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Green method for quantification of clindamycin in capsules by National Environmental Methods Index, Eco-Scale Assessment and Analytical GREEnness Metric

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Clindamycin (CLIN) is an antibiotic derived from lincosamide, produced from Streptomyces lincolnensis. Studies in the literature demonstrate that the evaluation of this drug, although effective, predominantly uses analytical conditions with the use of toxic solvents, which are against the principles of Green Analytical Chemistry (GAC). In this context, the objective was to develop and validate an eco-friendly method by spectrophotometry in the ultraviolet (UV) region for the quantitative evaluation of CLIN in capsules. In addition, the proposed method was evaluated for greenness by the National Environmental Methods Index (NEMI), Ecological Scale Assessment (ESA) and Analytical GREEnness Metric (AGREE). Purified water and ethanol (50:50, v/v), quartz cuvette and wavelength of 318 nm and potassium permanganate as an oxidizing agent were used. The method was linear in the range of 0.5 to 5 µg mL^{-1} (0.9998), precise (RSD < 5 %); selective through spectral overlap and forced degradation; accurate (99.85%); robust to changes in wavelength and cuvette capacity; content analysis was of 102.55 % and NEMI presented all 4 green quadrants; ESA, score of 79, which characterizes an excellent green analysis; and AGREE score of 0.8, thus characterizing it as a green method through the 12 GAC principles. The method was developed and validated and can be used for quantitative evaluation of CLIN in capsules. Furthermore, the method was considered green through the greenness profiling tools NEMI, ESA and AGREE.

Keywords: clindamycin, eco-friendly; UV; Eco-Scale Assessment; National Environmental Methods Index; Analytical GREEnness metric.

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Introduction

Clindamycin (CLIN, Figure 1) is an antibiotic derived from lincosamide, produced from *Streptomyces lincolnensis*, used to treat patients with pneumonia, osteomyelitis and infections caused by gram-positive bacteria (1). In the monograph described in official compendia (2, 4) for the evaluation of CLIN, High-Performance Liquid Chromatography (HPLC) is presented, and the most commonly used diluents are buffer, acetonitrile and methanol. The literature presents other analytical methods for its evaluation such as spectrophotometry in the ultraviolet and visible region, and liquid chromatography coupled to mass spectrophotometry, and the solvents of choice are also acetonitrile, buffers and methanol.

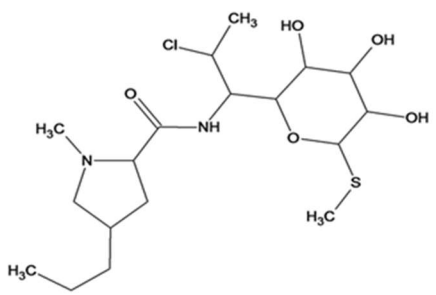

Figure 1. Chemical structure of clindamycin (CAS 18323-44-9).

Acetonitrile directly affects the health of the analyst and the environment, causing pulmonary and gastrointestinal problems because it is rapidly absorbed by these organs. Methanol, in turn, is a toxic and flammable solvent that affects the ecosystem and living beings, which in cases of poisoning is capable of causing metabolic acidosis and neurological damage, which can lead to death (5, 24).
Therefore, the literature presents a gap to be explored regarding the development of eco-efficient methods, with greener and less toxic alternatives, which generate less waste, for routine analyses in chemical-pharmaceutical laboratories, in accordance with the current demand (25, 30). Using analytical method evaluation tools is necessary to provide objective data regarding their greenness profile. The National Environmental Methods Index (NEMI), Ecological Scale Assessment (ESA), and Analytical GREEnness metric (AGREE) are examples of tools. The ESA takes into account the risks attributed to the reagents and quantities used, the risk words assigned to them, energy used, operational risks, and waste generation. The NEMI classifies methods according to four criteria: persistent, bioaccumulative and toxic (PBT), corrosive (pH < 2 or pH > 12), hazardous and residues (> 50g). Finally, the AGREE evaluates the greenness of analytical methods through the 12 principles of GAC (31, 34).
The objective of this study was to develop and validate an eco-friendly method by UV for quantitative evaluation of

CLIN in capsules, in addition to verifying the greenness of the analytical process.

