



Analytical study of amoxicillin and metronidazole co-loaded pharmacosomes for *H. pylori* eradication using DoE-based modeling of an HPLC-PDA technique

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ABSTRACT

Treating *Helicobacter pylori* (*H. pylori*) infection is challenging for the doctor due to the peculiar pathophysiology of bacteria that leads to antibiotic resistance. Pharmacosome is a novel formulation that could be a promise in treating the infection because of its ability to improve the bioavailability of antibiotics. The objective of this study is to develop and validate an high performance liquid chromatography (HPLC) -based analytical technique to evaluate the stability, % entrapment efficiency (% EE), and the drug release of amoxicillin (AMX) and metronidazole (MEZ) co-loaded pharmacosomes. To illustrate the effect of this method clearly, the design of the experiment tool was used to model the HPLC method. The independent variables were buffer ratio, pH, flow rate, and injection volume. The responses were retention time, drug peak area, and resolution between the drugs. The optimized method was validated as per ICH Q2(R2) guidelines. The linearity was plotted in the 0.5–20 µg/ml range for both AMX and MEZ. The linear regression equation showed an R^2 of more than 0.999, proving the linearity of the method. The method was used to check the stability, % EE, and % drug release of antibiotics in the pharmacosomes. The developed method's accuracy was between 99%–102% for both AMX and MEZ. The inter-day and intra-day precision of the developed method was within the limit for both AMX and MEZ, the %CV was less than 2%. The HPLC method was selective, sensitive, linear, and eco-friendly, with an Analytical GREENness Metric Approach and Software score of 0.66.

INTRODUCTION

Helicobacter pylori (*H. pylori*) is a Gram-negative pathogenic bacterium that grows in gastric mucosa causing gastric ulcers. Globally, 50% of the population has been infected with the bacteria [1]. *H. pylori* causes atrophy and chronic gastritis resulting in inflammation and ulceration of stomach

linings. Symptoms of the infection include abdominal pain and loss of hunger, and may also lead to lethal complications like bleeding in the stomach [2]. If the inflammation persists, it may also result in gastric cancer [3–6]. Immune thrombocytopenic purpura, iron deficiency anemia, and vitamin B12 insufficiency are also the other disease conditions of the infection [7,8]. The infection is transmitted orally, mainly by consumption of contaminated food products and water [9]. The infection may be asymptomatic in many infected individuals, yet they can be potential carriers for disease transmission.

Most bacteria entering the stomach will not survive its harsh acidic conditions. However, the peculiar pathophysiology in *H. pylori* enables the organism to survive in the acidic conditions of the gastric mucosa. The organism contains a

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special enzyme, urease, which hydrolyses urea in its vicinity to ammonium hydroxide, responsible for neutralizing the hydrochloric acid present in the stomach, creating a less acidic microenvironment for the bacteria to survive. *H. pylori* then swim into the inner layer of the gastric mucosa with the help of flagella and adheres to the mucosal surface with adhesins, resulting in bacterial colonization [10–12].

The current treatment strategy for eradicating *H. pylori* consists of a combination of antibiotics and a proton pump inhibitor. This includes first-line, second-line, and quadrupole therapies with various combinations of antibiotics. However, the special pathophysiology of the organism and ability to form coccoid helps the organism survive the treatment and develop resistance against antibiotics, preventing the eradication impossible [13–15]. Antibiotic resistance is caused by chromosomal mutation and physiological changes such as drug uptake, efflux mechanism, and biofilm formation, directly affecting the drug's effectiveness in the infection. Antibiotics generally used for treating *H. pylori* are amoxicillin (AMX), metronidazole (MEZ), clarithromycin (CLR), tetracycline (TET), and levofloxacin (LEV). Earlier researchers have studied the resistance of these antibiotics. The primary resistance rates to *H. pylori* for AMX, CLR, MET, LEV, and TET were reported to be 15.0%, 34.1%–55.2%, 69.4%–71.3%, 18.4%–27.9%, and 17.9%, respectively [8]. The medication's failure to reach the target site at the necessary concentration levels is the cause of this lack of effectiveness. Recent literature showed that resistance patterns vary based on the geographical area, age, gender, and prior use of the proton pump inhibitors.

AMX is a frequently used antibiotic due to its ability to re-secrete back to the gastric lumen, resulting in good bioavailability at the site where the bacteria resides [16,17]. Since *H. pylori* can survive in low-oxygen environments, doctors often prescribe antibiotics that are effective against anaerobic bacteria like MEZ in combination with AMX to treat *H. pylori* infection. AMX has bactericidal abilities and prevents the production of cell walls, whereas MEZ is used to treat infections produced by anaerobic bacteria. Both drugs have excellent tissue penetrability and good tolerability in the body [18–20]. The combination is considered to be an excellent choice for treating *H. pylori* infection [21,22]. The structure of AMX and MEZ is shown in Figure 1. The AMX and MEZ both are stable at gastric pH. Maintaining the antibiotic concentration

at the site of *H. pylori* infection is important. The bioavailability of AMX and MEZ is 60%–70% and 90%, respectively [23].

Pharmacosomes are lipid-conjugated vesicular structures that exist as hexagonal nano-sized micelles. These are used as drug carriers. Drug molecules with free hydroxyl, carboxyl, or amino functionality are converted to an ester, resulting in the formation of a pharmacosome. Pharmacological agents connect to lipids by electrostatic, covalent, or hydrogen bonding interactions. The complex possesses both lipophilic and hydrophilic properties, increasing the surface area of contact and thus the bioavailability [24–26]. Pharmacosomes also help in targeting the drugs to the site of infection [27–31]. As a part of our continuous research to find possible ways to eradicate *H. pylori*, we have prepared pharmacosomes containing AMX and MEZ.

To assess the stability, drug loading, % entrapment efficiency (% EE), and drug release profile of the antibiotics from the pharmacosomes, a validated analytical technique is required to quantify the selected antibiotics (e.g., AMX and MEZ) from the pharmacosomes. In this study, we have developed and validated a sensitive and accurate Reverse Phase-High Performance Liquid chromatography (RP-HPLC)-based analytical method employing modeling software such as design of experiment (DoE) [32–33]. DoE is a robust modeling approach used to predict chromatographic conditions that give the highest efficiency with fewer trials. It thereby also reduces the time and cost involved in the HPLC method development. Reducing the number of trials also saves solvent usage, thereby promoting the sustainability of the environment [34,35]. The consumption of organic solvents and other chemicals frequently employed in research labs is reduced in proportion to the number of experiments. This reduces the environmental effect of pharmaceutical research and development and is consistent with sustainable practice [36]. DoE helps researchers efficiently explore the relationships between multiple variables and their analytical responses [37,38]. The traditional HPLC method development strategy uses one factor at a time (OFAT) trials, which is time-consuming and expensive [39,40]. DoE helps reduce the number of trial runs and improves understanding of how these variables influence the overall method performance.

MATERIALS AND METHODS

Materials

A free sample of AMX (off-white powder; purity > 98%) was received from Sun Pharma, Gurgaon, India. MEZ

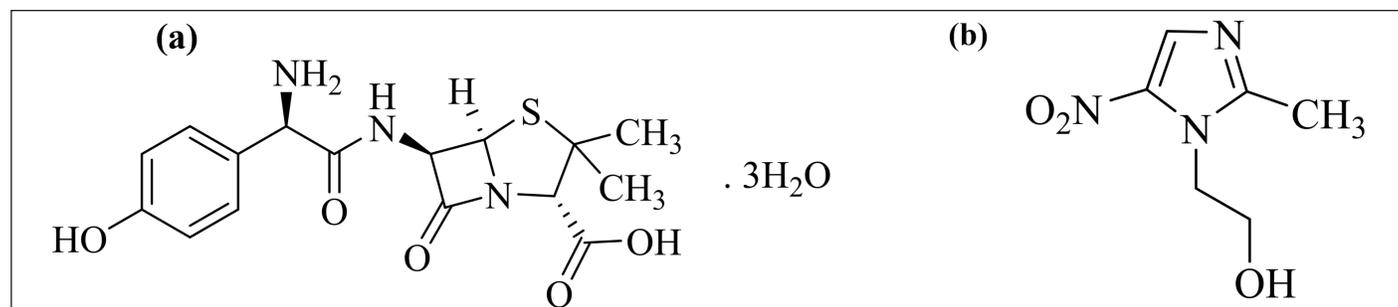


Figure 1. Chemical structure of (a) AMX and (b) MEZ.

(>98% purity) was purchased from Combi Blocks (CA, United States of America). Orthophosphoric acid (88%), HPLC grade acetonitrile, methanol, and hydrochloric acid (35%) were procured from Merck India Ltd, Mumbai, India. Milli-Q (purified water) was produced on-site using the instrument Direct Q3-UV system (Merck India Ltd, Mumbai, India). The membrane filter (0.45 μm) was procured from Merck India, Ltd, Mumbai, and the filtration assembly was procured from Riviera Glass Pvt. Ltd., Mumbai, India. The column of the stationary phase Luna octadecylsilane (ODS) C18 (250 \times 4.6 mm, 5 μm particle size) was acquired from Phenomenex, Hyderabad, India. Dichloromethane (DCM) (>98% purity) was procured from Molychem, Mumbai, India. Soya lecithin (30%) a phosphatidylcholine was procured from Himedia Laboratories Pvt. Ltd., Thane, India.

