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# *In-vitro* determination of ciprofloxacin - metronidazole interaction by derivative spectrophotometry with greenness profiling

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**Abstract**: **Background**: The study of DDIs has evolved significantly over the years, early *in-vitro* detections could help identify potential interactions before clinical use, enhancing drug efficacy and patient safety. **Objectives**: This work therefore focused on finding *in-vitro* DDI (incompatibility) between the two commonly prescribed antibiotics Ciprofloxacin (CFX) and Metronidazole (MTZ) in neutral, acidic and basic mediums using derivative spectroscopy. **Methods**: The spectral measurements were performed on UV-Visible spectrophotometer at 0, 4 and 24 hours. Standard solutions (100ppm) and working solutions (3-11ppm) of both drugs were prepared in water, while mixture contained 5ppm of each drug. AGREE calculator software was used to figure out the environmental impact of the used spectrophotometric method. A zero-crossing method was employed for simultaneous quantification of CFX and MTZ in admixture. **Results**: It was observed that a mixture having 5ppm CFX and MTZ showed a recovery percentage of 87.704% ± 0.001041 and 78.226% ± 0.002379 respectively in neutral medium at 4 hours, along with AGREE score of 0.8. The proposed method was greener (eco-friendly). **Conclusion**: The findings concluded that although the two drugs were compatible in acidic and basic mediums, these might be incompatible in neutral environment upon simultaneous administration.

**Keywords**: Ciprofloxacin; *In-vitro* techniques; Metronidazole; Spectrophotometry.

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## INTRODUCTION

The study of drug-drug interactions (DDIs) has evolved significantly over the years. DDIs can either increase or decrease the efficacy of the drug and arise when the presence of one drug alters the pharmacokinetics of another, impacting its absorption, distribution, metabolism and excretion (ADME). These interactions can lead to outcomes ranging from diminished therapeutic effectiveness to heightened toxicity**,** ultimately complicating both treatment strategies and patient safety (Zhao *et al.*, 2024). Initially identified through clinical observations, DDIs are now rigorously assessed using *in-vitro* models before reaching human trials. The development of standardized guidelines by regulatory agencies such as the FDA has further emphasized the importance of these studies in modern drug development (Marks, 2021). *In-vitro* DDI studies serve as an essential tool for early detection and mitigation of such risks, allowing researchers to evaluate the impact of drug candidates on metabolic enzymes and transporters before clinical exposure (Erceg and Antolovic Amidzic, 2025). Understanding drug-drug interactions is a critical aspect of drug development and patient safety. *In-vitro* DDI studies play a fundamental role in predicting potential interactions before clinical trials, helping to identify risks and optimize therapeutic strategies.

In clinical treatment, two or more drugs are usually required to cure a medical condition raising the concerns of poly-pharmacy. Ciprofloxacin (CFX) [1-cyclopropyl- 6-fluoro - 4 - oxo-7- (piperazin-1-yl) - quinoline-3-carboxylic acid], is a broad-spectrum synthetic antibiotic that is active against infections caused by gram-negative bacteria and gram-positive cocci. It also possessed activity against most strains of enterococci and some strains of the Methicillin-resistant *Staphylococcus aureus* and has reduced activity against anaerobes (Shariati *et al.*, 2022). Metronidazole (MTZ) [(2-(2-Methyl-5-nitro-1H-imidazol-1-yl) ethanol)], a nitro imidazole, has good anaerobic activity (Anjana, 2022). MTZ/CFX combination is mostly prescribed for enteric diseases, intra-abdominal or other poly-microbial infections. Also, the combination provides better treatment against mixed aerobic/anaerobic infections (Benson *et al.*, 2024). The chemical structures of CFX and MTZ are shown in fig. 1. Researchers have studied the spectrometric determination of CFX (Sebaiy *et al.*, 2023; Cicek and Erdogan, 2024) and MTZ (Abdel-Kader and Hashem, 2021; Rasheed, 2023; Sebaiy *et al.*, 2023) individually, in combination with each other (Sebaiy *et al.*, 2023) and with other drugs (El-Yazbi *et al.*, 2022; Xuan and Hoang, 2022; Tantawy *et al.*, 2023; Sadiq *et al.*, 2024). Though the existing investigations are still progressing to fully confirm their compatibility, here the current study also sought to contribute and further advance this area of research. The study therefore, aimed to analyze the compatibility of CFX and MTZ admixture without the need of prior separation.

The absorbance spectra of CFX and MTZ exhibit significant overlap, complicating their simultaneous determination using conventional spectroscopy. Derivative spectrophotometric method converts a zero-order spectrum

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into its derivatives through software processing, facilitating the resolution of overlapping spectra, suppression of background noise from impurities and enhancement of sensitivity and specificity (Attimarad *et al.*, 2021; Gupta *et al.*, 2022; Hagag *et al.*, 2023), making it a powerful tool for accurately determining CFX and MTZ in mixture.

***Experimental***

***Chemicals***

The commercial pharmaceutical preparations used for compatibility studies were:

*Ciproxin*: Ciprofloxacin 200mg/100ml injection, manufactured by Bayer Pakistan (Pvt) Limited.

*Flagyl*: Metronidazole 500mg/100ml injection, Manufactured by Sanofi Aventis Pakistan Limited.

***Study design***

The methodology was adopted from Vega and Sola (Vega and Sola, 2001). The experiment was performed in the fume hood. All the solutions were freshly prepared at ambient temperature (25 ± 5°C) under room light. The DDI was observed as 'physical incompatibility' (through visual inspection against a black-and-white background) and 'chemical incompatibility' between the two drugs. The concentrations observed prior to mixing were taken as 100%. After mixing, at time 0, 4 and 24 hours of the study, samples were measured as percentage of the initial concentration. A recovery below 90% of the initial concentration was taken as a significant loss (Tomczak *et al.*, 2021).

