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# Eco-friendly ultraviolet spectrophotometric methods for the determination of clotrimazole and tinidazole in bulk and ointment dosage form

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**A B S T R A C T**

Analyzing a drug over its overlapped spectra utilizes sophisticated instruments and more toxic solvents, which has a deleterious effect on environmental safety. There is an alarming need to develop a simple, novel, and cost-effective method for determining combined substances that are non-toxic to the environment. So, the study aimed to develop four simple, fast and eco-friendly spectrophotometric techniques for quantifying clotrimazole and tinidazole in bulk and ointment dosage form. Stock solutions produced at concentrations of 7 to 13 and 17.5 to 32.5 µg/mL of clotrimazole and tinidazole in 10% v/v ethanol and scanned in the UV-visible range 200-450 nm, and used for all methods. The methods were validated according to the International Council for Harmonization guidelines and found to be within limits. Additionally, the outliers were tested by using the Grubbers test and found within limits. Finally, green evaluation studies show that the method is more environmentally friendly, as confirmed by four assessment tools.



## 1. Introduction

Clotrimazole (CTZ) is chemically 1H-Imidazole, 1-[(2-chlorophenyl) diphenyl methyl]. (**Fig. 1a**) CTZ is an imidazole derivative that acts by changing the permeability of individual Candida or fungal cells. It clings to phospholipids mostly in the cell membrane, thus suppressing the manufacture of ergosterol and other essential sterols to form the cell membrane. Clotrimazole may inhibit fungal growth or lead to the death of fungal cells.[1]

Tinidazole (TDZ) is chemically 1H-Imidazole, 1-[2-(ethylsulfonyl) ethyl] -2-methyl -5-nitro (**Fig. 1b**). It is a medication that is used to treat protozoan infections. These are extensively used to treat various anaerobic amoebic and bacterial diseases across Europe and the developing world. TDZ may be an alternative to metronidazole in patients who are intolerant to metronidazole. TDZ is an antibiotic used to cure Amoebic dysentery, Giardia, Helicobacter pylori, and Trichomonas vaginalis. The half-life of clearance is 13.2 ± 1.4 hours. The plasma half-life is between 12 and 14 hours.[2–4]

A combination of CTZ and TDZ is used to treat vaginal discharge on a syndromic basis. It works by halting the development of germs and eliminating those that are already present and causing the illness. Vaginal infections may cause symptoms such as an increase in the volume of vaginal discharge or a change in the color of the vaginal discharge. Additionally, it may produce an unpleasant odor, itching, or discomfort in the vagina. These symptoms may be both humiliating and inconvenient. This combination of dose forms aids in the successful management of these symptoms by destroying the organisms that cause vaginal infection.

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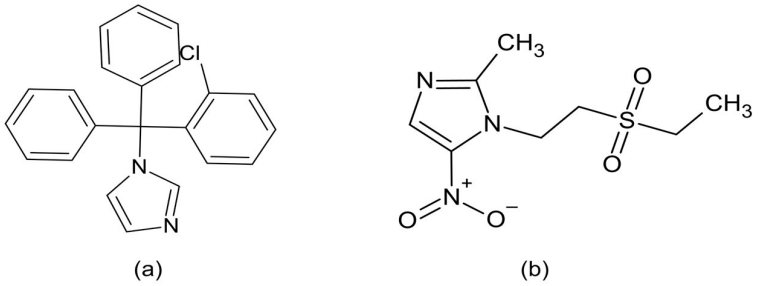



**Fig. 1.** Structure of Clotrimazole (a) Tinidazole (b)

Literature review states that no method was developed to estimate this combination in any dosage form. There are few methods available for estimation of these drugs individually by using hazardous solvents like 0.1 N HCl,[5] 0.1 N NaOH,[6] and $H_2SO_4$[7] for CTZ and TDZ respectively. A, few methods were developed for these drugs in other combinations using UV, [8–13] HPLC,[14] HPTLC.[15] So, the present study aims to develop a new eco-friendly method for the first time, which covers all the green analytical principles and is further assessed by using four assessment tools to ensure the developed method's greenness.

### *1.1. Derivative spectrophotometry*

Derivative spectrophotometry effectively resolves two overlapping spectra and minimizes vector external interference or interventions caused by an unclear shoulder on one end of maximum absorption. The transformation of a standard spectrum to a first, second, or higher derivative spectrum is called derivative spectrophotometry. The standard absorption spectrum is also known as the basic, zeroth level, or D0 spectrum in the context of derivative spectrophotometry. To produce a first, second, or higher-order derivative, the absorbance of a sample is differentiated concerning wavelength.

