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METHOD DEVELOPMENT AND VALIDATION OF AMOXICILLIN TRIHYDRATE BY HPTLC IN BULK AND PHARMACEUTICAL DOSAGE FORM

Kalyan Kumar Yadav, Jyoti Verma and Sanjay Kumar Kushwaha *

Bhavdiya Institute of Pharmaceutical Sciences and Research Sewar, Sohawal, Ayodhy - 224126, Uttar Pradesh, India.

Keywords:

Amoxicillin trihydrate, Method Development, Validation, HPTLC, HPLC

Correspondence to Author:
Dr. Sanjay Kumar Kushwaha

Director,
Bhavdiya Institute of Pharmaceutical Sciences and Research, Sewar, Sohawal, Ayodhy - 224126, Uttar Pradesh, India.

E-mail: sanjaykushwaha78927@rediffmail.com

ABSTRACT: Amoxicillin Trihydrate is an extensively used antibiotic, and the accurate determination of its content in bulk and pharmaceutical dosage forms is of greatest importance. This thesis focuses on the development and validation of a High-Performance Thin-Layer Chromatography (HPTLC) method for the analysis of Amoxicillin Trihydrate. The study begins with an inclusive review of the existing analytical methods for Amoxicillin Trihydrate, highlighting the advantages and limitations of each technique. HPTLC is selected as the method of choice due to its simplicity, cost-effectiveness, and potential for routine analysis in pharmaceutical laboratories. The Chromatographic separation was carried out on Merck, Germany, TLC plates of silica gel 60 $F_{254,}$ 10 cm x10 cm using n-Hexane: ethyl acetate in the ratio of 7:3 (% v/v) as a mobile phase followed by densitometric measurements at 230 nm. The following method was validated in terms of accuracy, precision, linearity, limit of detection, limit of quantification, and specificity according to the ICH (International Conference on Harmonization) guidelines. The calibration curve and results were found to be linear with regression equation of y = 1.2832x + 3341, between 2000 to 12000 ng/ band for Amoxicillin trihydrate respectively, with significantly high value of regression coefficient is (r^2) > 0.9936 with linear and homoscedastic residuals. The limit of quantification and limit of detection was found to be 1107.217 ng/band and 365.382 ng/ band respectively. The validated HPTLC method was successfully applied for the determination of Amoxicillin trihydrate in the commercial formulation.

INTRODUCTION: Amoxicillin Trihydrate is a widely used antibiotic belonging to the penicillin class of drugs. It is effective against various bacterial infections and is commonly prescribed in both outpatient and inpatient settings. Due to its wide-ranging spectrum of activity, low toxicity, and cost-effectiveness, Amoxicillin Trihydrate plays a critical role in the treatment of RTIs, UTIs, skin and tissue infections, and other common bacteriological infections [6].

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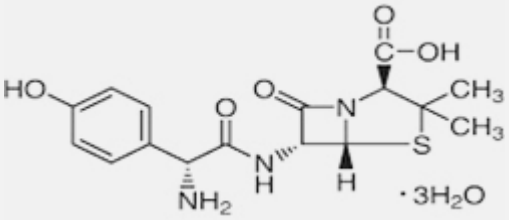
Accurate and reliable analysis of Amoxicillin Trihydrate is essential to ensure the quality and potency of pharmaceutical formulations. The finding of Amoxicillin Trihydrate in bulk ingredients and finished dosage forms is crucial for pharmaceutical manufacturers, regulatory authorities, and healthcare professionals.

Analytical methods that offer high sensitivity, selectivity, precision, and cost-effectiveness are in great demand to facilitate routine quality control and ensure the safety and efficacy of Amoxicillin Trihydrate-containing products [6]. Analytical chemistry is the branch of chemistry that focuses on the identification and quantification of substances and their properties [13]. It plays a critical role in various fields, including scientific research,

industry, environmental monitoring, and healthcare. The analytical chemist uses a variety of techniques and instruments to analyze samples, providing crucial information for decision-making and problem-solving [18].

