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Design of an Experimental Study for the Simultaneous Determination of Cefepime, Piperacillin and Tazobactam Using Micellar Organic Solvent-Free HPLC

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Abstract: Application of Sustainable analytical chemistry concepts has become crucial in order to remove the environmentally harmful impacts originating from the routine use of analytical techniques. Here, a new LC method is developed and its parameters are analyzed, depending on a mixed micellar mobile phase. This was primarily aimed at getting rid of the use of organic solvents in conventional routine analyses. Combinations of tazobactam (TZB) with piperacillin (PPC) or cefepime (CFM) are commonly used as effective antimicrobial therapies, especially for resistant strains. Therefore, the three drugs were separated and quantified using an organic solvent-free mobile phase. The mixed micellar mobile phase was comprised of 15 mM Brij-35 with 38 mM SDS, adjusted to pH 3.5. Separation was performed by HPLC on monolithic RP-C18 column Chromolith®Performance RP-18e (100 mm × 4.6 mm) at a rate of 1 mL per minute of flow in conjunction with a measurement wavelength 210 nm. The method was found valid and applicable in accordance of precision, and accuracy within ranges of 5–100 µg mL^{-1} for PPC and CFM and of 0.625–12.5 µg mL^{-1} for TZB. The quality-by-design technique was used to analyze the effect of modifying the mixed micellar ratios on separation efficiency and conclude their behavior. Finally, the suggested approach was assessed applying the green analytical procedure index against the greenest published methodology to show superiority.

Keywords: micellar liquid chromatography; design of experiment; cefepime; piperacillin; tazobactam

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1. Introduction

The significant emergence of antimicrobial resistance (AR) increased mortality rates all over the world. This can be attributed to the increased consumption of antibiotics which increased the defined daily dose by 65% between 2000 and 2015 [1]. For instance, according to a recent WHO report [2], ciprofloxacin resistance to *E. coli* increased from 8.4% to 92.9%, to *K. pneumonia*—from 4.1% to 79.4%. About 64% of patients infected with MRSA are susceptible to death [2]. If no further measures are taken, the resistance to second-line antibiotics is likely to be 72% higher in the period from 2005 to 2030 [1].

Combinations of tazobactam (TZB) with piperacillin (PPC) or cefepime (CFM) are commonly used as effective therapies. Several papers reported their excellent bactericidal power towards resistant bacteria, especially in cancer patients [3–5]. Therapeutic protocols of antibiotics for some critically ill hospitalized patients must be undertaken with care, especially for those who are susceptible to sub-therapeutic levels of antibiotics due to

kidney glomerular hype-filtration [6]. PPC is part of the penicillin family. It stops cell wall synthesis in bacteria by binding to certain proteins [7]. TZB is a suicide inhibitor to enzyme beta-lactamase which is produced by some resistant types of bacteria. TZB's main role is to render those bacteria less resistant to the antibiotic [7]. Meanwhile, CFM is a fourth-generation cephalosporin that can cross the blood–brain barrier. CFM has broad-spectrum activity against Gram-positive and Gram-negative bacteria than the first-generation drugs [8]. Chemical structures of the drugs under study are presented in Figure 1. The use of PPC/TZB and CFM is common for empirical therapies, especially in AR bacteria, due to extended-spectrum β-lactamase production [9].

