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Evaluation of graphical and statistical representation of analytical signals of spectrophotometric methods

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ABSTRACT

Simultaneous determination of miconazole (MIC), mometasone furaoate (MF), and gentamicin (GEN) in their pharmaceutical combination. Gentamicin determination is based on derivatization with of *o*-phthalaldehyde reagent (OPA) without any interference of other cited drugs, while the spectra of MIC and MF are resolved using both successive and progressive resolution techniques. The first derivative spectrum of MF is measured using constant multiplication or spectrum subtraction, while its recovered zero order spectrum is obtained using derivative transformation. Beside the application of constant value method. Zero order spectrum of MIC is obtained by derivative transformation after getting its first derivative spectrum by derivative subtraction method. The novel method namely, differential amplitude modulation is used to get the concentration of MF and MIC, while the novel graphical method namely, concentration value is used to get the concentration of MIC, MF, and GEN. Accuracy and precision testing of the developed methods show good results. Specificity of the methods is ensured and is successfully applied for the analysis of pharmaceutical formulation of the three drugs in combination. ICH guidelines are used for validation of the proposed methods. Statistical data are calculated, and the results are satisfactory revealing no significant difference regarding accuracy and precision.

1. Introduction

Local antifungals are mostly found in combination with antibacterials and corticosteroids. Miconazole (MIC) 1-[2-(2,4-dichlorophenyl)-2-[(2,4-dichlorophenyl)methoxy]ethyl]-1-*H*-imidazole the antifungal drug, inhibits the synthesis of ergosterol in fungal cell membranes [1]. Its official BP method [2] is a non-aqueous titrimetric method and an HPLC method for miconazole cream. According to the literature, MIC was investigated colorimetrically [3], spectrophotometrically [4–6], and by chemometric determination [7]. Literature shows the determination of MIC using HPLC [8–12] and HP-TLC [13–15].

Mometasone furaoate (MF) 9,2,1-dichloro-11b-hydroxy-16a-methyl-3,20-dioxopregna-1,4-dien-17-yl furan-2-carboxylate is a topical corticosteroid [1]. The official BP method is a direct spectrophotometric method [2]. It was also assayed by HPLC methods [16,17], and TLC methods [18,19].

Gentamicin (GEN) an aminoglycoside antibiotic [1]. The USP describes an HPLC method after derivatization with *o*-phthal aldehyde [20]. In literature it can be determined colorimatrically [21], and spectrophotometrically after derivatization with *o*-phthalaldehyde [22]. Several methods are described for the determination of GEN using HPLC [23–25], fluorimetric determination [26], gas chromatography (GC) [27], capillary electrophoresis (CE) [28], and ion selective electrodes [29].

The literature doesn't include any method for determining combinations of MIC, MF and GEN.

Spectrophotometric methods not only depend on substitution in regression equations to conduct quantitative analysis either conventional methods such as dual wavelength [30–32], derivative or derivative ratio [33], or novel ones such as ratio difference [34], derivative subtraction [35], and ratio subtraction [36]. In addition many methods are applied and widely used based on substitution in mathematical equations using the recorded absorbance values on the spectral charts, as absorption factor method [37], bivariate [38,39], Vierordt [40,41], and Q-methods [42]. Total concentration of components in pharmaceutical binary mixtures can be obtained by the aid of the isoabsorptive point, while another adjacent complementary spectrophotometric method

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must be used to determine the concentration of one of the two components separately [36,43]. As an isoabsorptive point is retained in the ratio spectrum as well as in derivative spectrum so can be used for the analysis of overlapped spectra [34,44,45]. A new approach utilizing the isoabsorptive point is also used for mixtures analysis namely, amplitude modulation method [46–48].

This work focuses on the use of mathematical techniques and how this can be adapted to resolve spectral overlap of MIC, MF in their mixtures after liquid-liquid extraction of GEN. Comparative study of the obtained results graphically on the spectrum chart after manipulation steps on the spectra manager software and those obtained after substitution in the computed regression equations is conducted, and then statistical analysis of the results is carried out to evaluate the efficiency of the method. In addition, since GEN contains primary amino groups on their skeleton so it is derivatized with OPA and measured spectrophotometrically with a sensitive linear range of concentrations. Consequently, comparative study of the results of the proposed methods with each other and with the official methods is applied to prove their effectiveness. The developed methods are also validated successfully for specificity, linearity, accuracy, precision and they are applied for quantification of these drugs in cream, avoiding interferences of excipients. The proposed methods are simple and reproducible; with minimal data manipulation that does not need any sophisticated device or specified computer software and can be easily used in laboratories where modern and expensive apparatus are not available.

1.1. Theories

1.1.1. Differential amplitude modulation (DAM)

This method is a modification for amplitude modulation method for binary mixtures X and Y with isoabsorptive point [47,48] where derivative spectra D^1 of Y is extended over that of X, for the ratio spectrum of the mixture is obtained using D^1 of normalized spectra of Y as a divisor to get P_Y from the plateau region (constant) at the extension .This constant is corresponding to concentration of Y while, the isoabsorptive point is used to yield the total amplitude ($P_X + P_Y$) which is corresponding to the total concentration of the mixture. Subsequently (P_X) which is corresponding to the concentration of X (C_X) is obtained by difference. The concentration of X or Y is calculated by substitution in the corresponding regression equation (showing slope of almost one and intercept around zero) representing amplitudes of P_Y or P_x versus their corresponding concentration.

1.1.2. Concentration value (conc. value)

This is a novel technique depending upon graphical representation of concentration on the spectra without any statistical manipulation for the results to compute the data into regression equation, where the concentration value of the drug is recorded directly on the spectral chart without the substitution in the regression equation to get direct recoveries of its concentrations. This method can be applied for single component after using derivatization reagent by using the normalized spectrum of the product as a divisor, in addition it can be used for analysis of mixture with isoabsorptive point which retains in derivative or ratio spectra. This is conducted for mixture by three different pathways using zero order absorption spectra (D^0) or first order derivative spectra (D^1) spectra as follows:

a) Dividing D^0 of the mixture by the D^0 of the normalized spectrum of the more extended component, the constant obtained from the plateau parallel to x-axis is found to represent the concentration of the extended component.
b) Dividing the D^1 of the mixture by the D^1 of the normalized spectrum of the more extended component, the constant obtained from the plateau represents the concentration of the extended component.
c) Dividing the mixture spectrum showing an isoabsorptive point {in zero order absorption spectrum} or isosbestic point (in derivative spectrum) by the D^0 or D^1 of the more extended component normalized spectrum. The constant obtained from the plateau region is equal to the concentration of the extended component; the peak amplitude at the isosbestic point is equal to the total concentration of the binary mixture. So by subtraction we get the concentration of the less extended component.

2. Experimental

2.1. Device and software

Spectrophotometric measurements were carried out by using a double-beam UV/Visible spectrophotometer model V-760, Jasco, Japan, connected to ACER compatible computer with software (Microsoft excel 2010). Spectra manager® software, version 2, Jasco Corporation was used. The absorption spectra of the solutions were carried out in a 1.00 cm quartz cells. Scans were carried out in the range from 200 to 400 nm at room temperature.

2.2. Reagents

2.2.1. Pure samples

MIC is supplied by Medical Union Pharmaceuticals Company with purity 100.02 ± 0.42 according to BP official method [2], MF and GEN is supplied by Sigma Company for Pharmaceuticals. MF purity 100.12 ± 0.52 according to BP official method [2], and GEN with purity 100.05 ± 0.23 according to USP official method [20].

2.2.2. Pharmaceutical formulation

Momenta® cream each 1 g contain 20 mg of MIC, 1 mg MF, and 1 mg of GEN manufactured by Jamjoom® Company for pharmaceuticals, is purchased from the Egyptian market.

