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Development, Validation and Greenness Assessment of Three Simple Spectrophotometric Methods for Determination of Two Combined Broad-Spectrum Antibacterial Agents

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Abstract

Three straightforward, valid, and environmentally friendly spectrophotometric techniques were established and validated for concurrent assay of a binary mixture of linezolid (LIN) and cefixime trihydrate (CEF) without prior separation. Ratio difference spectrophotometric method (Method A) utilized 6 µg/mL of CEF as a divisor and found that the subtraction of amplitudes at 250 and 222 nm in the ratio spectrum was proportional directly to LIN concentration. Similar to this, the ratio spectrum's amplitude difference between 222 and 250 nm was utilized to analyze CEF utilizing 8 µg/mL of LIN as the divisor. For the first order derivative ratio spectrophotometric approach (Method B), the previously constructed ratio spectra were subjected to a first order derivative manipulation process, the sum of peak and trough amplitudes at 240 and 260 nm and at 215 and 228 nm were selected to concurrently estimate LIN and CEF, respectively. Finally, utilizing mean centering of ratio spectra (MCR) (Method C) LIN and CEF were examined at 250 nmin the mean-centered previously created ratio spectra. All of the presented approaches were successful in estimating LIN and CEF in laboratory made dosage form revealing satisfactory recoveries. Furthermore, the presented methods were assessed for validation parameters in accordance with the International Council for Harmonization (ICH) recommendations and evaluated for greenness via both the analytical Eco-scale and the AGREE model.

Keywords: Linezolid; Cefixime; Ratio difference spectrophotometry; Ratio derivative spectrophotometry; Mean centering of ratio spectra; Greenness evaluation

1. Introduction

A major global issue is the spread of multidrug-resistant (MDR) bacterial diseases. The inability to be sensitive to at least one antibiotic from three or more distinct classes is referred to as multidrug resistance. Since MDR infections are challenging to be treated and commonly correlated with high mortality, therefore, the antibiotic combination therapy has been developed [1]. Although combination therapy has its negative sides, and its inappropriate use can exacerbate the already critical situation of antibiotic resistance, it is typically used for one or more of the reasons listed below. First, using multiple antibiotics broadens the antibacterial spectrum, guaranteeing that a minimum of one medication is targeting the pathogenic organism. Second, enhancing efficiency towards polymicrobial infections as in case of intra-abdominal infections with a rupture in the gut wall. Third, antibiotic combinations are employed for their synergistic activity. Many physicians favor combination antimicrobial therapy over monotherapy, highlighting that in-vitro experiments show improved outcomes due to synergistic effects. Fourth, the risks of developing resistance to two medications are lower when compared to a single drug [1].One of the evidences supporting the needfor

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and superiority of combination antimicrobial therapy over monotherapy for MDR pathogen infections is that antibiotic combination treatment strategy appears to be preferable to monotherapy for treating gram-negative multidrug-resistant bacteria [2]. This therapeutic trend necessitates the establishment of adequate analytical approaches for concurrent analysis of co-formulated or co-administered antibiotics.

On the other hand, in recent years, a new concept termed "green analytical chemistry" (GAC) (also recognized as environmentally-friendly analytical methods or clean analytical chemistry) has grown considerably, that was designed to make analytical procedures more environmentally benign and risk free [3]. Excessive chemical use results in a diverse range of health disorders, including toxicity, carcinogenicity, and mutagenicity, as well as different environmental problems such as pollution of air, water, and soil, as well as global warming.

GAC's goal is the reduction of the negative environmental influence of analytical procedures in four ways. First, is to make restrictions or limitations on the use of various chemical substances. Second, is to conserve energy. Third, is to reduce and appropriately disposethe generated waste. Fourth, is to enhance operator's safety [4]. The problem is to find a balance between improving the quality of the data and developing more environmentally friendly analytical methods. The presented work offers three simple and green analyzing methods for concurrent analysis of Cefixime trihydrate (CEF) in its binary combination with linezolid (LIN).

Cefixime trihydrate (CEF) is a cephalosporin antibiotic from the third generation. Chemically, CEF is known as (6*R*,7*R*)-7-[[(2Z)-2-(2-amino- 1,3-thiazol-4-yl) -2-(carboxymethoxyimino) acetyl] amino] -3- ethenyl-8-oxo-5-thia-1-azabicyclo [4.2.0]oct-2-ene-2-carboxylic acid; trihydrate (Figure 1) [5]. The antibacterial effect of cefixime owed to its ability to inhibit the production of mucopeptide in the cell wall of bacteria. After the preceding step, the autolytic enzymes that present in the bacterial cell wall as autolysins start to induce cell lysis.

An important feature of cefixime is that if beta-lactamase enzymes are present, it is found to be highly stable. Accordingly, many micro-organisms that resist some cephalosporins and penicillins due to beta-lactamases could be highly susceptible to cefixime [6]. It is commonly applied for treatment of many infections of the lower respiratory tract such as bronchitis, along with gonorrhea, pharyngitis, otitis media, and infections of the urinary tract.