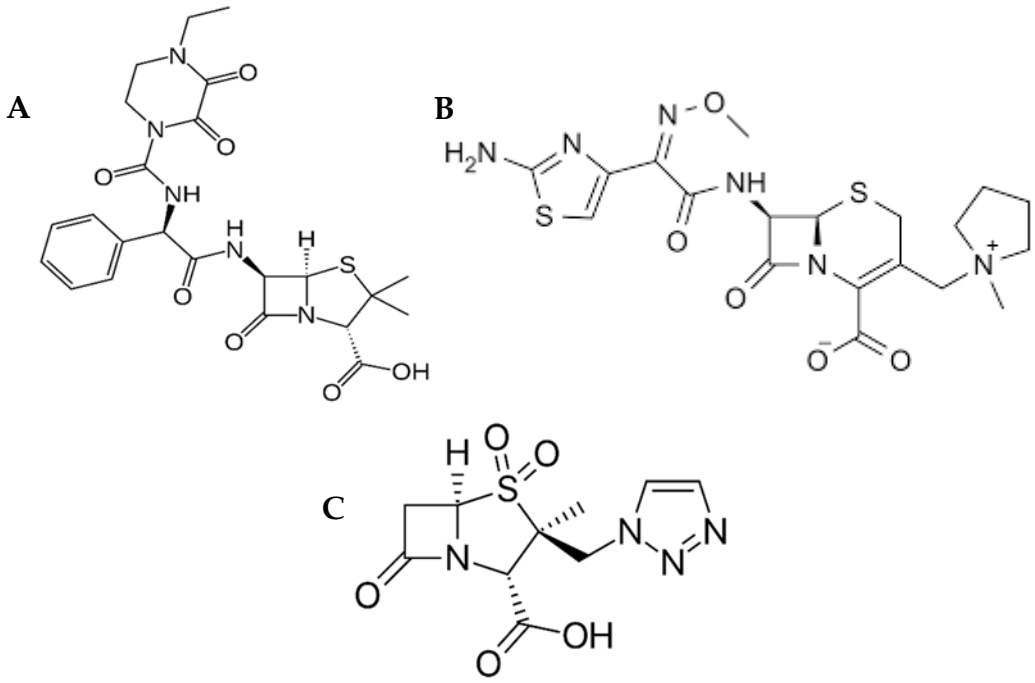


Figure 1. Chemical structures of (**A**) PPC, (**B**) CFM and (**C**) TZB.

Green analytical chemistry (GAC) approaches are aimed at designing of and using chemical products that reduce or eliminate the risk of chemical harm. GAC focuses on reducing, recycling or eliminating the use of toxic and hazardous chemicals in analytical processes by coming up with creative new ways that have less of an impact on the environment [10]. Micellar liquid chromatography (MLC) uses surfactants above their critical micellar concentration (CMC) as mobile phases. This technique is a good alternative to traditional RP-HPLC. It offers the benefit of enabling direct injection of physiological samples because proteins are dissolved in the surfactant [11], which makes them easier to remove. MLC has this advantage of replacing hazardous organic solvents in reversed-phase chromatography [12]. Design of experiments (DOE) is a recently established approach that uses a systematic way to find relationships between analytical process variables. It is a better way for understanding the effect of many factors which collectively constitute a crucial analytical process better than the one-factor-at-a-time (OFAT) method [13]. For multiple factors, DOE could also interpret whether these factors have interactive behavior on the analytical procedure response [14]. Therefore, DOE can experiment with several factors simultaneously, make trials within the experimental ranges, which in turn result in optimizing their combined effects.

Literature review revealed the reporting of different methods for the estimation of the PPC and TZB drugs either alone, together or in combination with other antibiotics. Several methods were reported for the determination of PPC/TZB [15,16] or CFM/TZB [17,18]. However, only few have been reported for the simultaneous determination of the three drugs under study using HPLC–MS detection in biological fluids [19–22], and one capillary zone electrophoresis (CZE) method [23]. Except for the CZE method [23], all other reported

methods used organic solvents that are ecologically harmful. Therefore, the main aim of the proposed study was to develop organic solvent-free methodology using mixed MLC. The study was enriched by the use of DOE to establish data for the combined variables on RP separations.

2. Materials and Methods

2.1. Instruments and Methods

An Alliance HPLC instrument was used from Waters, Milford, Massachusetts, the United States. The system was composed of a pump, auto-injector, and variable UV detector. The analytical data were processed using Waters Empower 3 software. A reversed-phase C18 monolithic column, Chromolith®Performance RP-18e (100 mm × 4.6 mm) purchased from Merck, Massachusetts, USA, has been employed. Adjustment of pH for the aqueous mobile phase was performed using an Adwa benchtop pH-meter, model AD1030, from ADWA, Szeged, Hungary.

DOE performed with the assistance of Design Expert® edition 11 from Stat-Ease Inc., Minneapolis, the United states. Experimental calculations were made using validated Excel 2013 software from Microsoft Office 2013, Microsoft Corporation, Redmond, Washington, the United states.

DOE was performed to test the effect of the three main variables on the separation efficiency of the three drugs under study. The effects of modulation of concentration ratios of the additives in addition to the pH value of the running mobile phase were studied. According to DOE results, the method validation was done using the optimal chromatographic conditions as shown in Table 1.

Table 1. Chromatographic conditions for quantification of TZB, PPC and CFM.

Parameter	Chromatographic Conditions
Stationary phase	Chromolith®Performance RP-18e (100 mm × 4.6 mm) Column Temp: 25 °C
Mobile phase	15×10^{-3} M Brij-35, and 38×10^{-3} M SDS adjusted to pH 3.5
HPLC	Speed rate: one mL per minute Setting wavelength: 210 nm

2.2. Materials and Reagents

Analytical standards of TZB (as sodium salt), PPC (as sodium salt) and CFM were supplied and certified from QPS laboratory, Cairo, Egypt. The reagents were all obtained of analytical grade. Sodium dodecyl sulfate is known as (CAS No. 151-21-3) was bought from Qualikems, India. Polyoxyethylene lauryl ether is known as Brij-35 (CAS No. 9002-92-0) was obtained from Alpha Chemika, India. Sodium hydroxide (NaOH; CAS No. 1310-73-2), heptane sulphonic acid sodium salt (CAS No. 22767-50-6) and phosphoric acid (CAS No. 7664-38-2) were purchased from Merck, USA. L-arginine (CAS No. 74-79-3), monobasic sodium citrate (CAS No. 18996-35-5) and disodium edetate (CAS No. 6381-92-6) were kindly provided by EIPICO, Tenth of Ramadan city, Egypt.