Experimental section

Material and reagents

CLIN standard, content 99.26%, and CLIN hydrochloride capsules 300 mg, as sample, were used. The reagents used were purified water (Gehaka®, Brazil), ethanol (Vetec®, Brazil) and potassium permanganate ($KMnO_4$) (Unipharm®, Brazil).

Equipment

Analytical balance model AUW220D (Shimadzu®, Japan), UV-Vis spectrophotometer model 840-297300 (Thermo Scientific®, USA), quartz cuvette with 3 mL capacity, ultrasound model USC-2800 (Unique®, Brazil), bath and UV light chamber were used.

Stock and sample solution preparation

To prepare the $KMnO_4$ solution, 100 mg was weighed on an analytical balance and transferred to a 50 mL volumetric flask, completing its volume with purified water, obtaining a solution with a concentration of 2 mg mL^{-1}. Furthermore, to determine the amount of $KMnO_4$ used for the oxidation reaction with CLIN, aliquots of 50, 100 and 150 µL of $KMnO_4$ were tested at concentrations of 1, 5, 10 and 15 µg mL^{-1} in order to observe the reaction formed between these compounds, verify the formation of a band in the spectrophotometer and which of them would present greater absorption. Thus, the 100 µL aliquot was chosen because it presented a greater absorption of CLIN using a smaller volume of the $KMnO_4$ reagent.

The standard CLIN stock solution was prepared by weighing an amount of 1.01 mg and transferred to a 50 mL volumetric flask with 25 mL of ethanol, which was subjected to 2 minutes of ultrasound and then the volume was completed with purified water, in order to obtain a stock solution of 20 µg mL^{-1}. From this stock solution, a working solution was prepared, transferring a 5 mL aliquot to a 10 mL volumetric flask together with 100 uL of the $KMnO_4$ solution to obtain a concentration of 10 µg mL^{-1} to perform the reaction time tests and compare the absorption spectra of CLIN without reaction, CLIN after reaction with $KMnO_4$ and blank. Aliquots of 0.125, 0.250, 0.500, 0.750, 1.00, 1.25 mL were taken to construct the linear range.

Twenty capsules were opened and the content was homogenized to obtain a pool. From this pool, a quantity equivalent to 1 mg of CLIN (1.57 mg considering the average weight of 471.45 mg) was transferred to a 50 mL volumetric flask with 25 mL of ethanol, which was subjected to 2 minutes of ultrasound, and then the volume was completed with purified water, in order to obtain a stock solution of 20 µg mL^{-1}.

Method development

Reaction between CLIN and $KMnO_4$

CLIN has only one hydroxyl group as a chromophore in its structure, which generates a weak absorption band and low detection sensitivity. Therefore, a derivatization with potassium permanganate was performed, as shown Figure 2.

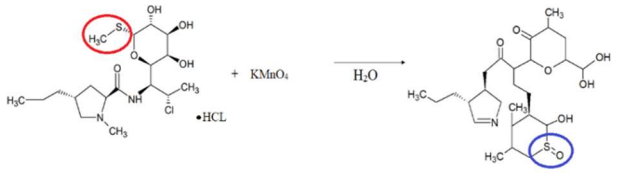

Figure 2. Simplified derivatization reaction of clindamycin hydrochloride and potassium permanganate in aqueous medium.

In the derivatization reaction, $KMnO_4$ partially oxidizes the thioester group (highlighted in red) present in the CLIN molecule, forming the sulfoxide group (highlighted in blue), and the permanganate is reduced to the manganese ion. Oxidation to sulfoxides alters the CLIN structure, increasing the presence of functional groups capable of absorbing UV light. This facilitates both spectrophotometric detection and chromatographic separation.

Method validation

Method validation was performed according to the International Conference of Harmonization (37) and National Health Surveillance Agency (ANVISA) (38) for linearity, limits of detection and quantification, precision, selectivity, accuracy and robustness.

Linearity

From the stock solution, aliquots were taken to prepare the solutions of 0.5, 1, 2, 3, 4 and 5 µg mL^{-1}. The linearity was performed on different days and in triplicate. The absorbance was measured at 318 nm. Data obtained were evaluated by the equation of the line, regression analysis, residue graph and analysis of variance (ANOVA).

Limit of detection (LOD) and Limit of quantification (LOQ)

The theoretical limits of detection and quantification were obtained from the calibration curves, done during the linearity, using Equations 1 and 2, respectively:

$$LOD = 3\,x\,\frac{SD}{a} \qquad \text{Equation 1}$$

$$LOQ = 10\,x\,\frac{SD}{a} \qquad \text{Equation 2}$$

Where, SD: standard deviation; a: average slope.