Linezolid (LIN) was the first oxazolidinone antibiotic to be developed, and chemically it is*N*-[[(5*S*)-3-(3-fluoro-4-morpholin-4-ylphenyl)-2-oxo-1,3-oxazolidin-5-yl] methyl] acetamide (Figure 1) [7]. Linezolid is mainly applied to cure different infections that are due to Gram-positive aerobic bacteria as community-acquired pneumonia, nosocomial pneumonia, and skin infections. Linezolid's antibacterial effect is mediated by its ability to prevent the start of bacterial protein production. It binds to a location on the subunit (50S) of the ribosomal RNA (23S) of bacteria and hinder the assembly of the functional initiation complex (70S), where that complex is necessary in bacterial reproduction and division [8].

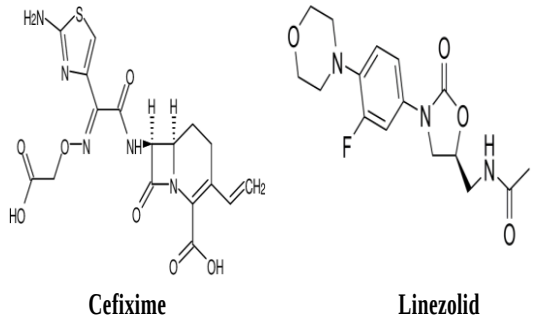


Fig. 1. Chemical structures of the compounds investigated in the study.

Reviewing all the available preceding publications revealed that numerous approaches for estimating Cefixime have been established, as spectrophotometry [9], the High Performance Thin Layer Chromatography (HPTLC) [10, 11], and the High Performance Liquid Chromatography (HPLC) [12-14] either individually or with other medicines in bulk, pharmaceutical formulations, or in biological matrices. Similarly, linezolid was reported to be estimated alone or in different combinations with other drugs, using different methods as spectrophotometry [15-17], HPTLC [18], and HPLC [19, 20].

The combined formulation of the tested antibiotics in market is Gramocef L® tablet, that contains 600 mg LIN and 200 mg CEF per each tablet. The concurrent assay of the tested medications in

pharmaceutical forms, was reported utilizing HPLC methods [21-28], different spectrophotometric methods including simultaneous equation (Vierodt's method) [29-33], Q analysis or the absorbance ratio method [24, 30, 34, 35], first order derivative spectrophotometry [36] and zero crossing second derivative spectrophotometry [32, 34].

Literature review revealed that there is no previously reported mean centering or ratio difference methods for analysis of the selected combination. Moreover, only one paper reported to use the ratio derivative method [32]. On the other hand, none of the early reported approaches were examined using any of the greenness evaluation tools.

The purpose of this work is to introduce three easily applied, smart and eco-friendly spectrophotometric approaches including, ratio difference spectrophotometry, ratio derivative spectrophotometry, and mean centering approaches for simultaneous estimation of the two selected analytes in their combined mixture with no need for a preceding separation step.

The three introduced methods fulfilled the International Council for Harmonization (ICH) guidelines concerning validation and were tested using two different greenness evaluation models, known as Analytical Eco-scale assessment tool and the Analytical GREEness metric (AGREE) [37, 38].

2. Experimental

2.1. Apparatus

A T80 UV–Vis 1800 UV Spectrophotometer (PG Instruments, UK) which is paired with a personal computer loaded with the UVWin version 5.2.0 software. Matlab™ software, version 7 for mean centering of ratio spectra method.

2.2. Materials and solvents

Cefixime powder was provided by Pharco Pharmaceuticals Company, Alexandria, Egypt, and LIN was supplied by EVA pharma Company, Alexandria, Egypt. Methanol of HPLC grade (Sigma-aldrich, Buchs, Switzerland) and distilled water were utilized as solvents.

2.3. Preparation of stock solutions

Using methanol, LIN and CEF stock solutions of 100 µg/mL final concentration were prepared separately.

2.4. General procedures and construction of calibration graphs

Accurately transferred portions of both LIN and CEF stock solutions were diluted independently with distilled water in two groups of calibrated volumetric flasks measuring 10 mL to actually achieve concentrations ranging between 1 and 20 µg/mL for both analytes. After utilizing a distilled water blank, recording and storing were applied to the absorption zero-order spectra of the early diluted solutions in wavelength ranging between 200 and 400 nm. All LIN absorption spectra were divided by the spectrum of 6 µg/mL CEF standard solution to develop the ratio spectra. Additionally, the CEF ratio spectra is constructed by dividing the recorded absorption spectra of CEF by the stored spectrum of 8 µg/mL LIN standard solution. Following the development of the ratio spectra for both tested drugs, the three introduced spectrophotometric procedures were carried out as follows.

2.4.1. Method A: Ratio difference spectrophotometric method

The regression equation and calibration graph were established for LIN by plotting the subtraction of readings between 250 and 222 nm in the previously saved LIN ratio spectra, against the corresponding LIN concentrations in µg/mL. Likewise, subtraction of readings in the stored CEF ratio spectra between 222 and 250 nm were charted against the equivalent concentrations of CEF in µg/mL.