2.2.3. Solvents and reagents

All are of analytical grade, methanol supplied by Carlo Erba. *o*-Phthalaldehyde supplied by Research-Lab Fine Chem Industries, boric acid, potassium hydroxide, and mercapto acetic acid (thioglycolic acid) is supplied by El-Nasr Pharmaceutical chemicals.

- Borate buffer solution (pH 10.4): Dissolve 2.473 g of boric acid into 100 mL volumetric flask with distilled water and adjust pH with 45% w/v solution of potassium hydroxide then complete with water till 100 mL [2].
- *o*-Phthalaldehyde reagent (OPA):
- Prepare according to BP [2] by dissolving 1.0 g of *o*-phthalaldehyde in 5 mL of methanol and adding 95 mL of borate buffer solution. To this, added 2.0 mL of mercaptoacetic acid (thioglycolic acid) and readjusted the pH to 10.4 with 50% w/v potassium hydroxide solution.
- Prepare 0.001 M OPA (For molar ratio) by dissolving 0.0134 g OPA then proceed as mentioned before according to BP [2].

2.2.4. Standard solution

- Standard stock solutions are prepared of 500 μg/mL of each, MIC and MF in methanol and GEN (0.001 M) in bidistilled water.
- Working standard solutions of 50 μg/mL of each, MIC and MF in methanol and GEN in bidistilled water.

2.3. Procedure

2.3.1. MIC, MF, and GEN spectra

Zero-order absorption spectra of 10 μg/mL of MIC, 10 μg/mL of MF, 10 μg/mL of GEN are obtained by scanning over the range 200–400 nm against methanol as a blank, then derivatized spectra

are obtained from the spectra manager software and stored on computer.

2.3.2. Linearity and calibration graphs

Portions equivalent to 10–130 μg/mL MIC, 10–170 μg MF, and 10–300 μg for GEN are transferred from working solutions (50 μg/mL) for MIC, MF, and GEN, into three series of 10-mL volumetric flasks. Complete to volume with methanol for MIC and MF, while GEN undergoes the derivatization procedure by adding 0.5 mL OPA solution, complete to the mark with methanol, and heat on a water bath 60 °C for 15 min. The spectra of the prepared standards are scanned against the appropriate blank in the range 200–400 nm and saved on the computer.

2.3.2.1. For derivative transformation of MIC, constant multiplication of MF, reaction product of GEN. Zero order absorption spectra (D^0) for MIC and MF are recorded directly, or after derivatization of GEN and saved on the computer. Calibration graphs are made by relating the λ_{max} of the scanned spectra at 219 nm, 247 nm and 333 nm for MIC, MF, and GEN, respectively and the corresponding concentrations to get the regression equations.

2.3.2.2. For first derivative (D^1) and derivative subtraction (DS) of MIC. For Derivative subtraction coupled with constant multiplication (DS-CM) and spectrum subtraction coupled constant multiplication (SS-CM) of MF. First derivative spectra are computed using $\Delta\lambda = 4$ and a scaling factor $= 10$. Calibration graphs are made relating the peak amplitude (P-Zero) of the D^1 spectra of MIC and MF at 233 and 274 nm respectively versus the corresponding concentrations to get the regression equations.

A calibration graph is made for MF by relating the peak amplitude ($P_{max-min}$) of the D^1 spectra between $P_{274-232}$ versus the corresponding concentrations and compute the regression equation.

2.3.2.3. For differential amplitude modulation (DAM). The obtained D^0 spectra of MIC and MF are divided by the normalized D^0 spectrum of MF, and the obtained ratio spectra are recorded. The calibration graphs are made by plotting the amplitudes of MF division spectrum or MIC division spectra at 232 nm and corresponding concentrations in order to compute the unified regression equation.

2.3.2.4. For constant value method (CV). D^1 spectra of MF spectra is divided by normalized D^1 1 μg/mL MF. Calibration graph is constructed by plotting amplitude at the plateau region 260–280 nm versus the corresponding concentration of MF to compute the regression equation.

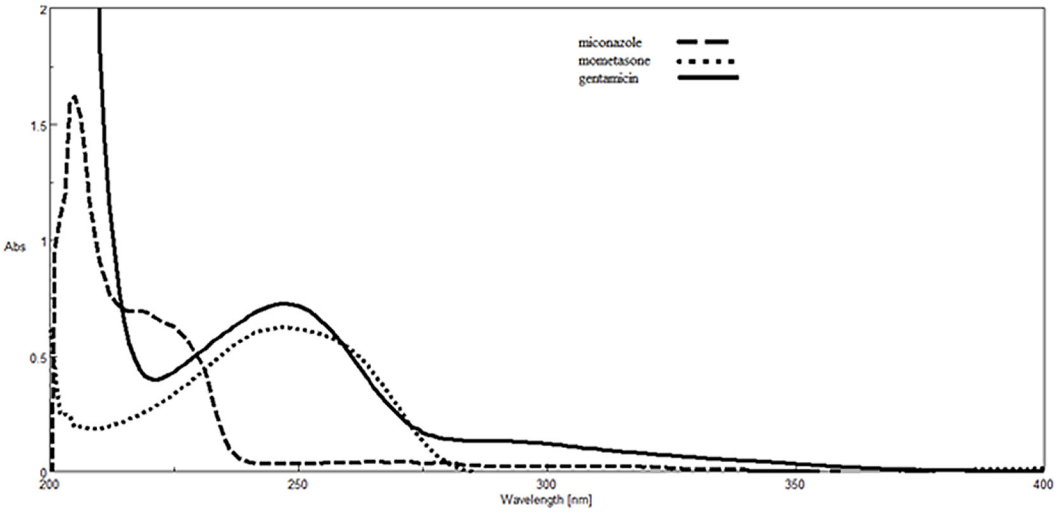


Fig. 1. (a). The zero order absorption spectra of miconazole 10 μg/mL, mometasone 10 μg/mL, and gentamicin 10 mg/mL, (b) zero order absorption spectrum of gentamicin sulphate 10 mg/mL showing λ_{max} at 247 nm and zero order absorption spectrum of derivatization product of gentamicin sulphate 10 μg/mL showing λ_{max} at 333 nm.

2.3.3. Applying the spectrophotometric methods for determining MIC, MF and GEN in laboratory prepared mixtures

Aliquots of MIC, MF, and GEN are separately transferred from their working solutions, methanol is added to reach required volume and get mixtures of different ratios of drugs under study, and proceed to get the binary mixture concentration of MIC and MF as GEN will not be interfering in low concentrations.

2.3.3.1. For MIC and MF. The concentrations of MF and MIC in each mixture are calculated by substitution in the corresponding regression equation for each method or on the spectrum chart via concentration value after performing the following manipulation:

A- D^1 measurement of MF: Get the D^1 of each the spectrum of each mixture then record the amplitude at peak maxima 274 nm.
B- Divide the obtained D^1 spectra of the mixtures by the derivative of the normalized spectrum of MF (1 μg/mL) to get the division spectra then the constant of each mixture is recorded from the plateau region 260–280 nm then proceed as detailed under each method as follows:

1- DS-CM: The measured constant of each mixture is either multiplied by the derivative of the normalized spectrum of MF (1 μg/mL) to get the D^1 spectrum of MF and the amplitude $P_{274–232}$ is recorded or subtracted from its corresponding division spectrum followed by multiplication with the divisor spectrum to get the D^1 spectrum of MIC in each mixture and the peak maxima at 233 nm is recorded.
2- SS-DS: For each mixture, the obtained D^1 spectra of MIC are subtracted from its corresponding D^1 spectrum of the mixture to obtain D^1 spectrum of MF from which $P_{274–232}$ is obtained.
3- Derivative Transformation (DT): For each mixture, the obtained D^1 spectrum of MF or MIC is multiplied by its decoding spectrum which is the division spectrum of normalized spectrum by its derivative to obtain zero order spectrum of MF or MIC then the absorbance is measured at 247 nm and 219 nm, respectively.
4- CV & DAM: The measured constant from the plateau region 260–280 nm can be used to get MF concentration using regression equation of CV or unified regression equation at 232 nm, while MIC concentration can be obtained by substitution in the same unified regression equation after subtraction the measured constant from the recorded amplitude at 232 nm.
5- Concentration value: For each mixture, The obtained D^1 spectrum of MF or MIC is divided by their corresponding D^1 normalized spectrum then the concentration of each is directly measured on the spectrum chart at plateau region 260–280 nm and 220–240 nm respectively, then Recovery % is calculated by division of recorded value by the claimed concentration and multiplied by 100. Using DAM procedure; MIC concentration can be obtained after subtracting the measured constant at 260–280 nm from the recorded amplitude at the isosbestic point 232 nm.

