Heliyon 9 (2023) e22891

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Development and validation of a high-performance thin layer chromatography method for the simultaneous determination of amoxicillin and clavulanic acid combinations in tablet dosage forms

Esubalew Asres [a,b], Thomas Layloff [c], Ayenew Ashenef [a,d,*]

[a] *Department of Pharmaceutical Chemistry and Pharmacognosy, School of Pharmacy, College of Health Sciences, Addis Ababa University, P.O. Box. 1176, Addis Ababa, Ethiopia*

[b] *Department of Pharmaceutical Chemistry, School of Pharmacy, College of Medicine and Health Sciences, Mizan-Tepi University, P.O. Box 260, Mizan, Ethiopia*

[c] *Consultant, P O Box 286, Granite City, IL, 62040-0286, USA*

[d] *Center for Innovative Drug Development and Therapeutic Trials for Africa (CDT-Africa), College of Health Sciences, Addis Ababa University, P.o. Box. 9086, Addis Ababa, Ethiopia*

ARTICLE INFO

Keywords:
Amoxicillin
Clavulanic acid
High-performance thin-layer chromatography (HPTLC)
Analytical method development
Validation

ABSTRACT

A simple and sensitive high-performance thin layer chromatography (HPTLC) method was developed and validated as per the International Council for Harmonization (ICH) guidelines for the simultaneous determination of amoxicillin (AMX) and clavulanic acid (CLA) combinations in tablet formulations. Chromatography was performed on precoated glass plates with normal phase silica gel 60 F_{254}. The mobile phase was acetone:ethyl acetate:glacial acetic acid:water (11:9:4:2 (v/v)). The plate was scanned at a wavelength of 428 nm after derivatization with ninhydrin. The validation of the method revealed that the linearity range lies between 400 and 1200 ng/band for AMX and 100–300 ng/band for CLA, with coefficients of determination of 0.9997 and 0.9966, respectively. