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Research article

A new selective colorimetric method coupled with a high-resolution UV method for the consecutive quantification of three drugs in semi-solid preparations

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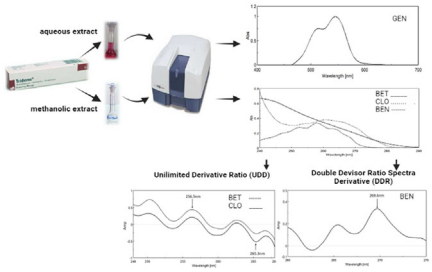
HIGHLIGHTS

- Green selective colorimetric method for increasing the sensitivity of Gentamycin quantification with no interference from accompanying drugs in Triderm®.
- The first colorimetric method for GEN quantification that doesn't require heating, long waiting, or solvent extraction.
- Introducing an efficient environmentally friendly surfactant in spectroscopic fields to aid the colored hydrophobic ion-pair solubility.
- Unlimited Derivative Ratio (UDD), a High-resolution green method for the concurrent quantification of the severely overlapped Clotrimazole and Betamethasone by the signal filtration process.
- Double Divisor Ratio spectra derivative method for Benzyl alcohol quantification.

GRAPHICAL ABSTRACT



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ABSTRACT

Triderm® cream and ointment contain clotrimazole (CLO), betamethasone dipropionate (BET), and the poor UV absorbing gentamycin (GEN), in addition to the preservative benzyl alcohol (BEN) which exists only in a cream preparation. A green, selective colorimetric approach was elaborated to increase the sensitivity of GEN quantification in Triderm® preparations, which depends on the immediate formation of a pink ion-pair between GEN and erythrosine (ERY) reagent in an aqueous acidic medium. The ion pair was made soluble in water with the assistance of the surfactant agent poloxamer 188 which is presented in this manuscript as an efficient solubilizing agent for the hydrophobic ion-pair. This surfactant agent has the feature of not affecting the native color of ERY, additionally the ease of preparing its aqueous solution with no need for heating or long waiting. The resulting complex GEN-ERY was measured directly at 545nm. This colorimetric approach was coupled with the Unlimited Derivative Ratio (UDD), which is a new smart UV method employed for the concurrent quantification of BET and CLO in Triderm® preparations without any intervention from BEN, due to its capability to resolve an extremely overlapped ternary spectrum that has no extended part, iso-absorptive point or robust zero crossing point. The newly developed UDD method depends on filtrating and measuring the signal of BET and CLO through calculating the equality factor(F) for CLO and BET after dividing their spectrum by BEN spectrum, derivatizing the resulting

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ratio spectrum, then constructing a regression equation employing the F factor for each BET and CLO. The overlapping excipient BEN was quantified via the Double Divisor Ratio spectra derivative method (DDR) relying on using a divisor comprising of a mix of BET + CLO. The advanced spectrophotometric approach validity was checked by confirming the linearity, accuracy, precision, and specificity in accordance with the ICH directions. NO notable difference when statistically comparing the newly established approach to the reference approach.

1. Introduction

The pharmaceutical formulation of (BET, CLO, and GEN) is utilized for fungal and bacterial infections associated with severe inflammation. It is the favorite choice when it is difficult to diagnose skin diseases of mixed infections [1]. Clotrimazole (CLO) IUPAC Name is 1-[(2-chlorophenyl) diphenylmethy]-1H-imidazole [2], Figure 1, an antifungal from the imidazole class that affects by damaging the permeability of the cell membrane [3] it was quantified via spectrophotometry [4, 5, 6], HPLC [7, 8, 9, 10], and TLC [11, 12, 13].

Betamethasone dipropionate (BET) IUPAC Name is 9-Fluoro-11b, 17,2 1-trihydroxy-16b-methylpregna-1,4-diene3,20- dione [2], Figure 1, a glucocorticoid with immunosuppressive and anti-inflammatory action [3], it was quantified via spectrophotometry [14, 15], HPLC [16], and TLC [17, 18].

Gentamycin (GEN) is an aminoglycoside bactericidal, Figure 1, works by inhibiting the synthesis of protein in bacteria [3], it was quantified via spectrophotometry [19, 20, 21, 22, 23, 24],spectrofluorimetric [25, 26], HPLC [27, 28, 29], and TLC [30].

Benzyl alcohol (BEN) IUPAC Name is phenyl methanol, Figure 1, utilized in topical pharmaceutical preparations as an antimicrobial preservative [31], it was quantified via spectrophotometry [32, 33, 34], HPLC [35], and micellar liquid chromatography [36].

The previous literature describes only one mathematical UV method [20] and five chemometric methods [37] for the analysis of pharmaceutical preparation containing only BET, CLO, and BEN without the presence of GEN, the USP describes an HPLC approach for the quantification of BET and CLO in cream preparations [38], another HPLC method [39] was reported for their quantification in a cream formulation.

Semi-solid preparations are a topical delivery system used to deliver drugs via the skin or mucous membranes, the incorporation of a combination of drugs into semi-solid formulations is intended to achieve more than one therapeutic goal at the same time and to get synergistic topical treatment for dermatological conditions. Unlike solid forms, the formulation of semi-solid preparations requires many auxiliary ingredients like preservatives, emulsifying agents, solubilizing agents, etc. to obtain a suitable drug carrier for topical delivery. This constitutes an obstacle to conducting rapid and economic daily analysis as a number of the used excipients interfere with drug analysis. To overcome this obstacle, it is important to supply the quality-control laboratories with a well-established analytical approach that allows rapid and inexpensive analysis of the drug combination without intervention with the excipients. As well as, to supply a correct sample preparation protocol to extract the entire drug substance from the semi-solid formulation, thus obtaining correct results for drug quantification.