Precision

Precision was evaluated in 3 levels: intraday, inter-day and inter-analyst. Intraday precision was performed on the same day, with the same analyst and same conditions, using 6 replicates. Inter-day precision was performed on different days, with the same analyst and same conditions, using 12 replicates. Inter-analyst precision was performed on the same day, with the same conditions and different analysts, using 12 replicates. The concentration used was 3 µg mL^{-1} and the results were analyzed by RSD (%).

Selectivity
Selectivity was determined by comparing the response obtained for CLIN standard and sample and placebo. The preparation of the placebo for the capsule pharmaceutical form was carried out through a pool with all the excipients present in the formulation: starch, talc, magnesium stearate, silicon dioxide and croscarmellose sodium and the solution was subjected to the same reaction with $KMnO_4$. The forced degradation test under acidic (HCl 0.01 M at 25 °C), basic (NaOH 0.01 M at 25 °C), neutral (purified water and ethanol at 25 °C) and photolytic (UV light at 25 °C) conditions at a concentration of 5 µg mL^{-1}, in order to obtain more defined spectra. Aliquots were collected after 1 hour in the degrading condition, and absorbance was measured and compared to time zero using 318 nm.

Accuracy
Accuracy was assessed by standard recovery test using 3 levels (80, 100, and 120 %) in triplicate, considering 3.0 µg mL^{-1} 100 %. Standard and sample stock solutions were prepared at a concentration of 20 µg mL^{-1}. From the standard CLIN stock solution, aliquots of 0.475, 0.625 and 0.775 mL were transferred to 5 mL volumetric flasks containing 0.125 mL of CLIN sample aliquot, in order to obtain a final concentration of 2.4, 3.0 and 3.6 µg mL^{-1}, respectively. Recovered standard (%) and RSD (%) were determined. Each condition was prepared in triplicate, and each was performed on 3 consecutive days.

Robustness
Robustness was studied by small modifications to the method, which were analyzed by the F-test and t-test. The modifications were: wavelength (316 nm, modified/ 318 nm, normal), quartz cuvette (700 µL capacity, modified/ 3.5 mL capacity, normal), ethanol brand (Ciavicco®, modified/ Vetec®, normal) and reaction time (no reaction time, modified/ 30 minutes, normal).

Content analysis
Standard and sample solutions at a concentration of 3 µg mL^{-1} were prepared in triplicate on 3 different days. The absorbance values were compared and the CLIN content in capsules was calculated. The content was analyzed according to the literature specifications (2, 3).

Greenness analysis method

National Environmental Methods Index
For evaluation using the NEMI tool, the Toxic Release Inventory (TRI) list established by the Environmental Protection Agency (EPA) was checked for PBT criteria with the reagents ethanol and $KMnO_4$, and the Resource Conservation and Recovery Act (RCRA) list was checked to determine the hazard. The pH of the solutions was measured, and the residues generated for the proposed method were calculated.

Eco-Scale Assessment
The ESA assesses the greenness of analytical methods, based on the assignment of penalties to parameters that do not meet GAC requirements, comparing the different stages of the analytical process with different parameters (31, 33). The penalty points were calculated, according to Equation 3:

ESA = 100 – [(chemical reagents pictogram x quantity of reagents x signal words) + energy + occupational hazard + (waste amount x waste characteristic)]

Equation 3

Analytical GREEnness metric
AGREE evaluates the greenness profile of the method through the 12 GAC principles, which are represented by segments that form a pictogram, and the weighting of each principle is represented by the width of the pictogram segment (34).

Results and Discussion

Method development

Reaction between CLIN and $KMnO_4$
The CLIN molecule has only one hydroxyl as a chromophore group; therefore, it was necessary to perform a reaction with $KMnO_4$, adding 100 µL to the standard solutions and CLIN sample (35-41). Figure 3 shows the different absorption spectra of CLIN at a concentration of 10 µg mL^{-1}, with (1) blank 100 µL of potassium permanganate at a concentration of 2 mg mL^{-1} in purified water: ethanol (50:50 v/v), (2) CLIN solution in purified water: ethanol (50:50 v/v) without reaction with $KMnO_4$ and (3) the reaction of CLIN with KMnO4 at a concentration of 10 µg mL^{-1}, in which it is possible to observe a sharp increase in absorbance and the formation of a new band distinct from those observed for the individual solutions, indicating the formation of oxidized products that absorb at different wavelengths.