Drug Profile:

TABLE 1: DRUG PROFILE OF AMOXICILLIN TRIHYDRATE

Name	Amoxicillin Trihydrate
Molecular Formula	$C_{16}H_{25}N_3O_8S$[24]
Molecular Weight	419.5 g/mol.[24]
Structure	
IUPAC Name	(2S,5R,6R)-6-[[(2R)-2-amino-2-(4-hydroxyphenyl) acetyl] amino]-3,3-dimethyl-7-oxo-4thia-1-azabicyclo [3.2.0] heptane-2-carboxylic acid; trihydrate[24]
Synonyms	Amoxicillin Trihydrate, BRL 2333, Wymox, Zimox, Larotid, Amoxil, Utimox, Alfamox Drg-0075, A-Gram, Dura AX
Drug Indication	Not recommended in conjunction with any beta-lactam medication that triggers a severe skin response or anaphylaxis (Stevens-Johnson syndrome). These reactions might be sensitive to cephalosporins or carbapenems cross-over.
Drug Absorption	Amoxicillin is approximately 60% bioavailable.8 A 250mg dose of oral amoxicillin reaches a Cmax 3.93±1.13mg/L with a Tmax 1.31±0.33h and an AUC of 27.29±4.72mg*h/L.4 A 875mg dose of oral amoxicillin reaches a Cmax 11.21±3.42mg/L with a Tmax 1.52±0.40h and an AUC of 55.04±12.68mg*h/L [4]
Mechanism of Action	Amoxicillin trihydrate competitively inhibits the penicillin-binding protein. Bacterial cell walls get cross-linked as a result of transpeptidase and glycosyltransferase processes that are triggered by penicillin-binding proteins.
Toxicity	Symptoms of an overdose include vomiting, diarrhea, rash, haematuria, oliguria, abdominal discomfort, and severe renal failure.
Protein binding	20%
Safety and Hazard	Danger
Pharmacodynamics	Penicillin-binding proteins are competitively inhibited by amoxicillin trihydrate.Its therapeutic spectrum is broad and its duration of action is prolonged [6]
Half-Life	About 62 minutes
Contraindication	Amoxicillin Trihydrate is used to treat certain type of susceptible bacterial infection of UTIs, Acute Gonorrhoea and Respiratory infection like Pneumonia etc. It also used various infections of ear, nose, throat and skin infections.

Chromatography: Chromatography is a technique used in mixture testing, refining, and separation. Chromatography is an analytical method that is frequently used to break down a mixture of chemical substances into its constituent parts so that each part may be properly examined. Although there are many different kinds of chromatography, such as gas, liquid, ion exchange, and affinity chromatography, they all use the same fundamental ideas [8].

TLC: Thin layer chromatography, or TLC, is a chromatography technique used to separate the constituent chemicals of a mixture into separate substances. There are two phases to this technique: a mobile phase and a contiguous fixed phase. By combining water, silica gel, and calcium sulphate, the stationary phase is prepared [8, 13].

HPTLC: High-Performance Thin Layer Chromatography, or HPTLC, is an enhanced and automated version of TLC. This is an effective analytical technique that may be applied to both qualitative and quantitative analytical tasks. Planer or flat-bed chromatography are other names for it [13].

Factors Influencing the TLC / HPTLC Separation and Resolution of Spots:

- Type of stationary phase [23]

- A layer of thickness /binder in the layer
- Mobile phase (solvent system)
- Size and saturation of the developing chamber
- Sample volume to be spotted
- The solvent level in the chamber
- Relative humidity
- Temperature (Rf values increase with rise in temperature)
- Separation distance

Importance of HPTLC: Sample and standard processing simultaneously demonstrates improved analytical precision and accuracy and reduces the requirement for internal standards.

✓ Multiple analysts are employed concurrently [15]

✓ Reduced maintenance costs

✓ Shorter analytical times and costs per analysis

MATERIAL AND METHOD:
Instruments:
Camag HPTLC system:

❖ Linomat- V applicator

❖ Camag TLC Scanner 3

❖ winCATS software V- 1.4.2

❖ Merck TLC plates precoated with silica gel 60 F_{254}

❖ Hamilton microlitre syringe

Double beam UV-visible spectrophotometer with single Monochromator (Shimadzu Model 1780):
Shimadzu Model AY-120 balance:
Calibrated Glassware was used for the study:
Chemicals and Reagents: A list of chemicals and reagents used in the study, along with their sources and specifications, is provided below:

Amoxicillin Trihydrate: High-quality Amoxicillin Trihydrate with a purity of ≥ 99% was obtained from Yarrow Chem Pharmaceuticals Mumbai. The specific grade and batch number of the compound were recorded**.**

Marketed formulation Details: Novamox – 250 Capsules Composition: Label claim: Each Capsule contains: Amoxycillin 250mg Manufactured by: Cipla Ltd

Methanol: HPLC grade methanol from Loba Chemicals was used as the organic solvent in the mobile phase. The methanol met the required purity standards and had a specified concentration of alcohol content.

Ethyl Acetate: HPLC-grade ethyl acetate obtained from Loba Chemicals was used as a component of the mobile phase. The ethyl acetate had a defined purity level and met the necessary specifications for use in analytical methods.