Because the spectrophotometric approach is preferable over the HPLC approach for being simple, quick, and inexpensive, a high-efficiency green colorimetric approach associated with the UV approach was utilized for the consecutive quantification of GEN, BET, and CLO in the existence of the overlapping excipient BEN.GEN was quantified by its reaction with erythrosine (ERY), which is the disodium salt of 2,4,5,7-tetraiodofluorescein utilized as a fluorogenic dye [40], ERY can be applied for the

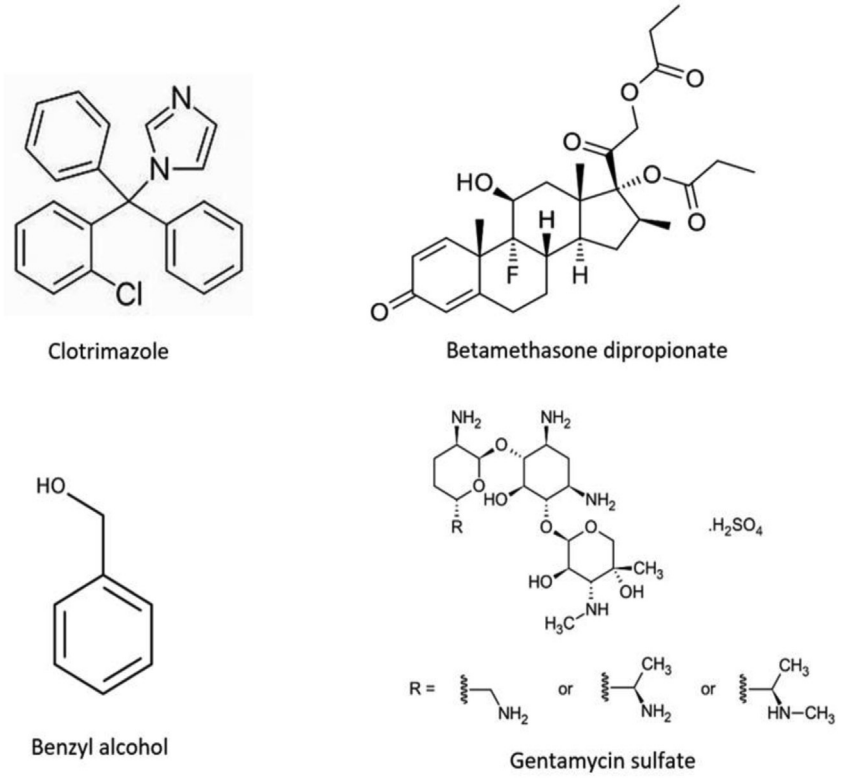

Figure 1. The analyzed drug's chemical structure.

spectrofluorimetric or spectrophotometric quantification of drugs by reacting with them in acidic medium to form a pink non-fluorescent ion-pair, which is determined Spectrophotometrically via measuring its color intensity, or spectrofluorimetric via measuring the decreasing of erythrosine native fluorescence [41, 42]. The resulting ion-pair between GEN and ERY was Poorly soluble in water, an attempt to resolve this issue is by adding poloxamer 188, which is a non-ionic poly-oxyethylene–polyoxypropylene copolymer, used primarily in pharmaceutical preparations as emulsifying or solubilizing agents [31], in this manuscript poloxamer 188 was first employed in the spectroscopic field as an attempt to resolve the issue of the ion-pair solubility in water without energy waste or prolonged preparation as in some surfactant, and without influencing the original color of ERY. This newly introduced approach outperforms the previous spectroscopic methods for GEN quantification, since it is the first colorimetric approach for its quantification with an instant reaction, no heating nor extraction of the formed complex is needed, besides its high sensitivity and selectivity.

In conjunction with the newly developed colorimetric approach, BET and CLO were quantified simultaneously without any intervention with BEN by the unlimited derivative ratio method (UDD) [43] Which depends on filtrating the signal of the compounds in the triple mixture through first applicating the derivative ratio process, and second by employing the concept of the equality factor (F), which works on removing the intervention of another drug by a simple mathematical process, therefore gaining a filtered signal related to the quantity of the intended drug only. This method offers an appropriate solution for the extremely overlay spectra in the absence of the iso-absorptive point, extended area, or proper zero crossing point. Also, the Double Divisor Ratio spectra derivative method (DDR) [44] was employed for detecting the quantity of BEN in the cream formulation via utilizing a double divisor, providing a good way to extract the signal of one substance from a triple mix with no intervention from the accompanying compounds and thus getting a separated spectrum with the possibility of selecting the appropriate wavelength for the quantitative estimation. This new spectral, none-chemometric protocol has the feature of electing the appropriate wavelength for the quantification process all over the spectrum, unlike the previous approach for BET, CLO, and BEN quantification namely dual-wavelength in ratio spectra (DWRS) [20] which is restricted by electing two specified wavelengths having an identical signal for the intervention component.

Due to the widespread use of Triderm® preparation for skin lesions, and the lack of an environmentally friendly spectrophotometric approach for its quick analysis, this manuscript provides a simple, efficient, and economic high-resolution protocol for the spectrophotometric quantification of BET, CLO, and BEN consecutively with GEN on the same spectrophotometric apparatuses without the need for additional procedures such as purchasing a specialized program, spectrofluorimetric measurements, heating, or long time waiting, also to presenting an appropriate sample preparation method to accurately extract these components from their semi-solid composition.

2. Theoretical background

2.1. Unlimited derivative ratio method (UDD)

This new approach can be applicated to a triple mix (A, B, C) with high overlaying spectra, it works by two consecutive steps of signal filtration:

2.1.1. 1st step: signal filtration by spectra manager software

Where C interference was canceled via dividing (A, B, C) spectrum by the C spectrum, and then selecting a proper order of derivation of the outcome ratio spectra (A + B + C/C).