2.3.3.2. For gentamicin determination. The solvent is evaporated and equal amounts of chloroform and water are added, shake in a separating funnel. Aliquots of 5 mL are accurately transferred into 10-mL volumetric flask, added to 0.5 mL of OPA reagent then completed to volume with methanol, heat on a water bath 60 °C for 15 min, cool. The absorbance of reaction product is measured at 333 nm against blank similarly prepared.

The spectrum of derivatized product of GEN with OPA is divided by its normalized spectra of the product which is absorptivity spectrum; obtained by division of the product spectrum by its concentration; record the concentration directly on the spectrum chart. The recovery % is calculated by division of recorded value by the claimed concentration and multiply by 100.

2.3.4. Application to pharmaceutical formulation

The concentrations of MF and MIC and GEN are calculated by substitution in the corresponding regression equation for each method or on the spectrum chart via concentration value after performing the following manipulation.

2.3.4.1. Methanolic extract for MF and MIC. Half a gram of Momenta® cream is weighed and dissolved in methanol by heating on a water bath 60 °C for 5 min. Remove and shake for 3 min then filter before transferring to 100-mL volumetric flask and the volume is completed to get a stock solution with concentration 100 μg/mL of MIC and 5 μg/mL of MF. Ten mL are taken from the stock solution and put in 100-mL volumetric flask and completed to the mark with methanol to get

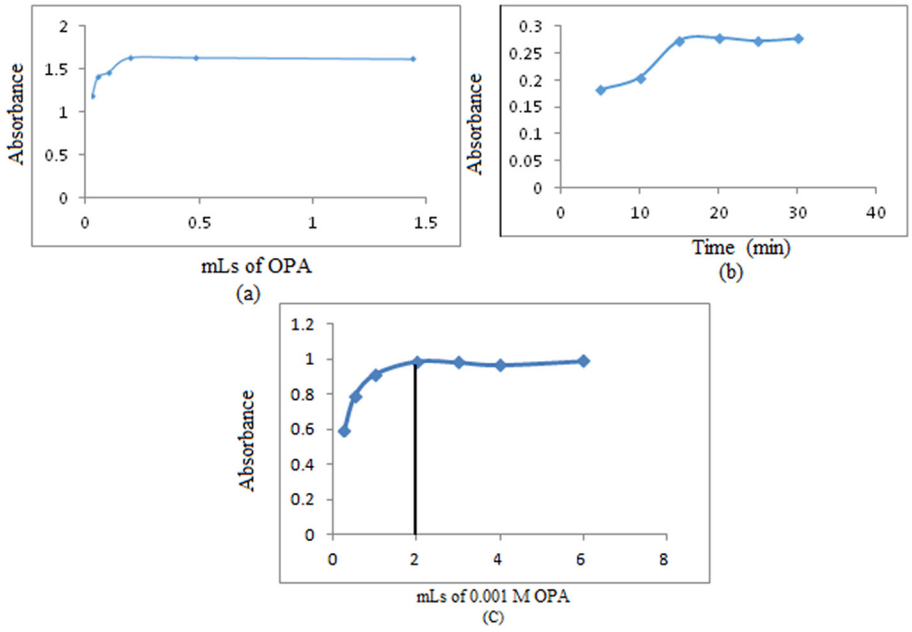


Fig. 2. (a) The effect of mLs of OPA solution on 30 μg/mL GEN, (b) the effect of time on 5 μg/mL GEN, in optimization of conditions of derivatization, (c) molar ratio of the derivatization reaction, where different aliquots of 0.001 M OPA solution reacted with 0.001 M GEN.

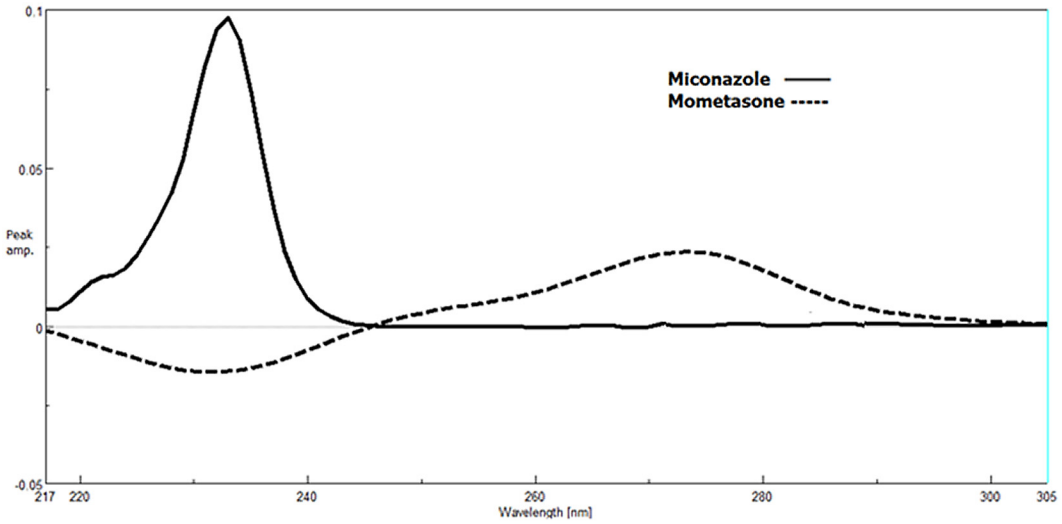


Fig. 3. The first derivative spectra of miconazole 10 μg/mL and mometasone furaoate 10 μg/mL.

a working solution with final concentration 10 μg/mL of MIC and 0.5 μg/mL of MF. One μg/mL of MF is added by spectrum addition technique.

2.3.4.2. Aqueous extract for GEN. Two grams of Momenta® cream are weighed and dispersed in 100 mL chloroform, then 100 mL bidistilled water are added, shake in a separating funnel 5 min and leave to stand 10 min. Take the aqueous layer, then aliquots of 5 mL are transferred into 10-mL volumetric flask where derivatization with OPA is applied using the specified experimental conditions, and complete to the mark to get a working solution with final concentration 10 μg/mL. Proceed as under analysis of laboratory prepared mixtures.

