C_X" rather than the meaningless amplitude value in the conventional constant center method. This modulation permitted the simple and direct determination of DIM.

The mathematical representation of ratio spectrum at 250 nm was presented as follow

$$P_{250} = \left(\frac{a_{X250}C_X}{\mathbf{a}_{\mathbf{Y250}}} \right) + C_Y \tag{6}$$

Difference between peak amplitude at 250 nm and 267 nm was calculated as follow

$$\Delta P_{(250-267)} = \left[\left(\frac{a_{X250}}{\mathbf{a}_{\mathbf{Y250}}} \right) - 1 \right] C_X \tag{7}$$

Eq. (7) represented a calibration plot between the difference in peak amplitudes "$\Delta P_{(250-267)}$" and the concentration of DIM "C_X", with a slope "$(a_{X250}/\mathbf{a}_{\mathbf{Y250}}) - 1$". This calibration was used to calculate the concentration of DIM in its binary mixture with CIN.

Table 2
Results of several experiments for the analysis of synthetic mixtures at different concentration ratios of DIM and CIN by the proposed RHPSAM.

Mix No.	Regression equation[a]	R	Taken (µg mL^{-1})		Found (µg mL^{-1})	
			DIM	CIN	DIM	CIN
1	$P_{220} = 1.0162\ C'_X + 17.958$ $P_{230} = 0.3921\ C'_X + 11.83$	0.9998 0.9997	10.00	8.00	9.82	7.98
2	$P_{220} = 1.0146\ C'_X + 23.118$ $P_{230} = 0.3923\ C'_X + 13.802$	0.9998 0.9996	15.00	8.00	14.97	7.93
3	$P_{220} = 1.0181\ C'_X + 28.427$ $P_{230} = 0.3834\ C'_X + 15.64$	0.9998 0.9995	20.00	8.00	20.15	7.92
4	$P_{220} = 1.0286\ C'_X + 20.324$ $P_{230} = 0.3819\ C'_X + 13.844$	0.9997 0.9994	10.00	10.00	10.02	10.01
5	$P_{220} = 1.0261\ C'_X + 25.399$ $P_{230} = 0.3756\ C'_X + 15.769$	0.9998 0.9994	15.00	10.00	14.80	10.21
6	$P_{220} = 1.0178\ C'_X + 30.581$ $P_{230} = 0.3777\ C'_X + 17.7$	0.9998 0.9998	20.00	10.00	20.12	10.10
7	$P_{220} = 1.0192\ C'_X + 22.012$ $P_{230} = 0.3992\ C'_X + 15.83$	0.9999 0.9997	10.00	12.00	9.97	11.85
8	$P_{220} = 1.0221\ C'_X + 27.14$ $P_{230} = 0.3971\ C'_X + 17.864$	0.9996 0.9995	15.00	12.00	14.84	11.97
9	$P_{220} = 1.0205\ C'_X + 32.24$ $P_{230} = 0.3943\ C'_X + 19.868$	0.9998 0.9997	20.00	12.00	19.76	12.08

[a] In regression equations 'C'_X' was the added concentration of DIM.

Simple subtraction of the obtained concentration of DIM from the total concentration of both drugs obtained at the isoabsorptive point, yielded the concentration of CIN "C_Y" in the specified mixture.

Calibration plot was performed between the difference between peak amplitudes at 250 nm and 267 nm on y-axis and the corresponding peak amplitudes at 267 nm (postulated amplitudes) on x-axis, from which the concentration of DIM was determined.

Simultaneous derivative ratio spectrophotometry "S^{1}DD"

Originally, derivative ratio spectrophotometry (^{1}DD) method was developed by Salinas et al. [21] to remove the interference of one component and determine the other. This method was then modulated to be simultaneous by coupling with amplitude modulation theory to generate simultaneous derivative ratio method (S^{1}DD) [20]. In S^{1}DD; before the derivatization step took place, the total concentration was firstly determined at the isoabsorptive point of the ratio spectrum. Then derivative of these ratio spectra was obtained to remove the constant generated in the division spectrum. Moreover, optimization of the proposed method was

more simple than the traditional ^{1}DD method, in which the influence of the divisor concentration should be examined for minimum noise and better sensitivity. However, the use of normalized spectrum as a divisor in S^{1}DD eliminated this optimization step, since normalized spectrum was a concentration independent spectrum.