Drug products were bought from the Egyptian marketplace. Tazocin® intravenous (I.V.) vials (labeled to contain 1.0/0.125 g PPC/TZB per vial) were manufactured by Sandoz, Basel, Switzerland. Forpar XP® I.V. vials (labeled to contain 1.0/0.125 g CFM/TZB per vial) were manufactured by Cipla, Munmbai, India.

2.3. Stock Standard Solution Composition

Stock solutions of TZB, PPC, as well as CFM were all made individually in distilled water at concentrations of 100.0, 800.0, as well as 800.0 μg mL^{-1}, in respective order. Standard working solutions were prepared from the stock solutions by dilution in the same solvent. A DOE standard solution was generated from the stock with ratios of 10, 80 and 80 μg mL^{-1} for TZB, CFM, as well as PPC, in respective order.

Linearity calibration curves were constructed using seven standard working solutions within concentration ranges of 5–100 μg mL^{-1} for PPC and CFM and within 0.625–12.5 μg mL^{-1} for TZB. Twenty microlitres of every concentration were fed 3 times into the HPLC system. Another 3 quality control (QC) working solutions were made, this time with lower (QCL), moderate (QCM), and higher (QCH) concentrations inside the specified ranges. QC standards were made by inoculating the drugs of investigation in a placebo solution at concentrations of 7.5, 10.0 and 12.5 μg mL^{-1} for TZB and at concentrations of 60.0, 80.0 and 100.0 μg mL^{-1} for PPC and CFM. The placebo solution was prepared by dissolving quantities of L-arginine, sodium citrate and sodium edetate which are commonly used excipients in I.V. infusions and vials at concentrations of 0.5, 0.5 and 0.1 g per liter in water. The three QC working solutions were employed to evaluate the precision and accuracy of the suggested method under the optimized chromatographic conditions.

2.4. Method Application on Pharmaceutical Dosage Forms

Tazocin® and Forpar XP® vials are labeled to contain 1.0 g of active antibacterial drugs PPC and CFM besides 0.125 g of TZB. The contents of each Tazocin® vial and Forpar XP® vial were separately transferred and dissolved with water up to 500 mL in a volumetric flask. The solutions were sonicated for 10 min and then filtered through 0.45 μm nylon membrane filters (Millipore, Milford, MA, USA); 1 mL aliquots of each solution were diluted to 25 mL using the mobile phase. The final concentration of diluted solutions contained 80 μg mL^{-1} of PPC or CFM besides 10 μg mL^{-1} of TZB. Then, the diluted solutions were analyzed under optimized chromatographic conditions. The average of three vials' determinations from each dosage form was calculated.

2.5. Design of the Experiment

The effect of three factors, SDS concentration, Brij-35 concentration and pH of the mobile phase on separation efficiency was investigated for the drugs under study. A central composite design (CCD) study was performed using 15 different mobile phase compositions (Table 2). Different compositions of mobile phases were generated by solubilizing equivalent concentration of Brij-35 and SDS in distilled water, and then adjusting the pH with one molar sodium hydroxide or 10% v/v ortho-phosphoric acid. The chromatographic column was conditioned for 10 min before each chromatographic run using the mobile phase at a flow rate of 1.0 mL min^{-1} at a detection wavelength of 210 nm. For each mobile phase, 20 μL were injected from the DOE standard in triplicate. The sustainability of the analytical method was improved by recycling the mobile phase in between the chromatographic runs. Before altering the composition of the mobile phase, the HPLC column was flushed with a 1:1 MeOH/water for ten minutes to remove any surfactant adsorbed on the stationary phase.

Table 2. CCD for the separation of PPC, CFM and TZB under the studied variables.

Composition	SDS (M)	Brij-35 (M)	pH
1	0.03	0.02	3
2	0.02	0.03	3.5
3	0.03	0.02	3.7
4	0.03	0.02	3
5	0.03	0.02	2.29
6	0.03	0.02	3
7	0.03	0.02	3
8	0.03	0.005	3
9	0.04	0.03	2.5
10	0.016	0.02	3
11	0.04	0.01	3.5
12	0.02	0.01	2.5
13	0.03	0.034	3
14	0.044	0.02	3
15	0.03	0.02	3

For each mobile phase composition, the average of responses for the resolution (Rs) between the critical pair of drugs (TZB–PPC), the symmetry of peaks (sym), theoretical plates (N), capacity factors (k) and retention times (t_R) were calculated. Designs (prediction formulas) relating the five responses to their essential factors were generated and analyzed. The optimization procedure centered on achieving a suitable Rs between the drugs under research in the lowest time of elution while keeping other system suitability parameters.

