JOURNAL OF PHARMACEUTICAL ANALYSIS

ANALYTICAL METHOD DEVELOPMENT AND VALIDATION OF AMOXICILLIN AND CLOXACILLIN IN API AND ITS DOSAGE FORM BY RP-HPLC

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Abstract

A simple, accurate, precise, sensitive and stability indicating reverse phase high performance liquid chromatography method has been developed for simultaneous determination of Amoxicillin and Cloxacillin sodium in bulk and in combined capsule dosage form. Chromatographic separation was performed on C18 column, with mobile phase consist of water, acetonitrile, and methanol in the ratio of 70:20:10 (v/v/v), at flow rate 1.4 ml/min. Quantification of both drugs was achieved at UV detector at 238.8 nm. The retention time of Amoxicillin and Cloxacillin sodium was found to be 8.240 and 14.036 minute respectively with run time 20 minutes. Amoxicillin and Cloxacillin followed the linearity in concentration range 20-100 µg/ml for both with correlation coefficient (r2) values 0.998 for Amoxicillin and 0.998 for Cloxacillin sodium. The proposed method was validated according to ICH guidelines in terms of linearity, accuracy, precision, LOD and LOQ. Percentage assay was found to be 98.92 % and 99.61 % for Amoxicillin and Cloxacillin respectively. In precision % RSD was found to be < 2% for both. The percentage recovery was found in range 99.18%-99.42%. The LOD and LOQ values were within limit. The degradation studies carried under condition of acid, base, neutral, oxidative, photolysis, thermal degradation and no attempt was made to identify the degradation product.

Key words: Amoxicillin, Cloxacillin sodium, RP-HPLC, validation, stability indicating assay method.

1. INTRODUCTION

In pharmaceutical industries, the validation of analytical methods is used to demonstrate that the method is fitted for its purpose; it must follow a plan which includes scope, performance characteristics, and acceptance limits. Analytical methods need to be validated or revalidated prior to their introduction into routine analyses (release of batch). The overarching philosophy in current good manufacturing practices (cGMPs) of the twenty - first century and robust modern quality systems is the quality that it has to be built into the product, and testing alone cannot be relied to ensure the quality of the product. From the analytical perspective, it will

mean that analytical methods used to test products should have quality attributes built into them. In order to apply quality attributes into the analytical method, fundamental quality attributes have to be applied by the bench - level scientist. This is a paradigm shift that requires the bench - level scientist to have a scientific and technical understanding, product knowledge, process knowledge, and/or risk assessment ability to appropriately execute the quality functions of analytical method validation. In addition, it requires the following procedures: (a) an appropriate training of the bench - level scientist to understand the principles involved with method validation, validate an analytical method, and understand the principles involved with the method validation, (b) proper documentation and understanding and interpreting data, and (c) cross – an understanding functional of the effect of their activities on the product and to customers (the patient). Management has a responsibility of verifying that gained skills from the training are implemented in routine analyses performance. This chapter gives a review and strategy for the validation of analytical methods in-house, recommendation in documentation and completion of method validation in the pharmaceutical environmental.

Amoxicillin:

Amoxicillin is a common antibiotic that works well for a variety of bacterial illnesses and is a member of the penicillin class. It kills or stops the growth of susceptible bacteria by preventing the synthesis of bacterial cell walls. Amoxicillin is frequently recommended due to its adaptability, comparatively low side effect profile, and availability in a number of forms, such as capsules, tablets, and oral suspensions. It is frequently prescribed for ailments such as ear infections, sinusitis, respiratory tract infections, urinary tract infections, and skin infections. It is commonly used as a first-line treatment for infections such as streptococcal pharyngitis and several forms of pneumonia in both adults and children. It does not work against viral infections, either, and abuse or overuse can lead to antibiotic resistance.

Mechanism of Action:

Amoxicillin is a beta-lactam antibiotic belonging to the penicillin class. Its primary mechanism of action involves inhibiting bacterial cell wall synthesis, which is essential for bacterial growth and survival. Amoxicillin binds to penicillin-binding protein (PBPs) located inside the bacterial cell wall. Inhibition of peptidoglycan cross-linking: PBPs are enzyme involved in the final stage of synthesizing and cross-linking peptidoglycan, a key structural component of bacterial cell wall Amoxicillin inhibits the transpeptidation reaction catalyzed by PBPs, preventing proper cross-linking.

Cloxacillin

Cloxacillin is a beta-lactam antibiotic with a narrow spectrum, classified under the penicillin group, and is mainly used to treat bacterial infections caused by penicillinase-producing staphylococci, including Staphylococcus aureus. It functions by blocking the synthesis of bacterial cell walls, resulting in the destruction of bacterial cells, and is effective in treating infections such as skin and soft tissue infections, bone infections (osteomyelitis), and endocarditis caused by susceptible organisms. Cloxacillin, available in both oral and injectable formats, is well-absorbed when taken orally and is generally prescribed every 6 hours, with the dosage tailored to the severity of the infection and patient-specific factors such as kidney function.