2.4.2. Method B: First derivative ratio spectrophotometric method

The first order derivative ratio spectra (^{1}DD spectra) were created by differentiating the previously generated LIN and CEF ratio spectra. To build the calibration curve for LIN, the sum of the absolute readings of ^{1}DD at 240 and 260 nm were charted against the corresponding LIN concentrations. On the other hand, the sum of the

absolute readings of ^{1}DD at 215 and 228 nm were tabulated for plotting of CEF calibration curve.

2.4.3. Method C: Mean centering of ratio spectra method

The Matlab software was used to mean-center the recorded ratio spectra of both tested medicines. Then, calibration curves created utilizing the mean-centered readings at 250 nm for either LIN or CEF.

2.5. Quantitative analysis of laboratory prepared mixtures

Four synthetically made mixtures of LIN and CEF in varying ratios were prepared for mimicking or being close to the actually found ratios in the combined formulation. Those mixtures were tested as initially described under the general procedure of every approach. The corresponding concentrations of both analytes were calculated utilizing the related regression equations in all methods.

2.6. Assay of laboratory prepared tablets of (LIN+CEF)

As the combined cefixime/linezolid tablets, as the brand Gramocef L®, are not available in the Egyptian market, the three introduced approaches were utilized for concurrent estimation of the tested medicines in laboratory-made tablets.

To emulate the brand Gramocef L®, ten laboratory made-tablets were produced to obtain 600 mg LIN and 200 mg CEF per tablet. Along with different excipients as magnesium stearate, flour, aerosil, and lactose, the quantity of LIN and CEF was measured, pulverized, and homogeneously blended. An exact weight of the produced tablets comprising 150 mg LIN and 50 mg CEF was mixed with 15 mL of HPLC grade methanol, vortexed for 5 minutes, and filtered finally in a calibrated flask measuring 50 mL. Rinsing of the residual powder was performed twice using 3 mL of methanol.

Washings were then added to the main filtrate solution. Final volume was then completed to 50 mL utilizing methanol to develop a stock solution of (3 mg/ml LIN and 1 mg/ml CEF) from the extracted sample. To get final concentrations within the desired linear ranges, distilled water was used to dilute different portions of the final extraction solution. The prepared dilutions were then handled as directed by "General Procedure". Regression data were utilized to calculate recovery values.

For standard addition test, spiking sample solutions with accurately transferred volumes of LIN and CEF were applied to reach final concentrations within the early specified linear ranges, then handled as under "General Procedure". Values that describing recoveries were estimated by comparing the response of each analyte to the increment response reached after standard addition.

3. Results and Discussion

3.1. Spectral characteristics and development of the three methods

Despite of the simplicity of using direct zero order spectra in the UV spectrophotometric analysis of drugs, it is not applicable when other compounds (medicines or excipients) with overlapping spectra coexist in the combined formulation.

Regarding the currently investigated binary mixture, both components LIN and CEF showed extensively overlapped spectra all over the wavelength range from 200 – 400 nm (Figure 2). As a result, determining those compounds in their binary mixture simultaneously requiresmathematical manipulation of the absorption spectra to eliminate the interference of a medicine while assaying the other one, without using complicated physical separation techniques as in the chromatographic methods.

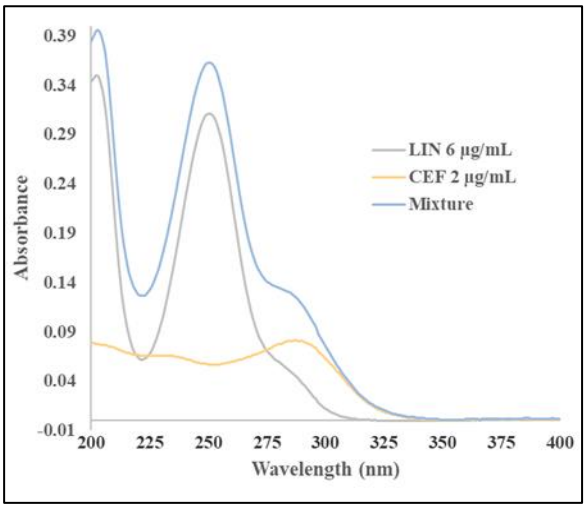


Fig. 2. Absorption spectra of 6 µg/mL LIN, 2 µg/mL CEF and their mixture in water.

The currently proposed three spectrophotometric methods depend on the development of ratio spectra of each drug. That fulfilled by dividing the absorption zero spectra of the tested medicine by the spectrum of an accurately selected concentration of the other drug as a divisor. This simple step helps to decrease the order of the interference. Then elimination of the interference could be easily performed using simple subtraction as in method A: Ratio difference spectrophotometric method [39, 40], first derivative order as in method B: First derivative ratio spectrophotometric method [40], or method C: mean centering of ratio spectra method [41].