Acetic Acid: Analytical Research grade acetic acid from Loba Chemicals was used for pH adjustment of the mobile phase. The acetic acid was of sufficient purity and met the required standards for analytical use.

Silica Gel 60F Plates: High-quality Silica gel 60 F254 plates with dimensions of 10 cm × 10 cm and a thickness of 0.2 mm were obtained from E. Merck KGa Chromatography. The plates had a uniform and consistent layer of silica gel, ensuring reproducible separation.

Toluene: HPLC grade toluene from Loba Chemicals was used as a constituent of the mobile phase. The toluene met the necessary purity requirements and had a specified composition suitable for the HPTLC analysis.

Ammonia Solution: A 25% v/v ammonia solution obtained from Loba Chemicals was used as an additive in the mobile phase. The ammonia solution had a specified concentration and met the required quality standards.

Orthophosphoric Acid: Analytical grade orthophosphoric acid from Loba Chemicals was used for the preparation of the mobile phase. The orthophosphoric acid had a defined purity level and met the necessary specifications for analytical use.

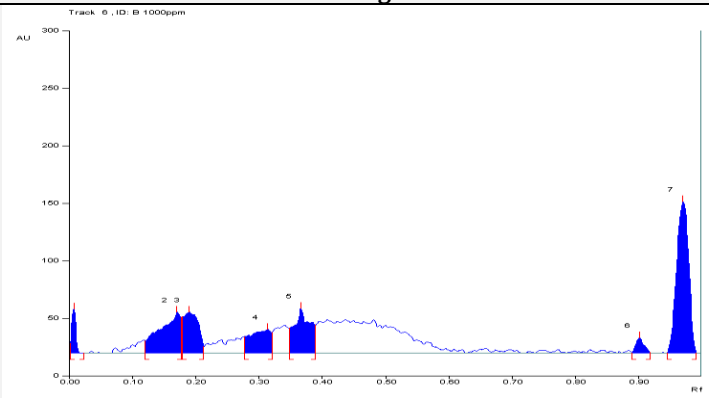
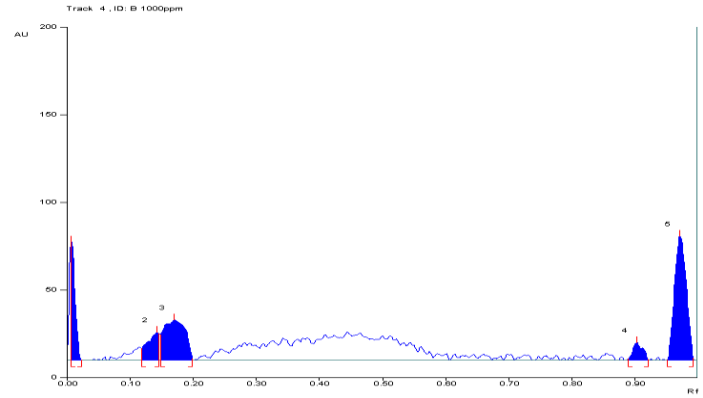
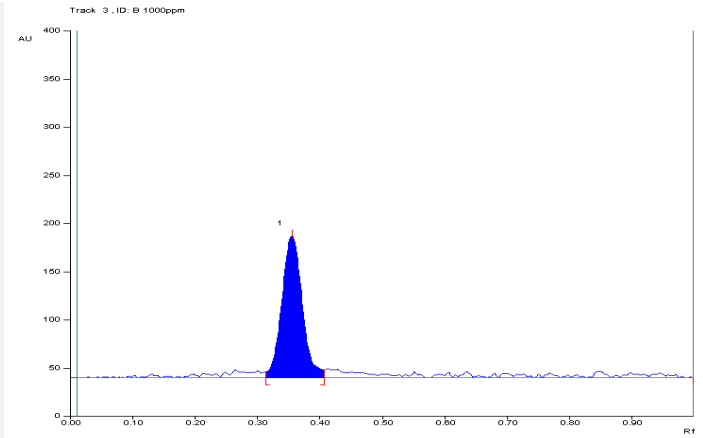
Deionized Water: High-quality deionized water was used for the preparation of solutions, dilution of samples, and cleaning of glassware. The water was obtained from a reliable source and underwent

appropriate purification processes to ensure its purity. The chemicals and reagents were carefully selected to ensure the accuracy and reliability of the analytical method.

METHOD DEVELOPMENT AND RESULT:

Trails for Amoxycillin Trihydrate:

TABLE 2: TRIALS OF MOBILE PHASE FOR AMOXYCILLIN TRIHYDRATE

Sr. no.	Mobile phase	Observations	Densitogram
1.	Chloroform: Methanol (6:4 v/v)	Peak moved to the solvent front	
2.	Chloroform: Methanol (9.5:0.5 v/v)	Peak moved to the solvent front	
3.	n-Hexane: Ethyl Acetate (7:3 v/v)	R_f – 0.36 with good peak shape, and acceptable peak parameters.	