Instrumentation

A Shimadzu HPLC system with an LC20-AD pump, SIL20-AC HT autosampler, CTO-10 ASVP column oven, and SPD-20A & SPD-M10A detectors with the LabSolution software was used for the optimization and validation of chromatographic methods. A calibrated Sartorius Mechatronics CP225D analytical balance (India) was used to precisely weigh the compounds used in the manufacture of standard solutions and buffers. A glass vacuum filtering assembly (Merck Millipore, Bangalore, India) was used to filter the mobile phase solution via a 0.22 μm membrane filter. The mobile phase solution was then degassed in an ultrasonic bath (GT Sonic, Guangdong GT Ultrasonic Co. Ltd, China). As needed, the pH of the mobile phase was measured and adjusted using a calibrated digital pH meter (Model: LI 617, ELICO, Telangana, India). To guarantee accuracy and precision in sample handling, calibrated variable micropipettes with capacities ranging from 0.2 to 10 μl , 10 to 100 μl , and 100 to 1,000 μl (Eppendorf, Germany) were used for various pipetting phases during sample processing.

Preparation of mobile phase and standard solution

The mobile phase combines 80% of 10 mM potassium dihydrogen phosphate buffer and 20% v/v methanol. The pH of the buffer was adjusted to 6.0 using orthophosphoric acid. The buffer was sonicated and passed through a 0.2 μm membrane filter after pH adjustment.

Primary stock solutions (1 mg/ml) of AMX and MEZ were prepared by dissolving 10 mg of pure standards in 10 ml of Milli Q water. Working standard solutions (100 $\mu\text{g/ml}$) of AMX and MEZ were prepared by dilution of the primary stock solution. Calibration solutions in the 0.5 to 20 $\mu\text{g/ml}$ range were prepared from the working standard solution.

DoE-aided modeling of the HPLC method

DoE is the modeling tool that helps to understand the effect of variables and the interactions that can affect the responses. To clearly illustrate the effect of this method DoE tool was employed to optimize the effects of various processing variables on the chromatographic method such as retention time (t_r), peak area, and resolution. The independent parameters included the mobile phase ratio, buffer pH, flow rate, and injection volume. Preliminary trials with OFAT were conducted to find the variables that affect the drug's separation. Box-Behnken design (BBD) and central composite design (CCD) are

the designs used for the optimization [41,42]. The BBD has the advantage that it requires less runs compared to the CCD. The BBD is frequently chosen over the CCD because of its efficiency and practicality. BBD usually necessitates fewer experimental runs, saving money while yielding reliable data for optimization. All experimental circumstances are safe and feasible since BBD stays away from extreme combinations of factor levels present in CCD. Better quadratic impact estimation is made possible by this design's emphasis on mid-level parameters, which eliminates the potential complexities that come with extreme values. Furthermore, BBD is especially helpful for well-informed procedures where the center of the design space is anticipated to be optimal. Regarding experimental design, BBD is useful since it provides a simplified method that strikes a compromise between thoroughness and resource efficiency. In the BBD model, we selected four chromatographic parameters as the independent variables for the five responses. The Design Expert[®], version 9.0.1. (Stat-Ease Inc, USA) was used to model the HPLC method. The input parameters in their lower and higher ranges were % aqueous (buffer) phase (A) ranging from 75% to 95%; buffer pH (B) ranging from 6.0 to 6.8; mobile phase flow rate (C) ranging from 0.8 to 1 ml/min; and injection volume (D) ranging from 10 to 20 μl . Based on the input, DoE suggested 29 trial conditions for the model development. We performed trials in HPLC with the recommended parameters and the responses obtained are provided in Table 1. The responses were the t_r of AMX (Y1), t_r of MEZ (Y2), peak area of AMX (Y3), peak area of MEZ (Y4), and resolution (Y5). The response was analyzed using ANOVA and the p value, determination coefficient (R^2), adjusted determination coefficient (adj R^2), and predicted determination coefficient (pred R^2) were analyzed. Surface analysis was carried out generating 3D-response surface graphs and perturbation plots. The 3D plot and perturbation plots provided information on the relationship between the variables and the responses. The model predicted 100 solutions with desirability (Supplementary Table S2), with parameters with the best desirability selected for the final chromatographic trial.

Method validation

The DoE-modeled method was validated as per the ICH Q2 (R^2) guidelines [43]. A system suitability study was performed by injecting six AMX and MEZ replicates at 1 mg/ml concentrations and determining the Tailing factor (TF), number of theoretical plate (N), and t_r . The method's selectivity was evaluated by checking the interference of blank solvent, matrix, and excipients with the drug's peak. The samples were injected in three replicates with blank diluent and analyzed for interferences at the t_r of AMX and MEZ.

The linearity of the developed method was evaluated by plotting a standard curve for the AMX and MEZ between the peak area and drug concentration. The linearity range for AMX and MEZ was from 0.5 to 20 $\mu\text{g/ml}$. The linearity samples were injected in three replicates. The accuracy of the HPLC method was checked at 3 different concentration levels (80%, 100%, and 120%). The inter and intraday precision CVs % of the method were evaluated. The robustness of the developed method was checked by changing the chromatographic parameters slightly such as pH of the buffer 6.0 \pm 0.2, temperature 25°C \pm 1°C,

flow rate 0.85 ± 0.05 ml/min, and injection volume 15 ± 1 μ l. The limit of detection (LOD) (Eq. 1) and limit of quantification (LOQ) (Eq. 2) of the developed method were determined from the slope and standard deviation of the responses. Here, σ is the standard deviation of the response and S is the slope of the calibration curve.

$$\text{LOD}=3.3 \sigma/s \quad (1)$$

$$\text{LOQ}=10 \sigma/s \quad (2)$$

Preparation and evaluation of AMX and MEZ co-loaded pharmacosomes

Preparation of pharmacosomes

AMX and MEZ-loaded pharmacosomes were prepared by solvent evaporation method [44,45]. The solvent fumigation

approach is frequently used because it may evenly distribute the lipid and drug components, encourage efficient complexation, and improve the final pharmacosomes stability. Controlled solvent evaporation made possible by this technique may result in increased drug loading and encapsulation effectiveness. Soya lecithin (80 mg) was weighed and dissolved in 60 ml DCM in a round bottom flask. AMX (50 mg) and MEZ (50 mg) were weighed, added to the above solution, and sonicated until complete dissolution. The solution was refluxed for 1 hour at an appropriate temperature of 37°C. The contents of the flask were evaporated at 35°C–40°C under vacuum in a rotary vacuum evaporator until the final traces of the solvent were removed. A vesicular suspension of pharmacosomes was created when the thin lipid layer that had developed at the bottom of the round-bottom flask was hydrated with 30 ml of phosphate buffer pH 7.4. The prepared pharmacosomes were lyophilized in a Labocon

Table 1. DoE recommended independent factors and their respected responses.

Runs	Trials suggested by the DoE model				Response obtained from the experimental trials				
	A	B	C	D	Y1	Y2	Y3	Y4	Y5
1	95	6.8	0.9	15	19.647	25.127	10.859	56.711	6.594
2	95	6.4	0.8	15	15.584	28.415	13.022	61.486	13.624
3	95	6.4	1	15	12.522	22.504	11.117	48.466	13.249
4	85	6.8	0.8	15	6.814	10.849	14.997	65.807	10.065
5	85	6	1	15	4.397	8.734	13.287	50.641	13.41
6	85	6.4	1	10	4.804	8.67	8.452	37.024	11.945
7	95	6.4	0.9	10	13.898	25.037	7.771	36.008	14.068
8	85	6.4	0.9	15	5.302	9.599	13.718	58.422	12.092
9	75	6.4	0.9	20	3.76	5.889	18.870	79.152	7.9
10	75	6.4	1	15	3.416	5.419	12.931	53.030	8.196
11	75	6.4	0.9	10	3.779	6.012	9.539	38.595	8.658
12	85	6.8	0.9	20	6.082	9.645	18.002	78.026	9.437
13	85	6.4	0.9	15	5.287	9.612	13.564	58.707	11.961
14	75	6.8	0.9	15	3.917	5.969	14.031	59.740	7.968
15	85	6	0.8	15	5.458	10.803	17.002	66.832	13.827
16	75	6	0.9	15	3.657	6.057	14.841	60.462	9.319
17	95	6	0.9	15	10.812	24.311	13.423	56.756	17.599
18	85	6	0.9	10	4.854	9.672	15.249	58.134	13.633
19	85	6.4	0.9	15	5.298	9.552	13.206	59.642	11.987
20	85	6.4	0.8	10	6.085	10.816	9.142	43.231	11.787
21	75	6.4	0.8	15	4.24	6.755	16.298	66.424	8.553
22	85	6.4	0.9	15	5.289	9.585	13.764	59.644	11.833
23	85	6.4	0.9	15	5.3	9.548	13.019	59.752	12.065
24	85	6.8	1	15	5.515	8.745	11.704	53.204	9.656
25	85	6.4	1	20	4.801	8.678	17.278	71.935	10.986
26	85	6.8	0.9	10	6.091	9.657	8.742	39.151	10.335
27	95	6.4	0.9	20	13.867	25.122	17.308	76.093	13.118
28	85	6	0.9	20	5.438	10.857	22.804	87.177	13.102
29	85	6.4	0.8	20	6.074	10.88	18.450	86.659	10.729

Note: – A indicates buffered phase, B indicates buffer pH, C indicates flow rate, D injection volume, Y1 indicates t_r of AMX, Y2 indicates t_r of MEZ, Y3 indicates peak area of AMX, Y4 indicates peak area of MEZ, and Y5 indicates resolution.