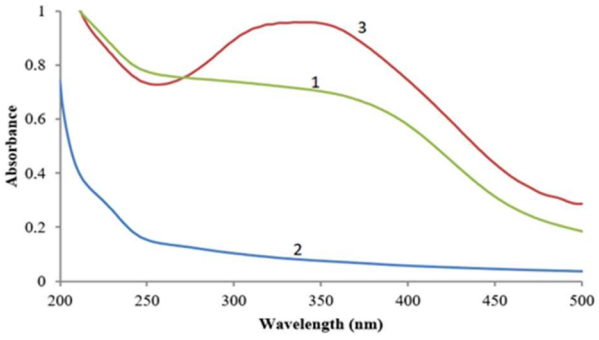

Figure 3. (1) Blank (100 µL of $KMnO_4$ at 2 mg mL^{-1} in purified water: ethanol (50:50 v/v)); (2) CLIN at a concentration of 10 µg mL^{-1} in purified water: ethanol (50:50 v/v); and (3) 100 µL of $KMnO_4$ in CLIN at a concentration of 10 µg mL^{-1} in purified water: ethanol (50:50, v/v).

Other studies that used potassium permanganate as an oxidizing agent verified the reaction time that is necessary to achieve maximum absorption (35-36). The reaction time was determined by monitoring the absorbance of the reaction product at different time intervals (10, 20, 30, 40,

50 min). Complete color development for the product was obtained after 30 min at 25°C, as shown in Figure 4. Furthermore, the reaction has been shown to be sensitive to temperature variations above room temperature at which $KMnO_4$ is consumed and there are extreme pH variations.

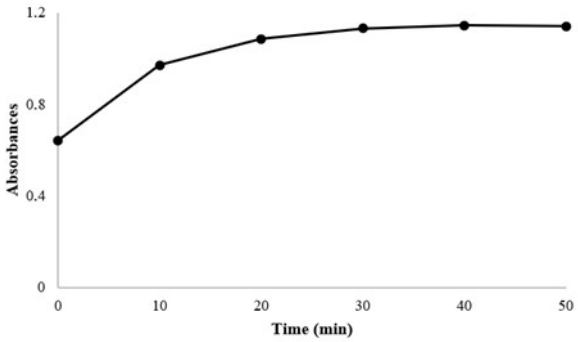

Figure 4. Reaction time between 100 µL of $KMnO_4$ at 2 mg mL^{-1} + CLIN standard solution at 10 µg mL^{-1}.

Method Validation

Linearity

Stock solutions at 0.5, 1, 2, 3, 4 and 5 µg mL^{-1} (Figure 5A) were prepared in triplicate on 3 different days. Data were validated by ANOVA (Table 1), which showed significant linear regression ($F_{calculated}>F_{critical}$) and no significant lack of fit ($F_{calculated}<F_{critical}$). Furthermore, the residue graph (Figure 5B) shows dispersion and no trend of the data. Therefore, the method can be considered linear.

Table 1. ANOVA results for evaluating the linearity of the method.

Parameters	318 nm
Linearity range (µg mL^{-1})	0.5 - 5
Slope	0.1004
Intercept	0.2356
Correlation coefficient (r)	0.9999
Regression	584.99* (4.75)
Lack offit	0.07 (3.26)

*Significant for $p<0.05$

Limit of detection (LOD) and Limit of quantification (LOQ)

Theoretical LOD and LOQ were, respectively, 0.10 and 0.29 µg mL^{-1}, showing the sensitivity of the method.

Selectivity

The selectivity of the method was evaluated in two ways (1) by comparing the spectra of the standard, CLIN sample and placebo, which demonstrated that the adjuvants do not interfere with the identification of CLIN (Figure 5C) and (2) by the forced degradation test. The spectra of each degraded condition can be seen in Figure 6 and the degradation percentage can be seen in Table 2. The degradation study showed stability of CLIN under basic (7.77 %) and neutral (7.28 %) conditions. Already in the conditions acidic (19.15 %) and photolytic (11.69 %) the degradation percentage was higher. It is worth highlighting that in the study carried out, the CLIN molecule underwent derivatization in order to facilitate its analysis by the spectrophotometric method in the UV region. Then, it was not possible to compare the degradation percentages in each stress condition with other data present in the literature, however, it allowed the evaluation of the sensitivity of the method for changes in the compound structure, reinforcing its usefulness for monitoring stability over time, proving it to be an indicative method of stability (42).