Mechanism of Action:

The bacterial cell membrane's penicillin-binding proteins (PBPs), which are enzymes involved in the last phases of peptidoglycan synthesis—a crucial part of the bacterial cell wall—are where it attaches itself. In particular, cloxacillin stops peptidoglycan chains from cross-linking by blocking the transpeptidation pathway. Osmotic instability, bacterial cell lysis, and eventually cell death result from this weakening of the cell wall. Because beta-lactamase enzymes, which some bacteria create to render other penicillins inactive, cannot break down cloxacillin, it is especially efficient against staphylococci that manufacture penicillinase. It actively destroys vulnerable bacteria, mostly gram-positive ones like Staphylococcus aureus (with the exception of MRSA), due to its bactericidal activity.

2. Methods and Materials:

Instrumentation and reagents

Chromatographic separation was performed on HPLC-system (model Shimadzu SCL- 10) C18 column (250 mm x 4.6 mm, 5 µm) UV Detector, equipped with a solvent delivery pump, sample injector and column thermostats. Lab solution (Version 1.25) software was applied for data collecting and processing. Water, acetonitrile, and methanol used were of HPLC grade. Pure drug sample of Amoxicillin and Cloxacillin was procured from Macleods Ltd., Daman.

Chromatographic condition

Mobile phase: Water: acetonitrile: methanol (70:20:10 v/v/v)
Column : C18
Detector wavelength : 238.8 nm
Injection volume: 20 µl
Flow rate: 1.4 ml/min
Run time: 20 minutes

Selection of detection of Wavelength

Accurately weighed 10 mg Amoxicillin and Cloxacillin were transferred into 100 ml volumetric flask separately, dissolved in mobile phase, sonicated and filtered through Whatman filter paper No. 41 and volume was made up to the mark with mobile phase. Pipette out 2 ml of this stock solution and diluted to 10 ml to get a concentration of 20 µg/ml of Amoxicillin and Cloxacillin each. The λ max was determined on Shimadzu UV-visible spectrophotometer (Shimadzu model UV 1800) in the range 200-400 nm. The overlain spectra showed iso-absorptive point at 238.8 nm was selected as wavelength. The overlain spectra of Amoxicillin and Cloxacillin.

Preparation of standard solution

Standard solution was prepared by transferring 50 mg of Amoxicillin and 50 mg of Cloxacillin in 50 ml of volumetric flask separately and dissolved in mobile phase. It was sonicated for 20 min to dissolve completely, and then volume was made up to the mark with mobile phase to get concentration 1000 µg/ml for both. From the above stock solution pipette out 1 ml and transferred to 10 ml volumetric flask and volume was made up with mobile phase and 20 µl was injected into HPLC system to develop the chromatogram. Typical chromatogram of Amoxicillin and Cloxacillin standard.

System Suitability test

System suitability was performed by injecting standard solution and determines the various parameters such as theoretical plates, tailing factor, resolution.

Method Validation

Method was validated as per ICH guidelines.

Linearity

Linearity was performed at five different concentration (20, 40, 60, 80, 100 µg/ml) for both Amoxicillin and Cloxacillin. Standard calibration curve was plotted between peak area against concentration of drug and results of linearity.

Accuracy

The accuracy of the proposed method was performed by recovery studies at three different levels (80%, 100%, and 120%) of concentration by adding a known Amoxicillin of standard drug to pre-analyzed sample. Each determination was repeated three times at each level and injected into HPLC system. Results of accuracy.

Precision

Precision of an analytical method is usually expressed as the standard deviation or relative standard deviation. The precision was determined at different parameter like repeatability, intermediate precision (intra-day, inter-day). Repeatability was determined by analyzing Amoxicillin (100 µg/ml) Cloxacillin (100 µg/ml) three times. Intraday precision was determined by analyzing same concentration of solution for three times within day and Inter day precision was determined daily for three days. Then % RSD was calculated and it was within limit (less than 2%).

Limit of detection (LOD) and Limit of quantification (LOQ)

LOD is the lowest concentration of analyte in sample that can be detected but not necessarily quantified. LOQ is the lowest concentration of analyte in sample that can be quantitatively determined with precision and accuracy.

LOD and LOQ was calculated by using following formulae

LOD = 3.3 σ/slope,

LOQ = 10 σ/slope (Where σ = the standard deviation of the response and S = slope of calibration curve).

Robustness

The robustness was performed by assaying test solutions after slight but deliberate change in analytical conditions. The study was performed by changing the flow rate, temperature and wavelength.

Analysis of Marketed formulation

Twenty capsules weighed, average weight was determined, crushed to a fine powder and mixed thoroughly. Accurately weighed powder equivalent to 50 mg of AMOXICILLIN and 50 mg of CLO was transferred into 50 ml of volumetric flask and dissolved in mobile phase. This was sonicated for 20 minutes, and then volume was made up to mark with mobile phase. Further dilution was done with mobile phase to get final concentration of 100µg/ml of Amoxicillin and 100µg/ml of CLO. The standard and sample solution was injected into HPLC system to

develop the chromatogram. Typical chromatogram of Amoxicillin and CLO is shown in figure 5. The content of Amoxicillin and CLO was calculated by using following formula.