LFD-BT-101 Bench-top freeze dryer and stored in a desiccator for further studies.

Assessment of % EE of pharmacosomes

The % EE of the prepared pharmacosome formulation containing AMX and MEZ was assessed utilizing the validated DoE-modeled reverse phase HPLC technique. Centrifuged the vesicular suspension of pharmacosome suspended in 1 ml water at 12,000 rpm for 30 minutes and subjected the supernatant for HPLC analysis to obtain the amount of unentrapped drug (free drug). Methanol was added to the above solution to break down the lipid layer of pharmacosomes and determine the total amount of drugs present. The EE % will be evaluated by the following equation:

$$\% EE = \frac{\text{Total amount of drug} - \text{Free amount of drug}}{\text{Total amount of drug}} \times 100 \quad (3)$$

Drug release study

The release behavior of the developed pharmacosomes was evaluated using a USP type 2 dissolution apparatus. Dissolution of AMX and MEZ from the prepared pharmacosomes at pH 1.2 and 7.4 was evaluated at a temperature of 37°C. The pH values of 1.2 and 7.4 were selected for our study because *H. pylori* bacteria reside in the stomach, and it is essential for pharmacosomes to effectively release the drug in the gastrointestinal tract to ensure complete eradication of the infection. The acidic pH of 1.2 simulates

the gastric environment, while pH 7.4 represents the more neutral conditions found in the intestines. We aim to optimize the formulation for targeted delivery and enhanced therapeutic efficacy against *H. pylori* infections by evaluating drug release at these specific pH levels. At predetermined time intervals, 2 ml of sample was withdrawn and analyzed for AMX and MEZ using the validated HPLC method. Taken at different time points. After the collection of samples, 2 ml of fresh media was added to the formulation's dissolution unit to maintain the sink condition [46,47].

Forced degradation studies

The stress-induced degradation study aims to assess a drug's stability in stressful conditions of formulation development. Acidic, alkaline, oxidative, thermal, and photolytic stresses were applied to the analytes. Forced degradation studies were conducted on pharmacosomes containing 1 mg/ml of AMX and MEZ. The DoE-modeled validated HPLC method was utilized to assess the drug concentration in the degradation samples. The assay was done in triplicate, and the percentage of deterioration was determined [20,48].

Acid hydrolysis of AMX and MEZ was evaluated at two different molar concentrations of HCl (0.1 N and 1 N). Degradation studies in an alkaline medium were checked and evaluated at two NaOH molar concentrations (0.1 N and 1 N). The oxidative stress-induced degradation study was evaluated using a 3% w/v H₂O₂ solution. The photolytic stability study was checked by exposing the prepared pharmacosomes to UV light for 24 hours. Thermal degradation study was evaluated in the oven at a temperature of 60°C for 24 hours [17,49].

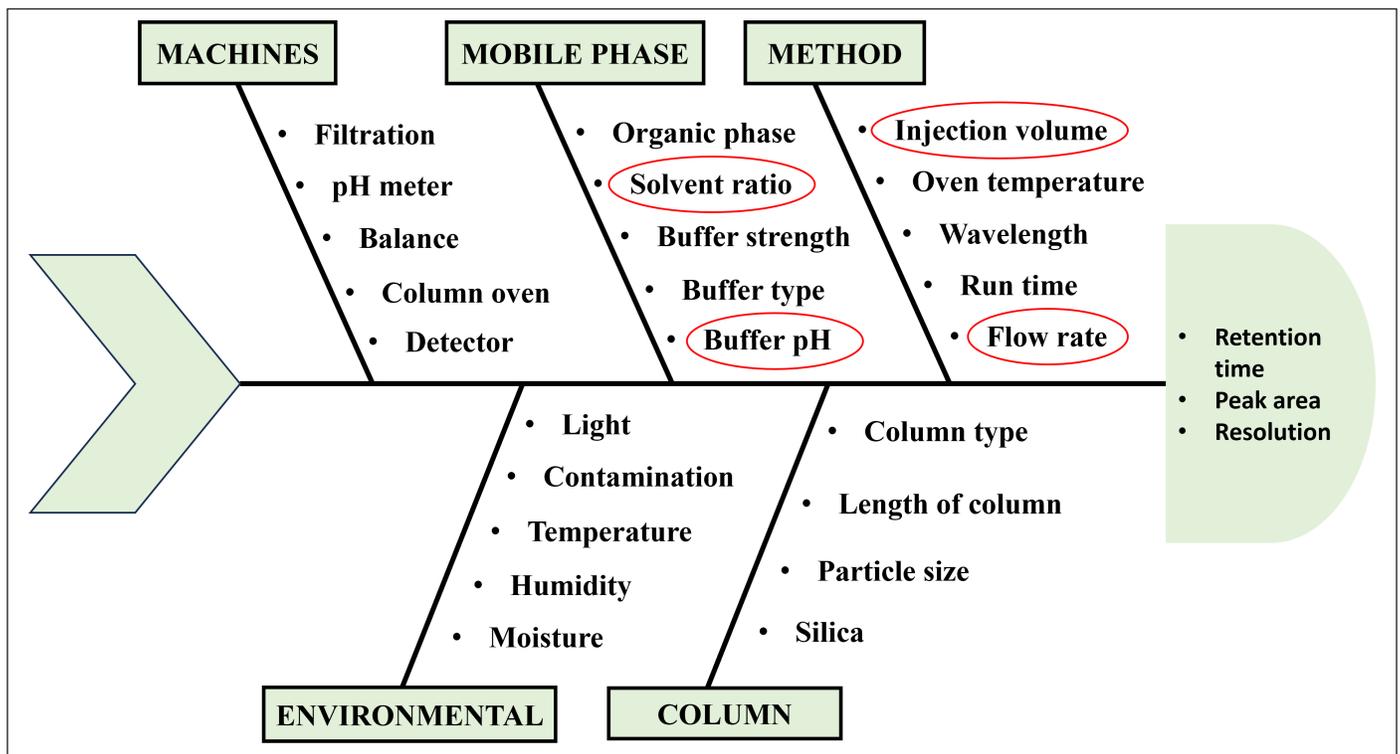


Figure 2. Ishikawa fishbone diagram for the identification of possible risk factors.

Greenness of the developed method

The greenness of the analytical technique was evaluated utilizing the AGREE software (Analytical GREENness Metric Approach and Software) [50]. This technique is designed to check the effect of the analytical method on the environment. This technique considers 12 factors, each given a score between 0 and 1, with higher numbers denoting a greener method.

RESULTS AND DISCUSSION

DoE-based modeling of the analytical method reduces the chances of trial failure and helps optimize chromatographic conditions, resulting in efficient separation of analytes. A photodiode array (PDA) detector was chosen for the separation considering the excellent absorption of AMX and MEZ at 228 and 320 nm, respectively. A Luna ODS C₁₈ column was selected as the stationary phase based on the drug chemistry (Fig. 1) and considering the LogP values of 0.87 and -0.02 for AMX and MEZ, respectively. The ODS stationary phase is hydrophobic in nature, facilitating the hydrophobic interaction between the non-polar group of the drug and the stationary phase. The AMX has the hydrophobic phenyl ring, and MEZ has the hydrophobic aromatic ring [51] which interacts with the ODS stationary phase. The strong hydrophobic interaction between the hydrophobic groups of drug analytes and the ODS stationary phase results in the increased t_R . An ODS C18 column was used to separate AMX and MEZ based on their respective LogP values of 0.87 and -0.02, which were determined by the literature and the chemistry of the medicines. Although AMX and MEZ are

mostly polar, interactions between the hydrophobic phenyl ring of AMX and the nitrogen group (NO₂) of MEZ with the hydrophobic ODS stationary phase are made possible by ODS's intrinsic hydrophobicity. Because of this hydrophobic contact, AMX and MEZ are less effectively retained in the stationary phase and can elute more readily. Accordingly, the Luna ODS C₁₈ column 250 × 4.6 mm, 5 μm column was employed in the preliminary OFAT trials. Phosphate buffer (10 mM), having a pH range between 6.2 and 8.2, was considered as a suitable choice for the aqueous phase considering the pKa values of AMX (7.1) and MEZ (2.8). This will help Initial OFAT trials using potassium dihydrogen phosphate buffer: methanol (80:20) at pH 6.5 produced a respectable resolution with t_R of 2.6 minutes for AMX and 4.0 minutes for MEZ [52–57].