2.3.5. Optimization of derivatization conditions

2.3.5.1. Effect of volume of o-phthalaldehyde (OPA). Aliquots equivalent to 30 μg/mL GEN are added to 0.025, 0.05, 0.1, 0.2, 0.5, 1, and 1.5 mL of OPA solution in 10-mL volumetric measuring flasks, The volume is completed to the mark with methanol. The solutions are heated on a water bath

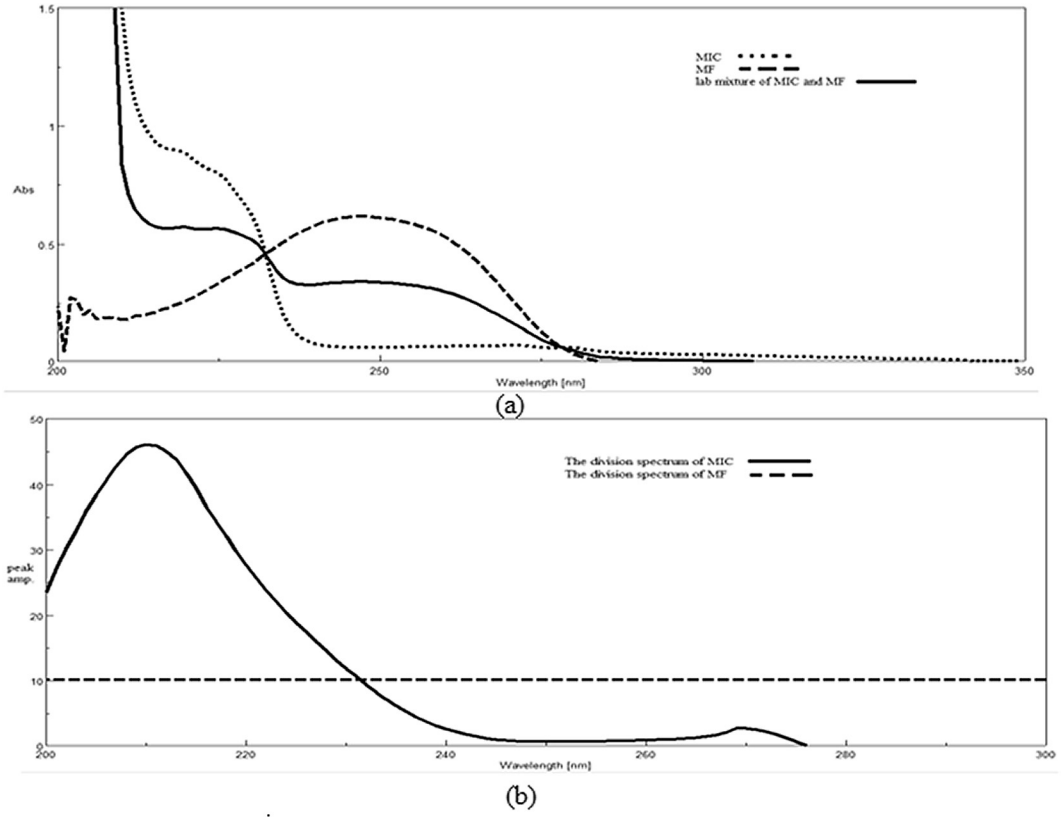


Fig. 4. (a) Zero-order spectra of 10 μg/mL of MIC and MF, separately in methanol, and binary of a mixture of MIC and MF,5 μg/mL of each. (b) The division spectrum of 10 μg/mL MIC and MF 10 μg/mL by normalized MF spectrum 1 μg/mL.

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

Drug name	MIC			MF					GEN
Method	D^0 (219 nm)	D^1 (233 nm)	AM (232 nm)	D^0 (247 nm)	D^1 ($P_{274–232}$)	D^1 (274 nm)	CV (260–280 nm)	AM (232 nm)	D^0 (333 nm)
Range (μg/mL)	1–13			1–17				1–13	1–30
Slope	0.0825	0.0082	1.0006	0.0573	0.0044	0.0025	0.998	1.0006	0.0398
Intercept	0.066	0.007	0.0436	0.0502	0.0008	0.0008	0.0311	0.0436	0.0759
Accuracy	100.64 ± 0.95	100.13 ± 1.29	100.22 ± 0.92	100.42 ± 1.069	100.16 ± 0.29	100.64 ± 0.73	100.09 ± 0.54	100.22 ± 0.92	99.20 ± 1.39
Correlation coefficient (r)	0.9999	0.9998	0.9999	0.9999	0.9999	0.9998	0.9999	0.9999	1.0000
**RSD[a]	0.762	0.840	0.514	1.129	0.682	0.672	0.816	0.514	1.098
**RSD[b]	1.296	1.874	0.728	1.265	1.147	1.377	0.981	0.728	1.308
Sandell's sensitivity (μg/cm^2)	0.0068	–	–	0.0091	–	–	–	–	0.0086
Molar absorptivity (abs/molar conc.)	445×10^5	–	–	31×10^6	–	–	–	–	235×10^5
LOD	0.006	0.084	0.006	0.035	0.107	0.149	0.002	0.006	0.021
LOQ	0.020	0.254	0.017	0.107	0.326	0.451	0.006	0.017	0.066

[a] **RSD: the intra-day ($n = 3$) relative standard deviation of concentrations 3, 5, and 10 μg/mL for MIC and MF and 5, 10, and 15 μg/mL for GEN.
[b] **RSD: the inter-day respectively ($n = 3$) relative standard deviation of concentrations 3, 5, and 10 μg/mL for MIC and MF and 5, 10, and 15 μg/mL for GEN.

60 °C for 15 min. The solutions are shaked gently and cooled before scanning to get the absorption graph of the reaction product the absorbance is measured at 333 nm against blank similarly prepared.

2.3.5.2. Effect of time. Aliquots equivalent to 5 μg/mL GEN, are added to 0.5 mL of OPA reagent in 10-mL volumetric measuring flasks, the volume is completed to the mark with methanol. The solutions are heated on a water bath 60 °C for 5, 10, 15, 20, 25, and 30 min. The solutions are shaked gently and cooled before scanning to get the absorption graph of the reaction product the absorbance is measured at 333 nm against blank similarly prepared.

2.3.5.3. Molar ratio. Aliquots equivalent to 0.001 M GEN, are added to 0.25, 0.5, 1, 2, 3, 4, and 6 mL of 0.001 M OPA regent. The volume is completed to mark with methanol. The solutions are heated on a water bath 60 °C for 15 min. The solutions are shaked gently and cooled before scanning to get the absorption graph of the reaction product the absorbance is measured at 333 nm against blank similarly prepared.

3. Results and discussion

Regression analysis is a statistical process; estimating relationships among variables such as linear regression that depends on some collected data and its direct relationship to concentration. The values of slope and intercept for each compound, within the specified range are statistically calculated through their respective response vs concentration plots. As regression analysis often depends on making assumptions about the process that can only be tested by the presence of sufficient quantity of data. While with minor tested effects, regression methods can give misleading inaccurate results [51]. In this work, by data processing steps via the spectra manager software using the divisor normalized standard curve, the obtained constant will be directly representing the corresponding concentration of the drug of interest then the R% is calculated relative to the actual concentration. This method is simple (no multistep resolution method or solving of mathematical equations), direct (the two components are measured at the spectrum chart) and rapid (no needs for statistical analysis of the results as it is directly recorded on the spectrum chart).

Regarding the mixture; zero-order absorption spectra (D^0) of MIC and MF are severely overlapped. But without any contribution of GEN up to 30 μg/mL as shown in Fig. 1a. Gentamicin has a very poor absorptivity at its λ_{max} 247 nm in a very high concentration range 2–10 mg/mL, in addition, it is highly soluble in water but insoluble in methanol and chloroform [2]. MF is insoluble in water, MIC is very slightly soluble in water, and both are soluble in methanol [2]. Mixtures of different concentrations of methanol: water cause turbid solutions upon dissolving the cream dosage form, so double extraction steps are used. Methanol only for dissolving the binary mixture of MF and MIC, while another portion of the dosage form is extracted in separating funnel using chloroform:water (50:50). The clear aqueous solution containing gentamicin is taken then derivatization with OPA is conducted which is selective for drugs containing primary amino group. Although MIC is very slightly soluble in water but it doesn't interfere with the gentamicin condensation reaction with OPA, since it doesn't have a primary amino group, so even if any portion will be present in the aqueous extract it will not interfere, beside if any portion is extracted in water having λ_{max} 219 nm and a contribution till 290 nm, so zero contribution at 333 nm where GEN after derivatization is measured.

