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Research Article

Green Analytical Rp-Hplc Method Development And Validation For Simultaneous Estimation Of Anti Biotic Drug In Bulk &Capsule Dosage Form

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ABSTRACT

The present study aimed to develop and validate a simple, accurate, precise, robust and green reverse-phase high-performance liquid chromatographic (RP-HPLC) method for the simultaneous estimation of Amoxicillin and Cloxacillin in bulk drug and capsule dosage form. The chromatographic conditions were optimized to achieve satisfactory separation of both drugs, followed by validation of the developed method for linearity, accuracy, precision, robustness and ruggedness. The method demonstrated excellent linearity with a correlation coefficient of 0.9998 for both Amoxicillin and Cloxacillin. The assay results were found to be 98.93% for Amoxicillin and 100.78% for Cloxacillin, with satisfactory precision and low percentage relative standard deviation. Robustness and ruggedness studies confirmed the reliability and reproducibility of the method under small deliberate changes in analytical conditions and between different analysts. The environmental performance of the method was evaluated using the Greenness Evaluation Metric of Analytical Methods (GEMAM) and Analytical GREEnness (AGREE) approaches. The developed method showed acceptable green analytical characteristics by minimizing solvent consumption, waste generation and potential environmental impact while maintaining analytical performance. Hence, the proposed green RP-HPLC method can be considered suitable for routine simultaneous estimation of Amoxicillin and Cloxacillin in pharmaceutical dosage forms.

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INTRODUCTION

Antibiotics play an important role in the treatment and prevention of bacterial infections. Amoxicillin and Cloxacillin are penicillin-class antibacterial agents used in pharmaceutical preparations. Reliable analytical methods are required for the quantitative determination of active pharmaceutical ingredients in bulk drugs and pharmaceutical dosage forms.

High-performance liquid chromatography (HPLC) is widely used in pharmaceutical analysis because of its sensitivity, selectivity and reproducibility. However, conventional chromatographic procedures may involve considerable quantities of organic solvents and generate chemical waste. Therefore, the development of analytical procedures that incorporate the principles of Green Analytical Chemistry is important for reducing environmental and occupational hazards.

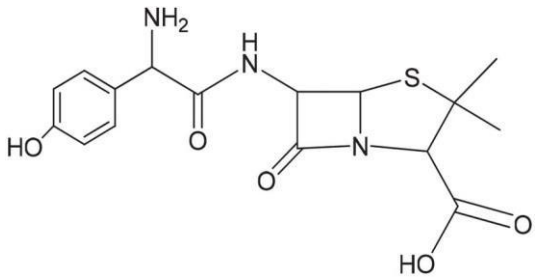
Green analytical chemistry aims to minimize the use of hazardous substances, reduce waste and energy consumption, and improve laboratory safety without compromising analytical quality. In the present study, a green RP-HPLC method was developed for the simultaneous estimation of Amoxicillin and Cloxacillin. The developed method was subsequently validated and its environmental performance was assessed using GEMAM and AGREE. The study objectives included reducing solvent consumption and waste generation while maintaining accuracy, precision, sensitivity and robustness.

DRUG PROFILE:

Drug name : Amoxicillin

Category : Antibiotic drug

Background : Amoxicillin is a broad-spectrum β-lactam antibiotic belonging to the aminopenicillin class.



Chemical structure:

Chemical name : (2*S*,5*R*,6*R*)-6-[[(2*R*)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid

Molecular formula : C16H19N3O5S

Molecular weight : 365.408 g/mol

Appearance : Off-white solid, Crystals from, Penicillin-type odour, Bitter taste

Melting point :194 °C

Route of administration : Amoxicillin is administered orally and is available in various forms, including immediate-release or extended-release tablets, chewable tablets, and suspension.

Storage : Store tablets and capsules at room temperature, away from moisture, heat, and light.

Approval date : 18 January 1974.

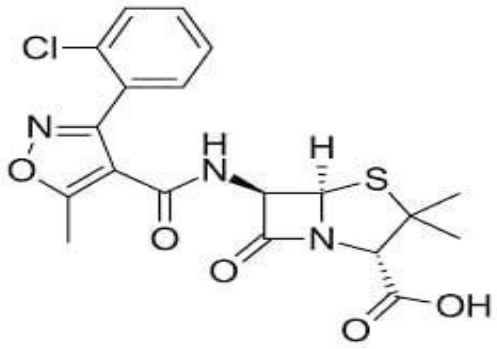
Mechanism of action : Amoxicillin belongs to the class of beta-lactam antimicrobials. Beta-lactams bind to penicillin-binding proteins, inhibiting transpeptidation — a crucial step in cell wall synthesis

involving cross-linking. This action activates autolytic enzymes in the bacterial cell wall, resulting in cell wall lysis and bacterial cell destruction.