2.2.2. 2nd step: mathematical filtration on an excel worksheet

By estimating the equality factor (F_B) using two wavelengths (λ_1, λ_2) on the derivative ratio spectrum, and multiplying it by P_{m2}, hence the signal (ΔP) is related only to A concentration:

$$\Delta P = P_{m\lambda 1} - F_B P_{m\lambda 2} = P_{A\lambda 1} - F_B P_{A2}$$

P_m: (A + B) amplitude on derivative ratio spectrum, P_A: A amplitude. F_B: $amp_{B\lambda 1}$/ $amp_{B\lambda 2}$. B concentration is estimated via the exact procedure after computing (F_A) at the two chosen wavelengths. For determining C concentration, the same procedure was pursued after dividing the D^0 spectrum (A, B, C) by A or B then derivative and continuing as previously mentioned.

2.2. Double divisor ratio spectra derivative method (DDR)

This approach is utilized for the concurrent quantification of the triple mix (A, B, C), it relies first on utilizing the mix of the A + B spectrum as a divisor, followed then by the derivation of the gained ratio spectrum to delete the influence of A and B, and finally getting a spectrum related only to C concentration, in an exact manner, A and B concentration could be estimated.

3. Experimental

3.1. Instruments and soft wares

JASCO V-650 spectrophotometer and a 1 cm quartz cell were utilized for scanning the D^0 spectra. Spectra manager® software, version 2, JASCO corporation was used to handle absorption and derivative ratio spectra.

3.2. Material and reagent

3.2.1. Pure samples

CLO, BET, BEN, and GEN were gained from FENGCHEN GROUP CO., LTD, China, with the purity of 99.95 ± 1.08, 100.09 ± 0.80, 99.25 ± 1.01, 100.24 ± 0.87 respectively.

3.2.2. Pharmaceutical preparations

Triderm® cream is labeled to contain 0.64mg BET (equivalent to 0.5 mg betamethasone), 10 mg CLO, 1mg GEN, and BEN (10–30 mg) in each 1 g of cream, Triderm® ointment is the same compositions as cream formulation except the presence of benzyl alcohol.

Triderm® cream with Batch number:19TRC22, and ointment with Batch number:19TRO1 were manufactured by UNIPHARMA for pharmaceuticals industries/DAMASCUS/SYRIA Under license from Schering-Plough Corporation/USA.

3.2.3. Solvents

Analytical grade Methanol was obtained from Panreac, Spain.

- Erythrosine (ERY) was gained from the Matheson company, it was prepared in distilled water having a concentration of 400 μg/mL equivalent to 4.546×10^{-4} mol/L.
- Poloxamer 188 was gained from Sigma Aldrich, it was Prepared in distilled water with a concentration of 1%.
- Acetate buffer pH 3 was prepared by mixing acetic acid 0.4 M with sodium acetate in a proper ratio to obtain pH 3.

3.2.4. Standard solutions

- Methanolic stock standard solution of 1000 μg/mL of CLO.

- Methanolic stock standard solution of 2000 μg/mL of BEN.
- Methanolic stock standard solution of 100 μg/mL of BET.
- Aqueous stock Standard solution of 100 μg/mL of GEN equivalent to 2.155×10^{-4} mol/L.
- Aqueous working standard solution of 10 μg/mL of GEN equivalent to 2.155×10^{-5} mol/L.

3.3. Procedure

3.3.1. Determining linearity and constructing calibration graphs

A set of six 10-mL volumetric flasks were produced by transferring portions equivalent to 0.5–4 μg/mL (1.07×10^{-6}–8.62×10^{-6} mol/L) of GEN from the working solution, adding 1.5ml poloxamer, then 1ml ERY solution, mixing gently, after that 1ml acetate buffer was added, mixed, and then completed with water to the mark, scanned in 400–700 nm range versus blank fitted as the same way of sample.

A set of six 5-mL volumetric flasks were produced by transferring portions equivalent to 100–500 μg/mL of CLO, 100–800 μg/mL BEN, and 5–50 μg/mL BET from the stock solution, and diluting with methanol to the line mark, then scanned versus the solvent in 200–400 nm range.

3.3.1.1. Colorimetric determination of GEN by ERY. The absorbance of the formed ion-pair was measured at 545 nm, and then GEN concentration was computed via the constructed linear equation between absorbance and concentrations at 545 nm.

3.3.1.2. Unlimited derivative ratio method for CLO and BET signal filtration

3.3.1.2.1. 1st step: signal filtration by spectra manager software. CLO D^0 spectra were divided via BEN 800 μg/mL standard spectrum, a derivation (first-order derivative, 13 data points, scale factor 10) was applied to the outcome ratio spectra and saved on spectra manager software.

3.3.1.2.2. 2nd step: mathematical filtration on an excel worksheet.. The equality factor of BET was computed by dividing BET amplitude (P_{BET}) at the two elected wavelengths (λ_1:256.5 nm, λ_2:265.3 nm), F_{BET} = (amplitude of BET at λ_1/amplitude of BET at λ_2) = −1.57, this computed factor was then multiplied by the amplitude of CLO (P_{CLO}) at 265.3 nm, and the linear equation between ($P_{CLO\lambda1}$-F_{BET} $P_{CLO\lambda2}$) and CLO concentrations was constructed on excel worksheet.

For BET quantification, the same two steps stated for CLO were pursued, and the equality factor of CLO was computed, F_{CLO} = (amplitude of CLO at λ_1/amplitude of CLO at λ_2) = −0.37, and multiplied by the amplitudes of BET at 265.3 nm, the concentration of BET was determined utilizing the linear equation between ($P_{BET\lambda1}$-F_{CLO} $P_{BET\lambda2}$) and correspondent concentrations.

3.3.1.3. Double divisor ratio spectra derivative approach for BEN estimation. BEN D^0 spectra were divided via a (400 μg/mL CLO + 20 μg/mL BET) spectrum, and the resulting ratio spectra went through a derivation (first-order derivative, 17 data points, scale factor 10), then a linear equation was built between BEN signals at 269.7 nm and its related concentrations.