***Preparation of standard and working solutions***

100 ppm standard solutions of both drugs were prepared separately using distilled water as a solvent. For direct measurement, further dilutions were made using the same diluent to get the neat solutions of 3, 5, 7, 9 and 11 ppm for CFX and MTZ respectively. The mixture of CFX and MTZ was prepared by combining 5 ppm solutions of both the drugs in 1:1 ratio. To account for the possibility of pH-dependent drug interactions, the analysis was conducted under both acidic and basic conditions. This approach ensures a comprehensive evaluation of the compatibility between CFX and MTZ, as change in pH can influence the chemical stability, solubility and interaction potential of the drugs (Gaohua *et al.*, 2021). To obtain acidic and basic solutions, appropriate concentration (0.1N) of hydrochloric acid (HCl) and sodium hydroxide (NaOH) were used.

The peak plasma concentrations of CFX (Junkert *et al.*, 2024) and MTZ (Shahzad *et al.*, 2025) were found to be 2-10 and 2-20 mg/L respectively. Therefore, the working concentration of 5 mg/L (5 ppm) was selected for this work. The use of clinically relevant concentration is important due to fact that the drug compatibility greatly affects its therapeutic efficacy and patient safety. Even a minor change in physicochemical compatibility at infusion-relevant levels (at ppm levels) may be problematic.

***Determination of UV spectrum (zero-order spectra)***

The spectral measurements of CFX and MTZ solutions and their mixture were performed on UV-Vis Specord 200 Plus Spectrophotometer using Aspect UV software with quartz cells of 1 cm path length. The absorption spectra of different dilutions (3-11ppm) of CFX, MTZ and their mixtures were recorded using distilled water as blank in the UV range (190-380 nm). The same procedure was repeated for the dilutions and mixture in acidic and basic environment. Each measurement was carried out in a triplicate. The absorption maxima weren't well-defined with interference within the UV range observed. Therefore, the analysis method based on derivative spectroscopy (1$^{st}$, 2$^{nd}$, 3$^{rd}$ and 4$^{th}$ i.e. D1, D2, D3 and D4 respectively) was utilized.

***Selection of zero-crossing point through derivative spectroscopy***

In order to obtain a wavelength for quantification of CFX and MTZ in admixture, a zero-crossing method was employed using derivative spectroscopy. Converting zero-order spectra into derivatives resulted in a weighty variant form. Here, the zero-crossing method was applied by determining the wavelength where zero absorption of one of the drug was found on the derivative curve of the other. From here, one of the derivative spectra was selected out of the four for simultaneous determination of drugs in mixture.

***Analytical parameters***

The prepared dilutions i.e., 3, 5,7, 9 and 11 ppm of both CFX and MTZ were used to determine if the solutions obey Beer-Lambert's law and was validated using Microsoft Excel 365. A regression equation ($y = ax + b$) for CFX and MTZ respectively was generated and the regression of analysis was calculated. These equations were further utilized in calculating the concentrations of drugs (recovery) in mixtures in neural, acidic and basic mediums.

***Greenness assessment using analytical GREEnness (AGREE) software***

In order to figure out the environmental impact of the spectrophotometric method used during the study, the greenness profile was also assessed using AGREE calculator software. This is a free, versatile and easy to operate tool that automates and expedites the evaluation process providing results within no time (Pena-Pereira *et al.*, 2020).

This metric system considered twelve parameters, known as SIGNIFICANCE principles of Green Analytical Chemistry (GAC) encompassing every step of the analytical procedure. After entering variables against each parameter, the tool generates a clock-like diagram which

has twelve sections; the width of each section presented the weight of the principle in consideration and can be adjusted depending on its importance. A central core displayed overall score of the analytical method and ranged from red (0) to yellow to green (1) with values close to 1 (darker green) indicating the method is greener. The whole process became even more easy as the AGREE software generates both a graph and a comprehensive assessment report for visual representation (Pena-Pereira *et al.*, 2020; El-Kafrawy *et al.*, 2022; Semysim *et al.*, 2025).

***Statistical analysis***

Paired (t-test) two samples for means was applied to calculate p-value using MS Excel 365. A p-value less than 0.05 (< 0.05) was used to indicate significant difference and indicated by (*) sign in the Table 1 and 2. This test was applied to monitor any change in concentration of the two drugs before and after mixing, avoiding any variation because of individual samples.

## RESULTS

During the study, no color change, precipitation or any other sign of physical incompatibility was seen. The spectrophotometric determination was given below.

***Determination of UV spectrum (Zero order spectra)***

The zero order (D0) spectra of CFX, MTZ and their mixture were recorded and presented in fig. 2. The absorption spectrum of CFX (5ppm) in neutral, acidic and basic solution showed two absorption bands; a strong band from 260-290 nm bands and a weak band at 310-350 nm with absorption maxima ($\lambda_{max}$) at 276 nm. The D0 spectra of neutral and basic solution of MTZ revealed a clear and strong absorption band at 287-355 nm with a $\lambda_{max}$ of 321 nm, whereas its spectrum in acidic environment showed a broad band ranging between 250-380 nm. Since it was evident that the spectra of both the two drugs overlapped in the region approximately 275-350 nm (especially in the case of acidic environment), making it difficult to measure CFX and MTZ through zero-order spectra simultaneously. Hence, the first (D1), second (D2), third (D3) and fourth (D4) derivative spectra of both the drugs were taken, recorded curves of derivatives for increasing concentrations of CFX, MTZ and their mixture in neutral solution were presented in Fig. 3.