DoE-assisted optimization of HPLC method

Evaluation of risk assessment

A cause-effect assessment was performed using the Ishikawa fishbone illustration (Fig. 2), to understand the link between the analytical method parameters and the analytical outcomes. The purpose of the Ishikawa diagram is to pinpoint potential risk factors and underlying reasons that might affect how well an operation is performed. It shows the fundamental relationship between the critical method parameters and the critical analytical attributes. Utilizing a risk-based strategy based on the risk associated with each parameter, the critical variable was identified. The key factors for the screening were the flow rate, injection volume, wavelength, oven temperature,

Table 2. Outcomes for ANOVA of the BBD.

Response	t_R of AMX (Y1)		t_R of MEZ (Y2)		Peak area of AMX (Y3)		Peak area of MEZ (Y4)		Resolution (Y5)	
F-value	47.81		478.99		13.46		23.54		20.08	
p-value	Model	<0.0001	Model	<0.0001	Model	<0.0001	Model	<0.0001	Model	<0.0001
	A	<0.0001	A	<0.0001	A	0.0122	A	0.1295	A	<0.0001
	B	0.0004	B	0.7806	B	0.0012	B	0.0636	B	<0.0001
	C	0.0091	C	<0.0001	C	0.0074	C	<0.0001	C	0.6652
	D	0.8630	D	0.4510	D	<0.0001	D	<0.0001	D	0.0661
	AB	0.0002	AB	0.3315	AB	0.5126	AB	0.9324	AB	<0.0001
	AC	0.2037	AC	0.0002	AC	0.5843	AC	0.9626	AC	0.9905
	AD	0.9944	AD	0.8203	AD	0.9382	AD	0.9529	AD	0.8995
	BC	0.8893	BC	0.9695	BC	0.8739	BC	0.6544	BC	0.9958
	BD	0.7291	BD	0.2041	BD	0.5242	BD	0.2305	BD	0.8093
	CD	0.9963	CD	0.9512	CD	0.8562	CD	0.2959	CD	0.9481
	A ²	<0.0001	A ²	<0.0001	A ²	0.2001	A ²	0.1115	A ²	0.0031
	B ₂	0.2747	B ₂	0.6933	B ₂	0.0376	B ₂	0.1494	B ₂	0.3518
	C ²	0.9925	C ²	0.3347	C ²	0.6599	C ²	0.4718	C ²	0.6237
	D ²	0.9549	D ²	0.4580	D ²	0.1600	D ²	0.1811	D ²	0.5312
<i>R</i> ²	0.9795		0.9979		0.9308		0.9592		0.9526	
Adjusted <i>R</i> ²	0.9590		0.9958		0.8617		0.9185		0.9051	
Predicted <i>R</i> ²	0.8820		0.9880		0.6069		0.7665		0.7278	

Note: –A specifies buffer ratio, B specifies buffer pH, C specifies flow rate, D specifies injection volume, AB specifies combination of buffer ratio and buffer pH, AC specifies combination of buffer ratio and flow rate, AD specifies combination of buffer ratio and injection volume, BC specifies combination of buffer pH and flow rate, BD specifies combination of buffer pH and injection volume, CD specifies combination of flow rate and injection volume.

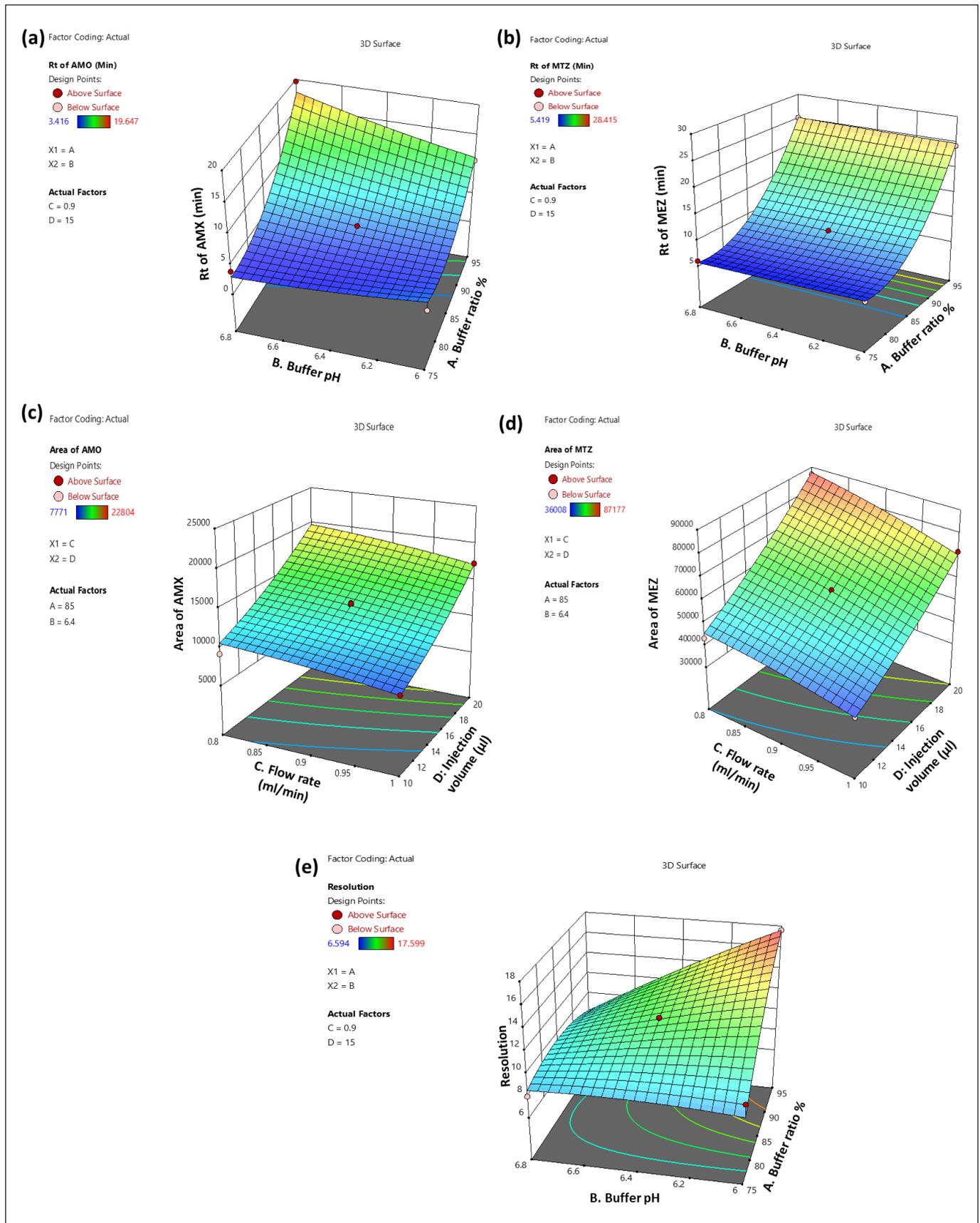


Figure 3. 3D surface response graph showing the influence of independent variables on the t_R of AMX (Y1), t_R of MEZ (Y2), peak area of AMX (Y3), peak area of MEZ (Y4), and resolution (Y5).