Drug name : Cloxacillin

Category : Antibiotic drug

Background : Cloxacillin is a β-lactam, penicillinase-resistant (anti-staphylococcal) penicillin developed to treat infections caused by penicillinase-producing staphylococci. It belongs to the isoxazolyl penicillin group and is stable against destruction by many bacterial β-lactamase enzymes.



Chemical structure:

Chemical name : sodium (2S,5R,6R)-6-[[3-(2-chlorophenyl)-5-methyl-1,2-oxazole-4-carbonyl]amino]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-2-carboxylate.

Molecular formula : C19H17ClN3NaO5S

Molecular weight : 457.9 g/mol

Appearance : white or almost white, odorless crystalline powder.

Melting point :168-172 °C

Route of administration : Cloxacillin is administered by oral, intravenous, intramuscular and is available in various forms, including capsule, oral suspension.

Storage : Store tablets and capsules at room temperature, away from moisture, heat, and light.

Approval date : August 1965.

Mechanism of action : Beta-lactam antibiotic. Inhibits bacterial cell wall synthesis by binding to penicillin-binding proteins.

MATERIALS AND METHODS:

Reagent and chemical used:

- Ethanol (HPLC grade)
- Milli-Q-Water (HPLC grade)
- Ammonium acetate (analytical grade)
- Glacial acetic acid (analytical grade)

Instrumentation

- Digital Balance
- SHIMADZU – HPLC-DLC20AD
- Sonica Ultra sonic cleaner – model 2200 MH
- Digital PH Meter

PROCEDURE:

Preparation of buffer solution:

0.77gm of ammonium acetate were dissolved in 800ml of milli-Q-water and adjust the PH for 3.5 by using glacial acetic acid make upto 1000ml

preparation of standard stock solution:

50mg of amoxicillin and 50mg of cloxacillin were dissolved in Milli-Q-water and made upto 100ml volumetric flask with solvent.

1ml stock solution was pipetted out and made upto the 10 ml volumetric flask with solvent. (50µg of amoxicillin & 50µg of cloxacillin\ml)

preparation of sample stock solution:

The average weight of 10 capsules was calculated as 112. 2mg.Then112.2mg of powder were dissolved in Milli-Q-water and made upto 100ml volumetric flask with solvent.

1ml stock solution was pipetted out and made upto the 10 ml volumetric flask with solvent. (50µg\ml)

METHOD DEVELOPMENT:

The developed method was fully validated for the parameters as per ICH guidelines.

Linearity

Weigh accurately and transfer about 50mg of brivaracetam into 100ml of volumetric flask dissolve and make up with solvent. Take 0.5ml, 0.75ml,1ml,1.25ml,1.5ml of stock solution is transfer into 10ml of volumetric flask and make up with solvent upto the volume. To make the linearity concentration of 50%, 75%,100%,125%,150%.

Accuracy

Weigh accurately tablet average weight of sample and transfer into 100ml of volumetric flask dissolve and makeup to the volume with the solvent. Take 0.8ml,1ml,1.2ml of stock solution was transferred into 10ml of volumetric flask and makeup with solvent upto the volume. To make the concentration of 80%, 100%,120%.

Precision

Weigh accurately tablet average weight of sample and transfer into 100ml of volumetric flask dissolve and makeup to the volume with the solvent.1ml of stock solution was transferred into 10ml of volumetric flask and makeup with the solvent upto the volume. Six-time replicate of injection to produce the similar of the result no maximum deviation.

Robustness

Small deliberate changes in method like flow rate, wavelength, mobile phase and ratio were made but there was no recognized change in the result and were within range as per ICH guidelines. Robustness conditions like flow minus, flow rate, wavelength decreasing, wavelength increasing, mobile phase decreasing, mobile phase increasing, ratio increasing and ratio decreasing was maintained and sample were injected in duplicated manner system suitability parameters were not much effected and all the parameters were passed. % RSD was within the limit.

Ruggedness

Ruggedness is a measure of reproducibility of test results under normal, expected operational conditions from laboratory to laboratory and from analyst to analyst. The method was fully validated for the parameters as per ICH guidelines.

RESULTS AND DISCUSSION:

The optimized chromatographic method provided satisfactory separation of Amoxicillin and Cloxacillin. The method demonstrated excellent linearity, with a correlation coefficient of 0.9998 for both analytes. The assay results were 98.93% for Amoxicillin and 100.78% for Cloxacillin. Accuracy and precision studies showed satisfactory recovery and low percentage relative standard deviation (%RSD). Robustness studies demonstrated that small deliberate changes in chromatographic conditions did not significantly affect the analytical performance.

Ruggedness studies also showed reproducible results between analysts.