***Derivative (D1 and D3) Spectra***

The D1 spectra of CFX in neutral solution at 0, 4 and 24 hours of the study was given in fig. 4. As per this, the D1 spectra of MTZ crossed zero at 266 nm while CFX displayed some absorbance at the positive side of the spectra. The recovery of 5ppm CFX neutral solution in mixture was then found to be 4.4840ppm (93.7552% ± 0.00227), 4.0723ppm (87.7041% ± 0.00104) and 4.740ppm (100.538% ± 0.000376) at 0, 4 and 24 hours of the study respectively. Likewise, the mixture having 5ppm neutral solution of MTZ was recovered as 3.35ppm (79.2415% ± 0.003548), 2.800ppm (78.226% ± 0.002379) and 3.877ppm (80.811% ± 0.001273) at CFX zero crossing points of 303 and 302 at 0, 4 and 24 hours respectively. In this way, simultaneous determination of CFX and MTZ was made possible using the first derivative technique.

In case of acidic environment, D3 spectra was selected, (Fig. 5). The 5ppm solution of CFX in a mixture was recovered as 5.052ppm (108.759% ± 0.00065), 4.825ppm (104.377 ± 0.000346) and 4.511ppm (106.553% ± 0.00033) at a zero-crossing point of 282nm at 0, 4 and 24 hours respectively. Similarly, at zero crossing points 304, 305 and 306nm, the recovery of 5ppm acidic solution of MTZ in a mixture was 5.159ppm (110.267% ± 0.000153), 4.761ppm (106.222% ± 0.000376) and 4.568ppm (101.351% 0.000145) at 0, 4 and 24 hours of the study respectively.

Likewise, the basic mixture having 5ppm CFX was measured at 267 and 266 nm (the zero-crossing point of MTZ with CFX absorbance at the positive side), the recovered solution was 4.845ppm (108.505% ± 0.00063), 4.526ppm (101.236% ± 0.00069) and 3.888ppm (100.90% ± 0.001217) at 0, 4 and 24 hours respectively. The 5ppm MTZ solution at zero crossing points of 297 and 298nm was measured as 4.505ppm (92.361% ± 0.000702), 4.545ppm (92.359% ± 0.0000944) and 4.308ppm (86.910% ± 0.00317) from the same mixture at 0, 4 and 24 hours of the study (Fig. 6).

***Analytical parameters***

The recovery of both the drugs in mixtures (in each medium) was obtained by plotting the absorbance (D1 and D3) values against respective concentrations and using subsequent regression equation ($y = a + bx$) (Table 1). It was observed that the linearity of 3-11ppm solutions of CFX and MTZ in neutral, acidic and basic mediums possessed a high degree of correlation coefficient $R^2 \approx 1$ (Table 1 and 2).

***Greenness assessment using analytical GREEnness (AGREE) software***

The graph of greenness assessment for *in-vitro* DDI determination between CFX and MTZ, as generated by AGREE software was shown in fig. 7. The cumulative score of 12 GAC principles was found to be 0.8 indicating that the used method aligned strongly with the green principles.

The AGREE metric system focused on twelve important practices of analytical method including preparation of the sample, the amount used for it, placement of analytical instrument, stepwise procedures, process automation, elimination of derivatization, analytic runs, wastes, energy consumption, hazardous materials and safety of users. Their overall scoring of these factors lied between 0-1. (Pena-Pereira *et al.*, 2020).