A constant value "C_y" in Eq. (3) was eliminated by obtaining the first derivative of this ratio spectra as follow

$$dP_\lambda = (a_{X\lambda}/\mathbf{a_{Y\lambda}})dC_X \qquad (8)$$

(Fig. 5) showed the obtained derivative ratio spectra of different concentrations of DIM. A correlation between the peak amplitudes at 275 nm and the corresponding DIM concentration was plotted from which the its concentration could be determined.

Ratio H-point standard addition method

Simultaneous determination of two components using plots of peak amplitudes of the ratio spectra at two wavelengths versus the added concentrations of one component is called ratio H-point standard addition method (RHPSAM) [20,22]. Any two analytical wavelengths along the ratio spectra can be chosen for

Table 3
Statistical comparison between the proposed and reported methods for the determination of DIM and CIN in pure powder.

Value	DIM				CIN		
	Proposed methods			Reported method [12][a]	Proposed methods		Reported method [12][a]
	SCC	S^{1}DD	RHPSAM		267 nm	RHPSAM	
Mean	100.07	99.87	99.90	100.80	99.96	99.92	100.24
SD	0.469	0.929	1.159	1.245	1.068	1.289	0.961
RSD	0.469	0.930	1.160	1.235	1.068	1.290	0.959
n	8	8	8	8	9	9	9
V (Variance)	1.257	0.863	1.343	1.550	1.141	1.662	0.924
Student's t test	1.328 (2.145)[b]	1.849 (2.145)[b]	1.609 (2.145)[b]	–	0.585 (2.120)[b]	0.597 (2.120)[b]	–
F	1.23 (3.79)[c]	1.80 (3.79)[c]	1.15 (3.79)[c]	–	1.24 (3.44)[c]	1.80 (3.44)[c]	–

[a] First derivative spectrophotometry for determination of DIM at 236 nm and CIN at 243.6 nm using methanol as a solvent.
[b] The corresponding theoretical values of t at (P = 0.05).
[c] The corresponding theoretical values of F at (P = 0.05).

Table 4
One-way ANOVA testing for the proposed methods used for the determination of DIM in its synthetic mixtures with CIN.

Source	D.F	Sum of Squares	Mean square	F value[a]	P value
Model	2	1.28347736	0.641738682	0.55697	0.58017
Error	24	27.6524692	1.15218622		

[a] Calculated F is less than the critical F value (3.403).

this method, and this was the main advantage of RHPSAM. (Fig. 6) showed the two selected wavelengths 220 nm and 230 nm where the measurements were done for higher sensitivity.

For DIM and CIN binary mixture, calibration plot was performed between the measured amplitudes in ratio spectra, using normalized CIN spectrum as a divisor, against the successive addition of DIM concentrations on the mixture, the following equation was concluded

$$P_\lambda = (a_{X\lambda}C_X/\mathbf{a}_{Y\lambda}) + C_Y + (a_{X\lambda}/\mathbf{a}_{Y\lambda})C'_X \tag{9}$$

where, P_λ was the peak amplitudes of the ratio spectra recorded at a given wavelength (λ) and C'_X was the added concentration of DIM. Other terms in the equation were the regression parameters of standard addition plot, where "$(a_{X\lambda}C_X/\mathbf{a}_{Y\lambda}) + C_Y$" was the intercept and "$aX\lambda_X/\mathbf{a}_{Y\lambda}$ " was the slope.

Applying Eq. (9) at the two selected analytical wavelengths (220 nm and 230 nm), the following two equations were obtained:

$$P_{220} = A + M_{220}C'_X \tag{10}$$

$$P_{230} = B + M_{230}C'_X \tag{11}$$

where A and B were the intercepts of the two regressions at 220 nm and 230 nm, respectively, such that $A = (a_{X220}C_X/\mathbf{a}_{Y220}) + C_Y$, and $B = (a_{X230}C_X/\mathbf{a}_{Y230}) + C_Y$, While M_{220} and M_{230} were the slopes of the regressions at 220 nm and 230 nm, respectively, such that $M_{220} = aX220/\mathbf{a}_{Y220}$, and $M_{230} = aX230/\mathbf{a}_{Y230}$

The plotted two calibration lines intersected at RH-point with a coordination $(-C_x, C_Y)$, (Fig. 7). Where, the X-coordinate was DIM concentration and Y-coordinate was CIN concentration. Accordingly, at this point the concentration of both drugs were simultaneously determined using the following equations

$$C_X = B - A/M_{220} - M_{230} \tag{12}$$

$$C_Y = M_{220}B - M_{230}A/M_{220} - M_{230} \tag{13}$$

Method validation

Validation of the proposed methods was performed according to ICH guidelines [23].