3.2. Optimization of the proposed spectrophotometric methods

3.2.1. Method A: Ratio difference spectrophotometric method

The ratio difference method initiated via recording zero order absorption spectra of LIN and CEF laboratory-made mixture solutions. Then by dividing the early recorded spectra (value by value at every wavelength between 200-400 nm) by a carefully selected CEF standard divisor = 6 µg/mL. The newly produced spectra represent LIN/CEF + constant, as shown in (Figure 3A). The constant CEF/CEF will be cancelled as a logical consequence after subtracting the readings at 250 and 222 nm. Then LIN concentration was obtained from the developed regression equation. Similarly, CEF can be assayed in the mixture utilizing standard LIN divisor = 8 µg/mL, then readings at 222 and 250 nm were subtracted (Figure 4 A). The resultant values were then used to obtain CEF concentration from the constructed regression equation.

3.2.2. Method B: First derivative ratio spectrophotometric method

The first order derivative spectra of LIN were easily constructed from the early recorded ratio spectra. This derivatization step helped to eliminate any interference from CEF. Then determination of LIN could be achieved by using the summation of absolute readings of ^{1}DD at 240 and 260 nm (Figure 3 B). For determination of CEF comparable steps were utilized. CEF could be determined from the first derivative ratio spectra using the summation of absolute readings of ^{1}DD at 215 and 228 nm (Figure 4 B). The obtained values were then used to calculate both LIN and CEF concentrations from the previously constructed regression equations.

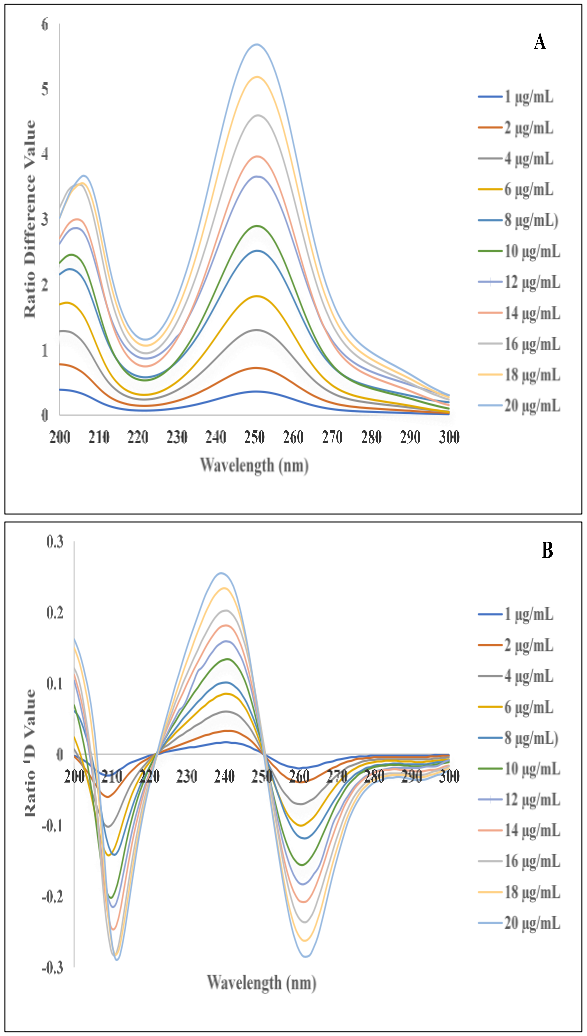
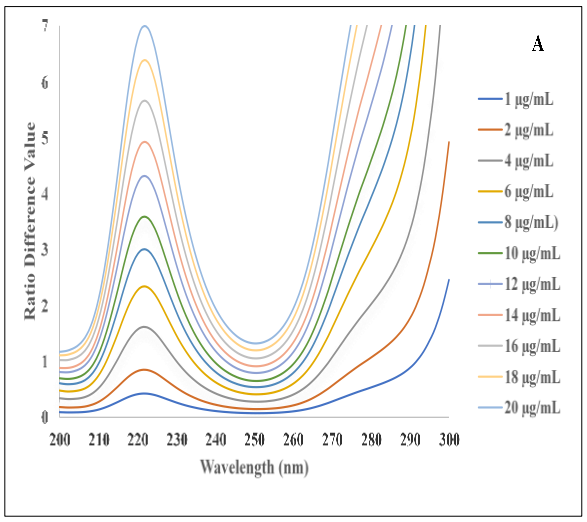


Fig. 3. Ratio spectra (A) and first derivative of ratio spectra (B) of 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 µg/mL LIN, using 6 µg/mL CEF as a divisor.



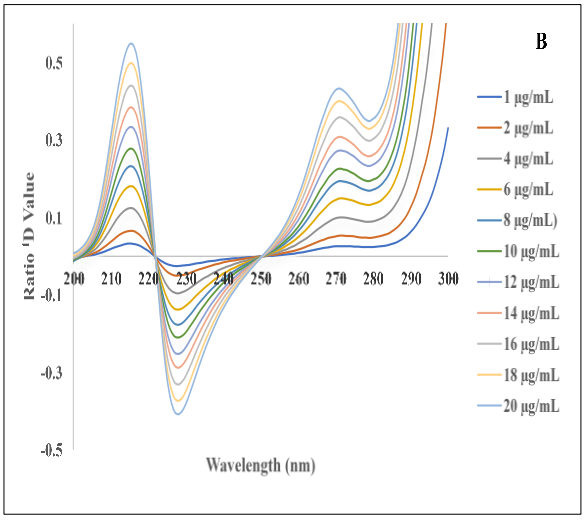


Fig. 4. Ratio spectra (A) and first derivative of ratio spectra (B) of 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 µg/mL CEF, using 8 µg/mL LIN as a divisor.