Method development:

Optimized chromatographic condition:

Mode of operation : Gradiant

Stationary phase : C18 Column (250 mm×5,4.5µ)

Mobile phase : Buffer: Ethanol (65:35)

Detection wavelength: 276 nm

Flow rate : 0.8ml/min

Column Temperature : 30℃

Retention time:

Amoxicillin :7.712minut

Cloxacillin :16.970minutes

Run time :15minutes

Injection volume : 50 mcg

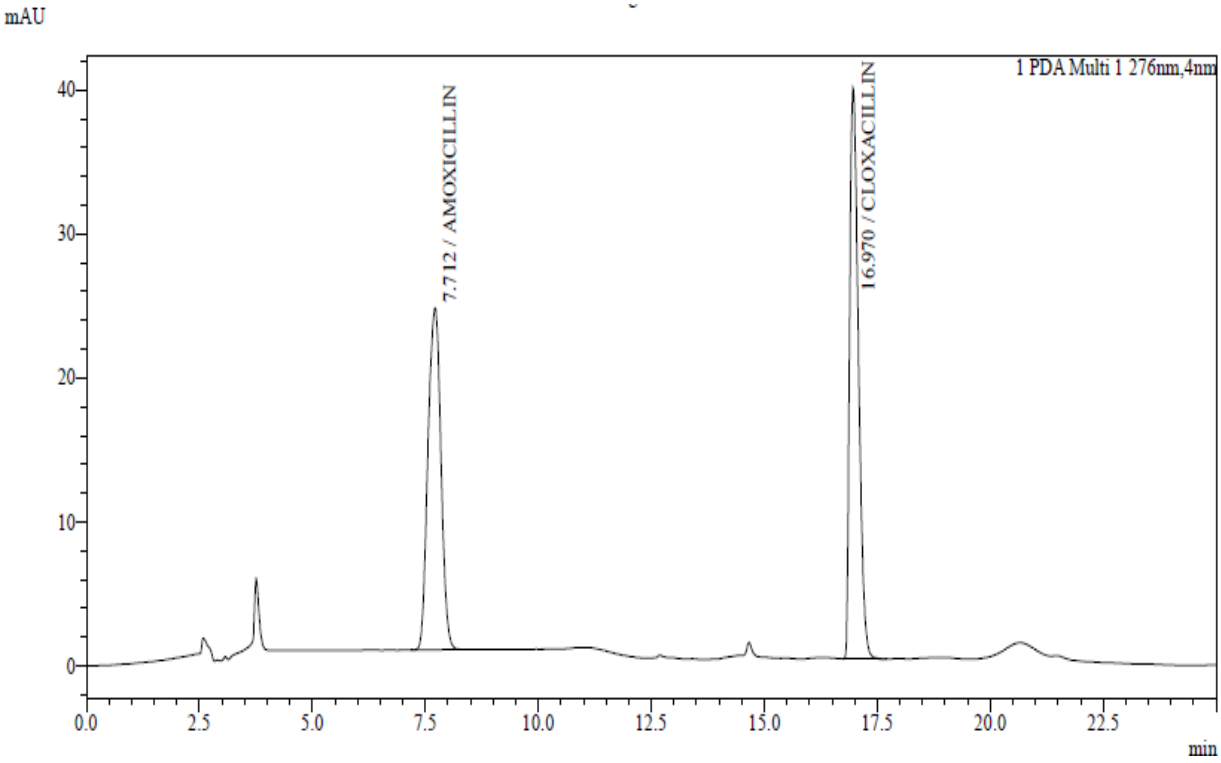


Figure 1: Optimized chromatogram

Peak table:

Peak	Drug name	Retention time	Area	Tailing factor	Theoretical plate
1	Amoxicillin	7.712	473298	1.023	3069
2	Cloxacillin	16.970	536140	1.438	31063