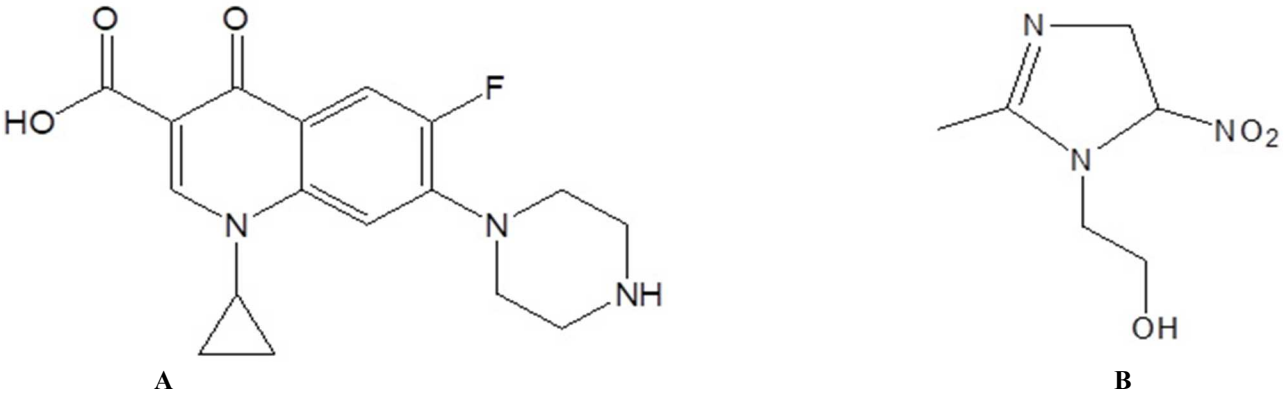



**Fig. 1**: Chemical structures of CFX (A) and MTZ (B).

**Table 1**: Determination of recovered CFX in mixture having MTZ using derivative spectroscopy

| | Neutral medium | | | Acidic medium | | | Basic medium | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 min. | 4 hrs. | 24 hrs. | 0 min. | 4 hrs. | 24 hrs. | 0 min. | 4 hrs. | 24 hrs. |
| λmax (nm) | 266 | 266 | 266 | 282 | 282 | 282 | 267 | 266 | 266 |
| Regression equation | y=0.0046x + 0.0035 | y= 0.0046x + 0.0035 | y = 0.0045x + 0.0035 | y = -0.0038x +0.0043 | y = -0.004x + 0.0038 | y= -0.0043x + 0.0031 | y = 0.0042x + 0.0007 | y = 0.0045x + 0.0015 | y = 0.0044x + 0.0027 |
| R2 | 0.9962 | 0.9926 | 0.992 | 0.9842 | 0.9799 | 0.971 | 0.97618 | 0.9746 | 0.968 |
| Recovery (ppm) | 4.4840 | 4.0723 | 4.740 | 5.052 | 4.825 | 4.511 | 4.845 | 4.526 | 4.477 |
| *Recovery (%) | 93.7554 ± 0.00227 | 87.704 ± 0.001041 | 100.538 ± 0.000376 | 108.759 ± 0.00065 | 104.377 ± 0.000346 | 106.535 ± 0.00033 | 108.505 ± 0.00063 | 101.236 ± 0.00069 | 100.900 ± 0.001217 |
| p-value | 0.4074 | 0.0932 | 0.90030 | 0.3743 | 0.7735 | 0.7220 | 0.4127 | 0.9174 | 0.9602 |
| %RSD | 1.65 | 0.81 | 2.62 | 2.61 | 2.60 | 2.49 | 2.25 | 2.45 | 1.94 |

*Values were presented as mean ± SEM

**Table 2**: Determination of recovered MTZ in mixture having CFX using derivative spectroscopy

| | Neutral medium | | | Acidic medium | | | Basic medium | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 min. | 4 hrs. | 24 hrs. | 0 min. | 4 hrs. | 24 hrs. | 0 min. | 4 hrs. | 24 hrs. |
| λmax (nm) | 303 | 303 | 302 | 304 | 305 | 306 | 297 | 298 | 298 |
| Regression equation | y= 0.0084x +0.0074 | y= 0.0085x +0.0074 | y= 0.0086x +0.0075 | y=-0.0022 x-0.001 | y=-0.0021x-0.0013 | y=-0.0022x-0.0012 | y= 0.0089x-0.0008 | y= 0.0094x-0.0026 | y= 0.0089x-0.0015 |
| R2 | 0.9976 | 0.998 | 0.9977 | 0.9799 | 0.9874 | 0.9808 | 0.9894 | 0.9931 | 0.9908 |
| *Recovery (ppm) | 3.35 | 3.800 | 3.877 | 5.159 | 4.761 | 4.568 | 4.505 | 4.545 | 4.308 |
| *Recovery (%) | 79.2415 ± 0.003548 | 78.226 ± 0.002379 | 80.811 ± 0.001273 | 110.267 ± 0.000153 | 106.22 2 ± 0.000376 | 101.351 ± 0.000145 | 92.361 ± 0.000702 | 92.359 ± 0.0000944 | 86.910 ± 0.00317 |
| p-value | 0.01835 | 0.1443 | 0.2087 | 0.6298 | 0.5791 | 0.9295 | 0.3294 | 0.2572 | 0.2572 |
| %RSD | 1.53 | 1.03 | 2.54 | 2.15 | 2.54 | 2.24 | 2.07 | 0.89 | 1.46 |

*Values were presented as mean ± SEM
p-value <0.05 indicates significant data

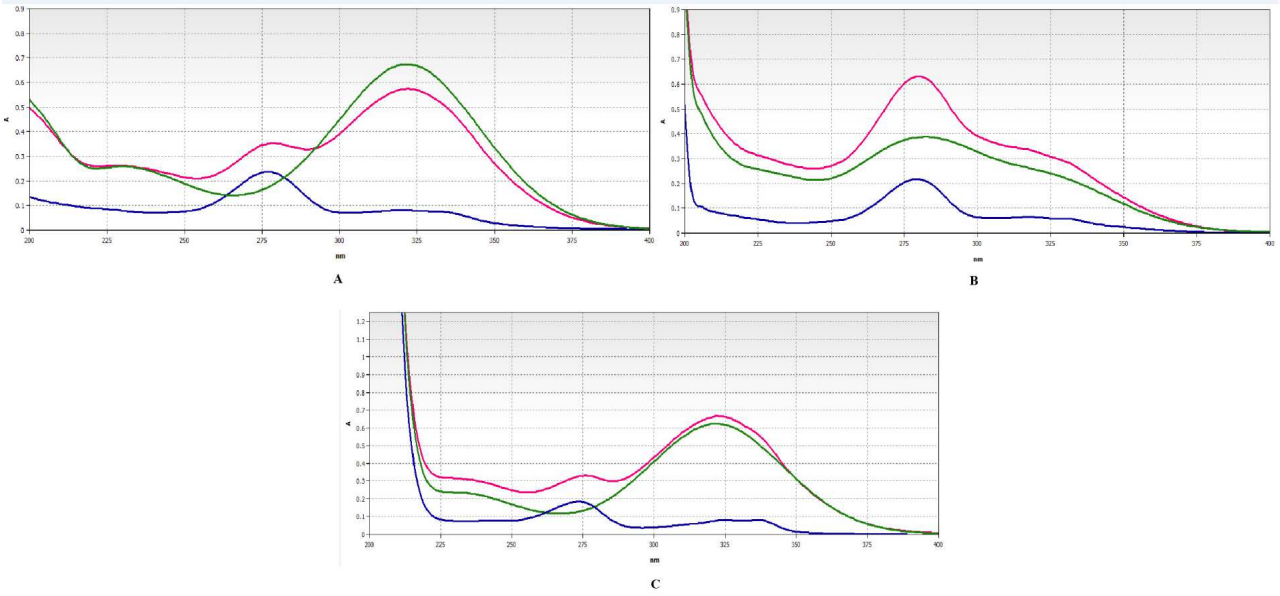



**Fig. 2**: Zero order spectra of in CFX (5ppm) in blue, MTZ (ppm) in green and their combination in pink in neutral medium (A) in acidic medium (B) in basic medium (C)