3.2.3. Method C: Mean centering of ratio spectra method

After applying mean centering on ratio spectra that early produced, readings at 250 nm were collected for either LIN or CEF (Figure 5 and 6). Those values were then utilized in calculating both LIN and CEF concentrations.

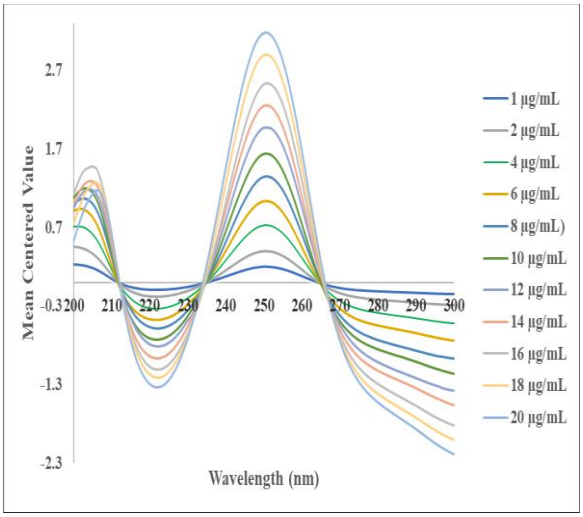


Fig. 5. Mean centered spectra of 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 µg/mL LIN, using 6 µg/mL CEF as a divisor.

3.2.4. Effect of divisor concentration

The influence of the divisor concentration on the resultant ratio spectra of the tested analytes was studied. The amplitudes of the absorbance ratios decreased or increased proportionally along with the increase or decrease in concentration of the divisor, respectively. Peak and trough positions, on the other hand, remained unchanged. Although, if the divisor has an insufficiently low concentration it could not be able to eliminate the interference and that proved by unacceptable recoveries. For the three introduced methods different divisor concentrations were tested in analysis of both LIN and CEF. Divisor concentrations that proved to produce the best results concerning measurement recoveries, accuracy, and repeatability were 6 µg/mL of CEF for analysis of LIN, and 8 µg/mL of LIN for analysis of CEF.

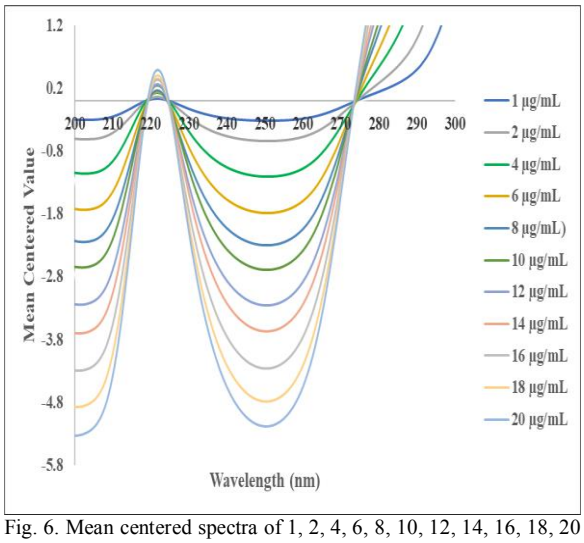


Fig. 6. Mean centered spectra of 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 µg/mL CEF, using 8 µg/mL LIN as a divisor.

3.3. Validation of the proposed spectrophotometric methods

Validation of the three presented approaches were assessed in accordance with ICH principles [42].

3.3.1. Linearity and concentration ranges

Linear responses of the three suggested methods were examined by analyzing serial concentrations of LIN and CEF within concentrations ranging from 1 to 20 µg/mL regarding both medicines. The assay was carried out in accordance with the experimental parameters specified for each procedure earlier. Table 1 summarizes several validation metrics such as correlation coefficients (r), slopes, and intercepts, with corresponding deviations. High correlation coefficients ($r > 0.9996$) and F values, as well as negligible intercepts and minor significance F values,

indicate good linear relationships for each approach. Additionally, the slopes' RSD% results that did not exceed 1% also prove sufficient linearity. For evaluating linearity parameters, all statistical computations, such as different standard deviation values, were performed, and the results were satisfactory.

3.3.2. Detection and quantitation limits

Standard deviations of both the intercept (S_a) and the slop (b) of each regression equation were utilized in determination of limit of detection (LOD) and limit of quantitation (LOQ) in accordance with the ICH regulations, where LOD = ($3.3S_a$/b) and LOQ = ($10S_a$/b). All LOD and LOQ of the presented approaches were listed in Table 1.