Amoxicillinunt of drug (mg) = At/As x Ds/Dt x Ws/Wt x A ……..…..(i)

% Estimation = At/As x Ws/Wt x Avg.wt (A)/Lable claim x 100 ……..(ii)

Where, At = Area count for sample solution

As = Area count for standard solution

Ds = Dilution factor for standard

Dt = Dilution factor for sample

Ws = Weight of standard (mg)

Wt = Weight of sample (mg)

A = Average weight of capsule

Forced Degradation Study

Stress degradation studies were performed to check the stability of the Amoxicillin and Cloxacillin on different conditions. The stress conditions for degradation study involved acid, base, neutral, oxidative, thermal, and photolytic degradation.

Acid degradation

Accurately weighed 20 mg of Amoxicillin and Cloxacillin was transferred to 100 ml volumetric flask, to it 20 ml mobile phase and 10 ml 0.1 N HCl was added. This flask was heated on water bath at 0 C for 4.30 hours. Solution was cooled and neutralized with 0.1 NaOH and volume was made up to mark with mobile phase, finally this solution was diluted with mobile phase to get concentration 20 µg/ml of Amoxicillin and 20 µg/ml of CLO. A 20 µl solution was injected into HPLC system and analyzed under chromatographic condition.

Base Degradation

Accurately weighed 20 mg of Amoxicillin and Cloxacillin was transferred to 100 ml volumetric flask, to it 20 ml mobile phase and10ml 0.1 N NaOH was added. This flask was heated on water bath at C for 4.30 hours. Solution was cooled and neutralized with 0.1 N HCl and volume was made up to mark with mobile phase. Finally this solution was diluted with mobile phase to get concentration 20 µg/ml of Amoxicillin and 20 µg/ml of Cloxacillin.A 20 µl solution was injected into HPLC system and analyzed under chromatographic condition.

Oxidative degradation

Accurately weighed 20 mg of Amoxicillin and Cloxacillin was transferred to 100 ml volumetric flask, to it 20 ml mobile phase and 10 ml 3% H2O2 was added. This flask was refluxed for 4 hours. Solution was cooled and volume was made up to mark with mobile phase, finally this solution was diluted with mobile phase to get concentration20 µg/ml of Amoxicillin and 20 µg/ml of Cloxacillin. A20 µl solution was injected into HPLC system and analyzed under chromatographic condition.

Photolytic degradation

Pure drugs were exposed to UV radiations for 12 hours. The sample after exposure to light were accurately weighed 20 mg of Amoxicillin and Cloxacillin was transferred to 100 ml volumetric flasks diluted with mobile phase to get concentration 20 µg/ml of Amoxicillin and 20 µg/ml of Cloxacillin O.A 20 µl solution was injected into HPLC system and analyzed under chromatographic condition.

Thermal degradation

Thermal degradation was carried out by exposing pure drugs to dry heat at 0 for 2 hours. The samples after exposure to heat were accurately weighed 20 mg of Amoxicillin and Cloxacillin, transferred to 100 ml volumetric flasks diluted with mobile phase to get AmoxicillIN 20 µg/ml and Cloxacillin 20 µg/ml. A20 µl solution was injected into HPLC system and analyzed under chromatographic condition.

Neutral hydrolysis

Accurately weighed 20 mg of Amoxicillin and Cloxacillin was transferred to 100 ml volumetric flask; to it 50 ml water was added. This flask was refluxed for 4 hours at 0, Solutions was cooled and volume was made up to mark with mobile phase. Finally this solution was diluted with mobile phase to get 20 µg /ml Amoxicillin and 20 µg/ml of Cloxacillin. A 20 µl solution was injected into HPLC system and analyzed under chromatographic condition.

3. Results and Dissusion:

Selection of detection wavelength

Standard solution of Amoxicillinxicillin and Cloxacillin sodium was prepared by using distilled water as a solvent. Then this solution was placed in UV and it was scanned at 200-400nm and the solutions shows λmax at 225nm so it was selected as wavelength.

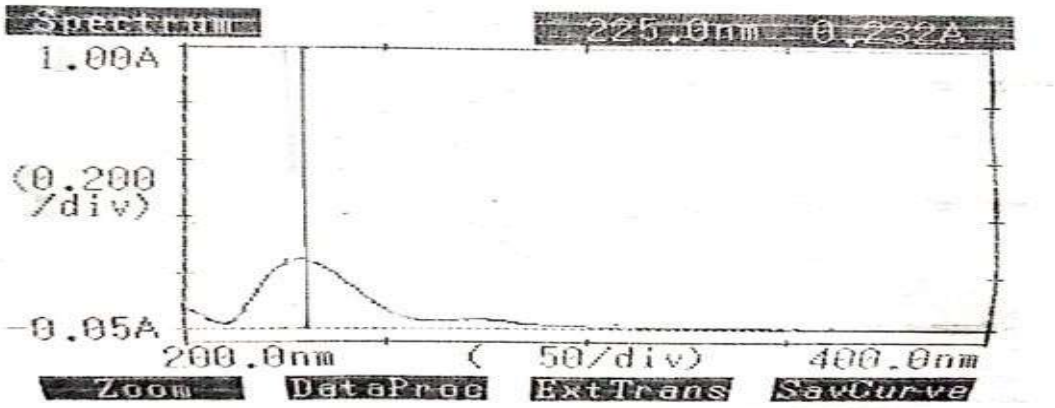


Figure 7.1: Selection of detection wavelength (10µg/mL)