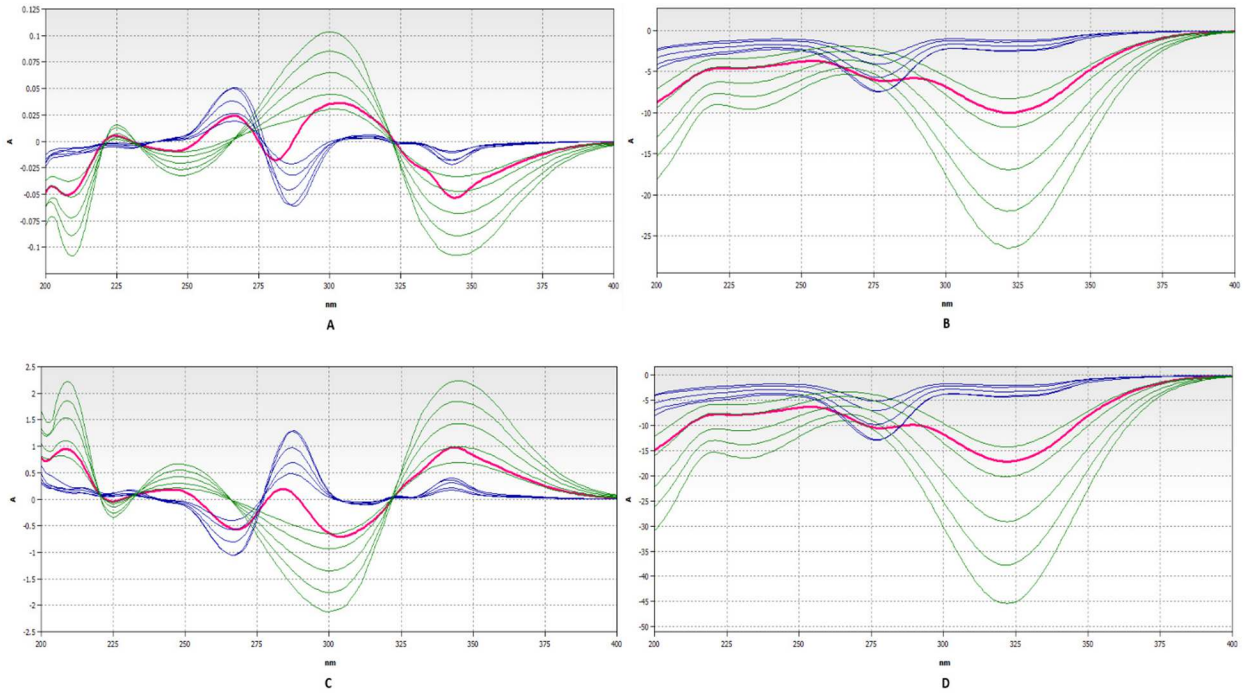



**Fig. 3**: Derivative Spectra of CFX (3-11ppm) in blue, MTZ (3-11ppm) in green and their combination in pink in neutral medium First derivative D1 (A), Second derivative D2 (B), Third derivative D3 (C) and Fourth derivative D4 (D).

## DISCUSSION

*In-vitro* drug interactions are often referred to as those taking place outside the body and include i) drug interactions due to compatibility issues up on co-administration ii) drug interaction with the packaging material iii) drug loss due to laboratory handling and iv) change in drug bioavailability due to formulation alterations (McElnay and Hughes, 2012). *In-vitro* drug incompatibilities have been classified as physical and chemical reactions, physical incompatibilities manifest through visible changes for e.g., discoloration, precipitation or turbidity. Chemical incompatibilities, on the other hand, referred to molecular transformation and became significant when greater than 10% of either of the drug's concentration degraded.