The λ max of the absorption band represents the location of the first derivative spectrum's maximum, minimum, and crossover point. The second derivative spectra are defined by two satellite peaks and an inverting band, the lowest of which corresponds to the fundamental band's maximum. [16]

### *1.2. Area Under Curve (AUC)*

The λ max of the absorption band represents the location of the first derivative spectrum's maximum, minimum, and crossover point. The second derivative spectra are defined by two satellite peaks and an inverting band, the lowest of which corresponds to the fundamental band's maximum.[17]

### *1.3. Absorption factor method*

This technique is dependent on the presence of two drugs A and B, with overlapping spectra. B exhibits interference at A's $K_{max}$ (k1), while X exhibits no absorbance at another wavelength (k2), i.e., abs k2X Y 14 absk2Y. A's quantitative estimate in combination (A + B) was accomplished by deducting the absorption due to B at $K_{max}$ of A from the absorption factor determined experimentally.

$$\text{Absorption of A in } \lambda_1 = \text{abs}\lambda_1(\text{A}+\text{B}) - \frac{\text{abs1}}{\text{abs2}'} \times \text{abs}\lambda_1(\text{A}+\text{B}) \quad (1)$$

Since: abs1, abs2 indicates absorbance of A in $\lambda_1$ and $\lambda_2$;

$\frac{\text{abs1}}{\text{abs2}'}$ is constant of B and absorption factor and

abs $\lambda_1$(A+B) and Abs $\lambda_2$ (A+B) indicates absorption of the mixture at particular wavelengths

To determine the concentration of A, the relevant regression equation should be used.

## 2. Results and Discussion

UV spectrophotometry is a simple, precise, and reliable instrument used for industrial purposes and academic purposes. The selection of four different methods which is based on one of the principles called Multi analytical technique and in the present study four methods has performed by using of a single solution indicates that the methods can be easily transferable form one method to other which confirms that these are best approaches towards green analytical chemistry principles. The common solvents used in the analysis of drugs using this instrument were methanol. The selection of methanol should avoid the analysis of compounds due to toxicity towards the environment and analysts. The solvent selected for this method

development was ethanol which is biodegradable and eco-friendly. The ethanol concentration in most of the experiments was used was 10% in water. The reason for selecting 10% ethanol was to decrease solvent consumption and make the method most affordable and economical. The two drugs were completely soluble in 10 % ethanol without any solubility issues. The recycling of the waste generated by the method is one of the principle in green analytical chemistry so, the whole solutions were used in the method has recycled by using simple distillation.

Once the drugs were prepared and scanned over a range of 200 to 450 nm, the CTZ spectra overlapped with the TZD spectra, and it made it challenging to analyze the CTZ in the presence of TZD using Zero-order spectra (**Fig. 2**). So to determine the drugs in the presence of another one, first-order spectra were applied to for the drugs individually and found that the two drugs' absorption were obtained in different regions where the other showed null absorbance at that juncture. Then the method has further extended to the second-order derivation and was found to have satisfactory results like the first order. The same was validated using the different concentrations of the individual and mixed spectrums.

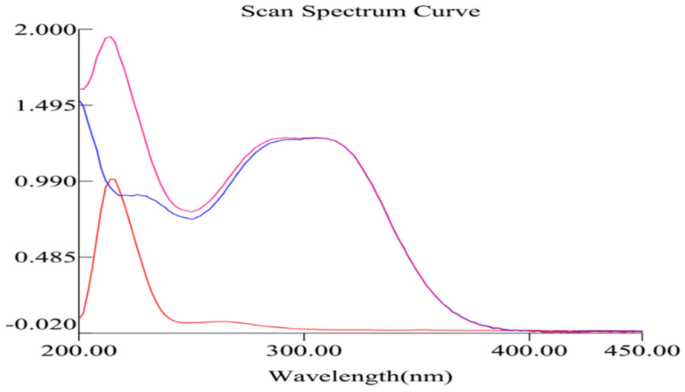



**Fig. 2.** Zero-order spectrum of CTZ (Red), TDZ (Blue), and Mixture (Rose)

*2.1. First-order derivative method*

The stored zero-order spectrums of individual drugs and mixtures were converted to first-order differential spectra using the win lab software via selecting the mathematical calculation to differential and finally to the first order. The obtained graphs were overlayed with the other concentrations to find the linearity. Further overlaying was done with the other drug to find the exact $\lambda_{max}$ was the one drug shows the maximum absorbance and the other drug shows zero (**Fig. 3**). This has been further confirmed by overlaying the mixed spectra (**Fig. 4**). Finally, the unknown sample was identified using the standard addition method, and the results were depicted in **Table 1**.