Optimization of chromatographic conditions

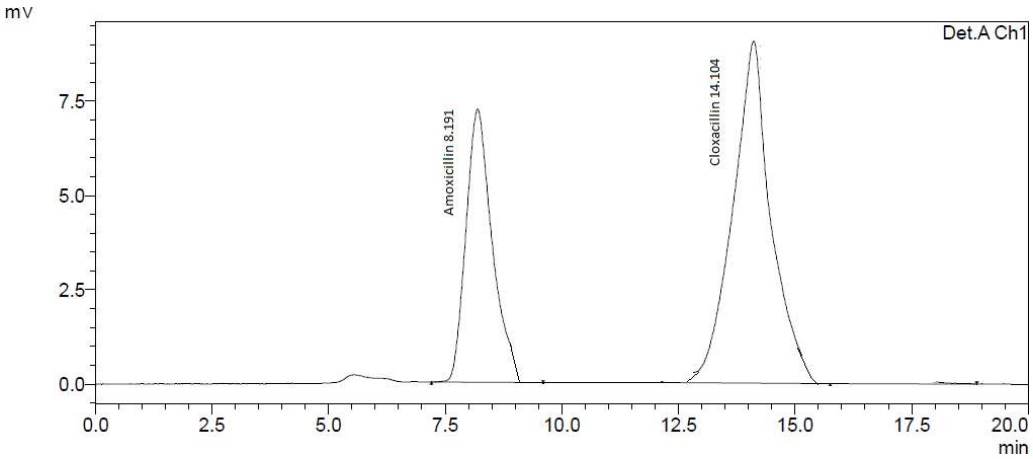
Various trials of columns and mobile phase compositions were performed at constant flow rate (1mL/min) and wavelength (225nm).

Various trials for optimization of chromatographic conditions

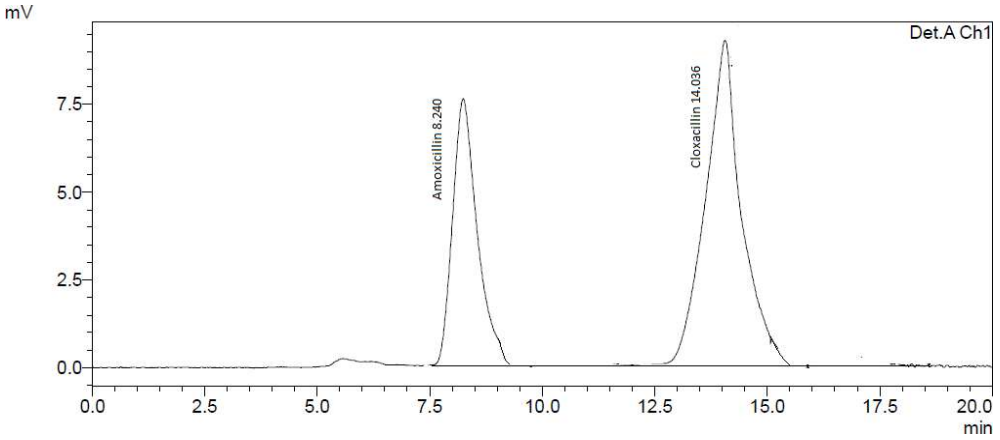
Trial No	Column	Mobile Phase	pH	Rt(min)	Result
1	Hypersil ODS, 100×4.6mm, 5µ	Buffer : Methanol (50:50)	3.5	5.09	Peak Merging

2	Hypersil ODS, 100×4.6mm, 5µ	Buffer : Acetonitrile (50:50)	3.5	1.81	Peak shape is not proper
3	X-bridge Shield RP18, 100×4.6mm, 3.5µ	Buffer : Acetonitrile (50:50)	3.5	1.23	Rt is too early
4	Inertsil ODS C18, 150mm×4.6mm, 5µ	Buffer : Acetonitrile (50:50)	3.5	4.76	Rt is too early
5	Inertsil ODS C18, 150mm×4.6mm, 5µ	Buffer : Acetonitrile (60:40)	3.5	9.95	Rt is not stable
6	Inertsil ODS C18, 150mm×4.6mm, 5µ	Buffer : Acetonitrile (60:40)	5	6.10	Early Rt
7	Inertsil ODS C18, 150mm×4.6mm, 5µ	Buffer : Acetonitrile (70:30)	5	9.99	Good peak shape

Various trails were performed on Inertsil column at flow rate 1mL/min and wavelength at 225nm. The system suitability parameters obtained for mentioned condition were within the range and it was suitable for further analysis.