3.3.3. Accuracy and precision

Within-day repeatability of all suggested approaches was evaluated via analyzing triplicates of three selected concentration levels of each medicine in one day. Additionally, all early selected concentrations were examined in three separate days to be capable of studying the between-days precision (intermediate precision). All relative standard deviation values (RSD%) were not greater than 2% for both compounds indicating adequate levels of repeatability and intermediate precision of the three introduced approaches (Table 2). Additionally, accuracy of all methods was affirmed via the adequate recoveries along with the acceptable values of percentage relative error (Er%) (Table 2).

3.3.4. Selectivity

Selectivity of all introduced approaches was examined via preparing different mixtures of both antibiotics in variable proportions included in their linear ranges (Table 3). These laboratory-made mixtures were tested utilizing the early described procedures. All findings in term of % recovery, RSD%, and Er% were sufficiently indicating the acceptable selectivity of the introduced methods and its potential in concurrent determination of both drugs.

3.3.5. Stability of solutions

The stability of standard and sample solutions was tested in water by storing them for 24 h at ambient temperature or for one week at 4°C. Additionally, the stability of stock solutions was also tested by refrigeration at 4°C for at least a week. Analysis showed that both of the tested medications were stable in those conditions as proven by the absence of any significant spectrophotometric changes.

Table 1

Analytical parameters for determination of LIN and CEF mixture using the proposed spectrophotometric methods.

Parameter	Method A: Ratio Difference Spectrophotometry		Method B: Ratio Derivative Spectrophotometry		Method C: Mean Centering of Ratio Spectra	
	LIN	CEF	LIN	CEF	LIN	CEF
Wavelength (nm)	250 - 222	222 - 250	240 + 260	215 + 228	250	250
Concentration range [a]	1 - 20	1 - 20	1 - 20	1 - 20	1 - 20	1 - 20
Intercept (a)	0.131	0.164	0.020	0.025	0.099	0.169
S_a [b]	0.021	0.026	0.002	0.004	0.011	0.020
Slope (b)	0.221	0.279	0.026	0.047	0.155	0.254
S_b [c]	0.002	0.002	0.0002	0.0004	0.001	0.002
RSD% of slope	0.905	0.717	0.769	0.851	0.645	0.787
r	0.9997	0.9997	0.9997	0.9997	0.9999	0.9998
$S_{y/x}$ [d]	0.036	0.046	0.004	0.007	0.018	0.035
F [e]	15903	15711	16696	16882	30165	22391
Significance F	6.31×10^{-16}	6.65×10^{-16}	5.06×10^{-16}	4.81×10^{-16}	3.54×10^{-17}	1.35×10^{-16}
LOD [f] (µg/mL)	0.314	0.308	0.254	0.281	0.234	0.260
LOQ [g] (µg/mL)	0.950	0.932	0.769	0.851	0.710	0.787

[a] Concentration is in (µg/mL)
[b] Standard deviation of the intercept
[c] Standard deviation of the slope
[d] Standard deviation of residuals
[e] Variance ratio, equals the mean of squares due to regression divided by the mean of squares about regression (due to residuals)
[f] Limit of detection
[g] Limit of quantification

Table 2

Precision and accuracy for determination of LIN and CEF in bulk form using the proposed spectrophotometric methods.

Method A: Ratio Difference Spectrophotometry							
Analyte	Nominal value [a]	Within-day			Between-days		
		Found[b] ± SD[c]	RSD(%)[d]	E_r(%)[e]	Found[b] ± SD[c]	RSD(%)[d]	E_r(%)[e]
LIN	6	6.01 ± 0.06	0.998	0.17	6.06 ± 0.01	0.165	1.00
	10	9.98 ± 0.05	0.50	-0.20	10.16 ± 0.20	1.97	1.60
	20	19.96 ± 0.02	0.10	-0.20	19.98 ± 0.09	0.45	-0.10
CEF	6	6.10 ± 0.11	1.80	1.67	6.01 ± 0.08	1.33	1.00
	10	10.07 ± 0.12	1.19	0.70	10.02 ± 0.08	0.80	0.20
	20	19.93 ± 0.18	0.90	-0.35	19.97 ± 0.30	1.50	-0.15
Method B: Ratio Derivative Spectrophotometry							
Analyte	Nominal value [a]	Within-day			Between-days		
		Found[b] ± SD[c]	RSD(%)[d]	E_r(%)[e]	Found[b] ± SD[c]	RSD(%)[d]	E_r(%)[e]
LIN	6	6.09 ± 0.07	1.15	1.50	6.04 ± 0.06	0.99	0.67
	10	10.13 ± 0.06	0.59	1.30	10.12 ± 0.08	0.79	1.20
	20	20.04 ± 0.02	0.10	0.20	20.04 ± 0.10	0.50	0.20
CEF	6	5.95 ± 0.05	0.84	-0.83	5.98 ± 0.04	0.67	-0.33
	10	9.91 ± 0.08	0.81	-0.90	9.97 ± 0.05	0.50	-0.30
	20	20.14 ± 0.37	1.84	0.70	19.76 ± 0.28	1.42	-1.20
Method C: Mean Centering of Ratio Spectra							
Analyte	Nominal value [a]	Within-day			Between-days		
		Found[b] ± SD[c]	RSD(%)[c]	E_r(%)[d]	Found[b] ± SD[c]	RSD(%)[c]	E_r(%)[d]
LIN	6	6.02 ± 0.07	1.16	0.33	6.08 ± 0.02	0.33	1.33
	10	10.09 ± 0.08	0.79	0.90	10.11 ± 0.19	1.88	1.10
	20	20.06 ± 0.03	0.15	0.30	19.98 ± 0.06	0.30	- 0.10
CEF	6	5.98 ± 0.05	0.84	- 0.33	6.03 ± 0.07	1.16	0.50
	10	10.03 ± 0.07	0.70	0.30	10.02 ± 0.06	0.60	0.20
	20	20.11 ± 0.32	1.59	0.55	19.94 ± 0.38	1.91	- 0.30