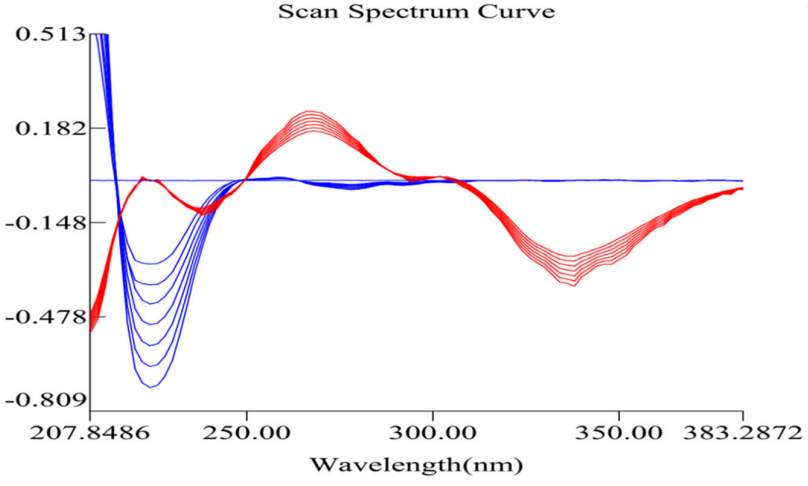



**Fig. 3.** First-order spectrum overlap of CTZ (Red) and TDZ (Blue)

*2.2. Second-order derivative method*

The win lab software converted the zero-order spectra of individual drugs and mixtures to differential second-order spectra. The mathematical calculation was changed to differential and then to second order. The resulting graphs were overlaid with the other concentrations to determine linearity and the other drug to determine the exact $\lambda_{max.}$ (**Figure 5**).

This was verified further by superimposing the mixed spectra (**Fig. 6**). Finally, the unknown sample was identified using the standard addition technique, and the findings are shown in **Table 1**.

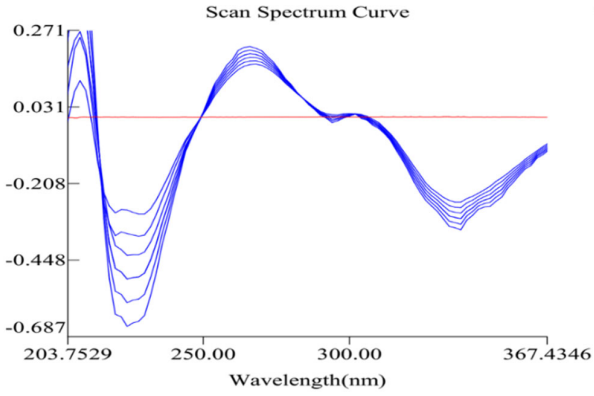



**Fig. 4.** First-order spectrum of mixture samples over a linear range

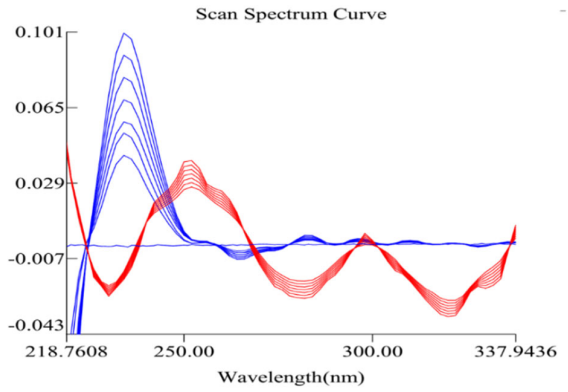



**Fig. 5.** Second-order spectrum overlap of CTZ (Red) and TDZ (Blue)

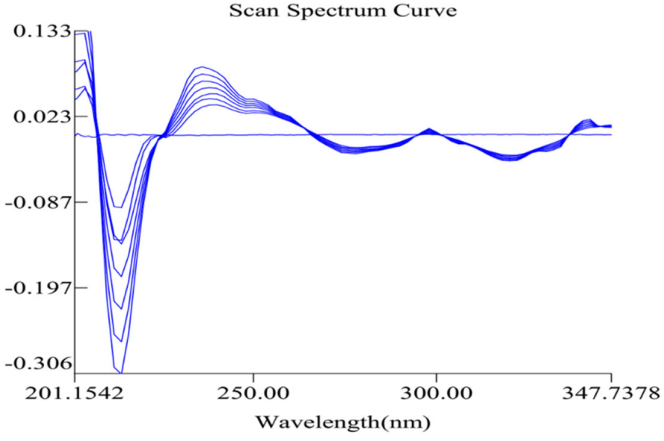



**Fig. 6.** Second-order spectrum of mixture samples over a linear range

*2.3. Area Under Cure method*

The zero-order spectrums of the drugs were overlapped and selected a range of wavelengths of around 300 to 310 nm for TDZ, as in this region, CTZ showed null or nearly zero absorbance. The second set of the range selected for CTZ was 207 to 215 nm, but in this case, both the drugs show the absorbance, so finding the exact area of CTZ in this range of nm can be calculated by subtracting the area of standard area of TDZ from the area of the mixture. The unknown sample concentration was found by comparing the standard spectrum with the sample—the area under the curve graph depicted in **Fig. 7**.