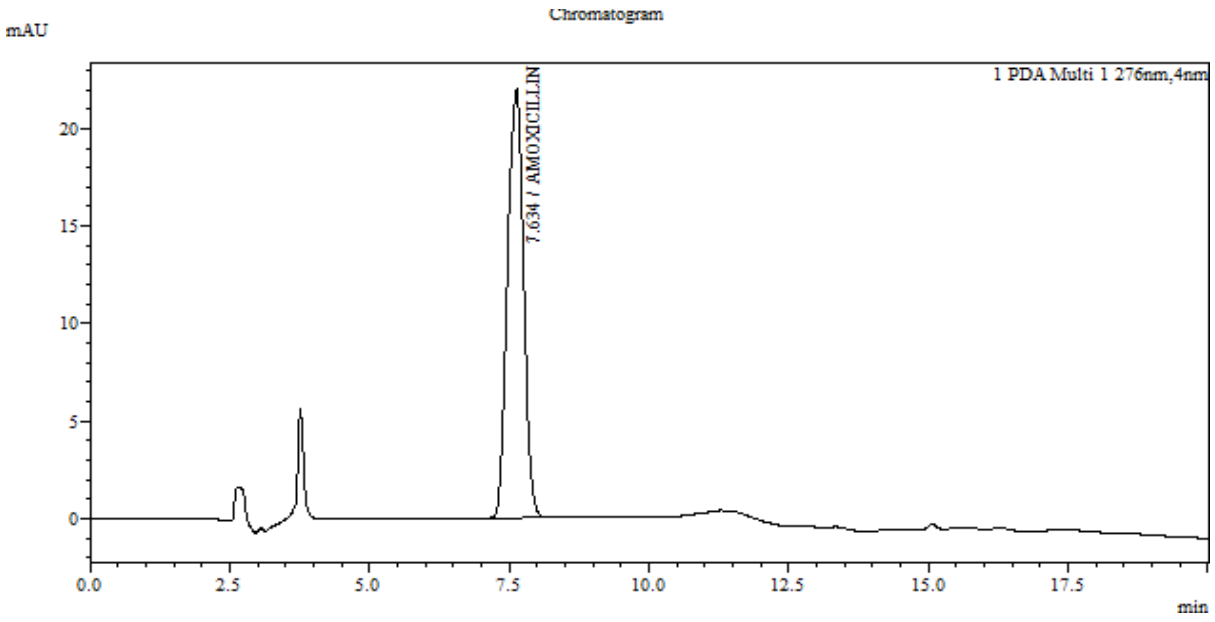


Figure 2: Optimized chromatogram of amoxicillin

Drug name	Retention time	Area	Theoretical plate	Tailing factor
Amoxicillin	7.634	470625	3186	1.042

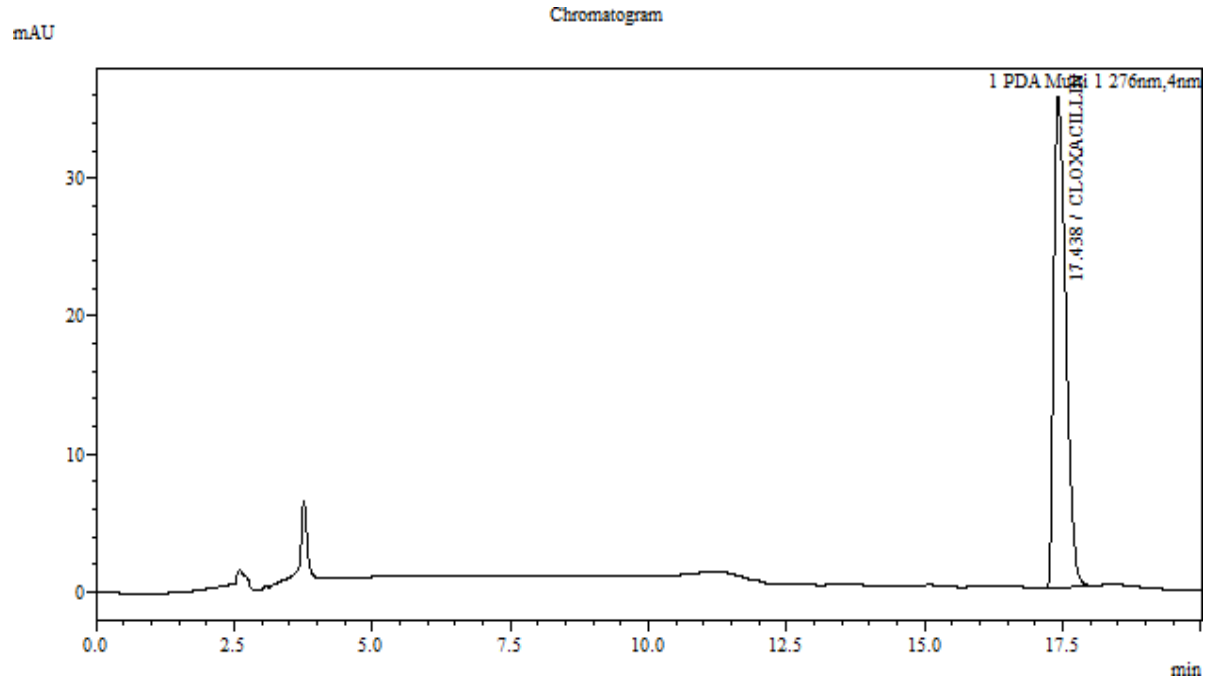


Figure 3: Optimized chromatogram of cloxacillin

Drug name	Retention time	Area	Theoretical plate	Tailing factor
Cloxacillin	17.438	624150	29981	1.425

Assay:

Table 1: Assay data for amoxicillin

Amoxicillin				
Injection	**Retention time**	**Area**	**Theoretical plate**	**Tailing factor**
Standard 1	7.712	473298	3069	1.023
Standard 2	7.724	473920	3133	1.028
Standard 3	7.721	473590	3128	1.028
Standard 4	7.721	475105	3137	1.035
Standard 5	7.723	475207	3163	1.036
Bracketing std	7.721	473704	3134	1.036
Sample 1	7.728	470625	3186	1.042
Sample 2	7.729	467515	3171	1.040

Average standard area	Percentage average	SD	%RSD
474137	98.93%	814.2918396	0.171741889