[a] Nominal value is in µg/ml

[b]Found value is in µg/ml

[c] Mean ± standard deviation for three determinations.

[d] % Relative standard deviation.

[e] % Relative error.

Table 3

Determination of LIN and CEF in laboratory-prepared mixtures using the proposed spectrophotometric methods.

Method A: Ratio Difference Spectrophotometry							
Nominal value [a]		Found [b] ± SD [c]		RSD (%) [d]		E_r (%) [e]	
LIN	CEF	LIN	CEF	LIN	CEF	LIN	CEF
4	4	4.03 ± 0.07	4.07 ± 0.04	1.74	0.98	0.75	0.75
6	6	5.99 ± 0.04	6.08 ± 0.06	0.67	0.99	-0.17	1.33
6	2	6.09 ± 0.06	1.99 ± 0.04	0.99	2.00	1.50	-0.5
12	4	12.04 ± 0.10	4.03 ± 0.02	0.83	0.50	0.33	0.75
Method B: Ratio Derivative Spectrophotometry							
Nominal value [a]		Found [b] ± SD [c]		RSD (%) [d]		E_r (%) [e]	
LIN	CEF	LIN	CEF	LIN	CEF	LIN	CEF
4	4	3.96 ± 0.06	4.03 ± 0.07	1.52	1.74	-1.00	0.75
6	6	5.94 ± 0.04	6.09 ± 0.08	0.67	1.31	-1.00	1.50
6	2	6.05 ± 0.08	2.01 ± 0.04	1.32	1.99	0.83	0.50
12	4	12.06 ± 0.18	4.04 ± 0.05	1.49	1.24	0.50	1.00
Method C: Mean Centeringof Ratio Spectra							
Nominal value [a]		Found [b] ± SD [c]		RSD (%) [d]		E_r (%) [e]	
LIN	CEF	LIN	CEF	LIN	CEF	LIN	CEF
4	4	4.07 ± 0.05	4.03 ± 0.02	1.23	0.50	1.75	0.75
6	6	6.11 ± 0.07	6.06 ± 0.07	1.15	1.16	1.83	1.00
6	2	6.11 ± 0.08	2.03 ± 0.04	1.31	1.97	1.83	1.50
12	4	12.19 ± 0.03	4.01 ± 0.03	0.25	0.75	1.58	0.25

[a] Nominal value is in µg/ml

[b]Found value is in µg/ml

[c] Mean ± standard deviation for three determinations.

[d] % Relative standard deviation.

[e] % Relative error.

3.4. Application of the validated methods in assay of tablets dosage forms

The three presented spectrophotometric approaches were effectively applied in analyzing LIN and CEF in laboratory-made tablets. Sample preparation was performed as described under "General Procedure", and then different portions were diluted by distilled water to give different concentrations in the previously mentioned linear range. External standard and standard addition manipulation process were conducted. Analysis findings as % recovery, SD, and RSD% values proved suitable accuracy and precision (Table 4). These findings indicate the applicability of the introduced methods for routine assay of LIN and CEF in the fixed dose combinations with satisfactory levels of accuracy, precision, and selectivity.

Table 4

Application of the proposed spectrophotometric methods forthe analysis of LIN and CEF in laboratory-prepared tablets.

Method A: Ratio Difference Spectrophotometry				
Laboratory prepared tablet	External Standard		Standard Addition	
	LIN	CEF	LIN	CEF
%Recovery ± SD[a]	100.41 ± 0.24	99.29 ± 0.75	100.18 ± 0.14	100.09 ± 0.26
RSD%[b]	0.23	0.76	0.14	0.26
Method B: Ratio Derivative Spectrophotometry				
Laboratory prepared tablet	External Standard		Standard Addition	
	LIN	CEF	LIN	CEF
%Recovery ± SD[a]	100.22 ± 0.13	99.09 ± 0.60	100.01 ± 0.30	99.78 ± 0.49
RSD%[b]	0.13	0.61	0.30	0.49
Method C: Mean Centeringof Ratio Spectra				
Laboratory prepared tablet	External Standard		Standard Addition	
	LIN	CEF	LIN	CEF
%Recovery ± SD[a]	100.61 ± 1.12	100.91 ± 1.02	100.10 ± 0.66	99.92 ± 0.24
RSD%[b]	1.11	1.01	0.66	0.24

[a] Mean ± standard deviation for five determinations.