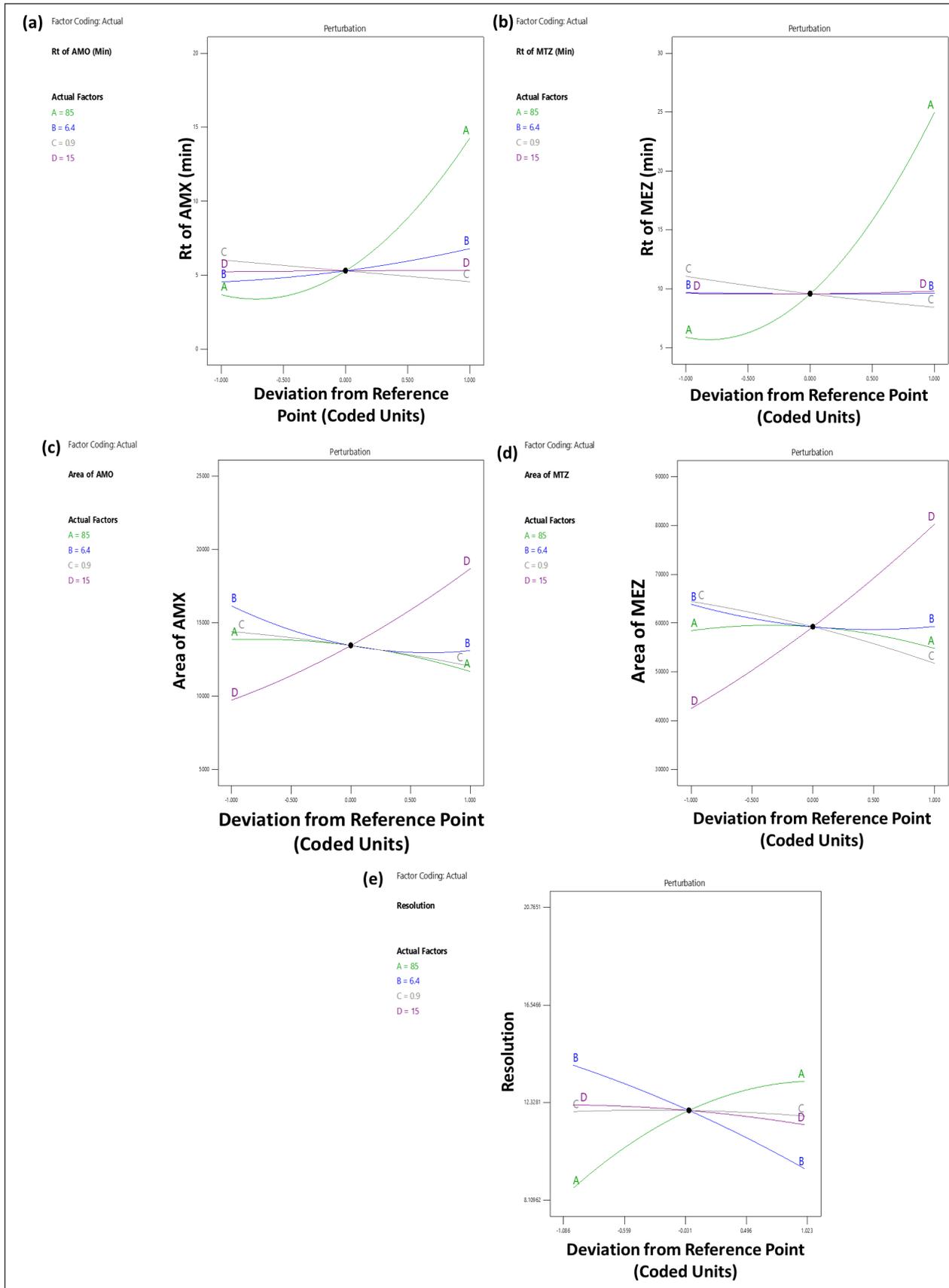


Figure 4. Perturbation plot depicting the impact of independent factors: (a) on the t_R of AMX (Y1); (b) t_R of MEZ (Y2); (c) peak area of AMX (Y3); (d) peak area of MEZ (Y4); and (e) resolution (Y5).

methanol: buffer ration, and buffer pH. Initial OFAT trials revealed that the buffer pH, buffer ratio, and mobile phase flow rate had a higher influence on the resolution, RT, and peak area.

BBD aided method optimization

The independent variables such as buffer ratio (A), buffer pH (B), flow rate (C), and injection volume (D) were optimized by DoE using the three-level BBD. The lower and higher limits for the variables were found using the OFAT technique. Accordingly, the range for the buffer ratio was from 75% to 90%, buffer pH was taken from 6.0 to 6.8, flow rate was taken from 0.8 to 1 ml/min, and injection volume was taken from 10 and 20 μ l, respectively. Based on this, the DoE software proposed 29 possible runs for these independent factors. The suggested 29 HPLC runs were performed, and the responses were analyzed for the optimized chromatographic conditions. The responses chosen for optimization were t_R of AMX (Y1), t_R of MEZ (Y2), peak area of AMX (Y3), peak area of MEZ (Y4), and resolution (Y5). Suggested trial conditions and the corresponding responses obtained in the HPLC trials are shown in Table 1. An ANOVA analysis was performed on this data to understand the variables having a significant influence on the response (Table 2). Each ANOVA result in our study represents the relative contribution of each component and how those components contribute to the response variables' overall variability. For instance, if a factor has a high F -value and a low p -value, it greatly impacts the result; if it has a

high p -value, it does not significantly affect the findings. By analyzing these numbers, we may determine which variables are essential to improving our circumstances and which are less important. 3D plots and perturbation plots were generated using the DoE software between the individual responses and the four variables that significantly influenced the response. The influence of each independent variable on the chromatographic responses is described in the following section. The software generated 100 solutions based on the various permutations and combinations of the chromatographic conditions (variables) and the most optimal conditions were selected from the desirability plot.

Influence of independent variables on t_R of AMX (Y1)

The 3D (Fig. 3a) and perturbation (Fig. 4a) graphs were used to predict the impact of independent variables on the t_R of AMX. The quadratic equation (Eq. 4) created by the ANOVA revealed that independent variables A and B have a significant impact on the t_R of AMX. An increase in factors A and B increased the t_R of AMX. The independent variable C has an opposite impact on the t_R of AMX, while the increase in C was found to reduce the t_R of AMX. The independent variable D did not significantly affect the t_R of AMX.

$$Y1 = +5.2952 + 5.29675 \times A + 1.12083 \times B - 0.733333 \times C + 0.0425833 \times D + 2.14375 \times AB - 0.5595 \times AC - 0.003 \times AD - 0.0595 \times BC - 0.14825 \times BD + 0.002 \times CD + 3.67677$$

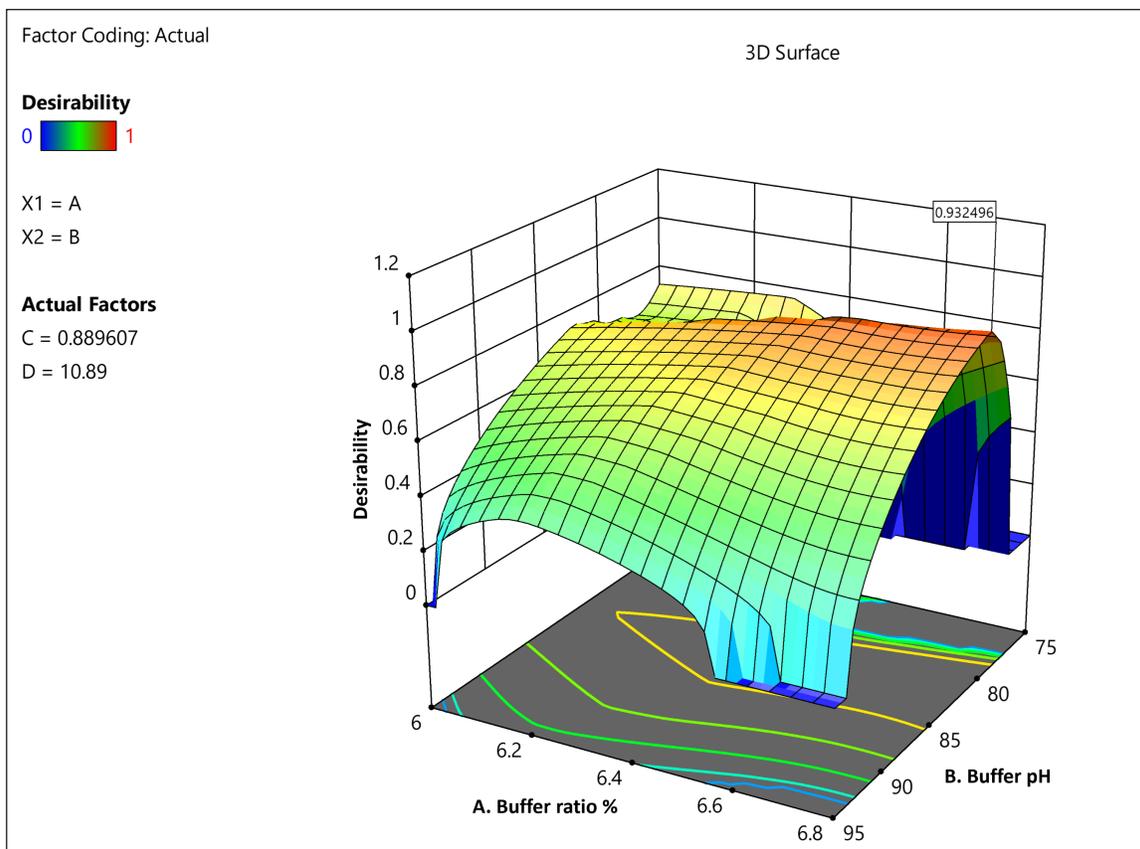


Figure 5. Desirability plot depicting the optimized conditions.

Table 3. Validation data of the optimized analytical method.