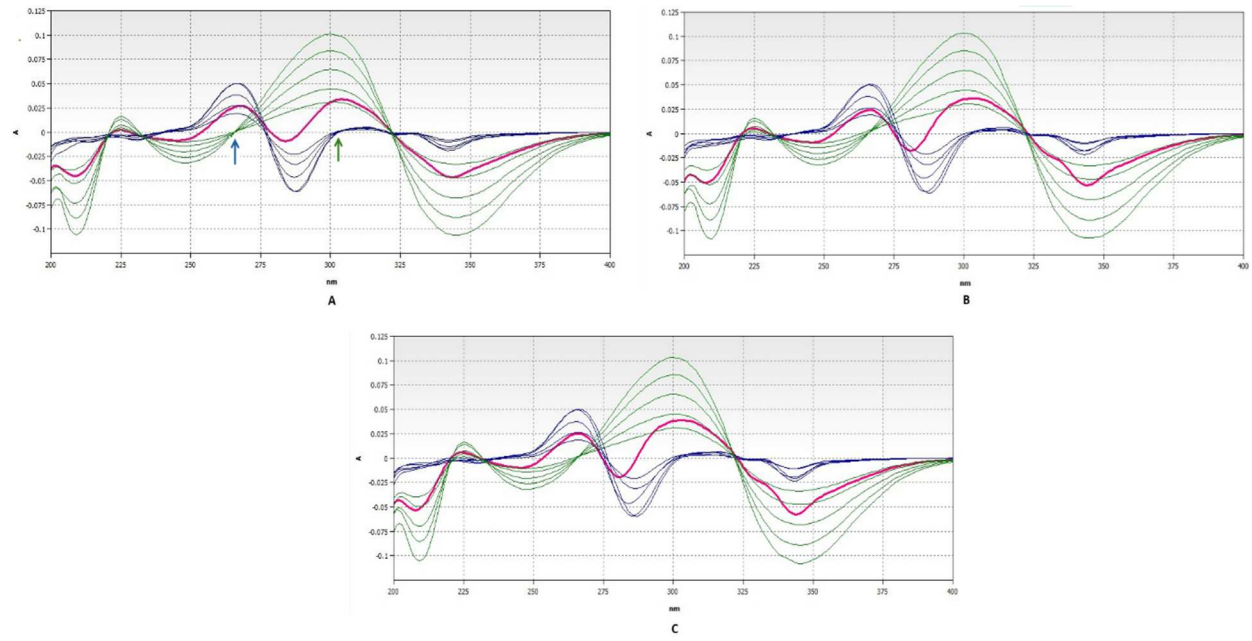


**Fig. 4**: D1 spectra of CFX (3-11ppm) in blue, MTZ (3-11ppm) in green and their combination in pink in neutral medium at 0 (A) 4 (B) and 24 hours (C). The blue arrow highlights zero crossing point for CFX and the green arrow highlights zero-crossing point for MTZ

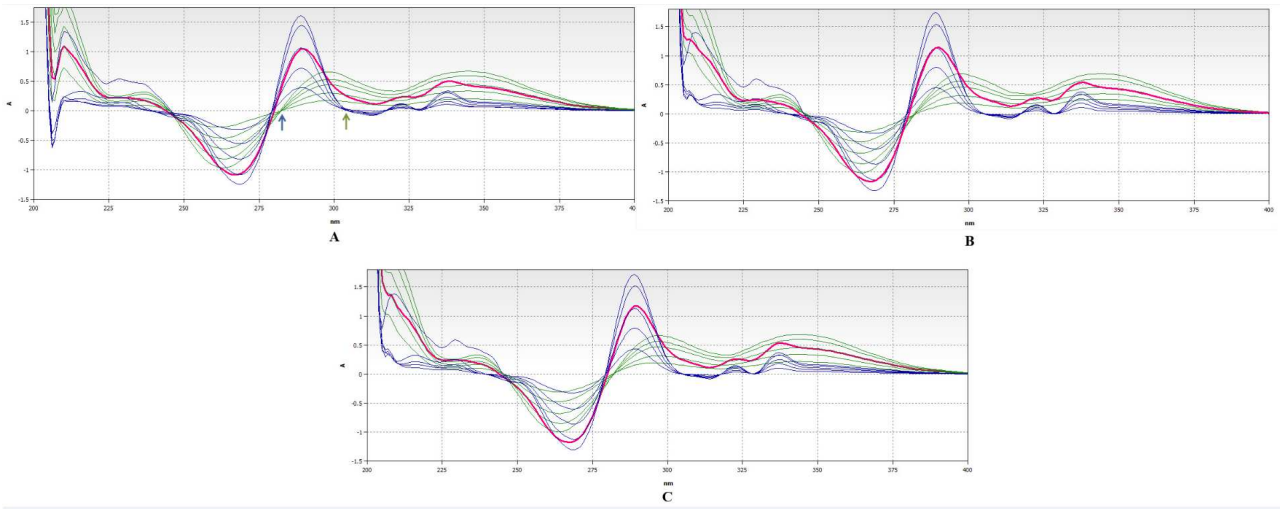


**Fig. 5**: D3 spectra of CFX (3-11ppm) in blue, MTZ (3-11ppm) in green and their combination in pink in acidic medium at 0 (A) 4 (B) and 24 hours (C). The blue arrow highlights zero crossing point for CFX and the green arrow highlights zero-crossing point for MTZ.

These interactions can result in diminished or even complete loss of therapeutic activity through formation of new, may be non-toxic or toxic species. In past studies, it was reported that two drugs can be considered "chemically compatible", if 90% of the drug remained unchanged (bioavailable) when simultaneously administered (Mohamed *et al.*, 2020; Tomczak *et al.*, 2021).

Therefore, the study aimed at exploring the *in-vitro* DDI between CFX and MTZ using spectrophotometry. CFX and MTZ are co-administered to treat mixed aerobic/anaerobic infections, raising the concerns of poly-pharmacy. Much work has already been done to determine the interaction of the two drugs using spectroscopic studies (Attia *et al.*, 2016; Jogdand *et al.*, 2022; Abdelwahab and Farid, 2023; Sebaiy *et al.*, 2023).