The derivatization reaction of amino groups of gentamicin (R-NH_2) with OPA (Ar-CHO) in an aqueous basic medium (pH 10.4) and in presence of mercaptoacetic acid ($HSCH_2COOH$) to form isoindole derivative of maxima at 333 nm [49,50] which is completely different than that formed in absence of mercaptoacetic acid with maxima at 252 nm. In addition, mercaptoacetic acid prevents the hydrolysis of OPA and it

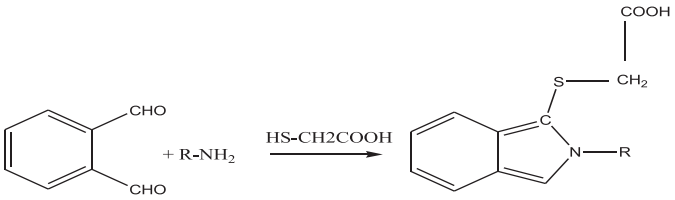
Table 2a
Determination of MF in laboratory prepared mixtures by the proposed methods.

Concentrations (μg/mL)			D^1 of MF						D^0 of MF		Division on normalized D^1 (1 μg/mL) MF					
MIC	MF	GEN	D^1 (274 nm)		CM ($P_{274–232}$)		SS ($P_{274–232}$)		Derivative transformation (233 nm)		CV (260–280 nm)		DAM (232 nm)		Concentration value	
			Found	R%	Found	R%	Found	R%	Found	R%	Found	R%	Found	R%	Constant	R%
5	**10**	5	10.19	101.98	10.09	100.92	4.95	99.13	10.01	100.06	10.05	100.59	10.11	101.08	**10.07**	100.70
10			3.01	100.26	2.97	99.04	10.03	100.39	2.97	99.31	3.05	101.83	3.00	100.06	**2.96**	98.66
5	**5**	5	5.02	100.58	4.91	98.38	4.97	99.56	4.94	98.98	4.96	99.35	5.02	100.41	**4.98**	99.78
10	**15**	7.5	15.27	101.83	14.74	98.31	9.81	98.15	14.94	99.60	15.10	100.67	15.13	100.90	**15.10**	100.67
4	**10**	5	10.15	101.50	10.03	100.35	4.01	100.21	10.01	100.08	10.03	100.30	10.08	100.78	**10.04**	100.415

Table 2b
Determination of MIC and GEN in laboratory prepared mixtures by the proposed methods.

Concentrations (μg/mL)			MIC										GEN			
			D^1		D^0		Division on normalized D^1 (1 μg/mL) MIC		Division on normalized D^0 (1 μg/mL) MF				After derivatization			
MIC	MF	GEN	DS (233 nm)		Derivative transformation (219 nm)		Concentration value		DAM (232 nm)		Concentration value		D^0 (333 nm)		Concentration value	
			Found	R%	Found	R%	Constant	R%	Found	R%	Constant	R%	Found	R%	Found	R%
5	10	**5**	5.04	100.86	4.99	99.82	**4.99**	99.80	5.12	102.41	**5.08**	101.60	5.01	100.36	5.02	100.40
10	3	**2.5**	10.04	100.44	10.01	100.07	**9.91**	99.10	3.02	100.73	**2.98**	99.33	2.49	99.81	2.48	99.20
5	5	**5**	4.99	99.91	5.01	100.17	**5.00**	100.00	5.02	100.41	**4.98**	99.60	5.03	100.60	5.01	100.36
10	15	**7.5**	9.93	99.31	10.13	101.31	**9.89**	98.90	10.07	100.70	**10.03**	100.33	7.45	99.39	7.35	98.00
4	10	**5**	4.07	101.86	4.01	100.28	**4.07**	101.75	4.09	102.23	**4.05**	101.21	5.01	100.36	5.03	100.60

stabilizes the reaction product. The general reaction of primary amine with OPA in presence of mercapto acetic acid (thioglycolic acid):



Different volumes (0.025–1.5 mL) are also tested. And OPA 0.5 mL is used in the subsequent experiments. A completion of the reaction is achieved (60 ± 2 °C), within 15 min. The molar ratio for the reaction to take place is conducted, using different ratios of GEN/OPA solution as shown in Fig. 2c, and it is practically proved that each mole of GEN reacts with two moles of OPA, so only two primary amino groups are involved in the reaction due to the steric hindrance effect.

In this work, GEN is determined after derivatization with (OPA) reagent at 333 nm as shown in Fig. 1b over a linearity range (1–30 μg/mL).

For laboratory prepared mixtures:

- For the determination MF and MIC:

Zero order absorption spectrum D^0 of MF shows maxima at 247 nm with linearity (1–17 μg/mL), while MIC shows a peak at 219 nm but it has a contribution till 290 nm as a straight line parallel to the wavelength x-axis (Fig. 2a). This complete overlapping of D^0 hinders the spectrophotometric methods based on the extension of one of the spectra. The first order derivative spectra D^1 of both drugs shows an extension of MF with maxima at 274 nm, where MIC has zero contribution Fig. 3.

1- Using the amplitude of D^1 spectra
The concentration of MF can be calculated after substitution in the corresponding equation. By applying derivative subtraction coupled with constant multiplication [35,52] or spectrum subtraction (SS) [53] the first derivative spectrum of each drug in pharmaceutical combination is obtained which act as spectral profile of the cited drug. For maximum sensitivity, the amplitudes of the D^1 spectra of MF can be measured peak to peak $P_{274–232}$ leading to higher amplitudes values and subsequently larger slope, while MIC is measured at P_{233}. Robustness of the method is very good since no critical point measurement either zero crossing point or coincident point. In addition, due to the application of ratio for the derivative spectra, this method has high signal to noise ratio.

2- Using the constant obtained from division by D^1 of laboratory prepared mixture by D^1 (1 μg/mL)normalized MF spectrum:

– Constant value (CV) [54,55] method is conducted where the constant is substituted directly in the equation representing the relationship between the constant in the plateau region 260–280 nm and the concentration of MF. The advantage of this method is its accurate results with minimum manipulation steps

– Differential amplitude modulation (DAM): This is a modification of amplitude modulation method [47,48] where the constant at the extended part is hard to be determined. While, the constant obtained from ratio spectra of the D^1 of the laboratory prepared mixture by the normalized D^1 of MF is easily measured at the extended part. The concentration of MIC and MF in each mixture is calculated by using the corresponding unified regression equation at 232 nm (Fig. 4). This method is applicable for progressive determination binary mixtures with D^0 severely overlapped spectra using a single divisor and one regression equation. The main advantage is that it doesn't require complementary method to measure one of the

Table 3
Results of determination of MIC, MF, and GEN in pharmaceutical dosage forms by the proposed methods and applying standard addition technique for GEN.

Method	MIC Found % ± S.D	MF Found[c] % ± S.D	GEN Found % ± S.D	Std add (GEN) Recovery % ± S.D
D^0	–	–	100.66 ± 0.88	99.77 ± 1.84
D^1	–	100.59 ± 0.58	–	–
CM (D^1)	–	100.63 ± 0.55	–	–
SS (D^1)	–	100.31 ± 0.41	–	–
CV	–	101.03 ± 0.38	–	
Concentration value	100.93 ± 0.53[a] 100.45 ± 0.57[b]	100.54 ± 0.27	100.48 ± 0.89	100.25 ± 0.30
DAM	100.90 ± 0.61	100.49 ± 0.36	–	–
DS	100.79 ± 0.75	–	–	–
Derivative transformation	100.80 ± 0.23	100.68 ± 0.51	–	–

[a] The constant obtained from dividing the D^1 spectrum of MIC by the D^1 normalized MIC.
[b] Subtracting the peak amplitude at the isosbestic point 233 from the MF concentration.
[c] Enrichment by spectrum addition of 1 μg/mL MF to the dosage form methanolic extract spectrum.