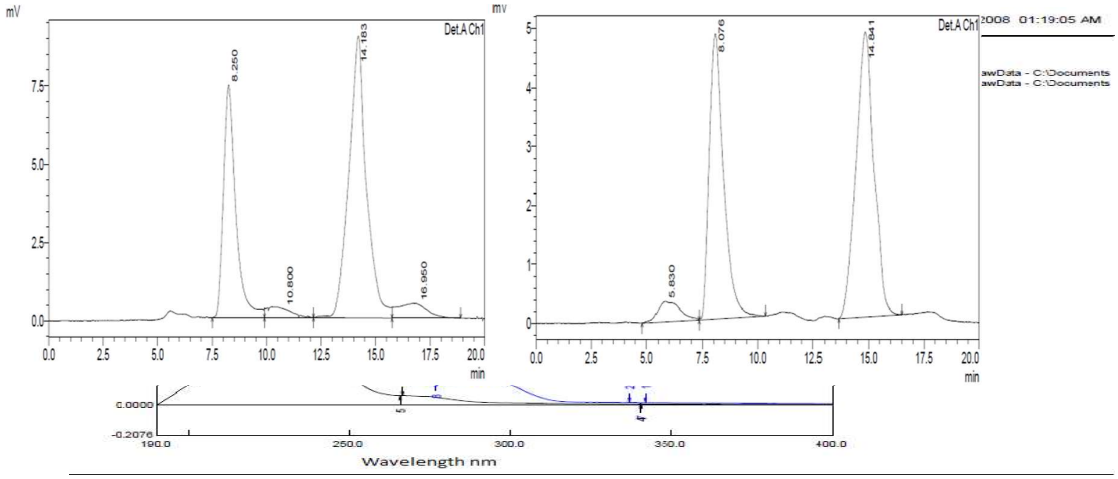


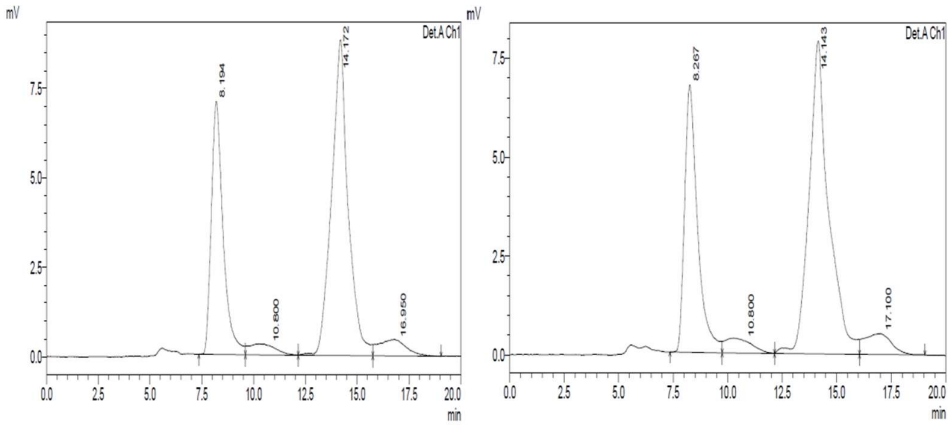
Trial 1- Chromatogram of Amoxicillin and Cloxacillin sodium (100µg/mL) Mobile phase – pH 3.5 Phosphate Buffer and Methane



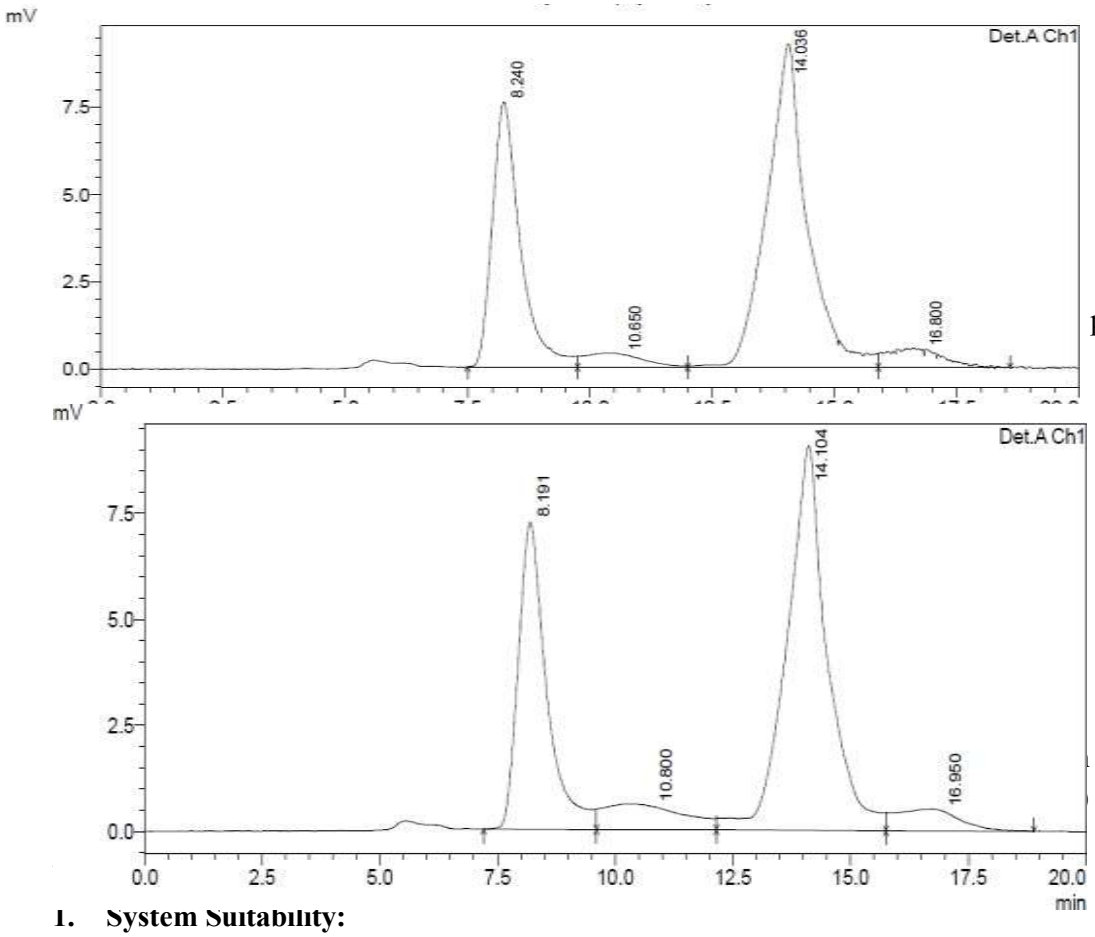
Trial 2- Chromatogram of Amoxicillin and Cloxacillin sodium (100µg/mL)