Chromatographic Conditions and Mobile Phase Selection: Amoxycillin Trihydrate 1000 µg/ml working standard solution was used for chromatographic separation experiments. To get the right R1 and shape for the drug peak, tests were first conducted on standard TLC plates with different solvents in different ratios. Following a few trials, the mobile phase of n-Hexane: Ethyl Acetate (7:3 v/v) was selected because it produced acceptable peak characteristics. Other chromatographic parameters that were optimized were run length, sample application volume, and chamber saturation time. Using a 100 µL sample syringe (Hamilton, Bonaduz, Switzerland) and a CAMAG Linomat 5 sample applicator (Switzerland), the samples were spotted in the shape of 6 mm wide bands with 8 mm between each band on precoated silica gel aluminum plate

60 F254 (10 ×10) with 250 µm thickness (E. MERCK, Darmstadt, Germany). A slit measuring 6 mm by 0.45 mm was used, and the scanning speed was set to 20 mm/sec. Using a 10 cm x 10 cm twin trough glass chamber (CAMAG, Muttenz, Switzerland) as the mobile phase, a linear ascending development was conducted. For the mobile phase, a 15-minute chamber saturation period was ideal. The chromatogram run measured 8 cm in length, and the development period took roughly 15 minutes. Using a hair dryer, TLC plates were dried in an air current. For every development, densitometry scanning was carried out using a CAMAG thin-layer chromatographic scanner running WINCATS software version 1.4.2 at 230 nm. The radiation source used was a deuterium lamp, which continuously emitted UV light with a wavelength of 200–400 nm.

Making the Typical Stock Solution: The typical stock remedy to achieve a concentration of 1000µg/ml, 10 mg of the medication was dissolved in 10 milliliters of methanol to create amoxicillin trihydrate. The working standard solution, which contained 100µg/ml of amoxicillin trihydrate, was made from the standard stock solution.

Preparation of Sample Solution: Sample solution preparation involved emptying twenty capsules containing 250 mg of amoxicillin trihydrate (Noamox - 250, Cipla Ltd.) and weighing the powder. A 10 ml volumetric flask containing 10 mg of powder was filled with methanol to achieve a concentration of 1000 µg/ml before being subjected to a 10-minute sonication. The mixture was filtered. A 4000 ng/band concentration was obtained by applying 4 l of the resulting solution to a TLC plate.

Analytical Wavelength Selection: The spectra were acquired by making further dilutions of the standard stock solution using methanol and scanning it over the 200–400 nm range. The medication was found to exhibit significant absorbance at 230 nm.

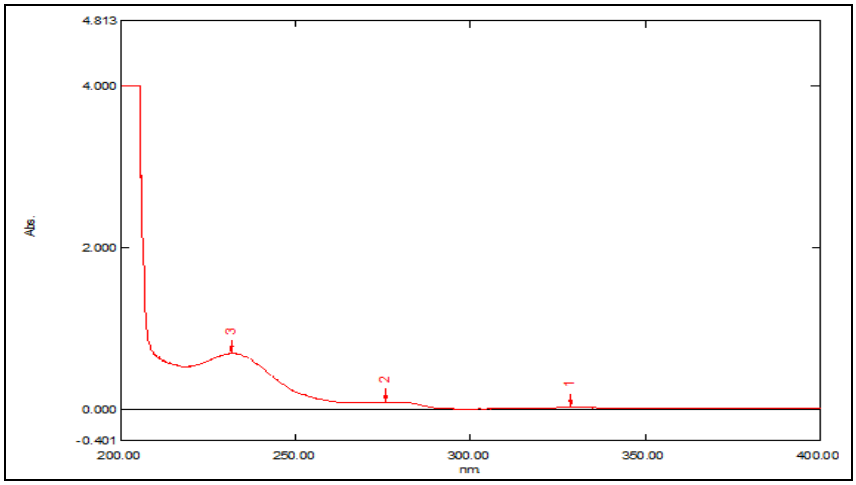


FIG. 1: UV SPECTRUM OF AMOXYCILLIN TRIHYDRATE (10µG/ML)