3. Results

3.1. Method Development and Optimization

CCD was performed in order to test the effect of different variables, such as Brij-35 and SDS ratio concentrations and pH, on the analytical performance of the proposed method. CCD can interpret the response of such variables as well as their possible interactions through a series of preliminary tests. CCD contains three design point runs: factorial, axial and center [24]. Eighteen runs were used to estimate the effect of the experimental variables with a total of 2^k factorial run, 2^k axial run in addition to 4 centroid runs at ($+\alpha$, +1, zero, -1, $-\alpha$) levels, where +1 and -1 are codes for the factorial runs representing low and high levels, $-\alpha$ and $+\alpha$ are the levels of axial point runs at the minimum and maximum levels and 0 codes the center runs [24].

Perturbation graphs in Figure 2 demonstrate the effects of pH, Brij-35 and SDS concentrations on peak symmetry and resolution. Responses were hypersensitive to factors with high curvatures. A straight line indicates a lack of responsiveness to changes in that one component. As far as the observed responses in the tested range are concerned, it was discovered that factors A (SDS C) and B (Brij C) were more critical than factor C (pH).

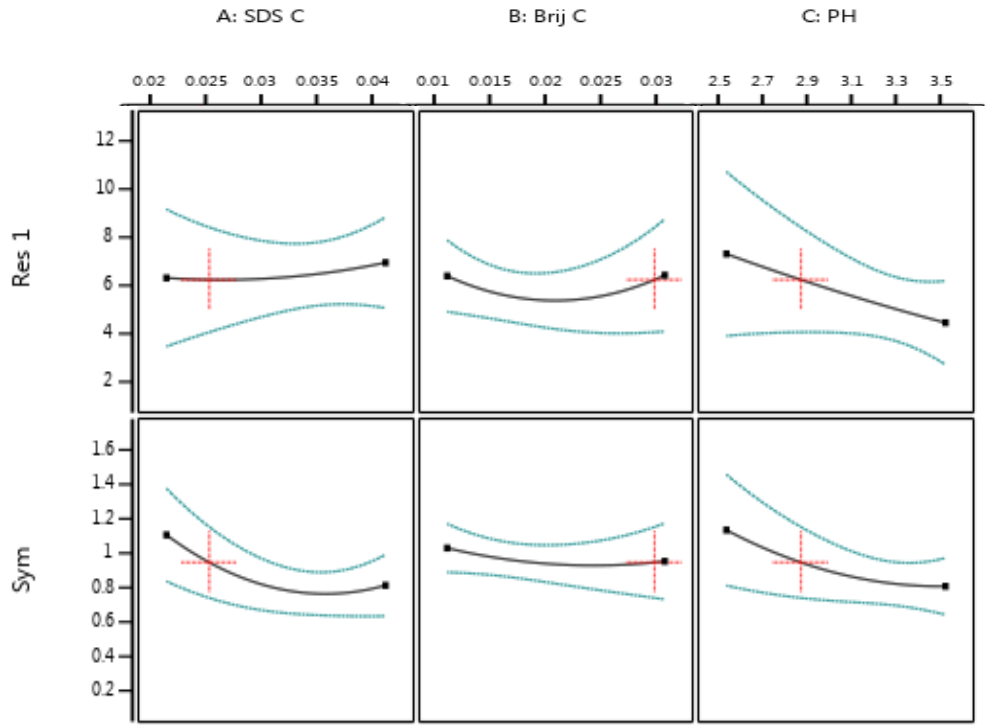


Figure 2. Plots of perturbation illustrating the impact of the parameters under study on resolution between the analytes and the symmetry factors of the peak (Black lines, result line; Green lines, upper and lower confidence intervals).

In preliminary investigations, high values of factor C (pH) caused considerable interference between the studied peaks, so higher pH values were omitted from the final CCD. As a result, responses are less sensitive to changes in pH.

Additionally, contour plots (Figure 3) were also used to examine the effects of applied factors on the target responses individually or collectively. Contour plots verified the perturbation plot results, but in a more obvious way. Factors A and B had opposing impacts on resolution and peak symmetry, as seen in Figure 3, while responses were optimal at greater values of factor A. Higher curvature of factor B's influence on resolution was evident, whereas lower and higher values may produce better resolution than intermediate values.