3.3.2. Applying the mathematical approaches for quantification of CLO, BET, and BEN in lab-prepared mixes

Various mixes with a diverse proportion of CLO, BETA, and BEN were set, the scanned D^0 spectrum of each mixture was divided by BEN 800 μg/mL standard spectrum, then the derivation (first-order derivative, 13 data points, scale factor 10) is performed on the resulting ratio spectrum, CLO was quantitively detected by multiplying the computed F_{BET} by the mixture amplitude (p_m) at 265.3 nm, then subtracting ($p_{m256.5\ nm}$-(−1.57*$p_{m265.3\ nm}$), and compensation in CLO constructed linear equation. For BET quantification the same stages of dividing and derivation were pursued as in CLO quantification utilizing the computed F_{CLO}, then the subtraction result of ($p_{m256.5\ nm}$-(−0.37*$p_{m265.3\ nm}$) was compensation in BET constructed linear regression.

For BEN quantification in the drug mixes, the scanned D^0 spectrum of each mixture was divided by (400 μg/mL CLO + 20 μg/mL BET) spectrum, then the derivation (first-order derivative, 17 data point, scale factor 10) is performed on the resulting ratio spectrum, BEN concentration was determined via linear equation constructed at 269.7 nm.

3.3.3. Application to pharmaceutical preparations

3.3.3.1. For GEN extraction

3.3.3.1.1. Ointment formulation. Weight precisely 1 g of Triderm® ointment, add 25 mL distilled water, heat it until completely melted, centrifuge it for 10 min, then filter it into 50 ml volumetric flask, wash the residue with three portions of bi-distilled water, and complete to 50 mL with it, transfer 1 ml from the filtered aqueous solution into a 10-mL volumetric flask, and continue the procedure as previously stated using ERY reagent.

3.3.3.1.2. Cream formulation. Weight precisely 1 g of Triderm® cream, add 25 mL distilled water, heat it until completely melted, add 10

Table 1. The previous studies of gentamycin quantification.

Method	Reagent	Procedure conditions	Linearity range	references
Spectrophotometric determination by forming a metal complex	$CuCl_2$.$6H_2$ O	____	51–261 μg/mL	[24]
Spectrophotometric determination by chemical derivation	Ninhydrin	Heating at 95 °C for 15 min	30–120 μg/mL	[19]
Spectrophotometric determination by oxidation by an excess of potassium permanganate and determination of unreacted oxidant by reacting it with different reagents	Amaranth dye	Heating at 100 °C for 25 min	4–8 μg/mL	[23]
	Acid orange		3–8 μg/mL	
	Indigo carmine		4–9 μg/mL	
	Methylene blue		5–9 μg/mL	
Spectrophotometric determination by ion-pair extraction	Methyl red	Extraction with chloroform	15–60 μg/mL	[22]
Spectrophotometric determination by the ion-pair formation	2,4,6-trinitrophenol	Alkalization of gentamycin sulfate and extraction of the formed base with chloroform	2.5–140 μg/mL	[21]
	2,4-dinitrophenol		2.5–100 μg/mL	
Spectrophotometric determination by Chemical Derivation	OPA	Heating at 60 °C for 15 min	3–30 μg/mL	[20]
spectrofluorimetric determination by charge transfer extraction	Safranin	Extraction with chloroform	4–50 pg/mL	[26]
spectrofluorimetric determination by Chemical Derivation	OPA	Heating at 60 °C for 15 min	0.25–1.25 μg/mL	[25]

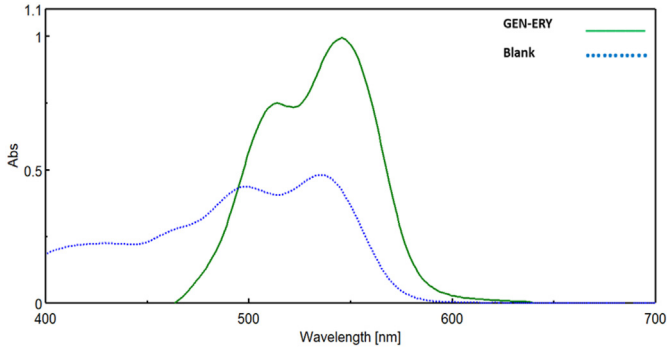

Figure 2. The absorption spectrum of gentamycin (4 μg/mL) ion pair with erythrosine reagent vs reagent blank.

mL chloroform to the aqueous solution, shake in a separating funnel, centrifuge the extracted aqueous layer and continue the steps as followed in the ointment formulation paragraph.

The quantity of chloroform added to the aqueous solution of the cream sample was utilized to extract the insoluble ingredients that turbid the aqueous solution, and which could not be eliminated by centrifugation or filtration, this technique of extraction is better than dispersing the

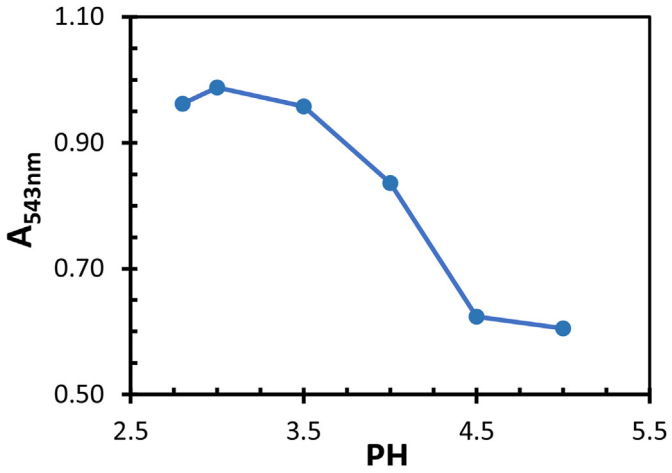

Figure 3. Effect of pH on the intensity of GEN-ERY ion pair.