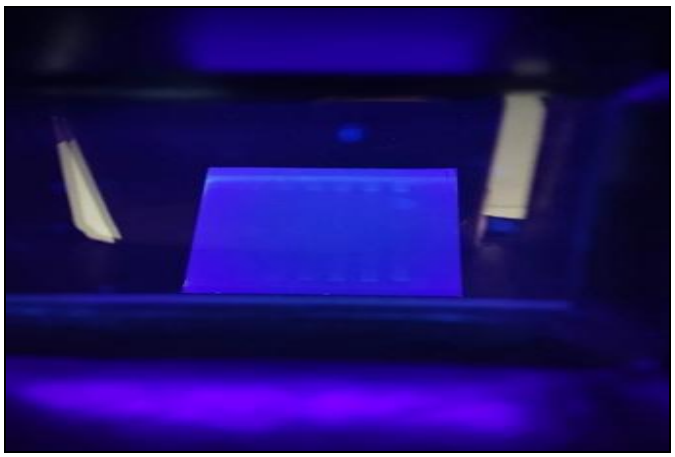


FIG. 2: DEVELOPED TLC PLATE

Densitogram and System Suitability Parameters of Drug: A 1000 µg/ml solution of amoxicillin trihydrate was made. On a pre-activated TLC plate, 4 µl (4000 ng/band) of the solution was applied using a Linomat 5 sample applicator and a Hamilton syringe (100 µl). For 15 minutes, the mobile phase was fully saturated in the development chamber. After being positioned in the saturated chamber, the spotted plate developed up to an 80 mm distance. After the plate was dry, it was scanned at 230 nm over an 80 mm distance. It was discovered that the retention factor was 0.36 ± 1.92.

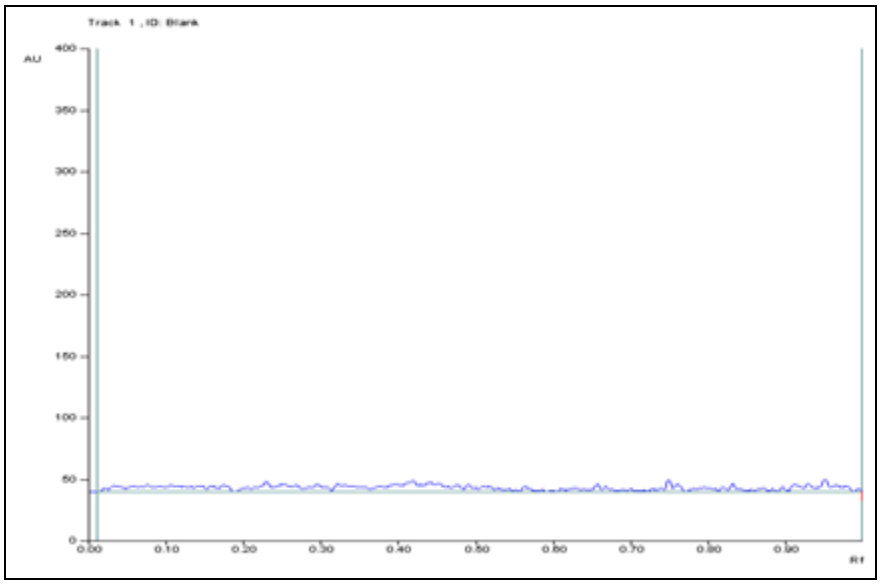


FIG. 3: DENSITOGRAM OF BLANK (METHANOL)

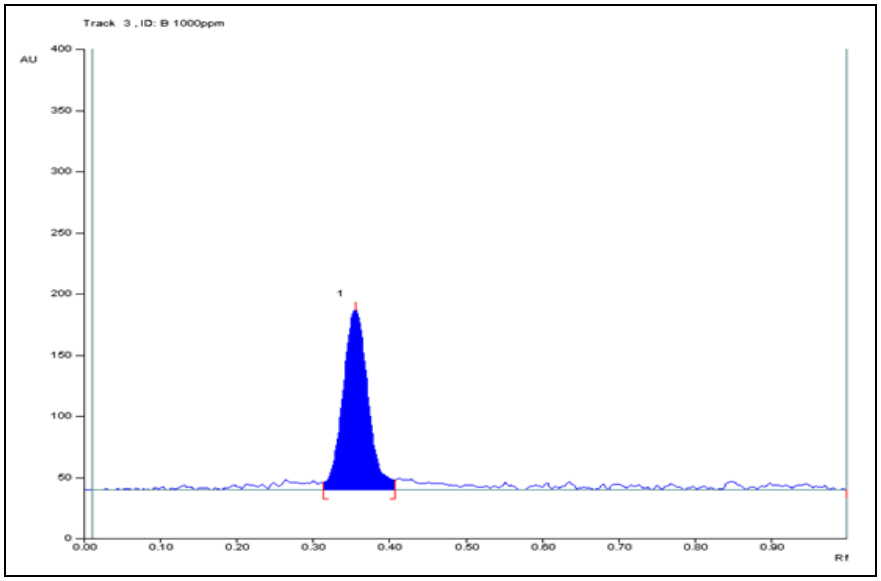


FIG. 4: DENSITOGRAM OF AMOXYCILLIN TRIHYDRATE STANDARD SOLUTION (4000 NG/BAND)