Table 2: Assay data for cloxacillin

Cloxacillin				
Injection	**Retention time**	**Area**	**Theoretical plate**	**Tailing factor**
Standard 1	16.970	536140	31063	1.438
Standard 2	16.988	538904	32392	1.398
Standard 3	17.010	538901	32491	1.402
Standard 4	17.022	540080	32531	1.428
Standard 5	17.034	541914	30873	1.460
Bracketing std	17.083	540051	32029	1.408
Sample 1	17.042	607966	28562	1.484
Sample 2	17.064	604150	29981	1.425

Average standard area	Percentage average	SD	%RSD
539331.6667	100.78%	1913.164748	0.374728805

Linearity:

Table 3: linearity data for amoxicillin

Amoxicillin				
Concentration	**Retention time**	**Area**	**Theoretical plate**	**Tailing factor**
50%	7.444	235551	3256	1.042
75%	7.559	362487	2943	1.025
100%	7.576	471667	3081	1.048
125%	7.581	583864	3077	1.053
150%	7.585	709012	3075	1.060

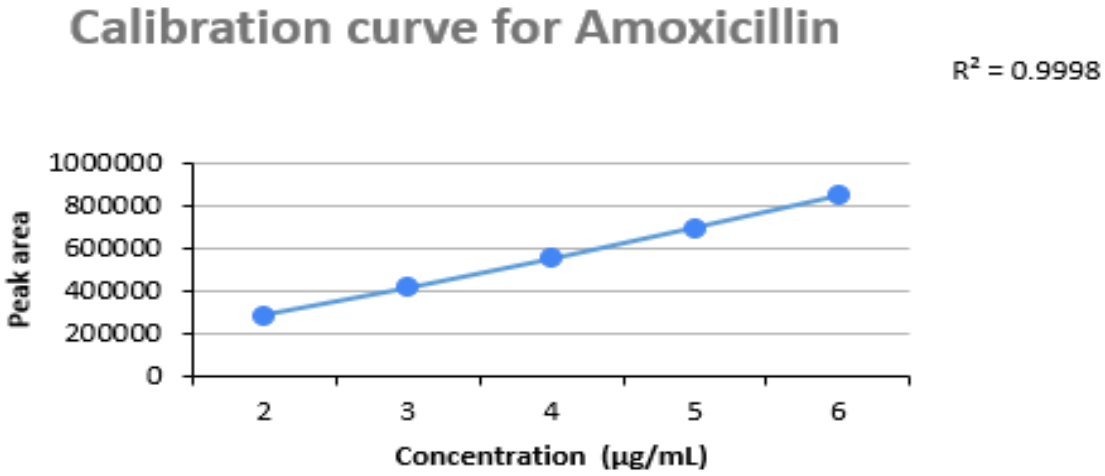


Figure 4: calibration curve for amoxicillin

Table 4: linearity data for cloxacillin

Cloxacillin				
Concentration	**Retention time**	**Area**	**Theoretical plate**	**Tailing factor**
50%	17.016	284981	37207	1.360
75%	17.025	415799	32429	1.398
100%	16.999	549245	29988	1.382
125%	16.963	694983	25862	1.467
150%	16.927	847574	23350	1.432

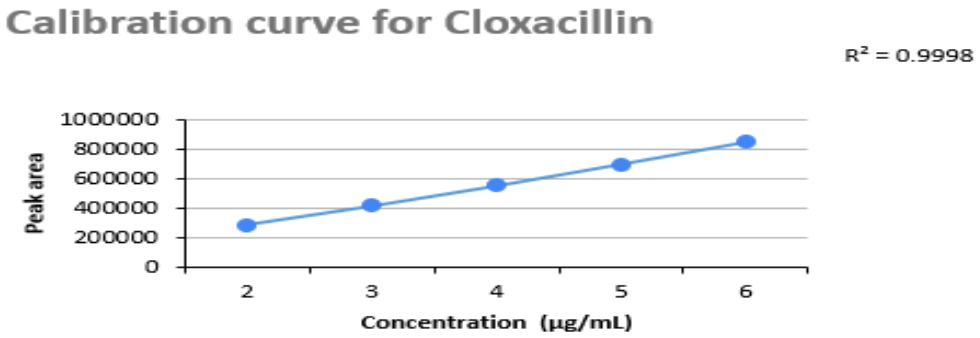


Figure 5: calibration curve for cloxacillin

Table 5: Linearity data

Parameters	Amoxicillin	Cloxacillin
Correlation coefficient R^2	0.9998	0.9998
Slope	116831.9	140135.4
Y intercept	-0.04154238	0.014632922