Table 4
Statistical comparison between results obtained by the proposed methods and the official methods for the determination of MIC, MF [2], and GEN [20] in pure powder form:

	MIC					MF							GEN		
Parameters	BP method[a]	D^0	D^1	DAM	Conc value[c]	BP method[b]	D^0	D^1	D^1 $P_{max} - P_{min}$	DAM	CV	Conc value[c]	USP method[a]	D^0	Conc value[c]
Mean	100.56	99.85	99.72	100.08	99.19	100.13	100.62	101.46	100.16	100.08	99.44	98.94	100.70	99.71	99.53
S.D.	1.05	0.75	1.22	0.91	1.12	1.71	1.94	1.35	1.29	0.91	1.68	0.78	0.78	1.42	0.73
N	6	6	6	6	6	6	6	6	6	6	6	6	6	6	6
Variance	1.11	0.56	1.58	0.83	1.25	2.92	3.81	1.84	1.66	0.83	2.82	0.60	0.61	2.03	0.53
F	–	1.95 (5.05)	1.42 (5.05)	3.48 (5.05)	2.33 (5.05)	–	1.30 (5.05)	1.58 (5.05)	1.75 (5.05)	3.48 (5.05)	1.03 (5.05)	4.84 (5.05)	–	3.31 (5.05)	1.15 (5.05)
Student's *t*	–	2.14 (2.57)	2.36 (2.57)	0.05 (2.57)	0.82 (2.57)	–	1.28 (2.57)	1.36 (2.57)	0.07 (2.57)	0.05 (2.57)	0.51 (2.57)	1.59 (2.57)	–	1.35 (2.57)	2.24 (2.57)

[a] HPLC method.
[b] Direct spectrophotometry at 249 nm.
[c] Without substitution in regression equation.

components singly and the results are not affected by the choice of divisor due to using of the normalized divisor.

- Concentration value is conducted as a new approach where the peak amplitude of the division spectrum of the laboratory prepared mixture at 232 nm represent the sum of concentrations of MIC and MF, and the constant obtained from the plateau region 260–280 nm of the division spectrum represent the concentration of MF. So, by subtracting them we get the concentration of MIC. The percentage recoveries using analytical signal is directly calculated. Also for each mixture, the obtained D^1 spectrum of MIC is divided by its corresponding D^1 normalized spectrum then the MIC concentration is directly measured on the spectrum chart at plateau region 220–240 nm.
  Recovery % = Recorded concentration / Claimed Concentration × 100
  Comparison of the results with those obtained by well-established methods reveal good recoveries and accurate results.
- Derivative transformation [56]. The D^0 absorption spectrum of MF and MIC in the mixture is obtained by derivative transformation method [56] via multiplication of the obtained derivative spectrum of either MF or MIC by its corresponding decoding spectrum then, the absorbance is measured at 247 nm and 219 nm, respectively. The concentration of MF or MIC is calculated using its corresponding regression equation. The advantages of the derivative transformation method that the zero-order spectrum of each drug in pharmaceuticals is obtained which act as spectral profile of each cited drug and robustness of the method is very good as the proposed drugs are measured at their maxima with maximum accuracy and precision.

3.1. For the determination of gentamicin sulphate (GEN)

Gentamicin is extracted in separating funnel using chloroform:water (50:50) in the aqueous layer followed by derivatization with OPA reagent under the optimum condition, then the absorbance at 333 nm is recorded. The concentration of gentamicin is calculated either using the corresponding regression equation or via concentration value method by division the reaction product spectrum by its corresponding normalized spectrum of reaction product corresponding to (1 μg/mL) GEN, where the plateau region (300–340 nm) is equivalent to the concentration. Recovery % = Recorded concentration / Claimed concentration × 100.

The high molar absorptivity and low Sandell's sensitivity (The concentration of the analyte in μg/mL which will give an absorbance of 0.001 in a cell of path length 1 cm of the resulting colored solution) indicated high sensitivity of the method.

3.2. Analysis of dosage form

For the analysis of Momenta® cream, two extraction steps should be applied successfully using methanol and (water/chloroform), respectively as extraction solvents, then determine both MIC and MF as a binary mixture in the methanolic extract via the proposed spectrophotometric resolution methods after enrichment of MF concentration via spectrum addition [47] of 1 μg/mL MF to have a total concentration within the proposed linearity range, while GEN is determined in aqueous extract after derivatization with OPA reagent at 333 nm under optimum condition. The results show good agreement between the amounts estimated and those claimed by the manufacturer.

Table 5
Results of ANOVA for comparison of the proposed methods and the official methods for determination of MIC, MF [2], and GEN [20]in pure powder form and those of MIC and MF in dosage form versus reported HPLC [10].

	Source of variation	Sum of squares	Degree of freedom	Mean square	F	*P*-value	F crit
MIC	Between groups	8.46	5.00	1.69	1.75	0.15	2.53
	Within groups	28.95	30.00	0.96			
	Total	37.41	35.00				
MF	Between groups	24.60	7.00	3.51	1.87	0.10	2.25
	Within groups	75.15	40.00	1.88			
	Total	99.75	47.00				
GEN	Between groups	4.80	2.00	2.40	2.26	0.14	3.68
	Within groups	15.89	15.00	1.06			
	Total	20.69	17.00				
Comparison with the reported HPLC method for determining MIC and MF in methanolic extract of dosage form							
MIC	Between groups	0.49	5.00	0.10	0.36	0.87	3.11
	Within groups	3.29	12.00	0.27			
	Total	3.79	17.00				
MF	Between groups	1.24	7.00	0.18	1.01	0.46	2.66
	Within groups	2.79	16.00	0.17			
	Total	4.03	23.00				

4. Validation: validation of the methods has been carried out according to ICH guidelines [57]

4.1. Linearity range

Linearity is calculated by making the different calibration graphs on three days. The calibration graphs are constructed within concentration ranges that support testing the dosage form. Replicates of three are performed for each data point. The concentration ranges, statistical parameters, molar absorptivities, and Sandell's sensitivity for each method are listed in Table 1.

4.2. LOD and LOQ

Limits of detection quantification for the proposed methods are calculated according to ICH [57] recommendations and the values are given in Table 1. LOD and LOQ are calculated by the aid of the slope of calibration graph and SD of intercept. The sensitivity of the proposed method confirmed by low LOD and LOQ values.

4.3. Accuracy

The accuracy of the results is checked by applying the proposed methods for determination of different blind samples of MF, MIC, and GEN within linearity range. The concentrations are obtained from the corresponding regression equations from which the percentage recovery suggested good accuracy of the proposed methods shown in Table 1.

The accuracy of the concentration value method is checked by calculation the percentage of error for six concentrations within Beer's law (true concentration- experimental concentration)/true concentration and showed satisfactory results, the small values E% 0.010, 0.0081 and 0.0046 for MF, MIC, and GEN respectively indicates good accuracy.

Accuracy of the methods for the analysis of pharmaceutical formulation is further assured by applying the standard addition technique for GEN, while to check the accuracy of methods of MF and MIC, their results are statistically compared to HPLC reported method [10] where good results are obtained, confirming the accuracy of the proposed methods.

4.4. Precision

Intra-day and inter-day precision for the methods checked by analyzing three different concentrations of each drug, within the linearity range, repeated in the same day or on three different days, results are stated in Table 1.

4.5. Specificity

Specificity is ascertained by analyzing different laboratory prepared mixtures of MIC, MF and GEN in different ratios within the linearity range. The mean ± SD show good percentage recovery with the lowest standard deviation among the other methods. Satisfactory results are shown in Tables 2a and 2b.