Trial 3 - Chromatogram of Amoxicillin and cloxacillin sodium (100µg/mL)





Trial 4 - Chromatogram of Amoxicillin and Cloxacillin sodium (100µg/mL)



phase –

1. System Suitability:

System Suitability Parameter

No. of Injection	Rt (min)	Area	Tailing factor	No. of theoretical plates
1	9.99	4120740	1.3	5064
2	9.98	4125182	1.4	5065
3	9.97	4110926	1.4	5060
4	9.98	4061319	1.3	5156
5	9.98	4083385	1.4	5114
Mean	9.98	4100310	1.4	5092
%RSD	0.07	0.7	0.98	0.82

Conclusion:

The system suitability parameters passes all the limits of %RSD, tailing factor and number of theoretical plates so it is suitable for use.

2. Specificity:

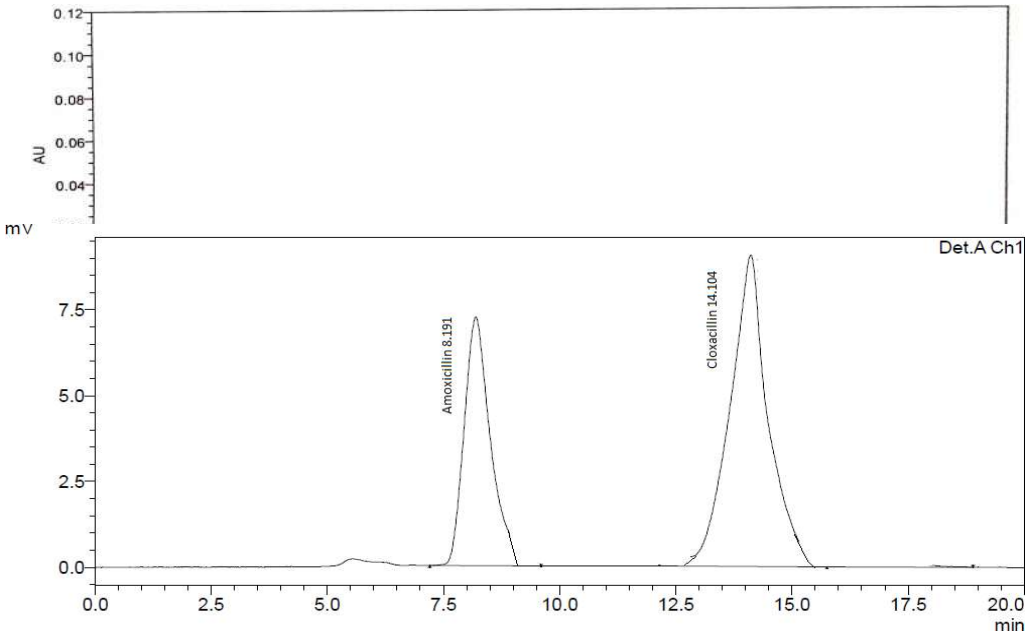


Fig 7.18: Blank solution chromatogram

Figure 7.19: Placebo chromatogram of Amoxicillin and Cloxacillin sodium

Conclusion:

There is no interference of any placebo and impurity peak at our main peak, so we can say that our method is specific.