Parameters	AMX	MEZ
t_R (min)	3.906 ± 0.015	6.834 ± 0.033
TF	1.470 ± 0.078	1.748 ± 0.034
Theoretical plate	4733 ± 31.594	3700 ± 63.871
Specificity	No interfering peaks at the t_R of AMX and MEZ	
Resolution	6.604 ± 0.054	
Linearity range (µg/ml)	0.5–20	0.5–20
Regression equation	$y = 8960.6x - 909.13$	$y = 22180x - 1108.7$
Correlation coefficient (r^2)	0.9999	0.9999
LOD (ng/ml)	105	65
LOQ (ng/ml)	225	110
	Accuracy (Recovery %)	
80%	101.81%	100.02
100%	99.63	99.99
120%	101.71	101.49
	Precision (CV %)	
Repeatability	0.55%	0.88%
Inter-day	1.53%	0.33%
	Stability (change %)	
Bench-top	-1.41%	-1.99%
Autosampler	-0.75%	-1.25%

$$\times A^2 + 0.37465 \times B^2 + 0.00315 \times C^2 - 0.018975 \times D^2 \quad (4)$$

Influence of independent variables on R_f of MEZ (Y2)

The 3D (Fig. 3b) and perturbation (Fig. 4b) graph were used to predict the impact of independent variables on the t_R of MEZ. The quadratic equation (Eq. 5) generated by the ANOVA showed that A and C significantly affect the t_R of MEZ. An increase in independent variable A reflected an increase t_R of MEZ, while an increase in independent variable C decreased the t_R of MEZ. The independent variables B and D did not significantly affect the t_R of MEZ.

$$Y2 = + 9.5792 + 9.53458 \times A - 0.0368333 \times B - 1.314 \times C + 0.100583 \times D + 0.226 \times AB - 1.14375 \times AC + 0.052 \times AD - 0.00875 \times BC - 0.29925 \times BD - 0.014 \times CD + 5.8449 \times A^2 + 0.071025 \times B^2 + 0.176275 \times C^2 + 0.13465 \times D^2 \quad (5)$$

Influence of independent factors on peak area of AMX (Y3)

The peak area of AMX was significantly affected by the independent variables C and D as can be seen in the 3D (Fig. 3c) and perturbation (Fig. 4c) graphs. The quadratic equation (Eq. 6) generated by the ANOVA showed that independent variables C and D significantly impact the peak area of AMX. While independent variables A and B has less significant effect on the peak area of AMX. Theoretically, an increase in injection

volume increases the number of moles of analyte available to emit a signal, which is associated with an increase in the area of AMX. Similarly, an increase in C decreased the analyte's interaction time with the stationary phase, which was associated with a slight decline in the response area of AMX.

$$Y3 = + 13454.2 - 1084.17 \times A - 1522.58 \times B - 1178.5 \times C + 4484.75 \times D - 438.5 \times AB + 365.5 \times AC + 51.5 \times AD + 105.5 \times BC + 426.25 \times BD - 120.5 \times CD - 689.142 \times A^2 + 1177.23 \times B^2 - 230.392 \times C^2 + 760.483 \times D^2 \quad (6)$$

Influence of independent factor on peak area of MEZ (Y4)

The quadratic equation (Eq. 7) generated by the ANOVA showed that independent factors C and D significantly affect the area of MEZ. The independent variables A and B have no significant effect on the area of MEZ. The 3D (Fig. 3d) and perturbation (Fig. 4d) graph showed that the peak area of MEZ was found to increase with an increase in D and a decrease in C.

$$Y4 = + 59233.4 - 1823.58 \times A - 2280.25 \times B - 6344.92 \times C + 18908.3 \times D + 169.25 \times AB + 93.5 \times AC - 118 \times AD + 897 \times BC + 2458 \times BD - 2129.25 \times CD - 2615.66 \times A^2 + 2349.09 \times B^2 - 1138.66 \times C^2 + 2167.09 \times D^2 \quad (7)$$

Influence of independent factors on resolution (Y5)

The resolution is the distance between the two nearby peaks. The generated ANOVA quadratic equation (Eq. 8) showed the effect of independent variables on the resolution. The 3D (Fig. 3e) and perturbation (Fig. 4e) graph showed that the independent variables A and B significantly affect the resolution. In contrast, the independent variables C and D have no significant effect on the resolution. An increase in A and a decrease in B lead to increased resolution between the peaks.

$$Y5 = + 11.9876 + 2.30483 \times A - 2.23625 \times B - 0.09525 \times C - 0.4295 \times D - 2.4135 \times AB - 0.0045 \times AC - 0.048 \times AD + 0.002 \times BC - 0.09175 \times BD + 0.02475 \times CD - 1.04463 \times A^2 - 0.282258 \times B^2 - 0.147008 \times C^2 - 0.188133 \times D^2 \quad (8)$$

Desirability

The chromatographic parameters with a desirability of 0.932 were confirmed as optimal chromatographic parameters, based on the desirability graph generated (Fig. 5). The most optimum conditions suggested by the desirability were as follows. Mobile phase ratio: methanol: potassium dihydrogen phosphate buffer (20:80) in isocratic mode; pH of the mobile phase 6.0; flow rate: 0.9 ml/min; injection volume: 10 µl; column oven temperature: 25°C. The above-suggested conditions were tried on the Phenomenex Luna C₁₈ column (250 × 4.6 mm, 5 µm particle size) with the detector wavelength set at 228 nm for AMX and 320 nm for MEZ. The chromatogram obtained under the above condition is shown in Figure 5. The reliability and reproducibility of the DoE-modeled method were further confirmed by performing full validation as per the ICH guideline.

Method validation

Validation of the analytical method assures that the method is accurate, reproducible, and fit for the purpose.

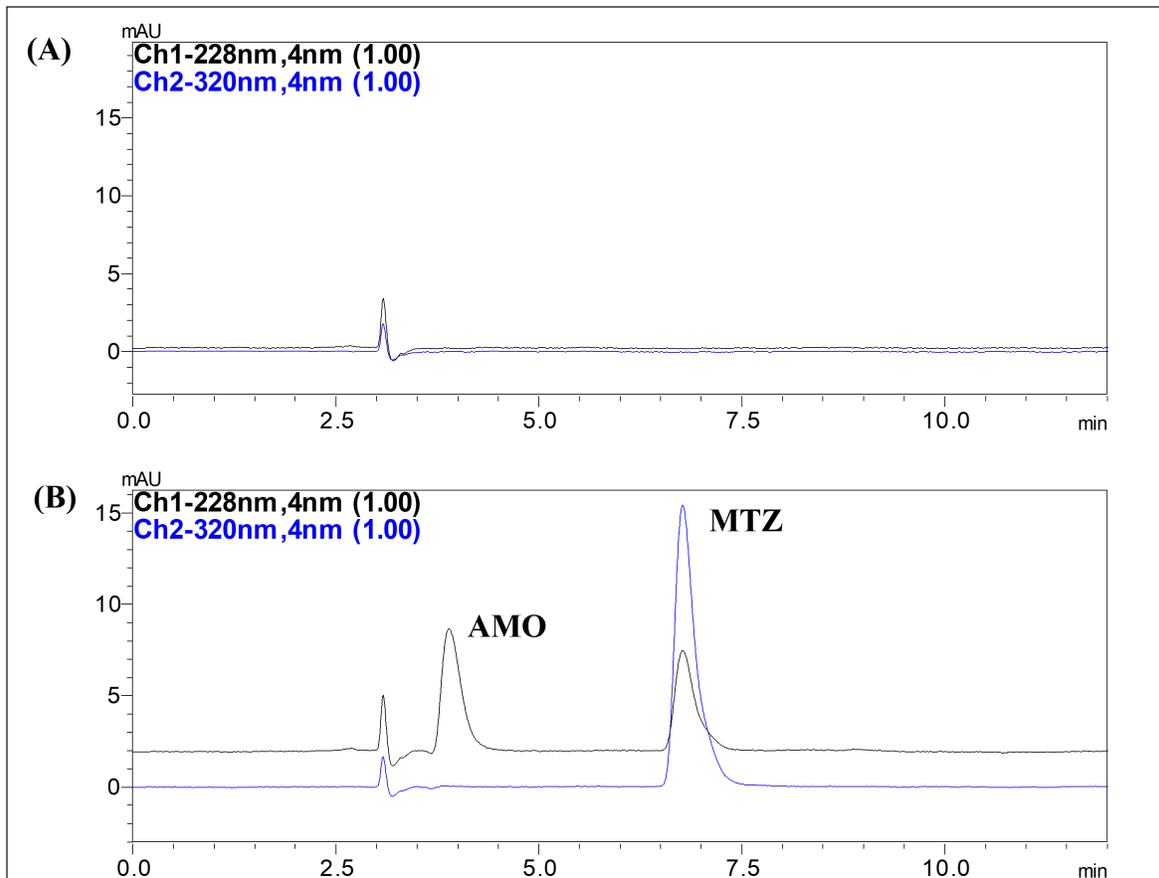


Figure 6. Chromatogram obtained at optimized conditions (A) Chromatogram of blank formulation; (B) Chromatogram of formulation co-loaded AMX and MEZ.

Table 4. Results of % EE of pharmacosomes co-loaded with AMX and MEZ.