**Table 1.** The suggested spectrophotometric techniques application of standard addition approach was used to confirm CTZ and TDZ in the marketed ointment.

| Marketed formulation | Contents | Present (µg/mL) | Added (µg/mL) | Standard addition FDM | SDM | AUC | AFM |
|---|---|---|---|---|---|---|---|
| | | | | Recovery % | Recovery % | Recovery % | Recovery % |
| Ointment (Infa VT) | CTZ | 10 | 5 | 100.06 | 99.68 | 100.56 | 99.24 |
| | | | 10 | 99.853 | 100.06 | 99.853 | 99.84 |
| | | | 12.5 | 100.06 | 99.853 | 99.13 | 100.43 |
| | TDZ | 25 | 12.5 | 98.386 | 99.853 | 100.54 | 99.42 |
| | | | 25 | 100.06 | 100.06 | 99.13 | 99.73 |
| | | | 35 | 98.386 | 99.853 | 99.853 | 98.96 |

FDM = First-order derivative method; SDM= Second-order derivative method; AUC= Area Under Curve; AF = Absorption Factor method

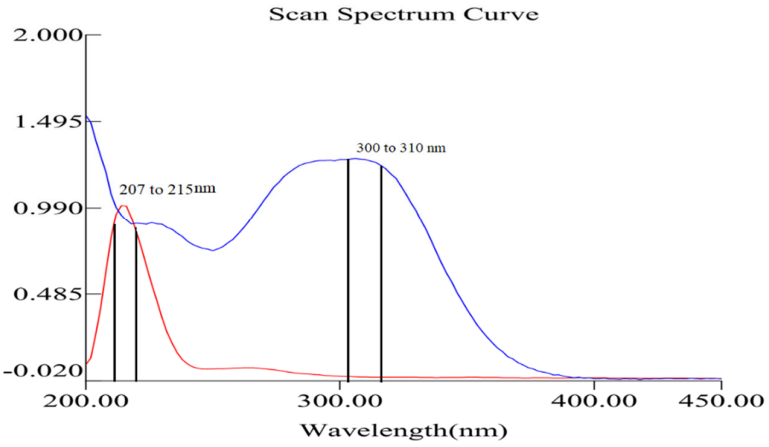



**Fig. 7.** Area under curve spectrum for CTZ and TDZ

*2.4. Absorption factor method*

The absorption spectra of various concentrations of TDZ standard solutions were obtained in the wavelength range 200–450 nm (**Fig. 8**). The absorption factor was determined to be 0.779. (the ratio between the absorbance of pure TDZ at 215 nm and 310 nm). CTZ was quantified in a mixture (CTZ + TDZ) by subtracting the absorption due to TDZ at CTZ's $K_{max}$ using the following equation:

$$\text{Abs of CTZ in 215} = \text{abs (mix) 215} - \frac{\text{abs of TDZ in 215}}{\text{abs of TDZ in 310}} \times \text{abs (mix) 310} \quad (2)$$

The concentration of CTZ was determined using the relevant regression equation, which was derived by plotting the absorption values for CTZ zero-order peaks at 215 nm against appropriate concentrations, and the overall results were depicted in **Table 1**.

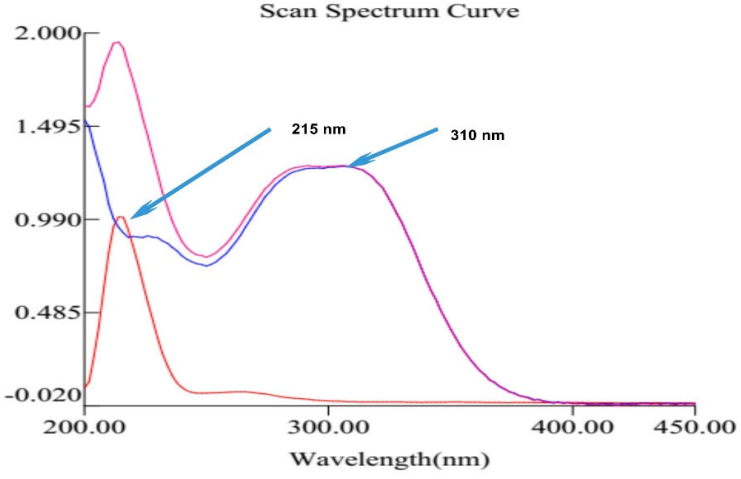



**Fig. 8.** Zero-order spectrum of CTZ (Red) (at 10 µg/mL), TDZ (Blue) (at 25 µg/mL) and Mixture (Rose)

*2.5. Effect of stressed conditions on spectrums*

*2.5.1. In acid condition:*

To 1 mL of standard solutions, 1 mL of 0.1 N HCl was added and made up to volume with diluent and scanned over a range of 200 to 450 nm using diluent with 1 mL of 0.1 N HCl as a blank and found that the CTZ showed a stable spectrum

like the standard. Still, the TDZ loses its spectra and shifts towards the hypsochromic shift tends to degrade a little (**Fig. 9a**). The overall Spectral absorption is depicted in **Table 2.**