3. Linearity and Range:

Table 7.6: Linearity concentration range with their area for Amoxicillin and Cloxacillin sodium

Linearity Level	**Conc. in µg/mL**	**Area**
Level 1	10	232513
Level 2	20	464300
Level 3	50	1157796
Level 4	80	1834828
Level 5	100	2288303
Level 6	120	2753031
Level 7	150	3428143

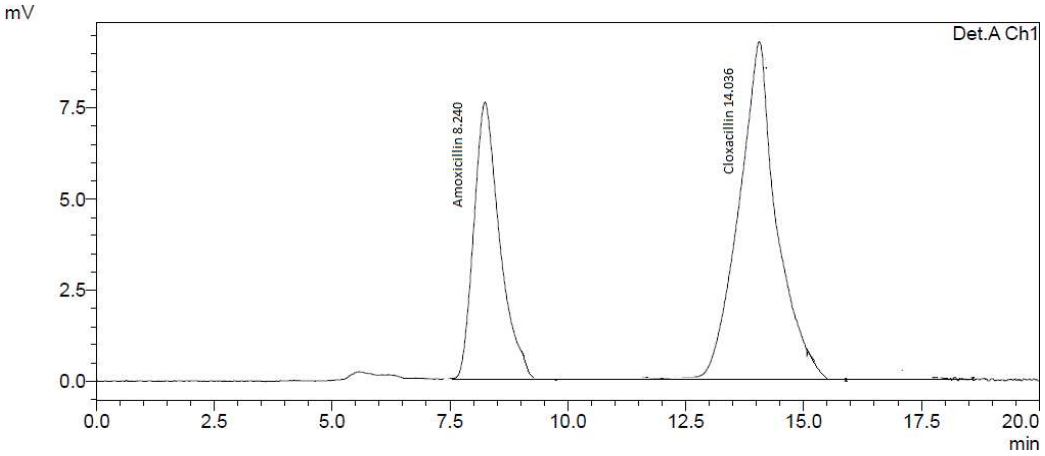


Figure 7.21: Linearity overlay chromatogram of Amoxicillin & Cloxacillin sodium at 225nm

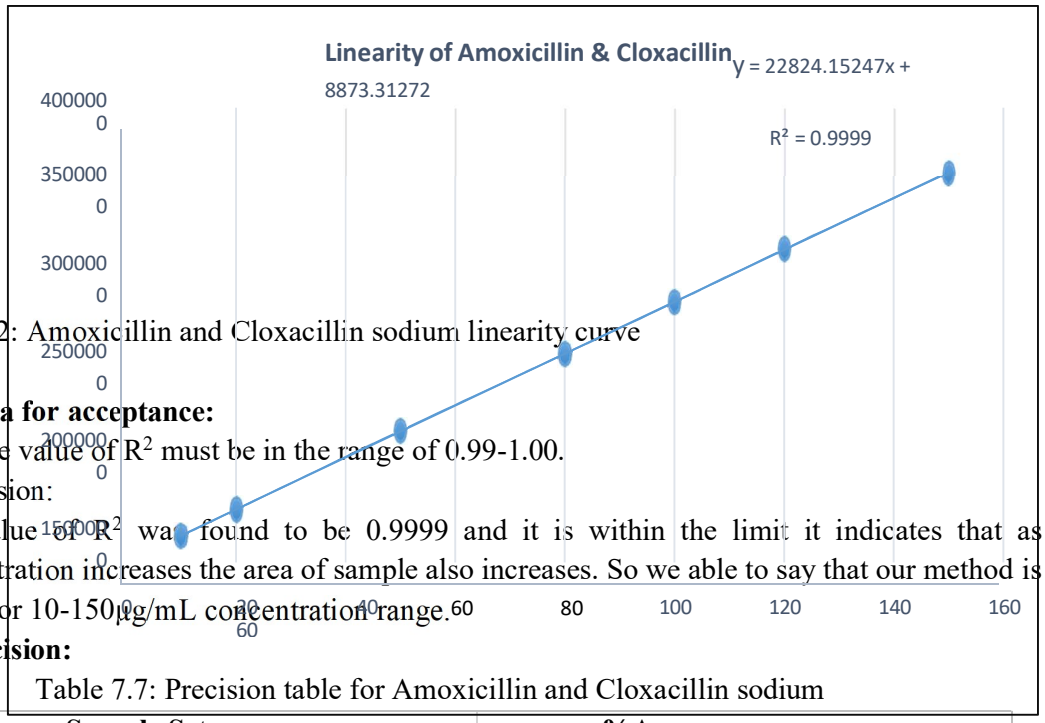


Fig 7.22: Amoxicillin and Cloxacillin sodium linearity curve

Criteria for acceptance:

- The value of R^2 must be in the range of 0.99-1.00.

Conclusion:

The value of R^2 was found to be 0.9999 and it is within the limit it indicates that as concentration increases the area of sample also increases. So we able to say that our method is linear for 10-150µg/mL concentration range.

4. Precision:

Table 7.7: Precision table for Amoxicillin and Cloxacillin sodium

Sample Sets	**%Assay**
Set-1	96.5
Set-2	96.1
Set-3	95.7
Set-4	96.2
Set-5	95.5
Set-6	95.5
Average	**95.92**
SD	**0.41**
%RSD	**0.43**

Conclusion:

➢ As per ICH guidelines, value of %RSD should be less than 2 and our observed RSD is also less than 2 so we can say that our method is precise.