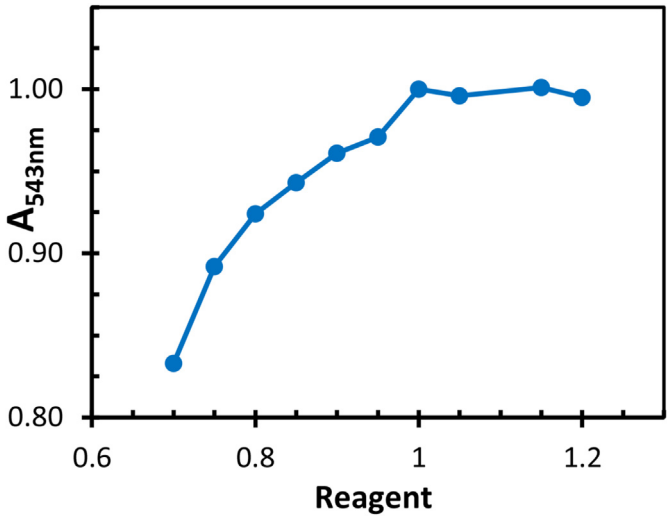

Figure 4. Effect of ERY volumes on the intensity of GEN-ERY ion pair.

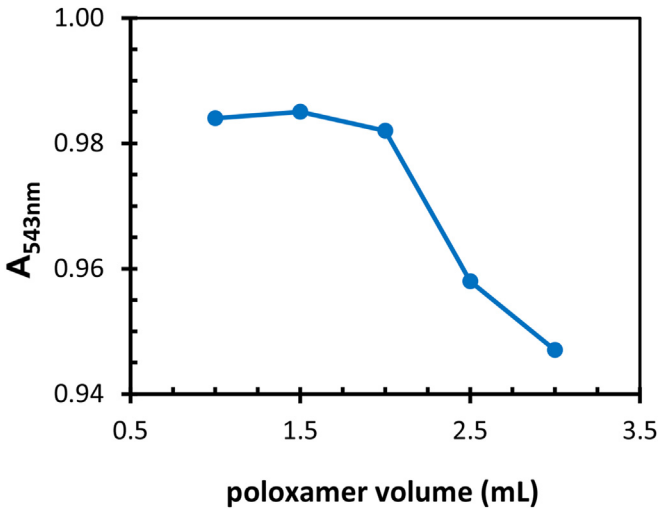

Figure 5. Effect of Poloxamer volumes on the intensity of GEN-ERY ion pair.

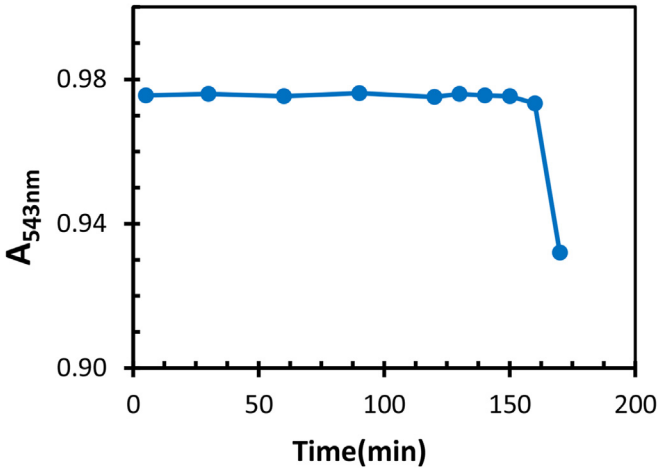

Figure 6. Effect of time (min) on the intensity of GEN-ERY ion pair.

weighted portion of cream in chloroform first, then extracting GEN with water, because of the reduce in the recovered amount of GEN upon using chloroform first to disperse the sample.

3.3.3.2. For CLO, BET, and BEN extraction. Weight precisely 1 g of Triderm® cream or ointment, add 10 mL methanol to it, and heat it until completely melted, transfer it to a capped test tube, shake it vigorously for 10 min, heat again, and shake vigorously for 10 min, put the capped test tube in the refrigerator for 20 min, then filter immediately while it is cold into 25 mL volumetric flask, wash the residue with methanol, and complete it with the same solvents to the mark, transfer 7.5 mL from the filtered methanolic solution into a 10-ml volumetric flask, and complete it with methanol to the line mark. The stated approach under lab-prepared mixes was then pursued.

4. Results and discussion

This manuscript describes a new protocol for resolving GEN, CLO, and BET in pharmaceutical preparation containing BEN as a preservative, by working in two separate stages using two different solvents to resolve the issue of finding a suitable solvent for the drugs in Triderm® pharmaceutical formulation, because of the dissimilarity in solubility of gentamycin and its accompanying components BET, CLO, and BEN, thus the

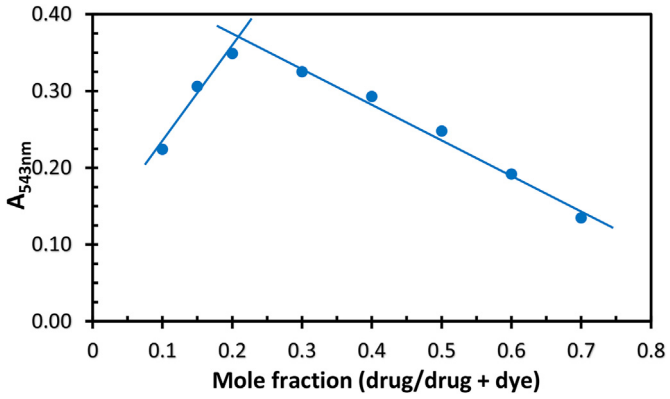

Figure 7. Continuous variation plots of the formed ion-pair between gentamycin and erythrosine reagent.

first step of analyzes including a selective colorimetric method depending on using water as a solvent to extract GEN from its pharmaceutical preparation and forming ion pair with ERY in an acidic medium, pursued by the other step which relies on using methanol to extract the three components CLO, BET and BEN from their pharmaceutical preparation and then applying the new developed mathematical approaches for the concurrent quantification of them.