Table 2. Absorbance values obtained in the forced degradation test for CLIN in capsules.

Degradation condition	Absorbance (time zero)	Absorbance (time 1 h)	Degradation (%)
HCl 0.01 M	0.248	0.200	19.15
NaOH 0.01 M	0.410	0.378	7.77
Neutral	0.482	0.447	7.28
Photolytic	0.472	0.417	11.69

Precision

Precision results are shown in Table 3. RSD (%) values were lower than 5.0 %, proving the precision of the method (37, 38, 43).

Table 3. Results for evaluating the precision of the method: Absorbance values at 318 nm.

Level	1	2	3	4	5	6	
Intra-day	0.536	0.527	0.540	0.527	0.536	0.528	1.07%
Inter-day	0.536 0.530	0.527 0.547	0.540 0.540	0.527 0.527	0.536 0.525	0.528 0.518	1.53%
Inter-analyst	0.536 0.545	0.527 0.534	0.540 0.523	0.527 0.528	0.536 0.553	0.528 0.538	1.62%

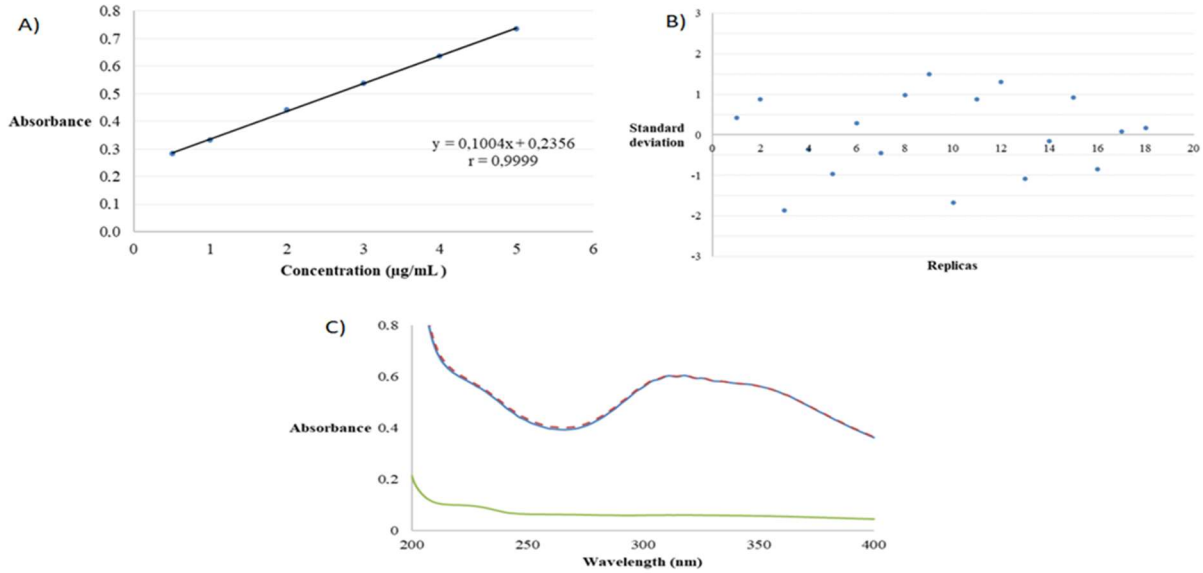

Figure 5. (A) Linearity graph, (B) residual graph and (C) overlay of the spectra referring to the solution of CLIN raw material (red), CLIN sample (blue) at a concentration of 3 µg mL^{-1} and placebo (green).