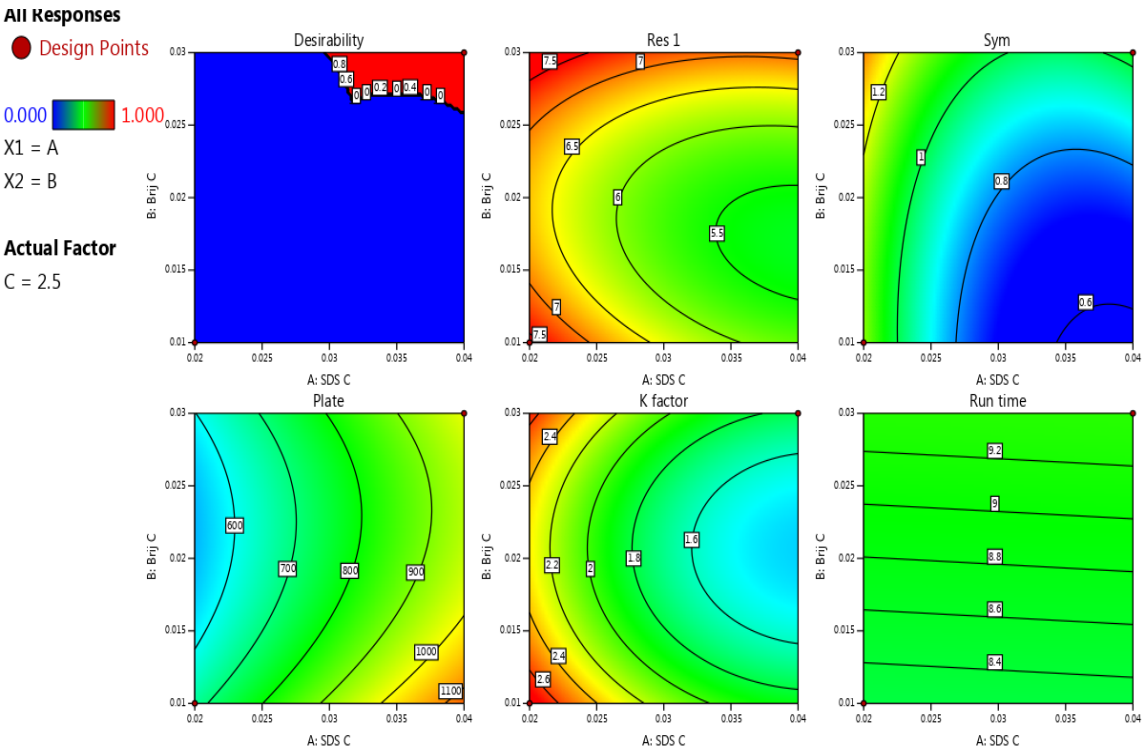


Figure 3. The contour plot depicts the interactive impacts of key parameters on all responses.

The overlay plot (Figure 4) was constructed so that the optimal responses might be chosen in reaction to a variety of different combinations of significant factors.

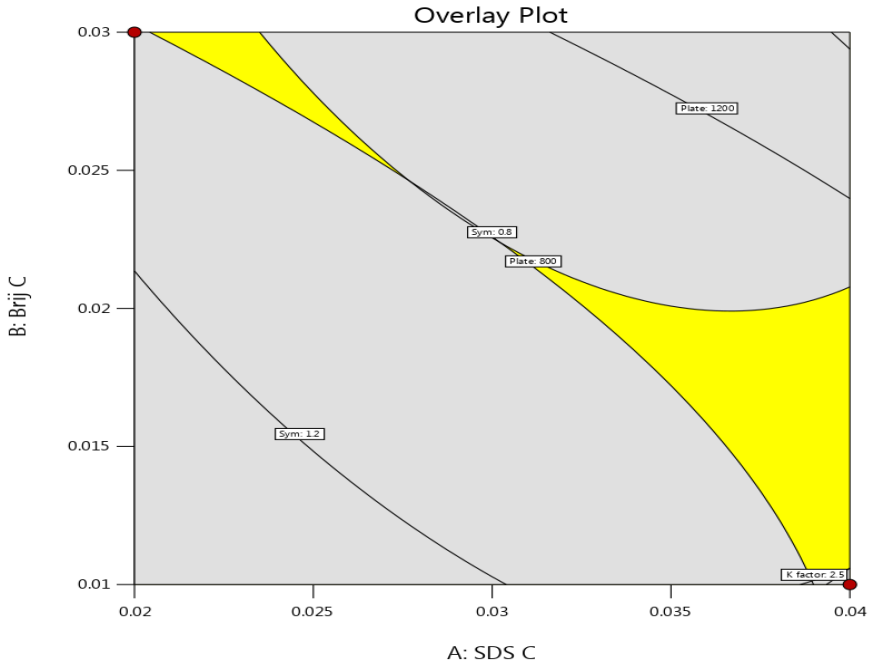


Figure 4. Overlay plot displaying the preferred responses within the appropriate parameters (sweet spot).

Five responses were optimized, including resolution (Rs), theoretical plates, peaks symmetry, selectivity (K factor) and total run time (t_R). In this step, several restrictions were specified in order to fit the highest desirability function for choosing the optimal chromatographic conditions [24], as shown in Figure 4. Optimizations focused on reaching

an appropriate Rs value (>2). The selected t_R of the analytes was set within 6 min; therefore, there was no need to target it. Graphical optimization was used, imposing the same restrictions to find the sweet spot as shown in Figure 4 (yellow region). On the other side, the grey region represents inappropriate parameters. The pH was 3.3–3.5, the Brij-35 concentration was 13–17 mM and the SDS concentration was 36–40 mM. Figure 4 also shows the permissible ranges.