*2.5.2. In basic condition:*

To 1 mL of standard solutions, 1 mL of 0.1 N NaOH was added and made up to the volume with the diluent and scanned over a range of 200 to 450 nm using diluent with 1 mL of 0.1 N NaOH as a blank and found that the CTZ showed a stable spectrum like the standard. Still, the TDZ loses its spectra completely and shifts towards the bathochromic shift to decrease spectral absorption a little (**Fig. 9b**). The overall Spectral absorption is depicted in **Table 2**.

*2.5.3. In oxidation:*

To 1 mL of standard solutions, 1 mL of 3% $H_2O_2$ was added and made up to volume with diluent and scanned over a range of 200 to 450 nm using diluent with 1 mL of 3% $H_2O_2$ as a blank and found that the CTZ loses its spectra completely and shifts towards the bathochromic shift tends to loose its spectral absorption a little. Still, the TDZ showed a stable spectrum like the standard (**Fig. 9c**). The overall Spectral absorption is depicted in **Table 2**.

*2.5.4. Photodegradation:*

The sample solutions at a concentration of 10 and 25 µg/mL of CTZ and TDZ was prepared and kept under UV light for 3 h and scanned for a range of 200 to 450 nm and found that the spectral properties in this condition showed stable peaks for both drugs (**Fig. 9d**).

**Table 2.** Results for CTZ and TDZ in various stressed conditions

| | CTZ | | | | TDZ | | |
|---|---|---|---|---|---|---|---|
| | | | % Assay | % decrease in Spectral absorption | | % Assay | % decrease in Spectral absorption |
| | Control | 1.008 | 100 | 0 | 1.278 | 100 | 0 |
| Acid* | 0 min | 0.994 | 98.611 | 1.3889 | 0.762 | 59.624 | 40.376 |
| | 30 min | 0.992 | 98.413 | 1.5873 | 0.761 | 59.546 | 40.454 |
| Alkali* | 0 min | 0.995 | 98.71 | 1.2897 | 1.534 | 120.03 | -20.03 |
| | 30 min | 0.991 | 98.313 | 1.6865 | 1.534 | 120.03 | -20.03 |
| Peroxide* | 0 min | 0.122 | 12.103 | 87.897 | 1.273 | 99.609 | 0.3912 |
| | 30 min | 0.116 | 11.508 | 88.492 | 1.271 | 99.452 | 0.5477 |
| Photo* | 3 h | 0.998 | 99.008 | 0.9921 | 1.274 | 99.687 | 0.313 |
| | 6 h | 0.997 | 98.909 | 1.0913 | 1.273 | 99.609 | 0.3912 |

* Mean =3

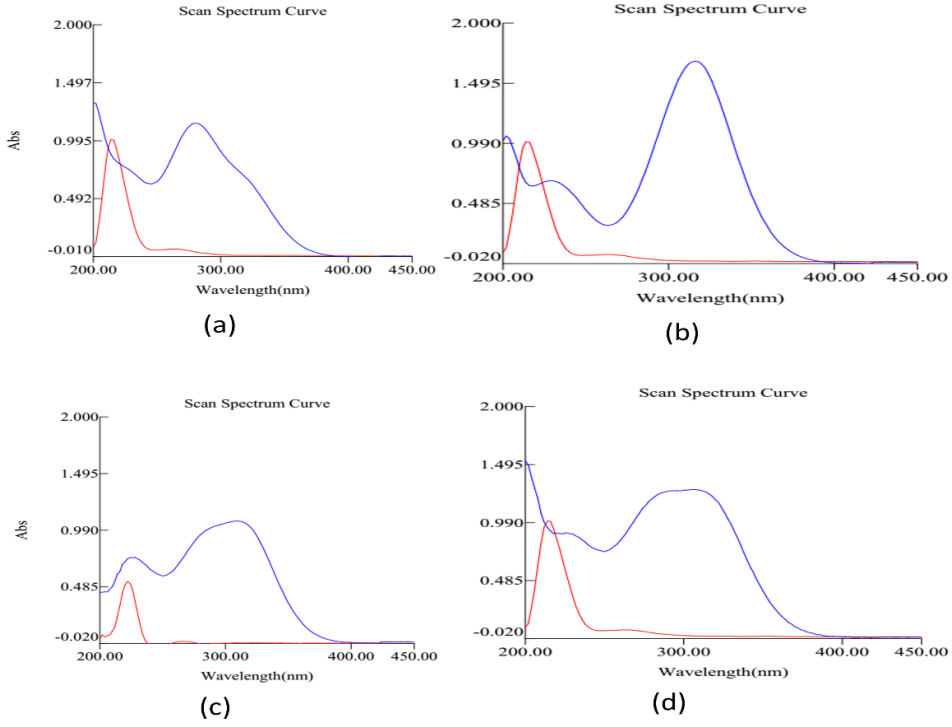



**Fig. 9.** Studies of CTZ (red) and TDZ (blue) in 0.1 N HCl (a) 0.1N NaOH (b) 3% $H_2O_2$ (c), Photodegradation (d) stressed conditions