[b] % Relative standard deviation.

3.5. Assessment of greenness of the proposed methods.

It is crucial to test the intended analytical approach from the perspective of green analytical chemistry (GAC) in order to minimize its potential environmental and health risky impacts. Various approaches have been introduced in recent years to examine the environmental influence of analytical methods. Logically, utilization of various greenness assessment techniques is preferable in order to generate a more informative comparison and to gather all accessible data to guarantee the greenness of the suggested procedure.

In our presented study two approaches known as the Analytical Eco-Scale and the new Analytical Greenness metric (AGREE) were utilized to evaluate the greenness of the three validated spectrophotometric methods [37, 38]. The three proposed methods were compared in term of greenness with two carefully selected reported methods, a spectrophotometric article [32] that only utilized a ratio derivative method for the studied binary mixture and a recently developed HPLC-UV [28] reported method.

The first greenness assessment tool is the Analytical Eco-Scale method. This tool is assessing the four primary parameters of the analytical method. These parameters are the used chemicals, energy, occupational hazards and waste. Considering the Analytical Eco-Scale approach, a total score that equals to 100 points was given to the most ideal green analytical procedure. Penalty points (PP) are set and subtracted from 100, for every deviation from the ideal situation. Finally, calculated scoresgreater than 75 prove excellent greenness, scores from 50 to 75 show acceptable greenness, and those below 50 represent insufficient greenness [37].

The analytical Eco- Scale score was utilized for assessing the three proposed spectrophotometric methods and the two selected early reported approaches (Table 5). All assessed approaches are regarded as having excellent greenness. However, the highest score of 97 belonged to the introduced methods, hence they are considered the greenest techniques, followed by the reported spectrophotometric procedure with a 91 score, and finally the HPLC method with a score of 90.

The second tool is the AGREE method. AGREE is the most recent and automated easily applied tool for greenness assessment. AGREE assess greenness of any analytical approach considering the 12 GAC fundamentals (SIGNIFICANCE) among the greenness assessment tool [38]. The ideal green method had a score 1 taking dark green color illustrated in the specific pictograms. The three proposed spectrophotometric techniques showed the perfect greenness with a score 0.89, then the reported spectrophotometric scoring 0.78 and finally the reported HPLC procedure scoring 0.71 as shown in Table 6.

Table 5

Penalty points of the proposed methods according to the Analytical Eco-scale in comparison with the two previously reported methods.

Evaluation Points	The three proposed spectro-photometric methods	The reported spectro-photometric method [32]	The reported HPLC method [28]
1- Chemicals			
Water	0	-	-
Methanol	-	6	6
2- Energy	0	0	1
3- Occupational hazards	0	0	0
4- Waste	3	3	3
PPs	3	9	10
Eco-scale score	97	91	90

Table 6

Greenness evaluation of the proposed spectrophotometric methods together with the two previously reported methods using AGREE Metrix.

Methods and chromatographic conditions	AGREE
The three spectrophotometric methods	
The selected reported spectrophotometric method(Thakkar D et. al.) [32]	
The selected reported HPLC method (Manwar JVet. al.) [28]	

3.6 Comparison of the proposed methods and the previously reported methods

To the extent that we are aware, there is no previously published ratio difference spectrophotometry or mean centering methods for analysis of the selected combination, along with only one paper reported to use the ratio derivative method [32]. It was found that the proposed methods were of higher or equal sensitivity compared to the different previously published spectrophotometric methods. Considering the only reported ratio derivative method [32], the three developed methods proved to be more sensitive and greener. Spectrophotometric methods, on the other hand, have been shown to be simpler, faster, more environmentally friendly, less expensive, and easier to use in daily routine quality control analysis than chromatographic methods. Furthermore, compared to the recent HPLC method [28] for analysis of both tested drugs, the proposed methods showed higher greenness properties using both the Analytical Eco-Scale and AGREE.

4. Conclusion

The suggested spectrophotometric procedures for analysis of LIN and CEF mixture allowed the concurrent quantification of both medicines in laboratory-made tablets without prior separation. These spectrophotometric approaches are inexpensive and simple alternatives to more sophisticated chromatographic processes. The presented spectrophotometric methods are easy to be performed, economic and green as assessed utilizing the analytical eco-scale and AGREE metrics. Additionally, the new approaches demonstrated excellent analytical performance in terms of various validation criteria. To the extent that we are aware, this is the first report for utilizing ratio difference and mean centering spectrophotometric methods in analysis of the binary mixture of the tested medicines. On the other hand, the presented paper is the first paper to evaluate greenness of the developed procedures for analysis of LIN and CEF compared with previously reported methods.

5. Conflicts of interest

There are no conflicts to declare

6. Formatting of funding sources

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