Following the post-analysis prediction, it was discovered that the expected average of responses was within 95% of the high and low probability ranges.

3.2. Validation of the Analytical Methodology

The proposed method was validated in accordance to the ICH guidelines [25]. According to the DOE results, the most ideal chromatographic separation was performed using a mobile phase composed of 15 mM SDS and 38 mM Brij-35 adjusted to pH 3.5 at a flow rate of 1.0 mL min^{-1}.

Specificity was evaluated for interferences from dosage forms' excipients. As shown in Figure 5, no interferences from excipients found in pharmaceutical formulations were found. System suitability parameters were calculated for retention time (Rt), theoretical plates (N), resolution between peak pairs and peaks' symmetry and are presented in Table 3.

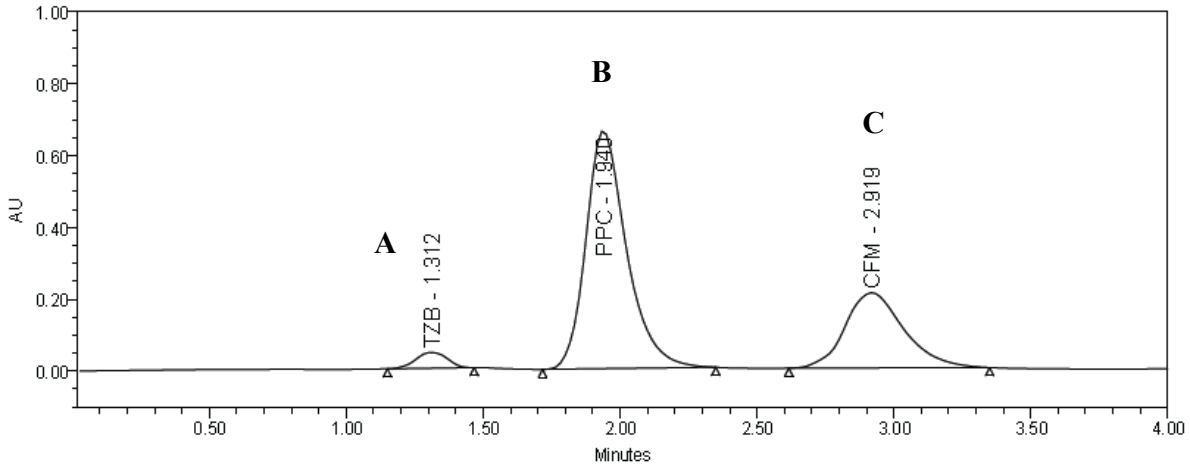


Figure 5. Chromatogram showing separation of (**A**) TZB, (**B**) PPC and (**C**) CFM in a laboratory-prepared mixture under the chromatographic parameters for validation. Mobile phase: 15 mM Brij-35, 38 mM SDS, pH 3.5 at one mL per min of flow rate.

Table 3. Linearity and system suitability results for the assessment of TZB, PPC as well as CFM utilizing the suggested method.

Parameter	TZB	PPC	CFM
Retention time (min ± SD)	1.31 ± 0.5	1.95 ± 0.5	2.92 ± 0.4
Resolution (Rs)	–	2.34	2.85
Theoretical plates, N *	1863	3210	5368
Symmetry factor	0.9	1.3	1.2
Linearity range (µg mL^{-1})	0.62–12.50	5.00–100.00	5.0–100.00
Linearity equation	y = 35.42x + 3.24	y = 87.21x − 33.72	y = 41.29x − 59.78
Correlation coefficient (R^2)	0.999	0.999	0.999
LOD (µg mL^{-1})	0.14	1.6	3.1
LOQ (µg mL^{-1})	0.5	4.9	9.5

* N per 1.0 m stationary phase.

Seven concentrations of CFM and PPC at 5.0, 10.0, 20.0, 40.0, 60.0, 80.0 and 100.0 µg mL^{-1} and of TZB at 0.62, 1.25, 2.5, 5.0, 7.5, 10.0 and 12.5 µg mL^{-1} were used for construction of linearity. The results of the average peak area responses were plotted against the

concentrations. Table 3 shows linearity parameters. Regression coefficient R^2 was more than 0.999 for all the analytes, indicating perfect linearity (see Figure 6). Limits of detection (LODs) and quantification (LOQs) were calculated based on the slope (S) of the calibration plots and the standard deviation (σ) of the regression lines. The LOD was calculated as 3.3 σ/S, while the LOQ was calculated as 10 σ/S. Table 3 show LODs and LOQs.