Trial No.	Soy lecithin (mg)	DCM (ml)	AMX (mg) and MEZ (mg) (1:1)	Entrapment efficiency of AMX (%)	Entrapment efficiency of MEZ (%)	Particle size of the pharmacosomes
1	80	60	50	77.50	75.18	150.30
2	90	60	50	79.26	76.85	152.89
3	100	60	50	80.14	76.24	154.62
4	110	60	50	80.64	77.64	156.34
5	120	60	50	82.04	78.84	161.54
6	130	60	50	85.39	82.55	165.98
7	140	60	50	86.23	83.87	177.28
8	150	60	50	86.619	84.66	192.44

Results of the validation of the developed method are given in Table 3. The results of the system suitability evaluation showed good resolution, TF, and N more than 2,000, ensuring that the analytical system is functioning efficiently. A blank chromatogram of the formulation showed no interfering peaks of the excipients or the degradation products at the t_R of AMX and MEZ demonstrating the selectivity of the method (Fig. 6). Linearity in the 0.5–20 $\mu\text{g/ml}$ range was obtained for AMX and MEZ with R^2 value > 0.999 . The linearity equation for the AMX was $y = 8960.6x - 909.13$ and for MEZ was $y = 22180x - 1108.7$. The calibration curve of AMX and MEZ is shown in Figure S1.

The accuracy evaluations of the developed method showed recovery results within $100\% \pm 2\%$ at all three concentration levels demonstrating the accuracy of the method. The precision % CV of $< 2\%$ seen for intra and inter-day precision studies shows that the method is highly reproducible for AMX and MEZ. The method's LOD and LOQ were 105 and 225 ng/ml, respectively, for AMX, while the values were 65 and 110 ng/ml for MEZ. Evaluation of the robustness of the method showed that slight variations to the temperature, injection volume, wavelength, pH of the buffer, and flow rate do not influence the separation efficiency. Robustness data

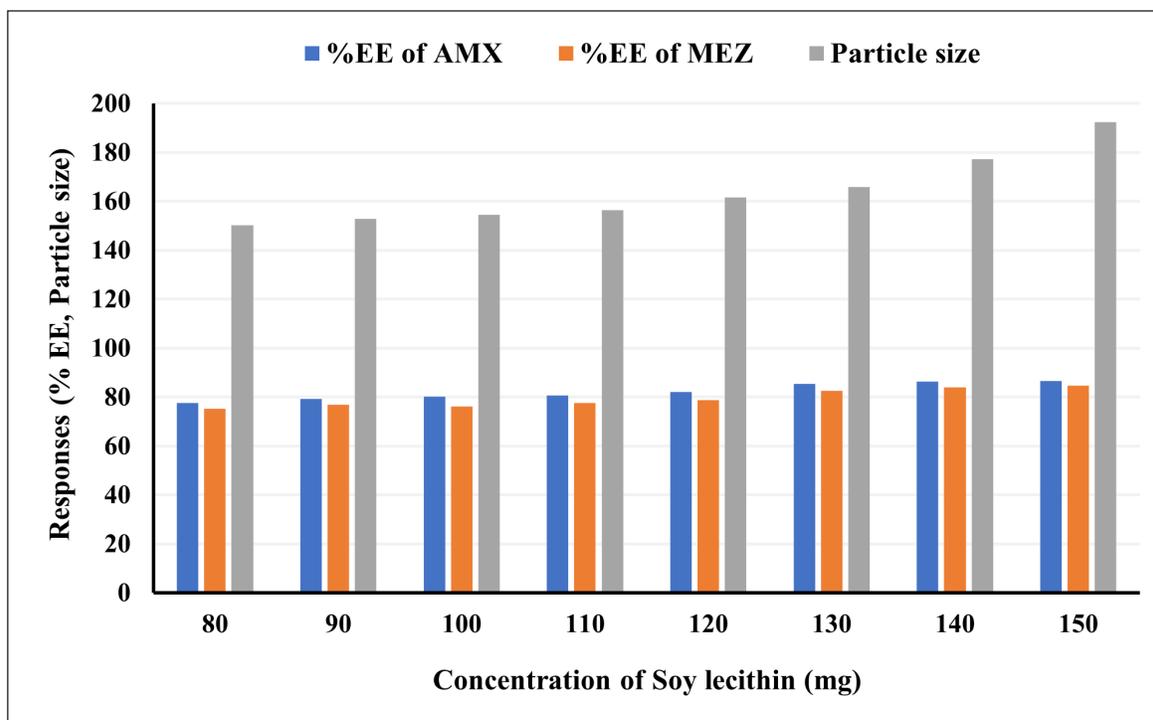


Figure 7. An illustration of the effect of increasing the ratio of soya lecithin on the entrapment efficiency and particle size.

are provided in Supplementary Table S1. The validation study confirms the method's suitability for quantifying these drugs from their formulations.

Application of the method for evaluation the developed pharmacosomes

EE % of pharmacosomes using the validated method

A critical formulation metric for assessing the efficiency of novel drug delivery systems is the evaluation of EE %. The capacity of a formulation to encapsulate the medicine within the carrier system is referred to as EE %. During formulation development, it is essential to identify the conditions that cause the highest entrapment of the drug. This determination is crucial for formulations such as mucoadhesive beads, liposomes, nanoparticles, and microspheres. We tried different ratios of soy lecithin and DCM to obtain maximum entrapment efficiency (Table 4, Fig. 7). As can be seen in the table and figure, we observed that the % entrapment is increasing with an increase in the ratio of Soy lecithin. However, the particle size of the pharmacosome also was found to increase with the increase in the ratio. The increase in the particle size was more prominent after the ratio of 130: 60. Accordingly, the final optimized pharmacosome is having a EE % of 85.39 % (AMX) and 82.55% (MEZ).

In-vitro drug release from the pharmacosomes

The developed method evaluated the drug release studies from the AMX and MEZ co-loaded pharmacosomes. The release studies were conducted at pH 1.2 and 7.4. Samples were collected at the following time points: 0.25, 0.5, 1,

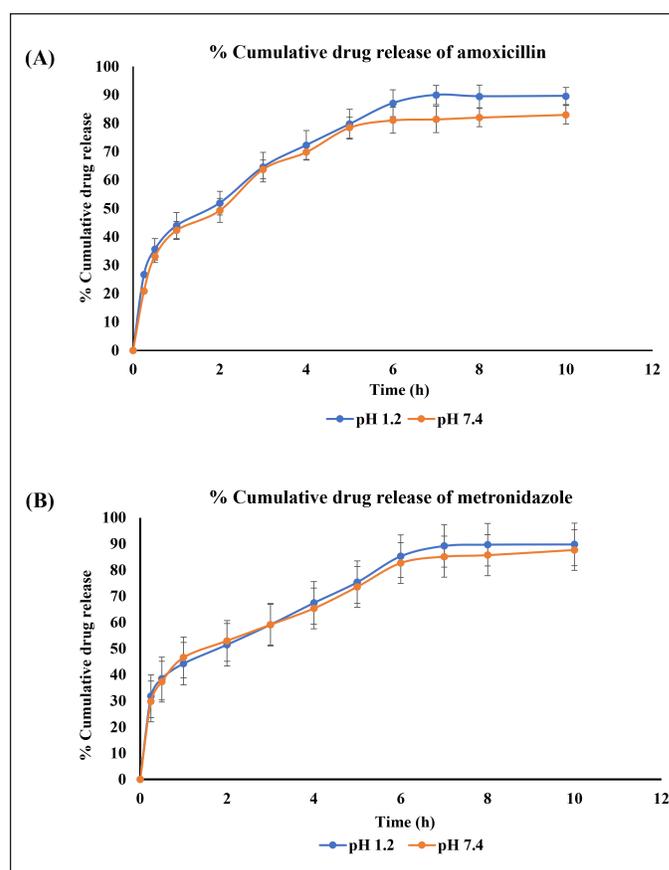


Figure 8. An illustration of % cumulative drug release of drugs at pH 1.2 and pH 7.4 (A) AMX and (B) MEZ.

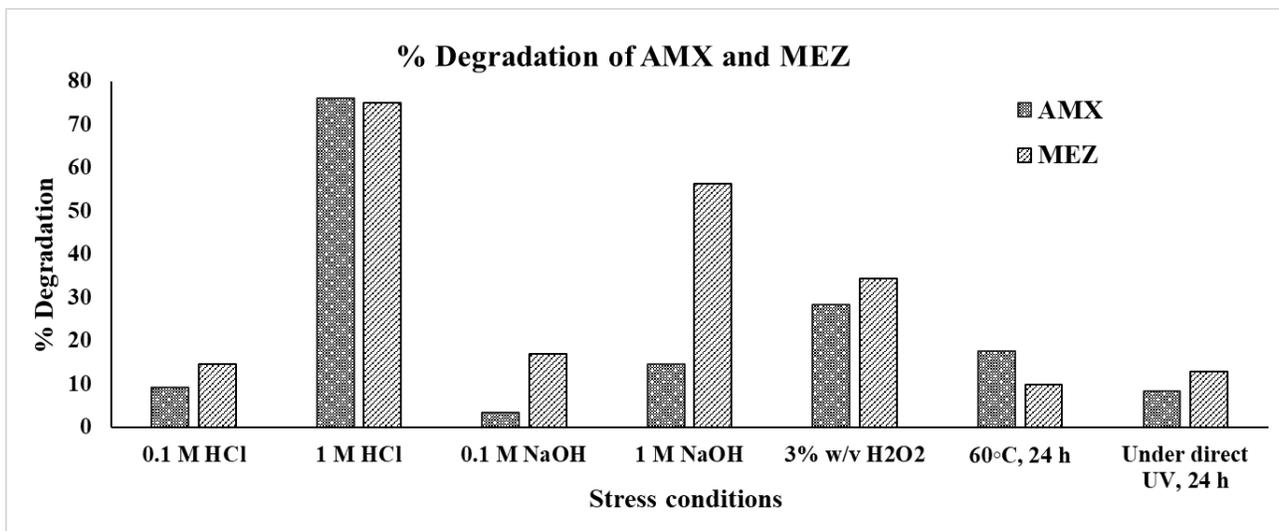


Figure 9. Forced degradation study results for AMX and MEZ illustrated in a column chart that displays the percentage of degradation under each condition.