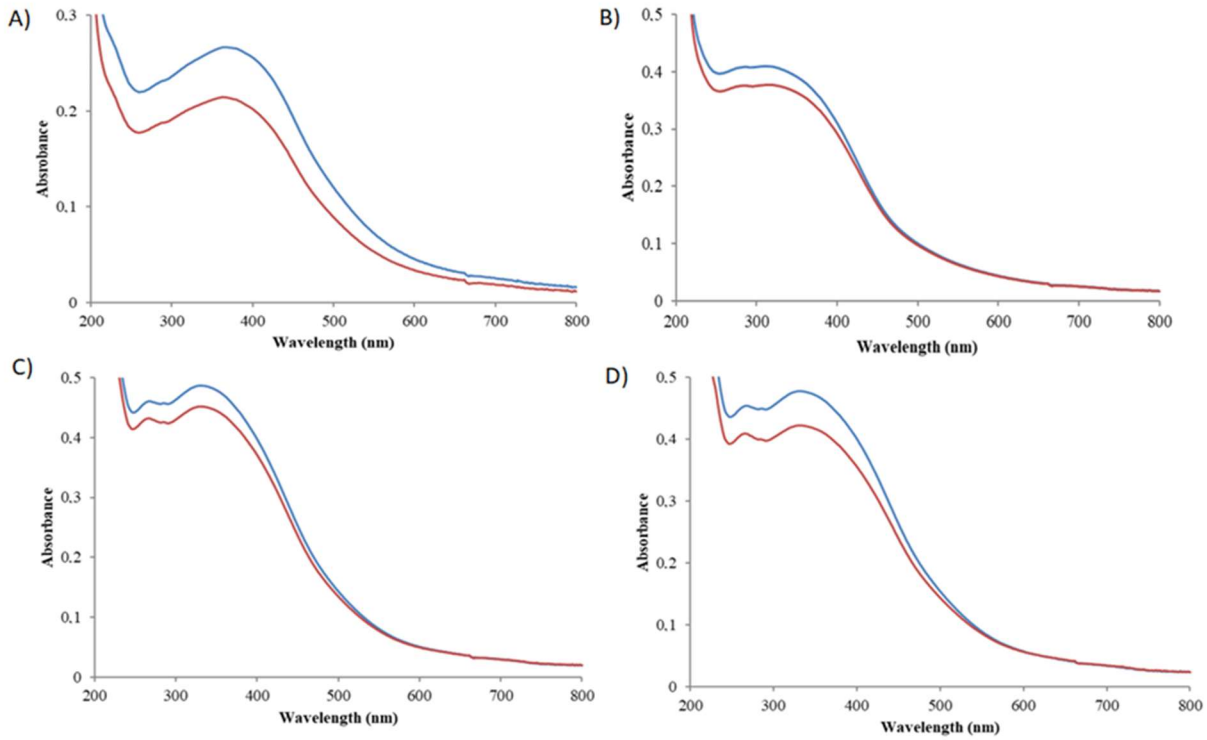

Figure 6. Absorption spectra of CLIN in capsules at time 0 (blue) and 1 h (red) under acidic (a), basic (b), neutral (c) and photolytic (d) conditions, all at a concentration of 5 µg mL^{-1} at 25 °C.

Accuracy

Standard recovery test results are shown in Table 4. The method can be considered exact, since the average recovery was 99.85 %, within the specification for pharmaceutical analyzes of 98 to 102 % (44, 45).

Table 4. Results for evaluating the accuracy of the method.

	CLIN standard added (µg mL^{-1})	CLIN standard recovered (µg mL^{-1})	Recovery* (%)	Mean recovery (%)	RSD (%)
R1	1.9	1.91	100.63		
R2	2.5	2.46	98.60	99.85	1.10
R3	3.1	3.11	100.32		

*Average of 3 determinations in triplicate

Robustness

Changes in wavelength (0.95<2.78) and changes in quartz cuvette (0.62<2.78) do not affect the method, since the calculated t was lower than the tabulated t at a significance level of 5%, as can be seen in Figure 7. However, changes in ethanol brand (23.83<2.78) and reaction time (42.12<2.78) significantly affect the method. Ethanol brands present different specifications for the test of substances reduced by $KMnO_4$, this variation is mainly associated with the presence of reducing impurities in some commercial ethanol formulations, such as aldehydes, secondary alcohols and other residual organic compounds from the distillation process, such substances can react with $KMnO_4$, promoting its partial or total reduction before the interaction with CLIN, compromising the intensity of the spectrophotometric response. The reaction time is essential to achieve maximum absorbance. In other words, if the reaction time is not waited, the absorbance will be lower than the true absorbance, with reaction time. These changes must be avoided, and information about these impacts must be included in the method execution procedure.