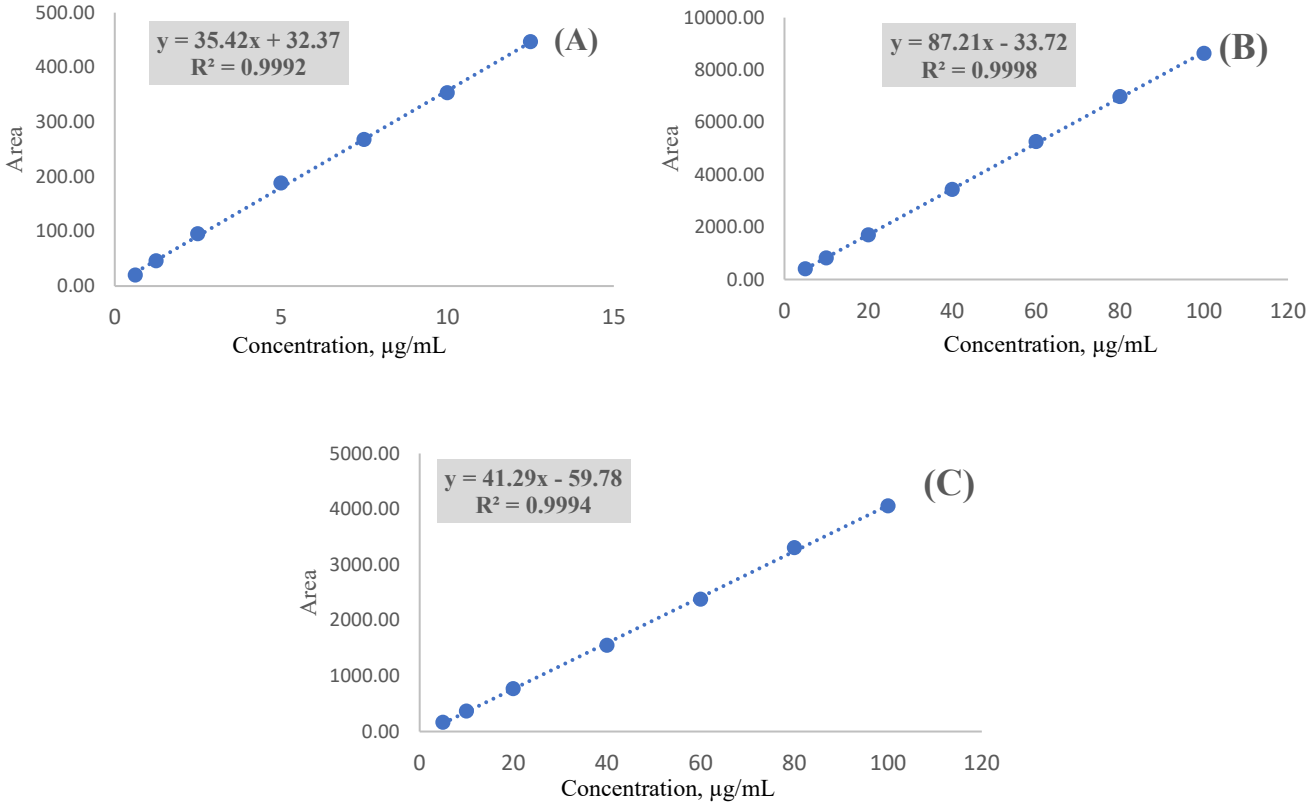


Figure 6. Linearity graphs showing regression coefficients and equations for (**A**) TZB, (**B**) PPC and (**C**) CFM.

Accuracy was assessed using the three QC standards (QCL, QCM and QCH) injected in triplicate for TZB, PPC and CFM. Accuracy results reported in Table 4 in terms of percentage recoveries for calculated and actual concentrations. The precision of the developed procedure was investigated by looking at its repeatability as well as its intermediate precision. Nine determinations were obtained using the QC standards covering the specified range for the procedure. Repeatability within the same day (referred to as intra-day) and intermediate precision within three different days (referred to as inter-day) were calculated and are presented in Table 4.

The robustness test on the proposed HPLC method was investigated during experimental design optimization and it was proved through the sweet spot in the design applying small changes in buffer pH (3.3–3.5), concentration of SDS (0.036–0.040 M) and concentration of Brij-35 (0.013–0.017 M). The results obtained in terms of recovery percentage for the QC standards indicated absence of any significant differences.

The stability of the standard solutions was evaluated as part of robustness, where the standard solutions were found stable for 2 days at room temperature (25 °C) and in cold (refrigerator, 2–8 °C). The change in the standard solution peak area response over 2 days was 1.86%, 1.29% and 2.12% for TZB, PPC and CFM, respectively, at room temperature.

Table 4. The results of the suggested method's accuracy and precision tests for determining TZB, PPC, and CFM are as follows.