Accuracy

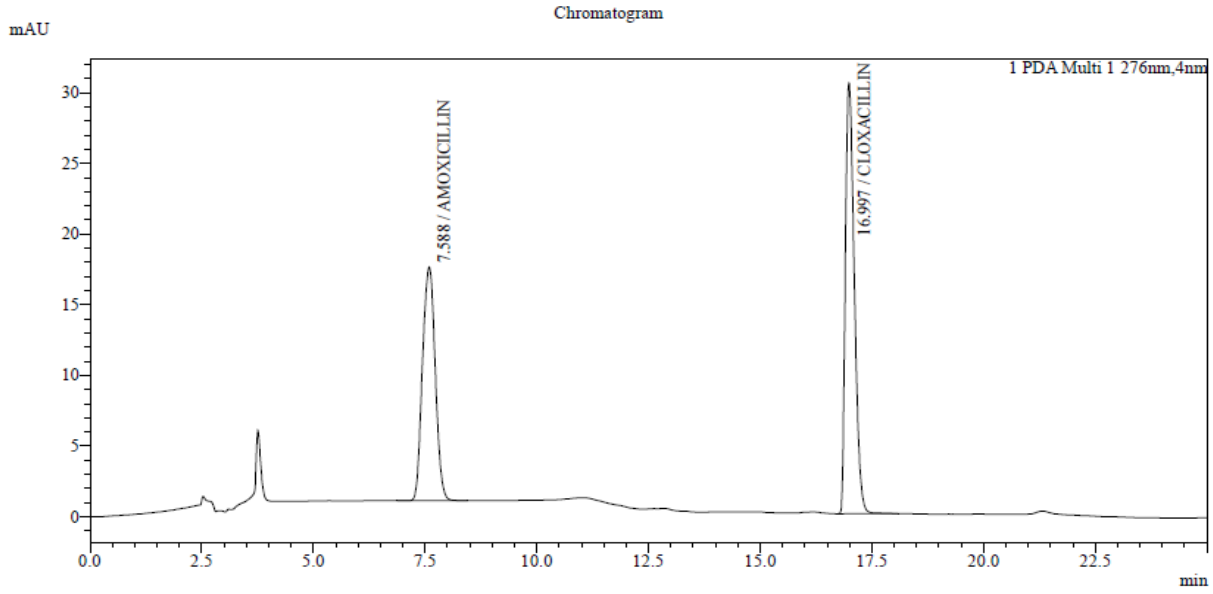


Figure 6: Accuracy-80%

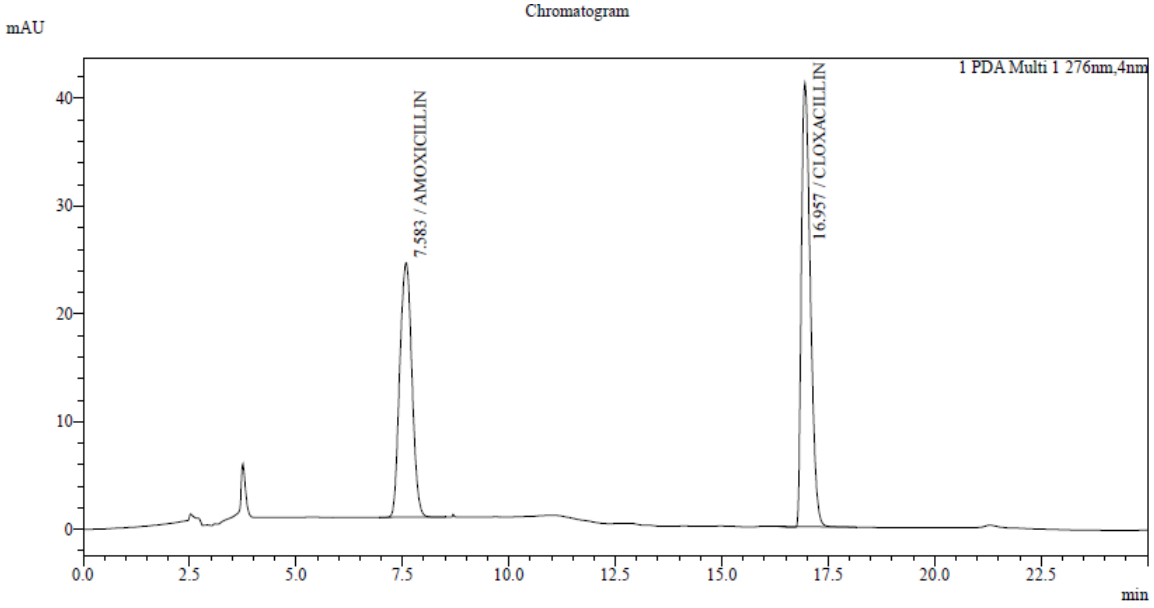


Figure 7: Accuracy-100%

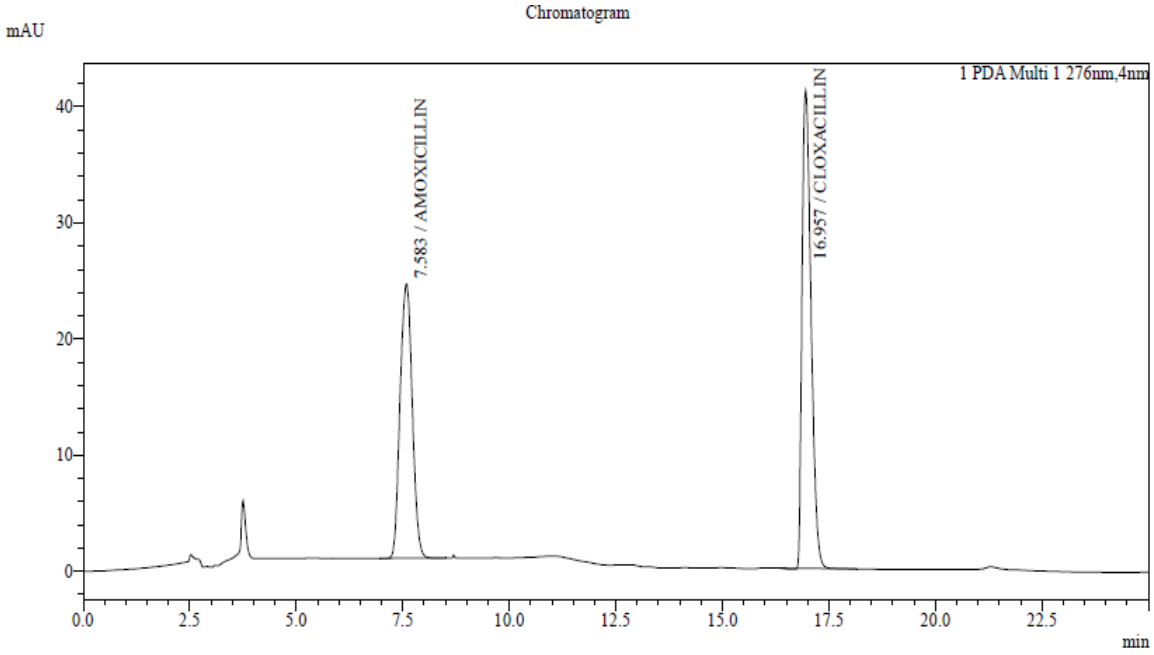


Figure 8: Accuracy-120%

Table 6: amoxicillin Data for accuracy

Amoxicillin				
Percentage concentration	**Percentage recovery**	**Mean recovey**	**SD**	**%RSD**
80%	100.12%	101.243%	1.2414	1.233
100%	101.00%			

120%	102.61%			

Table 7: cloxacillin Data for accuracy

Cloxacillin				
Percentage concentration	**Percentage recovery**	**Mean recovey**	**SD**	**%RSD**
80%	99.70%	%		
100%	100.66%			
120%	101.202%			

Precision:

Table 8: Data for precision

Amoxicillin					
Interday precision			Intraday precision		
Injection	Retention time	Area	Injection	Retention time	Area
1	7.571	475349	1	7.491	469800
2	7.576	474159	2	7.589	474516
3	7.576	474811	3	7.571	467498
4	7.704	472222	4	7.568	467657
5	7.710	470569	5	7.569	469775
6	7.720	471559	6	7.567	467855

	Average	SD	%RSD
Interday precision	473111.5	1931.72	0.408301485
Intraday precision	469516.33	2662.14	0.566994941

Table 9: Data for precision

Cloxacillin					
Interday precision			**Intraday precision**		
Injection	**Retention time**	**Area**	**Injection**	**Retention time**	**Area**
1	16.952	558576	1	16.987	550192
2	16.965	550893	2	17.002	554120
3	16.972	557510	3	17.002	543788
4	`7.080	550110	4	16.995	544371
5	17.082	548748	5	16.997	546329

6	17.087	550877	6	16.992	544641

	Average	SD	%RSD
Interday precision	552786.5	4159.76	0.75249665