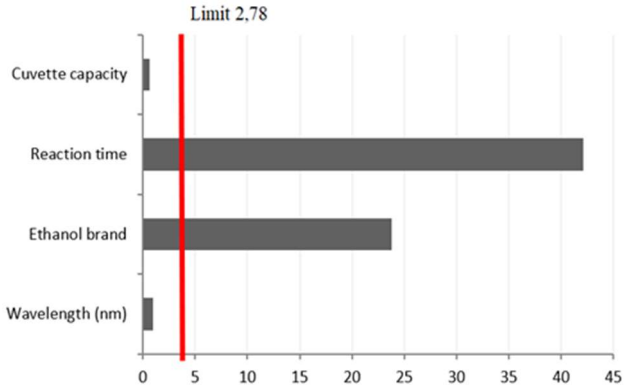

Figure 7. Effect of the variations studied on the robustness of the method.

Content analysis

CLIN content in the analysis of capsules was 102.66 %, within the specification of 90 to 110 % (2, 3).

Greenness analysis method

National Environmental Methods Index

Figure 8 shows the pictogram obtained for the proposed method, using NEMI tool.

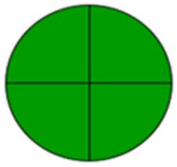

Figure 8. Qualitative evaluation for the proposed method in the UV region for quantification of CLIN in capsules.

The method presented a greenness profile with four green quadrants due to the absence of criteria such as PBT, hazard, corrosivity (pH = 6), high waste generation (approximately 15 ml) and danger.

Eco-Scale Assessment

According to Eco-Scale Assessment, the greenness of an analytical process is classified using the values: > 75 represents excellent green analysis; > 50 represents acceptable green analysis; < 50 represents inadequate green analysis (46). The proposed method is considered an excellent green analysis, since the greenness of the analytical process was 79, as shown in Equation 3 below:

$$ESA = 100 - [(1 \times 2 \times 1 \times 2 \times 2 \times 2) + 0 + 0 + (5 \times 1)]$$
$$ESA = 100 - 21 = 79$$

No method in the literature for evaluating CLIN in capsules by UV presents conditions as clean as the one proposed in this work. The available options require cyclohexane (24), methanol (5-7) and buffer (8-13). To summarize, some benefits of the proposed method include (i) excellent green analysis of CLIN in capsules compared to those that use more toxic organic solvents such as methanol and cyclohexane (6-18); (ii) rapid method with only 30 min of reaction time compared to methods that use 40 and 45 min, using room temperature compared to studies using up to 80°C (19-23); (iii) low cost because it does not require sample pretreatment (6-18); (iv) generation of less waste compared to works using cyclohexane and requiring special reagent treatment (19-23); (v) use of purified water and ethanol (50:50, v/v) only.

Analytical GREEnness Metric

The evaluation by the AGREEanalytical calculator considered all 12 GAC principles for the UV spectrophotometric method developed, as shown in Figure 9.

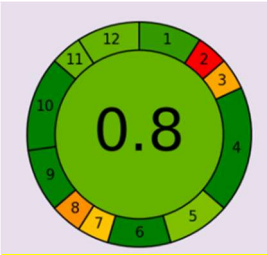

Figure 9. Pictogram generated by the AGREE tool to evaluate the greenness profile of the spectrophotometric method developed.

The method developed does not require external sample treatment; analyses are performed with small amounts of standard and sample on the bench in a non-invasive manner, in small concentrations; however, there are many preparations. The equipment is semi-automatic; sample preparation is performed in 2 distinct steps; $KMnO_4$ is not scored as a PBT reagent in the official lists or in the AGREE program, and does not score in this item; however, it generates waste that needs to be recycled and the sample yield is not significant due to the reaction time. Despite these considerations, the method can be considered green because it has a score of 0.8, since the closer to 1, the greener the method.

Conclusions

The proposed method is an excellent alternative for routine analysis in chemical pharmaceutical laboratories. It is an eco-friendly and stability-indicative method by spectrophotometry in the UV region for the evaluation of CLIN in capsules. It uses purified water and ethanol (50:50, v/v) as a diluent, quartz cuvette at 318 nm. The method was linear (0.5-5 µg mL^{-1}, r = 0.9999), selective, precise (RSD < 5 %), exact (99.85 %), robust and considered an excellent green analysis by the NEMI, ESA and AGREE criteria.

Acknowledgments

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Conflict of interest

The authors declare no conflicts of interest.

Sources of funding and Supplementary sections

Not applicable.

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