4.1. Quantification of GEN by reaction with ERY reagent

GEN has negligible absorption in the ultraviolet field, so many colorimetric approaches have been elaborated to quantify it. Still, they suffer from some drawbacks, like heating, long waiting for the reaction to complete, extraction with organic solvents, and low sensitivity as clarified in Table 1, this makes the newly presented approach more preferable, as it has good sensitivity and relies on the immediate development of the pink ion pair with erythrosine in the acidic medium in the existence of the nonionic surfactant poloxamer 188, the formed ion-pair was measured at 545nm as clarified in Figure 2. In addition to the high sensitivity of this newly displayed colorimetric approach, its eco-friendly feature and the speed of analysis process, it also proved its ability to selectively estimate gentamicin concentration in the existence of other

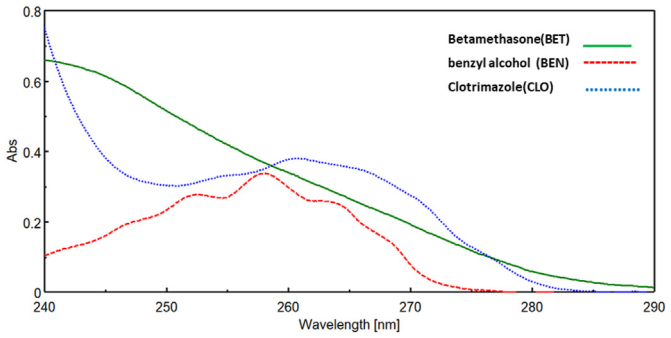

Figure 9. D^0 spectra of clotrimazole (200 μg/mL), betamethasone (20 μg/mL), and benzyl alcohol (200 μg/mL) in methanol.

accompanying drugs and excipients in Triderm® which what achieves the intended goal of this investigation.

4.1.1. Optimum reaction conditions

GEN molecule has four amino groups capable of forming an ion pair with ERY, various parameters affecting the ion pair creation, like pH, surfactant, temperature, the time required for complete reaction, and the concentration of ERY were investigated.

4.1.1.1. Effect of pH. The effect of some buffers on the color intensity was tested using phosphate, borate, Britton, and acetate buffers, the best buffer with the highest absorbance of the resulting ion-pair is the acetate buffer (0.4M), also the ion-pair formation was detected over pH range (2.5–4), at pH 3 the intensity of the pink color was maximum upon adding 1ml volume of the buffer as clarified in Figure 3.

4.1.1.2. Effect of the concentration of ERY reagent. The effect of different volumes (0.5–1.5) mL of ERY (0.04%) was examined, the signal of the developed ion pair was maximum and stable when utilizing 1mL volume of ERY with a final concentration of 40 μg/mL equivalent to 4.546×10^{-5} mol/L as clarified in Figure 4.

4.1.1.3. Effect of surfactant. The Previous studies describe some solutions to prevent the precipitation of the resulting ion pair, one of them

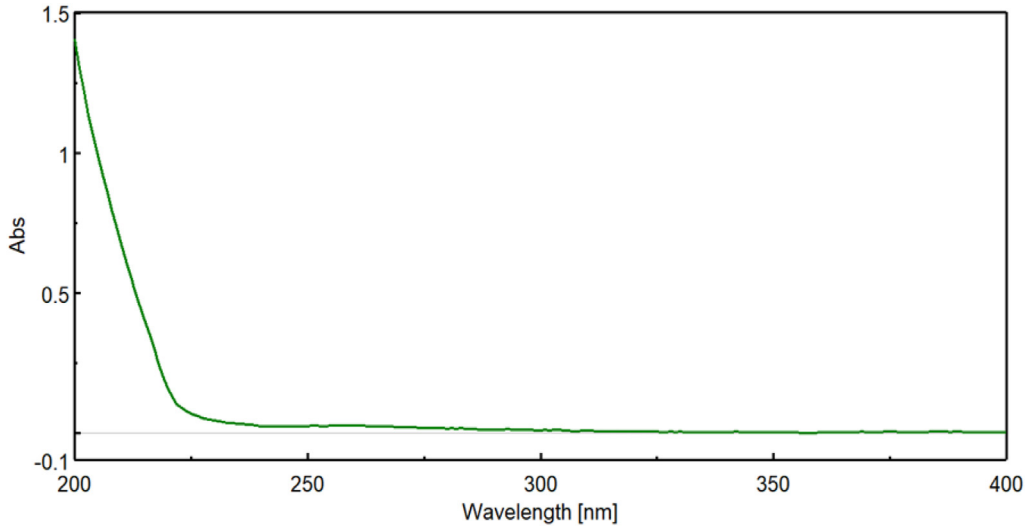

Figure 8. The UV absorption spectrum of the extracted clotrimazole in the aqueous layer.

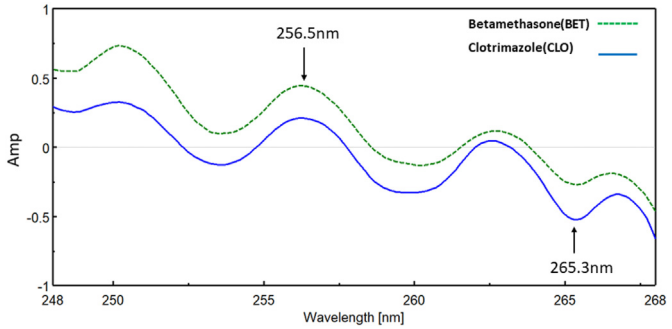

Figure 10. The first derivative ratio spectra of clotrimazole (200 μg/mL) and betamethasone (20 μg/mL) using (800 μg/mL) of benzyl alcohol as a divisor.