Standard	TZB		PPC		CFM	
	R% *	RSD **	R% *	RSD **	R% *	RSD **
			Repeatability			
QCL	101.10	0.48	100.30	0.49	98.84	0.53
QCM	99.50	1.08	101.00	1.08	100.70	1.39
QCH	99.20	1.31	99.79	0.49	101.00	1.41
			Intermediate precision			
QCL	99.20	1.81	99.53	1.03	101.50	2.98
QCM	98.50	1.94	99.47	2.03	101.40	2.45
QCH	100.80	1.83	99.49	0.81	101.40	0.93
			Accuracy			
QCL	99.61	0.49	101.18	0.29	98.43	0.42
QCM	98.80	1.39	100.54	0.33	101.80	0.94
QCH	100.12	1.83	99.37	0.25	99.72	0.90

* Average recovery % = calculated conc./actual conc. ×100 (n = 3), ** RSD: relative standard deviation.

3.3. Application on Pharmaceutical Preparation

The proposed method was applied for determination of the studied drugs in their marketed pharmaceutical dosage forms. Table 5 lists the tablet dosage form names, compositions and the calculated percentages of the actual found concentrations to the labeled concentrations. The recovery results were found acceptable according to the proposed validated method.

Table 5. The outcomes of applying the suggested method on drug products.

Drug Products (Company)	Analyte	Concentration (mg)	R% * ± RSD
Tazocin® vial (Sandoz)	TZB	125.0	99.20 ± 3.62
	PPC	1000.0	100.30 ± 1.84
Forpar XP® vial (Cipla)	TZB	125.0	98.80 ± 1.16
	CFM	1000.0	100.80 ± 0.13

* Average percentage recoveries and relative standard deviations (n = 3).

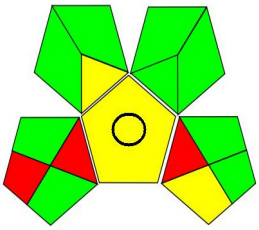
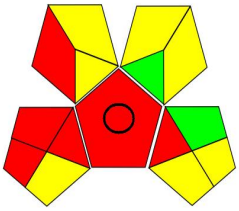
3.4. Evaluation of Greenness in Relation to Other Previously Published Analytical Methods

The evaluation of greenness of the analytical procedures became of absolute importance in order to minimize the amount of hazardous reagents waste. For instance, a conventional HPLC system can generate up to 0.5 L of organic solvents daily [26]. Therefore, several metrics were developed within the past decade for assessing the impact of analytical procedures on the environment. In order to evaluate the proposed method, it was compared to a previously described method. [19].

The green analytical procedure index (GAPI) [27] is one recent metric that had been cited in more than 380 articles before the writing of this manuscript (June 2022). The GAPI divides assessment into five pentagrams comprising 15 parts, each representing a different stage in the process of analysis. The GAPI uses a color-coded system to analyze the influence in accordance of green, yellow and red representing lower, moderate as well as higher harms, accordingly. Table 6 shows the evaluation of the two methods. The GAPI pictogram for the proposed method, shown in Table 6, outperforms the method introduced by D'Cunha et al. [19]. No solvent was required for sample preparation or methodology of the proposed method compared to the non-green solvent, acetonitrile [28], required for both in the comparison method. The run time of the proposed method is shorter than that of the comparison method. Moreover, the proposed method uses an isocratic technique instead of the gradient technique introduced by D'Cunha et al. [19]. This means less overall time when adding the time required for column reconditioning in gradient techniques,

which requires at least seven times the void volume time. In terms of energy consumption, although both methods use HPLC instrumentation, the proposed method does not require the high energy consumed by a sensitive MS detector. In summary, the 9 green regions of the proposed method in the GAPI pictograph demonstrate that it outperforms the other reported method.

Table 6. Comparative analysis between the developed and previously published LC methods for the measurement of the analytes under examination.

	Proposed Method	**Reported Method [19]**
Technique	HPLC-UV	HPLC-UV
Optimization technique	RSM *	OVAT *
Application	Pharmaceutical dosage forms	Plasma
Stationary phase	RP-C_{18}, monolithic	Phenomenex Kinetex RP-C_{18}
Organic modifier	Totally free	Acetonitrile
Analytes similarity	TZB, PPC and CFM	TZB, PPC, meropenem and CFM
Elution, run time	Isocratic, 4.0 minutes	Gradient, 7.0 minutes
GAPI * evaluation		

* RSM, response surface methodology; OVAT, one variable at a time; GAPI, green analytical procedure index.

4. Conclusions

Response surface methodology and QbD were applied for development and optimization of a micellar LC. The proposed method is the first solvent-free LC for simultaneous determination of cefepime, piperacillin and tazobactam. By applying an adjustable central composite design study, the method could be optimized for attaining the best resolution between the analytes within the shortest analysis time. The method was effectively employed in order to determine the analytes in their drug products. Finally, the new developed method was analysed for greenness on novel metrics and found to be in harmony with the concepts introduced by green analytical chemistry.

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