Linearity

The linearity of each proposed method was evaluated by analyzing different concentrations of DIM (10–45 µg mL^{-1}) and CIN (4–20 µg mL^{-1}). The analyses were performed according to the experimental conditions previously described. The parameters of linear equations along with their relative standard errors were summarized in (Table 1).

Table 5
One-way ANOVA testing for the proposed methods used for the determination of CIN in its synthetic mixtures with DIM.

Source	D.F	Sum of Squares	Mean square	F value[a]	P value
Model	2	0.0537242686	0.0268621343	0.01070	0.98936
Error	24	60.2438576	2.51016073		

[a] Calculated F is less than the critical F value (3.403).

Range

The calibration ranges were established, through the consideration of practical range necessary for each method, according to Beer's law and the concentration of DIM and CIN present in the pharmaceutical combinations to give accurate and precise results, (Table 1).

Accuracy

Accuracy was checked by analysis of blind pure samples of DIM and CIN by the proposed methods, where satisfactory results were obtained. The mean recoveries and RSD of pure samples analysis were provided in (Table 1).

Selectivity

Selectivity of the proposed methods was evaluated by the analysis of nine different laboratory prepared mixtures of DIM and CIN. The recovery percentage and RSD were acceptable enough to assist the selectivity as shown in (Table 1).

Moreover, (Table 2) showed the two regression equations, obtained for each laboratory mixture analysis using RHPSAM, from which concentrations of DIM and CIN were determined in the nine mixtures.

Precision

Repeatability, three different concentrations of DIM (10.0, 20.0 and 30.0 µg mL^{-1}) and CIN (8.0, 10.0 and 12.0 µg mL^{-1}) were analyzed three times intra-daily using the proposed methods. The relative standard deviations were calculated in (Table 1), showing low deviations and high repeatability.

Reproducibility, the previous procedures were repeated inter-daily on three different days for the analysis of the three previously chosen concentrations. The relative standard deviations were calculated in (Table 1), showing high intermediate precision.

Application of the proposed methods in assay of pharmaceutical formulation

The proposed methods were successfully applied for the determination of the studied drugs in their pharmaceutical dosage forms, the mean recoveries and RSD were shown in (Table 1). Moreover standard addition technique was applied to assist the validity of the methods.

Statistical analysis

The results obtained by the proposed and reported [12] spectrophotometric methods were statistically compared. The calculated t-value and F-value were less than theoretical ones, therefore no significant difference between the proposed and reported methods regarding both accuracy and precision, as shown in (Table 3).

Selectivity of the proposed methods for the determination of DIM and CIN binary mixtures was statistically compared using one-way ANOVA. The calculated F-values were less than the critical one, at the 0.05 level for DIM and CIN determinations, (Tables 4 and 5). Therefore, the population means were not significantly different and no difference between the proposed methods regarding their selectivity for both drugs.

Conclusion

Severely overlapped spectra of DIM and CIN binary mixtures were simultaneously resolved by obtaining ratio spectra using the normalized CIN spectrum as a divisor. Normalized spectrum provided a smart tool in spectral resolution by modulating the

peak amplitudes at isoabsorptive point into the total concentration, as the case in SCC and S^{1}DD. Moreover, CIN normalized spectrum was used as a divisor to modulate the generated constants in ratio spectra into CIN concentrations, as the case in RHPSAM. The normalized spectra manipulating methods are characterized by simple and moderate procedures compared to other conventional ratio manipulating spectrophotometric methods. In addition, the proposed methods were accurate and precise for the determination of DIM and CIN binary mixtures and can be applied in quality control laboratories without preliminary separation step.

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