### *2.6. Method validation*

The developed methods were further validated using linearity, the limit of quantification, detection, accuracy, and precision. The results were further depicted in Table 3 for the parameters. Numerous statistical techniques could well be influenced by outliers. For example, a single wildly error in data point may affect the fundamental mean and standard deviation calculations. Any data research should include an outlier identification method as a normal process. Potential outliers should be analyzed to determine their likelihood of being erroneous. If a data point consists of an error, it should be corrected or, if this is not possible, deleted. If there is no cause to believe the outlying point is wrong, it should not be carelessly eliminated. However, more stringent procedures may be required. Robust techniques often mitigate the effect of outlying points without completely removing them. To test for outliers in this research, the grubbers test was used, and it was determined that we could not reject the null hypothesis and thus conclude that no result is an outlier at the 0.05 level of significance. Table 4 summarizes the findings obtained with the grubbers test for the three medications. The methods were further compared using t-test and f- test and found no significant difference in the developed approach in the analysis of the binary mixture.

**Table 3.** Validation parameters for the proposed methods

| Parameters | First-order | | Second-order | | Area Under Curve | | Ratio absorption method | |
|---|---|---|---|---|---|---|---|---|
| | CTZ | TDZ | CTZ | TDZ | CTZ | TDZ | CTZ | TDZ |
| Wave Length | 224.19 | 266.6 | 236.13 | 280.01 | 207-215 | 300-310 | 215 and 310 | 215 and 310 |
| Y*= | 0.0637x - | 0.0067x + | 0.0087x - | 0.3946x - | 0.1033x - | 0.0531x + | 0.06 07x - | 0.104x + |
| $R^2$ | 0.9987 | 0.9995 | 0.9997 | 0.9987 | 0.9993 | 0.999 | 0.9991 | 0.9994 |
| LOD | 0.277 | 0.454 | 0.13 | 0.706 | 0.222 | 0.677902 | 0.23 | 0.219652 |
| LOQ | 0.84 | 1.376 | 0.392 | 2.139 | 0.672 | 2.054248 | 0.695 | 0.665611 |
| **Accuracy** | | | | | | | | |
| Mean± SD* | 99.89±0.79 | 99.58±0.56 | 98.63±0.85 | 98.45±0.89 | 98.24±0.63 | 99.46±0.33 | 99.57±0.53 | 98.58±0.49 |
| **Precision** | | | | | | | | |
| % RSD* | 0.716 | 0.975 | 0.746 | 0.948 | 0.652 | 0.952 | 0.396 | 0.964 |

* Mean =3

**Table 4.** Grubbers test results for the determination of outliers

| | First-order | | Second-order | | Area Under Curve | | Ratio absorption | |
|---|---|---|---|---|---|---|---|---|
| | CLT | TDZ | CLT | TDZ | CLT | TDZ | CLT | TDZ |
| **Data Point with Largest** | 0.707 | 0.252 | 0.095 | 0.024 | 1.301 | 1.787 | 0.342 | 1.409 |
| **Data Index with** | 7 | 7 | 7 | 7 | 7 | 7 | 7 | 7 |
| **G Statistic** | 1.3876 | 1.3814 | 1.4022 | 1.4261 | 1.3816 | 1.4105 | 1.3699 | 1.3922 |
| **Critical Value** | 2.01997 | 2.01997 | 2.01997 | 2.0199 | 2.01997 | 2.01997 | 2.01997 | 2.01997 |
| **Approximate P Value** | 1.0096 | 1.0252 | 0.972 | 0.9121 | 1.025 | 0.9509 | 1.0560 | 0.9975 |
| **Significance** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |

### *2.7. Green assessment for the proposed method*

The whole method was developed based on the 12 principles of green analytical chemistry.[18–26] To claim an analytical approach as eco-friendly, it should be proved with the help of assessment tools. Four assessment tools were used (available to date) in assessing the developed method as follows. NEMI and AES have calculated manually; however, GAPI assessment was performed using software called GAPI chart generator version 1.0, and AGREE metric calculation using AGREE calculator version 0.5 beta.