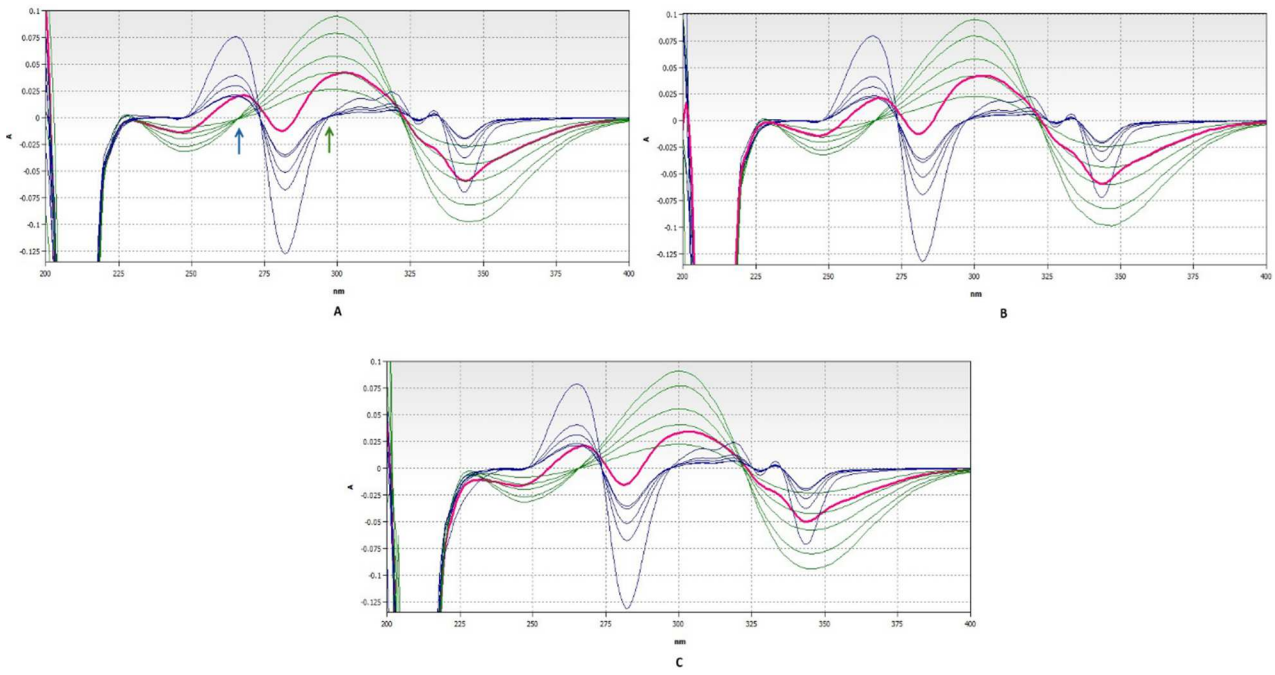
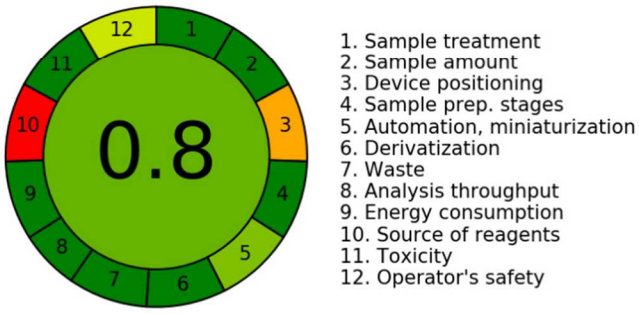



**Fig. 6**: D1 spectra of CFX (3-11ppm) in blue, MTZ (3-11ppm) in green and their combination in pink in basic medium at 0 (A) 4 (B) and 24 hours (C). The blue arrow highlights zero crossing point for CFX and the green arrow highlights zero-crossing point for MTZ




| Criteria | Score | Weight |
|---|---|---|
| 1. Direct analytical techniques should be applied to avoid sample treatment. | 1.0 | 4 |
| 2. Minimal sample size and minimal number of samples are goals. | 1.0 | 4 |
| 3. If possible, measurements should be performed in situ. | 0.33 | 4 |
| 4. Integration of analytical processes and operations saves energy and reduces the use of reagents. | 1.0 | 4 |
| 5. Automated and miniaturized methods should be selected. | 0.75 | 4 |
| 6. Derivatization should be avoided. | 1.0 | 4 |
| 7. Generation of a large volume of analytical waste should be avoided, and proper management of analytical waste should be provided. | 1.0 | 4 |
| 8. Multi-analyte or multi-parameter methods are preferred versus methods using one analyte at a time. | 1.0 | 3 |
| 9. The use of energy should be minimized. | 1.0 | 4 |
| 10. Reagents obtained from renewable sources should be preferred. | 0.0 | 4 |
| 11. Toxic reagents should be eliminated or replaced. | 1.0 | 4 |
| 12. Operator's safety should be increased. | 0.6 | 4 |

**Fig. 7**: Greenness assessment by AGREE software calculator for simultaneous determination of CFX and MTZ in mixtures in neutral, acidic and basic environments. (1= remote sending without sample damage, 2 = 0.005, 3 = at-line, 4 = 3 or fewer, 5 = semi-automatic & miniaturized, 6 = none, 7 = 0.002, 8 = 1 & 30, 9 = UV Vis and 0.03 (0.5hrs * 0.6KW), 10 = none of the bio-based, 11 = no, 12 = toxic to aquatic life, corrosive)

Spectrophotometric approaches, in most cases, served as the best alternate to advanced chromatographic methods offering additional benefits of being easy to use, cost-effective, simple, efficient and superior greenness scores (Abdulwahab *et al.*, 2021; Vaikosen *et al.*, 2023; Akbel, 2024; Katamesh *et al.*, 2024).

When the D0 spectra of neutral and basic mixtures having both active ingredients were scanned, it was observed that the MTZ did not show much interference with the strong band of CFX, permitting it to be quantified in presence of MTZ. Conversely, the D0 spectra of MTZ possessed noteworthy overlapping with the weaker band of CFX, imposing constraints in analyzing MTZ in combination with CFX. The presence of both drugs in acidic solution further intensified the problem. Derivative spectroscopy effectively addressed this problem where overlapping signals can not only be separated by derivatising the D0 spectra but also helped in removing unwanted noise in the sample. Hence for each CFX, MTZ and their mixtures in neutral, acidic and basic solutions, first, second, third and fourth orders were taken for selection of the suitable derivative spectra. The derivative spectra showing positive absorbance values for CFX and MTZ at their respective zero-crossing points were selected for simultaneous determination. For this reason, D1 spectra for neutral and basic medium while D3 spectra for acidic medium was selected for further DDI analysis at 0, 4 and 24 hour of study.