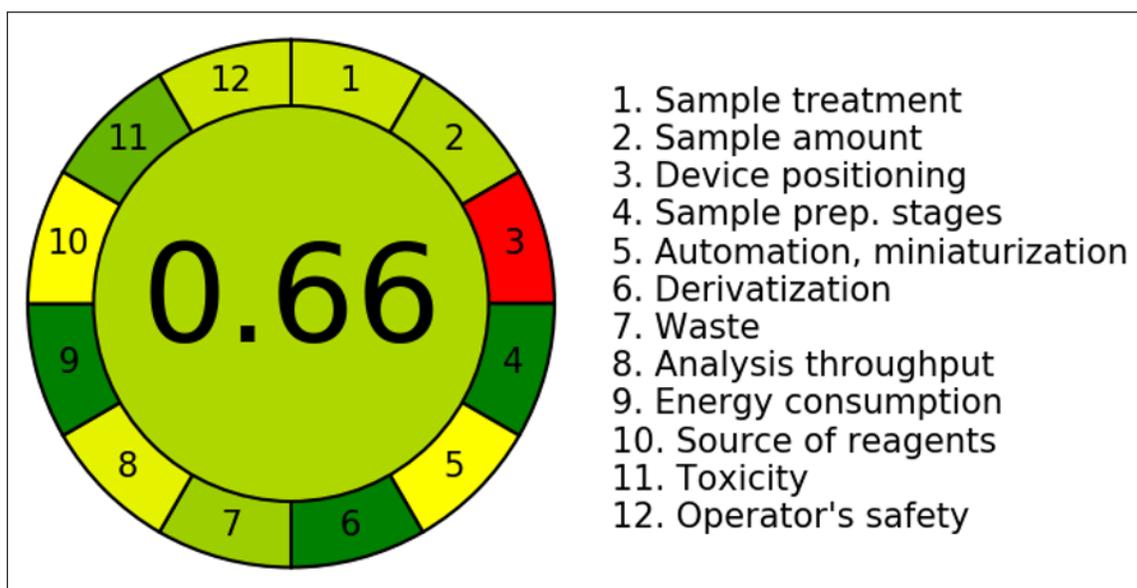


Figure 10. Pictorial illustration showing the greenness of the analytical method.

2, 3, 4, 5, 6, 7, 8, and 10 hours. The results showed that the pharmacosomes were stable at both pH levels tested. Complete release of both drugs were observed by 6 hours at all the pH levels studied. There was a burst release of around 30% of the drug within 30 minutes, followed by the linear release of the drug for up to 6 hours, after which no release was observed, as can be seen in [Figure 8A and B](#). The release profile showed that the highest release obtained from pharmacosomes was $89.62\% \pm 3.04\%$ of AMX and $89.84\% \pm 2.01\%$ of MEZ at pH 1.2, while at pH of 7.4, they were $83.01\% \pm 3.29\%$ of AMX and $87.69\% \pm 3.59\%$ of MEZ.

Forced degradation study results

A degradation study was conducted to assess the stability of AMX and MEZ throughout formulation

development. [Figure 9](#) demonstrates the whole degradation analysis done as part of the study under various stress situations. AMX was shown to be degraded by more than 72% in strongly acidic conditions (1 N HCl); however, the degradation was less than 10% under moderately acidic conditions (0.1 N HCl). Conversely, in alkaline conditions and on exposure to sunlight, degradation of AMX was negligible. The degradation of MEZ was 20% in mildly alkaline (0.1 N NaOH) and 59% in strongly alkaline (1 N NaOH) conditions. The degradation of MEZ by exposure to sunlight was less than 20%. The study showed that the drug will be stable at conditions employed for its formulation, which does not involve higher acidic and alkaline conditions. The pictorial representation of the chromatogram and peak purity graph of AMX and MEZ is shown in [Figures S2 and S3](#).

Greenness of analytical method

The aim of green analytical chemistry (GAC) is to evaluate the impact of analytical procedures on the atmosphere, worker safety, and health is the aim of GAC, mainly when the procedure requires utilizing organic solvents in the mobile phase. In recent years, there has been a tremendous increase in interest in GAC, making it feasible to rate the greenness of various analytical approaches utilizing a range of green evaluation tools. Here we would like to highlight that the solvents used in this investigation were chosen with care to reduce their negative effects on the environment while preserving analytical effectiveness. To minimize the use of improved, giving preference to aqueous-based systems and eco-friendly substitutes whenever feasible. By minimizing solvent waste and guaranteeing safer laboratory procedures, this is consistent with the tenets of GAC. Furthermore, to give a better picture of the sustainability of the process, we have now given solvent consumption in ml each run. The method's solvent consumption efficacy is demonstrated by the total volume of mobile phase utilized each run, which is 10.8 ml to further increase the method's environmental friendliness, efforts were undertaken to reduce solvent usage without sacrificing chromatographic performance. Using the AGREE program, we created an illustration (Fig. 10) that showed how environmentally friendly, the AGREE score of 0.66 showed that the developed method was environment friendly, the developed RP-HPLC technique for the measurement of AMX and MEZ was determined to be environmentally friendly.

CONCLUSION

The three-level BBD was used to create a reliable HPLC-PDA analytical method for simultaneously measuring AMX and MEZ from its formulations. To clearly illustrate the effect of this method, the DoE tool assisted in understanding the effects of the interactions between the independent factors on the results of chromatographic separation. The model-predicted method was found to be linear, precise, and accurate. It demonstrated selectivity for the AMX and MEZ from the excipients of the formulation and degradation products. The pharmacosomes are amphiphilic complexes loaded with hydrophilic and lipophilic drugs, which improves their solubility and stability. The unique characteristics of pharmacosomes such as small size, high drug entrapment efficiency, and amphiphilicity permit them to better interact with biological membranes, facilitating drug absorption. One of the significant advantages of pharmacosomes is their ability to improve the dissolution and permeation of drugs, leading to increased bioavailability. Adding more lecithin increases efficiency, but it can also have an impact on formula stability and particle size. Larger particle sizes may result from enhanced vesicular aggregation and bilayer formulation at higher lecithin concentrations. In particular, large particles may slow down the pace at which drugs are absorbed in biological systems, which might impact drug absorption. Because smaller particles dissolve more rapidly and are absorbed more efficiently in the gastrointestinal tract, this is especially important for oral formulation. The developed method was utilized to calculate the formulation's *in-vitro* drug release and EE %, which allowed for the optimization of the

excipient ratio to provide the maximum EE % and required drug release. The method was also applied to evaluate the % of degradation successfully. The model-predicted method is also very ecofriendly. The method can be applied further to analyze AMX and MEZ, commonly used as first-line treatment in *H. pylori* infection in other combination therapies in clinical settings. Additionally, investigating the effectiveness of these drugs in different formulations such as nanoparticles or sustained-release forms could provide insights into overcoming antibiotic resistance. In conclusion, we have developed and fully validated an HPLC-based analytical technique for the quantification of AMX and MEZ from its formulations. The method could be used to assess drug loading, EE %, release profile, and drug assay from several formulations of AMX and MEZ. The developed method is eco-friendly, economical, robust, accurate, and more precise than the previous method.

LIST OF ABBREVIATIONS

AMX, Amoxicillin; BBD, Box-Behnken design; CAAs, Critical analytical attributes; CCD, Central composite design; CMPs, Critical method parameters; DoE, Design of experiment; EE %, % Entrapment efficiency; GAC, Green analytical chemistry; HPLC, High Performance Liquid chromatography; *H. pylori*, *Helicobacter pylori*; LOD, Limit of detection; LOQ, Limit of quantification; MEZ, Metronidazole; N, Number of theoretical plate; OFAT, One factor at a time; PDA, Photodiode array; TF, Tailing factor; t_R , Retention time.

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AUTHOR CONTRIBUTIONS

All authors made substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVAL

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

PUBLISHER'S NOTE

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USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

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