Results for analyzing the three drugs in momenta cream are shown in Table 3. The results show that the proposed methods are adequately accurate and there is no interference of pharmaceutical excipients.

5. Statistical comparison

Table 4 shows statistical results obtained by comparing the proposed methods and the official method of MIC, MF [2], and GEN [20]. Calculated *t* and F values ensure no significant difference between the proposed and the official methods. One-way ANOVA is applied, Table 5 ensure that the graphical method namely concentration value has no significant difference regarding to other proposed methods and official methods for the determination of MIC, MF, and GEN.

6. Conclusion

Resolution techniques for the analysis of the ternary pharmaceutical mixture of MIC, MF, and GEN are introduced. The novelty in this work is the use of differential amplitude modulation methods which is applied to analyze overlapped spectra without extraction of one of the components. In addition, a novel technique is introduced to get the concentration of the cited drugs namely, concentration value depending on the graphical data of the spectra, so the concentration value is directly recorded on the spectral chart. The use of the physical separation via extraction followed by mathematical manipulation permits the determination of the components in this complex mixture with minimum manipulation steps and satisfactory results. Applying the optimal reaction conditions for the quantitative determination of gentamicin with OPA. The proposed methods accordingly can be successfully applied for routine analysis of the studied drugs either in their pure bulk powders or pharmaceutical formulation in quality control laboratories.

References

[1] S.C. Sweetman, Martindale: The Complete Drug Reference, Pharmaceutical Press, London, 2005.
[2] British Pharmacopoeia, The Stationery Office on be- Half of the Medicines and Healthcare Products Regulatory Agency (MHRA)@ Crown Copyright, 2013.
[3] N. Alizadeh, R. Ansari, N.K. Rezaee, Extractive spectrophotometric determination of miconazole nitrate in pure and pharmaceutical preparation, J. Pharm. Health 1 (4) (2012) 45–52.
[4] A.A. Salem, H.A. Mossa, B.N. Barsoum, Application of nuclear magnetic resonance spectroscopy for quantitative analysis of miconazole, metronidazole and sulfamethoxazole in pharmaceutical and urine samples, J. Pharm. Biomed. Anal. 41 (2) (2006) 654–661.
[5] C.P. Yanping, Determination of miconazole nitrate capsules by UV spectrophotometry, Drug Stanoaros of China 2002, p. 3.
[6] M.L.A. Nein Erk, Spectrophotometric resolution of metronidazole and miconazole nitrate in ovules using ratio spectra derivative spectrophotometry and RP-LC, J. Pharm. Biomed. Anal. 25 (1) (2001) 115–122.
[7] H.M. Heneedak, I. Salama, S. Mostafa, M. El-Sadek, HPLC and chemometric methods for the simultaneous determination of miconazole nitrate and nystatin, J. Chromatogr. Sci. 50 (10) (2012) 1–7.
[8] T.S. Haggag, Gradient HPLC-DAD stability indicating determination of miconazole nitrate and lidocaine hydrochloride in their combined oral gel dosage form, J. Chromatogr. Sci. (2012) 4–16.
[9] N. O'Connor, M. Wharton, P. Sweetman, The determination of miconazole and its related production impurities together with basic solution stability studies using a sub 2 μm chromatographic column, J. Chromatogr. Sci. 50 (2012) 199–205.
[10] R.I. El-Bagary, M.H. Tammam, A. Abo Elmaaty, Simultaneous determination of miconazole and hydrocortisone or mometasone using reversed phase liquid chromatography, Eur. J. Chem. 3 (4) (2012) 421–425.
[11] C. Akaya, S.A. Özkan, Z. Şentürkc, Ş. Cevheroğlud, Simultaneous determination of metronidazole and miconazole in pharmaceutical dosage forms by RP-HPLC, Farmaco 57 (11) (2003) 953–957.
[12] M.M. De Zan, M.S. Camara, J.C. Robles, S.V. Kergaravat, H.C. Goicoechea, Development and validation of a simple stability-indicating high performance liquid chromatographic method for the determination of miconazole nitrate inbulk and cream formulations, Talanta 79 (3) (2000) 762–767.
[13] I. Salama, M.S. Gomaa, Comparative determination of miconazole, nystatin, hydrocortisone and neomycin by HPTLC/HPLC-DAD, Eur. J. Chem. 4 (2013) 1.
[14] D.B. Meshram, S.B. Bagade, M.R. Tajne Pak, Simultaneous determination of metronidazole and miconazole nitrate in gel by HPTLC, J. Pharm. Sci. 22 (3) (2009) 323–328.
[15] M.Čakar, G.Popovic, D.AgbabA, High-performance thin-layer chromatography determination of some antimycotic imidazole derivatives and preservatives in medicinal creams and a gel J. AOAC Int., 88(5), (2005),1544–1548.
[16] K.A. Shaikh, A.T. Patil, Stability indicating HPLC method for the determination of mometasone, oxymeazoline, phenyl ethanol, and benzalkonium chloride in nasal drops, J. Trace Anal. Food Drugs 1 (2013) 14–21.
[17] X. Wei Teng, K. Foe, K.F. Brown, David J. Cutler, Neal M. Davies, High-performance liquid chromatographic analysis of mometasone furoate and its degradation products: application to in vitro degradation studies, J. Pharm. Biomed. Anal. 26 (2) (2001) 313–319.
[18] A.A. Kulkarni, R. Nanda, M.N. Ranjane, Simultaneous estimation of Nadifloxacin and mometasone in topical creams, Pharma Chem. 2 (3) (2010) 25–30.
[19] L. Wulandari, T. Kiau Sia, TLC densitometric determination of mometasone furoate in topical preparations: validation, J. Liq. Chromatogr. Relat. Technol. 26 (1) (2003) 109–117.
[20] The United States Pharmacopoeia, The National Formulary, 25th ed. United States Pharmacopeial Convention, Rockville, MD, 2007.
[21] A. El-Didamony, A. Amin, Ahmed Ghoneim, A. Telebany, Indirect spectrophotometric determination of gentamicin and vancomycin antibiotics based on their oxidation by potassium permanganate, Open Chem. 4 (4) (2006) 2391–5420.