In neutral mixture, in the presence of MTZ, the recovery of CFX was reduced from the start till 4 hours and then a subsequent increase was observed at 24 hours of the study. Surprisingly in combination with CFX, a same pattern was seen in case of MTZ i.e., an initial reduction that continued till 4 hours after which it was increased by 24 hours. Since the mixture had less than 90% of both CFX and MTZ at 4hrs in neutral medium, one could say that the two drugs might be incompatible upon simultaneous administration. It was interesting to observe that the acidic mixture of CFX containing MTZ showed a gradual improvement in the recovery over 24 hours. The presence of CFX in the same environment would appear to progressively reduce the recovery of MTZ during 24 hours. If we talk about the basic medium in which CFX was present along with MTZ, an initial decline in its recovery was observed up to 4 hours followed by minimal change at 24 hours. Whereas MTZ demonstrated steady recovery till 4 hours afterward a noticeable reduction was observed by 24 hours. At this point, in basic mediums, CFX was found incompatible with MTZ, prompting concerns over DDIs.

This non-linear response of incompatibility likely suggests a transient physicochemical interaction (rather than a true degradation) due to an initial ion-ion pairing or hydrogen-bond interaction between CFX carboxylate/piperazinyl group and MTZ nitroimidazole moiety. These effects persist briefly and resolve over time, with equilibration restoring CFX and/or MTZ signals again during the study. In addition, amplitude measurement of peak-to-baseline in D1and D3 spectra showed excellent proportionality to the concentrations in the mixture.

In order to promote sustainable practices, the assessment of any chemical process on the environment is deemed crucial and follows several techniques. Green Analytical Chemistry, in this regard, presents noteworthy aptitude in minimizing the harmful impression of analytical processes on environment and ultimate users (Vaikosen *et al.*, 2023). This tool offers methodological approach to designing and implementing greener as well as eco-friendly analytical methods through regular investigation of chemical usage, energy consumption, potential hazards and waste generation (Mohyeldin *et al.*, 2023; Shi *et al.*, 2023; Meher and Zarouri, 2025). In modern era, use of software and spreadsheets-based computational tools for greenness evaluation has gained much attention due to their simplicity, availability, precision and ease of comparison. A range of software solutions has been developed to estimate the sustainability of analytical methods including NEMI, ComplexGAPI, AGREE, AGREEprep, RGB 12 Alogrithm and ChlorTox Scale (Yin *et al.*, 2024; Semysim *et al.*, 2025).

Among them, AGREE, introduced in 2020, remains one of the valuable tool in recent research (Moema *et al.*, 2023; Mohyeldin *et al.*, 2023). This software used a red-yellow-green color scale to illustrate the performance across 12 GAC principles in visual form indicating their strengths and weaknesses. It was said that an environmentally favorable analytical method typically achieves a numerical score above 0.6 (Abdelgawad *et al.*, 2022; Moema *et al.*, 2023). Since our method yielded a score of 0.8 (green) for estimating *in-vitro* DDI between CFX and MTZ using first order derivative spectroscopy, we could rank it as eco-friendly. The method employed an at-line approach, where the drugs solutions of both CFX and MTZ, were prepared manually for analysis and then transported to the instrumental area. In addition, the solution preparation was started with a very few amount of the drug (0.005g), using distilled water as a solvent and involved simple steps such as mixing and filtration without any other treatment. Likewise, the instrument used for studying DDI was UV-Vis Spectrophotometer that's why the total energy consumption would be expected to be less than 0.1kWh. Moreover, in order to achieve acid and basic mediums, HCl and NaOH were used which were corrosive and toxic to aquatic life; none of the chemicals were form bio-based origin. This highlighted the need of designing and implementing further greener approaches in these areas.

In comparison to conventional chromatographic techniques, the derivative spectrophotometric method utilizing aqueous medium served as a greener alternative by eliminating hazardous organic solvents, reducing waste and maintaining adequate sensitivity within the acceptable quantitative limits. Though the chromatographic methods

such as and UPLC and RP-HPLC remained selective and provide lower detection limits, these are highly dependent on the use of organic mobile phases, which ultimately account for their lower greenness scores (≤ 0.75) (Ahmed-Anwar *et al.*, 2023; Kannaiah *et al.*, 2025). On the other hand, spectrophotometry typically achieves values >0.70–0.75 (Alqahtani *et al.*, 2024), especially in our case the use of pure water at 5ppm further minimizes environmental impact while retaining clinically relevant sensitivity.

In future, different test such as HPLCA/LC-MS could be performed for exact quantification, followed by pH-dependence, dilution and salt addition tests to examine any reversible complexation or ion-pairing mechanism. A forced acid/base hydrolysis, photolysis and oxidative stress would also be considered to determine and distinguish degradation pathways

## CONCLUSION

The proposed spectrophotometric approach enables precise and interference-free wavelength selection for simultaneous determination of CFX and MT, offering accurate, precise and cost-effective routine tool as an alternate to more resource –intensive chromatographic technique. A 24 hours *in-vitro* interaction study using pharmaceutical preparations of ciprofloxacin and metronidazole indicated drug incompatibility between them in neutral medium. Furthermore the increase or decrease in drug's availability when they are present simultaneously underlined the clinical relevance of spectroscopically detected incompatibilities and raised concerns about potential DDI, which may result in reduced efficacy or therapeutic failure. In addition, an AGREE score of 0.8 also validated strongly with 12 principles of green analytical chemistry.

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