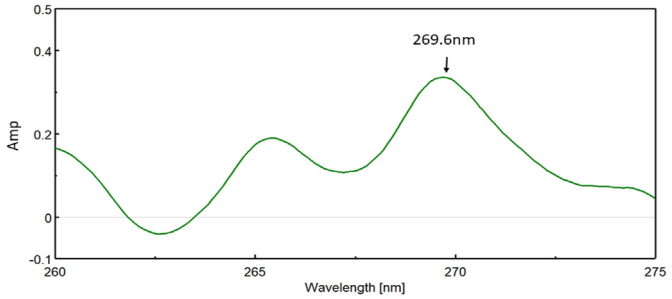

Figure 11. The first derivative ratio spectrum of benzyl alcohol (200 μg/mL) using (400 μg/mL CLO + 20 μg/mL BET) as a divisor.

is the technique described by El-Brashy [45], which depends on diluting the sample solution to the maximum, and then adding the reagent at a neutral solution, good mixing is required before adding the acidic buffer, this described method failed in preventing the precipitation of GEN-ERY ion pair as it accrued after 10 min of following this procedure, another method depending on utilizing non-ionic surfactant was tried such as CMC, MC, PVA, PVP, Tween 80, PEG, and Poloxamer188 which was first tested in spectroscopic studies, only poloxamer188 succeeded in this task and none of the rest gave any satisfactory results considering that CMC and MC require heating and long preparation time, also PEG failed to give a clear solution, and upon using PVA and tween an inhibition in ERY fluorescence happened. The non-ionic surfactant Poloxamer188 was chosen as it keeps the solutions clear, doesn't affect the ERY fluorescence, very soluble in water, and doesn't need a long-time preparation.

Moreover, different volumes of poloxamer188 were tested to select the optimal one, 1.5 mL of poloxamer188 was sufficient to obtain a clear solution with no notable impact on the ion-pair absorbance as clarified in Figure 5.

Table 3. Resolving results of the lab-prepared mixes by the suggested approach.

components ratio (ug/ml) BET: CLO: BEN	BET	CLO	BEN
	UDD method (Mean% ± SD)	UDD method (Mean% ± SD)	DDR method (Mean% ± SD)
10:400:600	100.66 ± 0.11	100.39 ± 0.22	98.01 ± 0.53
13:200:200 [a]	101.32 ± 0.51	99.78 ± 0.50	99.23 ± 0.65
15:300:300	101.83 ± 0.66	100.43 ± 0.64	99.39 ± 0.41
20:200:600	100.45 ± 0.77	101.15 ± 0.55	99.36 ± 0.71
30:200:400	98.74 ± 0.40	99.66 ± 0.71	98.94 ± 0.83

a: the ratio of drugs presented in Triderm® preparation.

4.1.1.4. Effect of reaction time and temperature. Various time intervals were tested to ensure the complete formation of the complex, at 5 min the complex was completely formatted and remained stable for 2.5 h as clarified in Figure 6. Raising the temperature caused the solution to become cloudy, so the measurements were achieved at 25 °C (room temperature).

4.1.1.5. Effect of order of addition. The best order of additions is a drug-surfactant-dye-buffer which gave the best absorbance, repeatability, and stability.

4.1.1.6. Stoichiometric ratio. Job's method was applied by preparing standard solutions set where the drug and the reagent concentration were changed while their sum remained constant, the signal of the resulting ion-pair was estimated at 545 nm, and it was figured that the molar ratio of the drug to dye in the ion-pair complex was 1: 4 as clarified in Figure 7.

4.1.2. Effect of interferences

The possibility of forming ion pair between ERY and CLO, BET, and BEN was studied Since these drugs are conjugated with GEN in Triderm® pharmaceutical preparations, this was done by developing mixtures containing a disparate ratio of the drugs, methanol was then evaporated, and an equal amount of water and chloroform were added, and Shaked in a separating funnel, the aqueous layer was separated and a specified volume was conveyed to a volumetric flask, and the procedure was followed as formerly stated. BET and BEN didn't interfere with the ion pair formation since they don't consist of the amino group, also they are not soluble in water so they were extracted by chloroform from the mixtures, CLO contains a ternary amino group, and it is slightly soluble in water, so a small amount of CLO can exist in the aqueous layer with GEN as Figure 8 shows, even though it doesn't interfere with GEN determination

Table 2. Parameters and validation data of the suggested approaches for CLO, BET, BEN, and GEN quantification.

Compound name	CLO	BET	BEN	GEN
Method	UDD	UDD	DDR	Ion- pair complex with ERY
wavelength	256.5 nm, 265.3 nm	256.5 nm, 265.3 nm	269.7 nm	545 nm
	F = −0.37	F = −1.57		
Linearity range	100–500 ug/mL	5–50 ug/mL	100–800 ug/mL	0.5–4 ug/mL
Slope	−0.003	0.0158	0.0016	0.2385
Intercept	−0.0053	0.0068	0.0203	0.0378
Correlation coefficient	0.9997	0.9999	0.9997	0.9998
Mean%±SD[a]	100.94 ± 1.37	99.02 ± 0.72	100.21 ± 1.09	101.03 ± 1.59
Repeatability[b]	0.884	1.276	1.005	1.234
Intermediate Precision [b]	1.476	1.366	1.611	1.934

a: accuracy is expressed as mean%± standard deviation of three concentrations of (CLO, BET, BEN, GEN).
b: Repeatability and Intermediate Precision are expressed as the relative standard deviation of three concentrations of (CLO, BET, BEN, GEN).