[22] J. Krzek, H. Woltynska, U. Hubicka, Determination of gentamicin sulphate in injection solutions by derivative spectrophotometry, Anal. Lett. 42 (3) (2009) 473–482.
[23] K. Vučićević-Prčetić, N. Radulovic, Development and validation of liquid chromatography tandem mass spectrometry methods for the determination of gentamicin, lincomycin, and spectinomycin in the presence of their impurities in pharmaceutical formulations, J. Pharm. Biomed. Anal. 56 (4) (2011) 736–742.
[24] A.R. Arul Joseph, Development and validation of a RP-HPLC method for the determination of gentamicin sulfate and its related substances in a pharmaceutical cream using a short pentafluorophenyl column and a Charged Aerosol Detector, J. Pharm. Biomed. Anal. 51 (3) (2010) 521–531.
[25] D. Löffler, T.A. Ternes, Analytical method for the determination of the aminoglycoside gentamicin in hospital wastewater via liquid chromatography–electrospray-tandem mass spectrometry, J. Chromatogr. A 1000 (1–2) (2003) 583–588.
[26] A.A. Al-Majed, F. Belal, M.A. Abounassif, N.Y. Khalil, Fluorimetric determination of gentamicin in dosage forms and biological fluids through derivatization with 4-chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), Microchim. Acta 41 (1–2) (2003) 1–6.
[27] N. Isoherranen, S. Soback, Determination of gentamicin after trimethylsilylimidazole and trifluoroacetic anhydride derivatization using gas chromatography and negative ion chemical ionization ion trap mass spectrometry, Analyst 125 (9) (2000) 1573–1576.
[28] L. Yuan, H. Wei, S. Li, Direct determination of gentamicin components by capillary electrophoresis with potential gradient detection, Electrophoresis 26 (1) (2004) 196–201.
[29] E.G. Kulapina, V.V. Baraguzina, O.I. Kulapina, Rapid potentiometric determination of aminoglycoside antibiotics in drug dosage forms and biological fluids using ion-selective electrodes, J. Anal. Chem. 60 (2005) 523–527.
[30] N.K. Ramadan, H.M. Mohammed, A.A. Mostafa, Simultaneous determination of rabeprazole sodium and domperidone, J. Appl. Pharm. Sci. 1 (9) (2011) 73–80.
[31] N.S. Abdelwahab, Spectrophotometric methods for simultaneous determination of carvedilol and hydrochlorothiazide in combined dosage form, Arab. J. Chem. (2011).
[32] J.J. Berzas Nevado, C.G. Cabanillas, F. Salinas, Spectrophotometric resolution of ternary mixtures of salicylaldehyde, 3-hydroxybenzaldehyde and 4-hydroxybenzaldehyde by the derivative ratio spectrum-zero crossing method, Talanta 39 (5) (1992) 547–553.
[33] A. Afkhami, M. Bahram, Successive ratio-derivative spectra as a new spectrophotometric method for the analysis of ternary mixtures, Spectrochim. Acta A 61 (2005) 869–877.
[34] H.M. Lotfy, M.A. Hegazy, Comparative study of novel spectrophotometric methodsmanipulatingratio spectra:anapplication onpharmaceuticalternary mixture of omeprazole, tinidazole and clarithromycin, Spectrochim. Acta A Mol. Biomol. Spectrosc. 96 (2012) 259–270.
[35] H.M. Lotfy, S.M. Tawakkol, N.M. Fahmy, M.A. Shehata, Successive UV-spectroscopic resolution as a novel technique for the analysis of ternary mixtures of pharmaceuticals, Spectrochim. Acta A 121 (2014) 313–323.
[36] M.G. El-Bardicy, H.M. Lotfy, M.A. El-Sayed, M.F. El-Tarras, Smart stabilityindicating spectrophotometric methods for determination of binary mixtures without prior separation, J. AOAC Int. 92 (2) (2008) 299–310.
[37] C.V. Patel, A.P. Khandhar, A.D. Captain, K.T. Patel, Validated absorption factor spectrophotometric and reversed-phase high-performance liquid chromatographic methods for the determination of ramipril and olmesartan medoxomil in pharmaceutical formulations, Eurasian J. Anal. Chem. 2 (3) (2007) 159–171.
[38] Y. El-Saharty, Simultaneous determination of Piracetam and Vincamine by spectrophotometric and high-performance liquid chromatographic methods, J. AOAC Int. 91 (2008) 311–321.
[39] L. López-Martínez, P.L. López-de-Alba, L.M. de- León-Rodríquez, M. Yepez-Murrieta, Simultaneous determination of binary mixtures of trimethoprim and sulfamethoxazole or sulphamethoxypyridazine by the bivariate calibration spectrophotometric method, J. Pharm. Biomed. Anal. 30 (1) (2002) 77–85.
[40] S. Altınöz, S. Toptan, Simultaneous determination of Indigotin and Ponceau-4R in food samples by using Vierordt's method, ratio spectra first order derivative and derivative UV spectrophotometry, J. Food Compos. Anal. 16 (4) (2003) 517–530.
[41] N. Erk, Determination of active ingredients in the pharmaceutical formulations containing hydrochlorothiazide and its binary mixtures with benazepril hydrochloride, triamterene and cilazapril by ratio spectra derivative spectrophotometry and Vierordt's method, J. Pharm. Biomed. Anal. 20 (1–2) (1999) 155–167.
[42] K.P. Bhusari, P.B. Khedekar, S. Dhole, V.S. Banode, Derivative and Q-analysis spectrophotometric methods for estimation of hydrochlorothiazide and olmesartan medoxomil in tablets, Indian J. Pharm. Sci. 71 (5) (2009) 505–508.
[43] M.R. El-Ghobashy, N.F. Abotalib, Spectrophotometric methods for the simultaneous determination of binary mixture of metronidazole and diloxanidefuroate without prior separation, J. Adv. Res. Cairo Univ. 1 (2010) 323–329.
[44] K.U. Ratnasingham, S.M. Andreani, D. Light, B. Patel, Meta-analysis of the use of glyceryl trinitrate ointment after haemorrhoidectomy as an analgesic and in promoting wound healing, Int. J. Surg. (Lond.) 8 (8) (2010) 606–611.
[45] E. Dinç, F. Onur, Application of a new spectrophotometric method for the analysis of a ternary mixture containing metamizol, paracetamol and caffeine in tablets, Anal. Chim. Acta 359 (1–2) (1998) 93–106.
[46] H.M. Lotfy, S.M. Tawakkol, N.M. Fahmy, M.A. Shehata, A comparative study of novel spectrophotometric resolution techniques applied for pharmaceutical mixtures with partially or severely overlapped spectra, Spectrochim. Acta A 136 (2015) 937–952.
[47] H.M. Lotfy, Absorbance subtraction and amplitude modulation as novel spectrophotometric methods for the analysis of binary mixtures, Int J Pharm Pharm Sci 6 (1) (2014) 735–741.
[48] H.M. Lotfy, S.S. Saleh, N.Y. Hassan, H. Salem, A comparative study of novel spectrophotometric methods basedon isosbestic points; application on a pharmaceutical ternary mixture, Spectrochim. Acta A Mol. Biomol. Spectrosc. 126 (2014) 112–121.
[49] M.A. Ramadan, *o*-Phthalaldehyde based spectrophotometric method for determination of Sitagliptin in tablets, Int. J. Pharm. Pharm. Sci. 6 (2) (2014) 125–129.
[50] R. Srikanth, D. Raghunadha Reddy, C.S. Venkatesan, Development and validation of RP-HPLC pre-column derivatisation for the trace level determination of tertbutylamine in tigecycline drug substance, Int. J. Pharm. Bio. Sci. 4 (2) (2013) 522–531.
[51] D.A. Freedman, Statistical Models: Theory and Practice, Cambridge University Press, 2005.
[52] H.M. Lotfy, Determination of simvastatin and ezatimibe in combined tablet dosage forms by constant center spectrophotometric method, Int. J. Pharm. Pharm. Sci. 4 (2012).
[53] A. Samir, H.M. Lotfy, H. Salem, M.A. Al Kawy, Development and validation of simultaneous spectrophotometricand TLC-spectrodensitometric methods for determinationof beclomethasone dipropionate and salbutamol in combined dosage form, Spectrochim. Acta A Mol. Biomol. Spectrosc. 128 (2014) 127–136.
[54] H.M. Lotfy, M.A. Hegazy, M.R. Rezk, Y.R. Omran, Novel spectrophotometric methods for the simultaneous determination of timolol and dorzolamide in their binary mixture, Spectrochim. Acta A Mol. Biomol. Spectrosc. 126 (2014) 197–207.
[55] S.S. Saleh, H.M. Lotfy, N.Y. Hassan, H. Salem, A comparative study of progressive versus successive resolution techniques applied for pharmaceutical ternary mixtures, Spectrochim. Acta A Mol. Biomol. Spectrosc. 132 (2014) 239–248.
[56] M.A. Hegazy, H.M. Lotfy, M.R. Rezk, Y.R. Omran, Novel spectrophotometric determination of chloramphenicol and dexamethasone in the presence of non labeled interfering substances using univariate methods and multivariate regression model updating, Spectrochim. Acta A Mol. Biomol. Spectrosc. 140 (2015) 600–613.
[57] ICH Q2B, Note for guidance on validation of analytical methods methodology, International Conference on Harmonization, IFPMA, Geneva, 1997.