#### *2.7.1. National Environmental Methods Index (NEMI)*

The NEMI evaluations are made using a circular pictographic depiction split into four quadrants. As the first quadrant represents persistent, bioaccumulative, and toxic (PBT) list, if the chemicals used in the method were listed in this list, the part of the pictogram would be colorless. The second quadrant, hazardous wastes from the Environmental Protection Act (EPA), if the chemicals used in the method were listed in this list, then the part of the pictogram will be colorless. Third quadrant, pH values between 2 and 12 if the chemicals used in the method were outside the range of 2- 12, then the pictogram will be colorless. As a fourth quadrant, waste generation should be less than 50 mL or grams. If the chemicals wastage in the method were more than the allotted limit, the pictogram would be colorless. The reagent employed in this study was 10% ethanol, which is not classified as a hazardous chemical on either PBT or EPA list. As a result, the first and second quadrants were highlighted in green. Because the pH of the ethanol was between 2-12 so the third quadrant was colored green. Finally, since the waste produced by analyzing a single sample is less than 50 mL, the final quadrant was colored green. The suggested method's overall NEMI pictogram is shown in **Fig. 10 a**.

*2.7.2. Green Analytical Procedure Index (GAPI)*

GAPI is a qualitative indicator used to assess an analytical technique's greenness. The GAPI value is shown in color coding in the final pictogram. The GAPI pictogram has three times the number of components as the NEMI pictogram, making it more suited for assessing greenness in analytical chemistry. Additionally, it incorporates updated NEMI principles. Further, it includes several sample phases, making it superior to NEMI in green analytical chemistry evaluation. The GAPI evaluation is composed of 15 stages that are well-organized inside the GAPI programmer. The proposed approach was evaluated holistically using GAPI software, and the findings are shown in **Fig. 10 b**.

*2.7.3. Analytical Eco-Scale (AES):*

The AES evaluation is based on the chemicals' penalty points (PP). PP was created using a pictorial depiction of the chemicals involved in the procedure. It consists mainly of four assessment stages and the total computation of AES = 100 - PP.

Step 1: Based on the pictographic representation of chemicals, ethanol coded with 2 pictograms, so the overall PP= 4.

Step 2: Because the quantity of solvent or reagent used was less than 10 mL per sample, the PP = 1 × 4 (pictogram score) = 4.

Step 3: Given that the UV energy consumption for a single analysis is 0.1 kWh, PP = 0.

Step 4: waste of solvents that have an adverse impact on the environment based on the wastage recycling employed in this method, implying that PP = 0.

Finally, since the total PP loss for the developed technique was 4, the developed method's AES score was 96.

*2.7.4. Analytic GREEnness (AGREE):*

It is a sophisticated technique for determining the greenness of analytical procedures. It is a qualitative evaluation of how nature interacts with the surroundings. The result of AGREE will be a circular pictogram split into 12 parts, each representing one of the twelve principles of green analytical chemistry. Each principle or component gets a score between 0 and 1, with 1 being the most environmentally friendly. The total average will be shown in the dial's center, and the value closest to 1 indicates that the technique is ecologically friendly. The software was programmed to implement the created methodology in its entirety, and the resulting results reflect the most environmentally friendly implementation of the proposed method. **Figure 10 c** summarizes the AGREE findings.

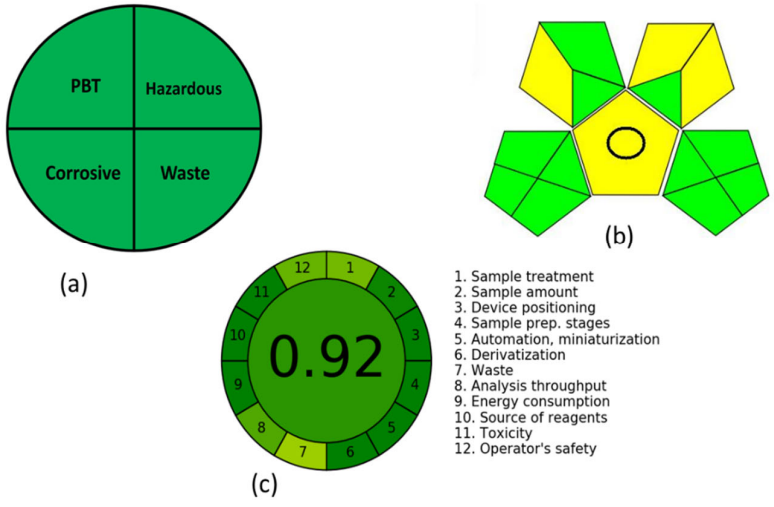


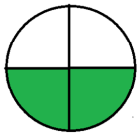
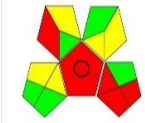
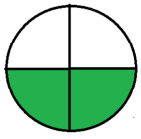
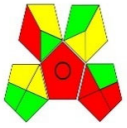
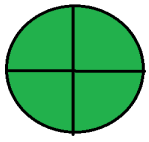
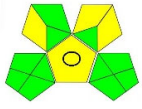

**Fig. 10.** Green assessment for the developed methods NEMI (a) GAPI(b) and AGREE (c)

*2.8. Method comparison*

The developed method was compared with the reported HPLC methods regarding the method efficiency. Greenness indicates that the developed method has no lag in efficiency and is superior in ecological safety towards the environment.