Intraday precision	547240.11	4093.19	0.748013642

Robustness

Table 10: amoxicillin Data of robustness (change in flow rate)

Amoxicillin				
Flow rate	**Retention time**	**Area**	**Theoretical plate**	**Tailing factor**
0.9ml	8.545	545838	3166	1.058
1.1ml	7.706	485259	2891	1.067

Table 11: cloxacillin Data of robustness (change in flow rate)

Cloxacillin				
Flow rate	Retention time	Area	Theoretical plate	Tailing factor
0.9ml	18.7612	626371	11639	1.630
1.1ml	17.831	560881	12722	1.563

Ruggedness

Table 12: amoxicillin Data for ruggedness

Amoxicillin				
Analyst	**Average percentage**	**Average standard area**	**SD**	**%RSD**
Analyst-1	99.259	47413.333	814.81670	0.171852468
Analyst-2	98.560	475441.1667	917.54267	0.192987638

Table 13: cloxacillin Data for ruggedness

Cloxacillin				
Analyst	Average percentage	Average standard area	SD	%RSD
Analyst-1	101.600	546061	2694.0768	0..4933655

Analyst-2	100.480	546220	3836.7613	0.70242056

Established method greenness analysis:

Green assessment can be defined as the absence or minimal usage of hazardous chemicals, the elimination of waste, and the decrease of energy consumption the suggested method's greenness has been investigated by assigning penalty points to all of the specified parameter for the approach by applying a "Greenness evaluation metric of analytical methods (GEMAM)", after that given a score for every of the twelve green analytical chemistry elements as shown below in "Analytical GREEnness (AGREE)".