5. Solution Stability:

Table 7.8: Standard Solution Stability table for Amoxicillin and Cloxacillin sodium

Standard Solution Stability			
Time (Hrs)	Standard Area	Difference	%Difference
Initial	2150 295	NA	NA
6	2155 741	5446	0.2 5
12	2166 942	1664 7	0.7 7
18	2180 724	3042 9	1.4 2
24	2187 742	3744 7	1.7 4
30	2204 414	5411 9	2.5 2
36	2211 471	6117 6	2.8 5
42	2224 827	7453 2	3.4 7
48	2239 451	8915 6	4.1 5

Table 7.9: Sample solution Stability table for Amoxicillin and Cloxacillin sodium

Sample Solution Stability			
Time (Hrs)	Sample Area	Difference	%Difference
Initial	2192 211	NA	NA
6	2189 360	2851	0.1 3
12	2192 523	312	0.0 1
18	2196 986	4775	0.2 2

24	2197 718	5507	0.2 5
30	2205 420	1320 9	0.6 0
36	2201 775	9564	0.4 4
42	2212 577	2036 6	0.9 3
48	2213 234	2102 3	0.9 6

Acceptance criteria:

- %difference should be less than 2.

Conclusion:

- The standard solution is stable up to 24hrs while sample solution is stable up to 48hrs.

6. Accuracy:

Table 7.10: Determination of Accuracy of Amoxicillin and Cloxacillin tablet

Level (%)	Amoxicillinunt of flucloxacillin sodium taken (mg)	Diluted up to in volumetric flask(mL)	Volume taken (mL)	Diluted up to in volumetric flask(mL)	Concentration (ppm)	Mean Recovery
50	2768.62	500	5	50	50	96.9
100	5536.95	500	5	50	100	97.6
150	8304.47	500	5	50	150	98.2

Acceptance criteria:

- %Recovery at each level should be 95-105% Conclusion:
- As per ICH guidelines %recovery should be 95-105% and observed %recovery is within the range so we can say that our method is precise.

7. Filter Compatibility:

Table 7.11: Determination of filter study of Amoxicillin and Cloxacillin sodium

Filter Study			
Name of filter	Average Area	Difference	%Difference
Centrifuge	2185502	NA	NA
Agilent Nylon 0.45µ 3mL discard	2187697	2195	0.10

Agilent Nylon 0.45µ 5mL discard	2190259	4757	0.22
Agilent PVDF 0.45µ 3mL discard	2210865	25363	1.16
Agilent PVDF 0.45µ 5mL discard	2197557	12055	0.55
Valuprep PVDF 0.45µ 3mL discard	2193846	8344	0.38
Valuprep PVDF 0.45µ 5mL discard	2189880	4378	0.20

Acceptance criteria:

%difference should be less than 2.

Conclusion:

So we can say that all types of 0.45µ filters can be used

8. Robustness:

Parameter	Factor	Mean area ± SD	%RSD
Buffer pH	pH 4.8	4410648 ± 25011	0.57
	pH 5.2	4283627 ± 64365	1.50
Flow rate (mL/min)	0.8	4375375 ± 19840	0.45
	1.2	4250483 ± 27184	0.64

Criteria for acceptance:

The percentage relative standard deviation should not be greater than 2.

Conclusion:

We can say that our method is robust as percentage relative standard deviation was found to be below 2%.DISCUSSION

Method Development

Selection of Detection wavelength

Bactericide and Larotid 10 mg were dissolved in the mobile component. The spectra of the solution were obtained and scanned from 200 to 400 nm. The wavelength for Larotid and the bactericide was selected using the overlay spectrum. The detecting wavelength was chosen to be the isobestic purpose.

Validation Parameters

Precision

Precision of the strategy was administered for each sample and the customary solutions as delineated beneath experimental work. The corresponding

chromatograms and results are shown below.

System Suitability

The system suitability of the strategy was checked by injecting five completely different preparations of the Larotid and its antibacterial effect. The parameters of system suitability were checked.

Intermediate Precession (Ruggedness)

At completely different levels of huskiness, such as day-to-day and system-to-system variation, there was no significant change in the assay content and system quality metrics.

Accuracy

Sample solutions at totally different concentrations (50%, 100% & 150%) were ready and also the recovery was calculated.

CONCLUSION

- Based on the data which are obtained we reach a conclusion that chromatographic method for Amoxicillin and Cloxacillin was successfully develop using Inertsil ODS C18 (150mm×4.6mm, 5µ) column, flow rate was 1mL/min, mobile phase was pH 5 Phosphate buffer: Acetonitrile (70:30 v/v).
- This method was developed and validated in accordance with the ICH guidelines.
- This proposed method was linear between 10-150µg/mL concentration range and having correlation coefficient value was 0.9999.
- The developed method was accurate, precise, specific, robust and in within the specifications limits as per the ICH guidelines.

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