An overview of the chosen chromatographic parameters:

TABLE 3: CHROMATOGRAPHIC PARAMETERS

Sr. no.	Parameter	Conditions Used for Analysis
1	Stationary phase	TLC aluminium plate precoated with silica gel 60 F_{254}
2.	Mobile phase	n-Hexane: Ethyl Acetate (7:3 v/v)
3.	Detection Wavelength	230 nm
4.	Saturation time	15 min
5.	Bandwidth	6 mm
6.	Time of development	15 min

TABLE 4: SYSTEM SUITABILITY PARAMETER

Name	RT (Min) Mean ± % RSD	Concentration (µg/ml)	Area	Asymmetry
Amoxycillin Trihydrate	0.36 ± 1.92	4000	8871.6	1.06

Verification of the Analytical Process:

The Procedure was Verified by ICH Q2 (R1) Standards:

Specificity: The method's specificity was determined by peak purity profiling experiments [17, 19]. The peak purity values were determined to be greater than 0.996, indicating that no other peak of a degradation product or impurity interfered with them.

Linearity: Amoxycillin Trihydrate 1000 µg/ml solution yielded five replicates per concentration. Six concentrations of amoxicillin trihydrate were analzsed over the concentration range of 2000–12000 ng/band to determine the linearity (relationship between peak area and concentration).

To create the calibration curve, the peak regions were plotted against the relevant concentrations, as **Fig. 5** illustrates. According to the regression equation, y = 1.2832x + 3341, the findings are linear.

TABLE 5: LINEARITY STUDY OF AMOXYCILLIN TRIHYDRATE

Replicate	Concentrations of Amoxycillin Trihydrate (ng/band)					
	2000	4000	6000	8000	10000	12000
1	5749.2	8871.6	11008.5	13079.3	16222.9	19176.4
2	5525	9204.9	11028.5	13039.9	16226	19091
3	5574.7	9008.8	11418.1	12718.6	16950.1	18872
4	5776.1	8936.3	11216.6	13053.6	16130.7	19039.5
5	5722.2	8799.1	11258	13017.4	15548.3	18440
Average	5669.440	8964.140	11185.940	12981.760	16215.600	18923.780
SD	112.205	155.352	170.516	148.811	498.033	292.359
% RSD	1.979	1.733	1.524	1.146	3.071	1.545

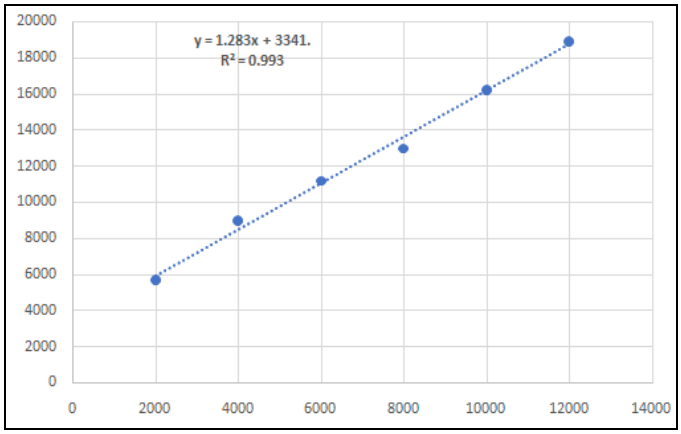


FIG. 5: CALIBRATION CURVE FOR AMOXYCILLIN TRIHYDRATE

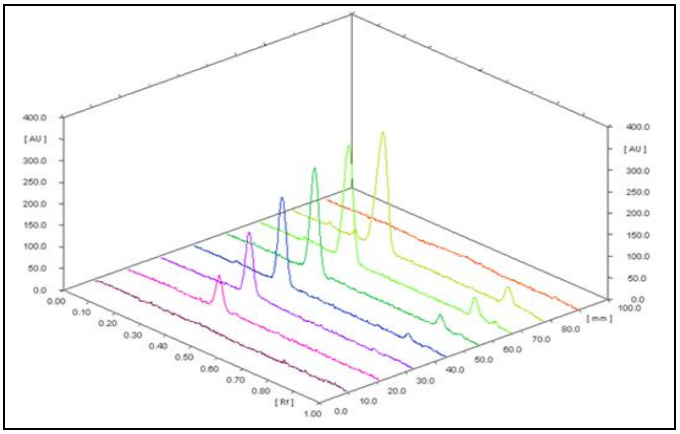


FIG. 6: DENSITOGRAM OF LINEARITY FOR AMOXYCILLIN TRIHYDRATE

Range: Amoxycillin Trihydrate = 2000 -12000 ng/band.