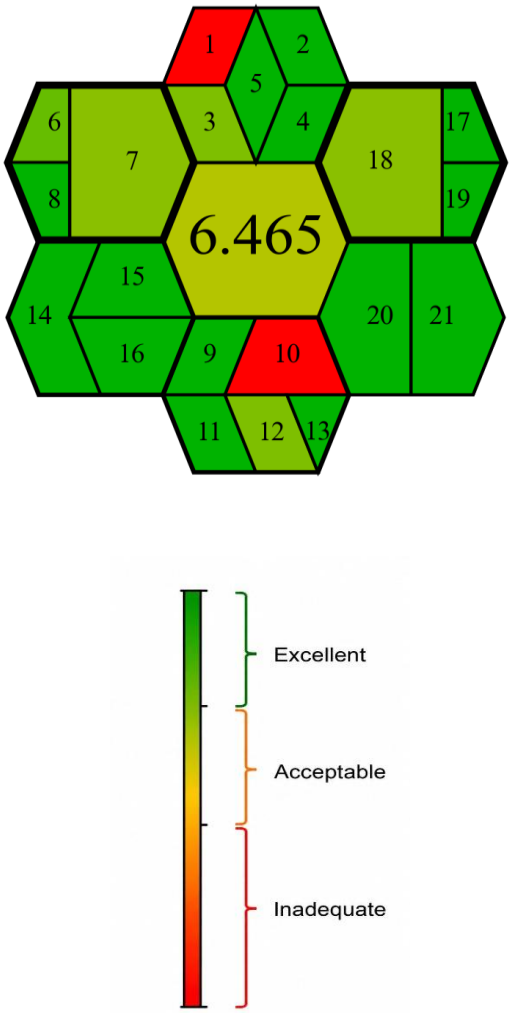


Figure 9: The general result of the greenness evaluation metric of analytical methods (GEMAM) assessment (left) and the corresponding colour scale for reference (right)

Table 14: Different sections & different criteria of GEMAM

Sections	Criteria
	1. Sample preparation site
	2. Whether the sample is damaged during sample preparation

sample	3. Range of extraction when sample preparation
	4. The size of sample
	5. Storage of sample
Reagent	6. Description of the ideal green derivation
	7. The amounts of reagents
	8. The score of reagents
Methods	9. Number of analytes determined in a single run (or analysis parameter)
	10. Sample throughput (per hour)
	11. Number of main steps in the analysis process
	12.Ratio of the mass of sustainable and renewable materials to the total mass of materials used
	13. Economic benefits of the methods
Instruments	14. The energy consumption per analysis should be minimized
	15. Automatic of instruments
	16. Miniaturization of instruments
	17. Waste treatment
Waste	18. The amounts of wastes
	19. The score of wastes
Operation	20. Hermetic sealing of analytical process
	21. Noise generation of analytical process

Figure 10: The general result of Analytical GREEnness (AGREE)

1. The sample procedure
2. Amount of sample
3. Positioning of analytical device
4. Degree of automation
5. Sample preparation
6. Derivatization agents
7. Amount of waste
8. Number of analytes determined in single run
9. Samples analyzed per hour
10. The most energy-intensive technique
11. Type of reagents
12. Operator's safety-threats not avoided in the method

CONCULUSION

The present study successfully developed and validated a simple, accurate, precise, robust and rugged **green RP-HPLC method for the simultaneous estimation of Amoxicillin and Cloxacillin** in bulk drug and capsule dosage form. The optimized

chromatographic conditions provided satisfactory separation of both drugs with good retention characteristics.

The developed method exhibited excellent linearity with a correlation coefficient of **0.9998** for both Amoxicillin and Cloxacillin. The assay results were satisfactory, with **98.93% for Amoxicillin and 100.78% for Cloxacillin**, accompanied by low %RSD values. The accuracy studies demonstrated satisfactory percentage recovery, while precision studies showed good reproducibility with low %RSD values.

The robustness and ruggedness studies confirmed that the method can withstand small deliberate changes in chromatographic conditions and variations between analysts without significant changes in the results. Therefore, the developed method is suitable for routine quantitative analysis of Amoxicillin and Cloxacillin in pharmaceutical dosage forms.

Furthermore, the method was evaluated using green analytical assessment approaches, including **GEMAM and AGREE**, to assess its environmental impact. The method aims to minimize solvent consumption, waste generation, energy consumption and exposure to hazardous chemicals while maintaining analytical performance. Thus produce the acceptable and excellent green analytical values.

Hence, the developed green RP-HPLC method can be considered a **reliable, efficient and environmentally conscious analytical method** for the simultaneous estimation of Amoxicillin and Cloxacillin in bulk and capsule dosage forms.

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