The overall methods were compared towards the environmental safety and found that the developed method has scored high greenness value than the other methods and the results were depicted in **Table 5.**

**Table. 5.** The summary of the results

| S.No | Instrument | Mobile phase/ solvent/ chemicals | NEMI | GAPI | Eco-scale | AGREE |
|---|---|---|---|---|---|---|
| 1 | HPLC | Acetonitrile and Phosphate buffer |  |  | 16 + 1 + 0 + 3 = 20 Σ = 80 |   |
| 2 | HPLC | Phosphate buffer: Acetonitrile |  |  | 16 + 1 + 0 + 3 = 20 Σ = 80 |   |
| 3 | UV (proposed method) | Ethanol (10%) |  |  | 4+ 0 + 0 + 0 = 4 Σ = 96 |   |

Eco-Scale represented as PP for pictograms + Instrument energy + occupational hazard + waste reduction = total PP; Σ = 100 – Total PP

## 3. Conclusions

The quadra procedures were easy, accurate, and repeatable, with minimal manipulation approaches for detecting CTZ and TDZ in bulk and ointment dose form. The technique is entirely eco-friendly and sustainable due to the usage of ecologically benign ethanol. According to ICH standards, the three methods were verified and shown within limitations, with reliability and reproducibility of less than 1.5 % relative to the percent RSD. The fact that the four approaches produced comparable outcomes shows that the difference in method selection was negligible. The technique was further assessed using green evaluation methods and provided the most environmentally friendly results when ethanol was used as a solvent. As this method has no adverse effect on the environment, it is easily adaptable for the analysis of CTZ and TDZ drugs, as well as for the analysis of other drugs, as these drugs are soluble in ethanol so, this method is easily adoptable by the pharmaceutical industries and QC department for routine research and sustainable development.

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## 4. Experimental

4.1. *Materials and Chemicals used:*

The CTZ and TDZ pure drugs were gifted by the Shree icons, Goregaon, Vijayawada India, 98.9 to 101.1% pure with a label certification. The purity of CTZ and TDZ is determined as 99.66% and 99.64 %, respectively, using the spectrophotometric techniques described.

Marketed formulation containing CTZ (2%) and TDZ (5%) was obtained from the local pharmacy.
Ethanol – HPLC grade, Hyman (German).

*4.2. Instruments used:*

UV VIS spectrophotometer (3092 double beam) - Lab India (Gujarat India), and
UV software - Win Lab Version 5.1.1.
Unichrome ultrasonic (Gujarat -India).

*4.3. Diluent preparation:*

10% ethanol in HPLC grade water was used for the analysis.

*4.4. Method development:*

A stock solution of 10 µg/ml of CTZ and TDZ in diluent solution was made. Stock solutions diluted with diluent in the range of 7 to 13, and 17.5 to 32.5 µg/mL of CTZ and TDZ. 7 concentrations were run through a 200 to 450 nm using diluent as a blank and stored for further use. The exact solutions were used for the calibration in different methods.

*4.5. Preparation of marketed formulation:*

Each commercial formulation was precisely and accurately weighed to an equivalent of 20 mg and 50 mg of CTZ and TDZ then 50 ml of ethanol was added to a 100 mL volumetric flask. After shaking, the resulting mixture was filtered, and the volume was appropriately adjusted. This standard preparation method was replicated by implementing several aliquots tested throughout the concentration range of optimal.

*4.6. Application of methods*

To the scanned and stored Zero-order spectra over a concentration range of individual standards and sample mixtures, First and second-order derivatives were applied by keeping the number of points and coefficient as 5 and 100, respectively, for all the spectra. AUC method was applied to the standard zero-order spectra and identified the concentration of the unknown sample concentration.

*4.7. Effect of stressed conditions on the spectrum*

The samples were set to stressed studies at various conditions like acid, base, photo, and peroxide degradations. To a series of volumetric flasks, 1 mL of sample along with 1 mL of 0.1 N HCl, 0.1 N NaOH, and added 3% $H_2O_2$ were added and scanned over a range of 200 to 450 nm for the sample solution using diluent as a blank. Photodegradation was achieved by using a UV light for 3 h and scanned for the degradation of the two solutions correspondingly.

*4.8. Method Validation:*

The proposed methods were validated according to ICH guidelines for various parameters like linearity, accuracy, the precision limit of detection, and quantification.

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