Precision: The exactness of the technique was proven by tests of fluctuation within and between days [19]. Three duplicates of each concentration were examined on the same day in the intra-day investigations, and the % RSD was computed. Three replicates of three concentrations were tested on three consecutive days for the interday variation studies and the percentage RSD was computed. The results for both intraday and interday precision are displayed in **Table 6.**

TABLE 6: INTRADAY AND INTERDAY VARIATION STUDIES DATA FOR AMOXYCILLIN TRIHYDRATE

Concentration (µg/ml)	Intra-day Precision			Inter-day Precision		
	Average area	% Recovery	Mean ±% RSD	Average area	% Recovery	Mean ± % RSD
	8549.1	101.461		8519.1	100.877	

4000	8536.3	101.212	101.896±0.958	8486.3	100.238	100.209± 0.681
	8628.8	103.014		8449.1	99.513	
	10988.5	99.325		10971.6	99.105	
6000	11066.4	100.336	99.589 ±	11087.8	100.614	99.725 ± 0.792
	10971.6	99.105	0.659	10998.5	99.454	
	13679.3	100.705		13618.6	100.114	
8000	13539.9	99.347	100.052±0.680	13553.6	99.481	99.810 ± 0.318
	13617.4	100.102		13589.9	99.834	

Limits of Quantification (LOQ) and Limit of Detection (LOD): LOD and LOQ are calculated from the formula:

$$LOD = 3.3\ \sigma / S$$

$$LOQ = 10 / S$$

Where, σ = standard deviation of Y-intercept

S = slope of the calibration curve.

LOD = 365.382 ng/ band

LOQ = 1107.217 ng/band

Assay of Marketed Sample: Novomox (250 mg) capsule formulation analysis was done according to the instructions in the section on sample solution preparation. The process was carried out six times. After applying a sample solution, the area was noted. The sample was selected with a base concentration of 4000 ng/band from the tablet solution. Using a linear equation, concentration and recovery percentage were calculated. **Table 7** displays the assay findings that were obtained.

TABLE 7: ASSAY OF MARKETED FORMULATION

S. no.	Peak Area	Amount Recovered (µg/ml)	% Recovery	Mean ± %RSD
1	8479.8	4004.442	100.111	
2	8535.1	4047.537	101.188	
3	8549.5	4058.759	101.469	100.764 ±1.042
4	8575.7	4079.177	101.979	
5	8514.2	4031.250	100.781	
6	8425.5	3962.126	99.053	

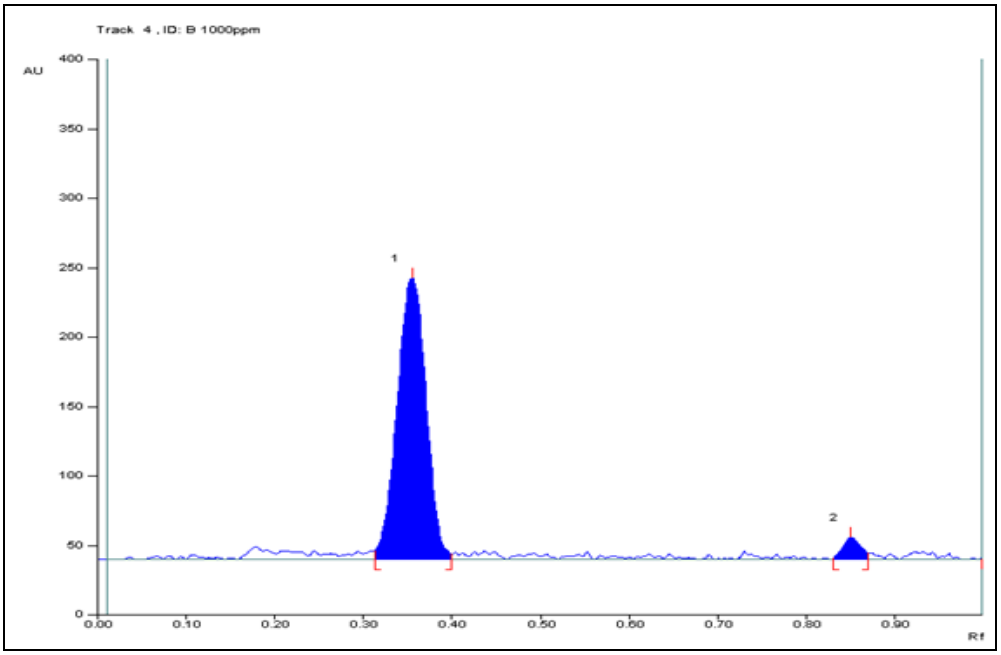


FIG. 7: REPRESENTATIVE DENSITOGRAM FROM SAMPLE ANALYSIS (FORMULATION ANALYSIS)

Precision: Recovery studies were conducted by spiking the standard medication into the tablet solution at three different concentrations: 50%, 100%, and 150%, to verify the correctness of the approach. The linearity equation was used to calculate the percentage of recovery. The sample's basic concentration was 4000ng/band. Table 8 displays the acquired results.