Table 4. Results of applying the suggested method and the reference method on Triderm® pharmaceutical preparation.

drug	CLO		BET		BEN		GEN	
Triderm® formulation	cream	ointment	cream	ointment	Cream	ointment	cream	Ointment
Proposed method [a]	97.05 ± 0.34	102.93 ± 0.70	97.22 ± 1.47	102.75 ± 1.50	97.67 ± 0.48	____	102.72 ± 0.82	102.27 ± 0.81
Standard added [a]	101.57 ± 0.95	98.23 ± 0.52	102.80 ± 1.02	98.61 ± 1.51	98.94 ± 0.83	____	101.36 ± 0.25	101.87 ± 0.43
Reference method [b]	98.06 ± 0.61	101.29 ± 1.73	97.87 ± 0.64	101.45 ± 0.93	98.63 ± 1.02	____	102.48 ± 0.34	102.16 ± 0.52
f -value [c]	3.141	6.172	5.094	2.632	4.607	____	1.023	2.430
t -value [c]	2.512	1.524	0.700	1.280	1.468	____	0.136	0.187

a: mean%±SD of three determinations of each component.
b: USP HPLC method for CLO, BET, and BEN quantification, UV method for GEN quantification [20].
c:f(0.05)19, t(0.05)2.776.

Table 5. Robustness of the suggested assay method of Gentamycin.

parameters	pH		Poloxamer volume		Erythrosine volume	
	3.1	2.9	1.4 mL	1.6 mL	1.01 mL	0.99 mL
Mean%±SD [a]	98.94 ± 1.50	98.59 ± 1.78	100.36 ± 1.07	99.78 ± 0.34	101.68 ± 0.59	101.27 ± 1.28
assay with no variations [b]	99.85 ± 1.5					

a: mean% of three determinations ±standard deviation.
b: mean% ± standard deviation of three determinations of gentamycin using pH3, poloxamer 1.5 mL volume, erythrosine 1 mL volume.

because it couldn't form ion pair with ERY, so ERY reacts selectively with GEN in the existence of the formulated components in Triderm® preparations.

4.2. Quantification of CLO, BET, and the preservative BEN

By scanning and overlaying the D^0 spectra of CLO, BET and BEN as in Figure 9, it was observed that no helping spectral features for the quantitively analysis of them are presented, so the employment of the unlimited derivative ratio approach (UDD) using BEN D^0 spectrum as a devisor was necessary to cancel it's interfering from the ternary combination spectrum, and consequently the ability of CLO and BET quantitively detection in their mixtures, for applying this method a number of BEN concentration were tested to select the best devisor that give the best recovery of CLO and BET from their disparate mixes, the spectrum of 800 ug/mL of BEN was chosen as a devisor as it achieved the intended purpose, derivation process was done using (first order derivative, 13 data point, scale factor 10) as shown in Figure 10, 256.5 nm, 265.3 nm were elected for calculating the equality factor of CLO and BET as they gave the finest repeatability and recovery for the determined compound, CLO concentration was detected by multiplying the computed F_{BET} via the mixture amplitude at 265.3 nm then calculating the difference ($pm_{256.5\ nm}$-(F_{BET}*$pm_{265.3\ nm}$), and substituting in the linear equation, the same process was repeated for BET quantification utilizing F_{CLO}. BEN concentration in the drug mix could be detected utilizing the double divisor ratio spectra approach as shown in Figure 11, using the spectrum of (400 μg/mL CLO + 20 μg/mL BET) as a devisor as it gave the best recovery of BEN from its mixtures with CLO and BET, the measurements at 269.7 nm gave the finest results of BEN quantification.

The advanced approach has the feature of resolving extremely overlapped spectrum with minimal steps involved, and by simple calculations giving satisfactory results without the need for complicated software.

5. Validation and statical comparison

The validation of the newly develop approaches was achieved following the directions of ICH [46], using the experimental conditions clarified above, linearity was confirmed by constricting the calibration graphs for the three drugs in the concentration ranges listed in Table 2, accuracy was assured by applying the proposed approaches on the pure compounds as well as on pharmaceutical preparations via the technique of standard addition as in Table 4, the calculated recoveries% showed good results with RSD <2, precision was checked by obtaining accepted RSD value upon applying the proposed approaches on three concentrations levels of pure drugs in the same day, or on three days as stated in Table 2, method specificity was confirmed via applying the introduced approaches on drug mixes with divers mixing proportion across the linearity range as in Table 3, also by applying on pharmaceutical preparation as in Table 4, the computed mean%+SD of each compounds were satisfying confirming the absence of intervention from the other Accompanying components or excipients, robustness of the stated approach for GEN quantification was achieved via making small change on the experimental circumstances such as pH, reagent volume and surfactant volume, none of these variables had a noticeable effect on GEN quantification, that was confirmed by mean%+RSD values shown in Table 5. Statical comparability was made by computing the f and t value as stated in Table 4 confirming that no important variation between the newly developed approach and the reported one exists upon applying to Triderm® pharmaceutical preparations.

6. Conclusion

This manuscript displays the power of an advanced eco-cordial protocol for handling semisolid forms containing complicated formulations with the existence of the UV none absorbance gentamycin, and the interfered excipient benzyl alcohol that impedes the quantification of clotrimazole and betamethasone. Gentamycin signal was improved by reacting with erythrosine reagent to develop a water-soluble ion pair with the aid of the green surfactant poloxamer188, the resulting GEN-ERY ion pair was distinguished by being instantaneously formed without the need for extraction with organic solvents, and it was measured at 545 nm. the obstruction of benzyl alcohol was eliminated by using its spectrum as a divisor followed by derivation and applying a simple mathematical technique for CLO and BET signal filtration. The developed approaches are characterized by their capability to the quick determination of the studied components without the help of additional apparatuses or complicated programs, making them preferred methods over HPLC and chemometric methods for conducting daily analyzes in drug analysis laboratories.

Declarations

Author contribution statement

Amir Alhaj Sakur: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Analyzed and interpreted the data.

Duaa AL Zakri: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

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Data will be made available on request.

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The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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