TABLE 8: RECOVERY STUDIES OF AMOXYCILLIN TRIHYDRAT

Level	Amount of sample taken (ng/band)	Amount standard spiked (ng/band)	Area	% Recovery	Mean ±% RSD
50 %	4000	2000	10986.1	99.293	99.489
			11066.8	100.342	±0.777
			10950.6	98.832	
100 %	4000	4000	13607.6	100.007	99.794 ±
			13516.4	99.118	0.600
			13633.4	100.258	
150 %	4000	6000	16046.4	99.011	99.334
			16089.9	99.350	±0.317
			16127.2	99.641	

Robustness: By doing the study in an environment where the scanning wavelength was changed, the robustness of the method was assessed. The impact on the area was recorded when the time was switched from spotting to development and development to scanning. The approach was proven to be robust. **Table 9** displays the acquired results.

TABLE 9: ROBUSTNESS STUDY

Sr. no.	Parameters	Variation	Concentration (ng/band)	%RSD
1.	Scanning wavelength	230± 1 nm	4000	1.391-1.909
			8000	0.427-0.695
			12000	1.624-1.993
2.	Duration between application and development	(0, 30, 60 min.)	4000	1.235-1.951
			8000	0.427-1.183
			12000	1.993-2.009
3.	Duration between development and scanning	(0, 30, 60 min.)	4000	1.391-1.774
			8000	0.427-0.937
			12000	1.369-1.993

TABLE 10: SUMMARY OF VALIDATION PARAMETERS

Sr. no.	Parameter	Amoxycillin Trihydrate
1	Linearity	$y = 1.2832x + 3341$; $R^2 = 0.9936$
2	Range	2000 – 12000 ng / band
	Precision	%RSD
3	Intraday	0.659 – 0.958
	Interday	0.318 – 0.792
4	% Assay	100.764 ±1.042
	Accuracy	Mean ±% RSD
	50%	99.489 ±0.777
5	100%	99.794 ± 0.600
	150%	99.334 ± 0.317
6	LOD	365.382 ng/ band
7	LOQ	1107.217 ng/band
8	Specificity	Specific
9	Robustness	Robust

CONCLUSION AND DISCUSSION: The present study focused on the development and validation of an HPTLC method for the quantification of Amoxicillin trihydrate in bulk and pharmaceutical dosage forms. The successful development of this analytical method is crucial for ensuring the accurate determination of this widely used antibiotic. Throughout the method development process, parameters such as mobile phase composition, plate pre-washing, and sample application were systematically optimized to achieve the best separation, resolution, and sensitivity for Amoxicillin trihydrate. The selection of an appropriate mobile phase consisting of solvents with different polarities played a critical role in achieving optimal separation and peak resolution. The finalized method demonstrated clear, well-defined peaks with adequate retention and separation of the analyte from potential interfering substances. Validation of the developed method was carried out following ICH guidelines, ensuring its reliability, accuracy, precision,

linearity, and robustness. The obtained validation parameters, including linearity within the specified range, precision (% RSD), accuracy (% recovery), and robustness, validated the suitability of the developed HPTLC method for the quantification of Amoxicillin trihydrate in both bulk and pharmaceutical formulations. The method exhibited excellent linearity over the concentration range studied, showcasing its ability to precisely quantify varying amounts of the drug.

The application of the validated method to pharmaceutical dosage forms confirmed its applicability for routine analysis, as evidenced by the consistency and accuracy of results obtained from commercially available formulations. Moreover, the method's robustness was confirmed by deliberate variations in method parameters, affirming its reliability even under minor experimental fluctuations. In conclusion, the developed and validated HPTLC method offers a rapid, precise, and reliable approach for the quantification of Amoxicillin trihydrate in bulk and pharmaceutical formulations. Its successful application in pharmaceutical analysis indicates its potential for routine quality control assessments, ensuring the potency and quality of Amoxicillin-containing products in the market.

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CONFLICTS OF INTEREST: The authors declare no conflict of interest.

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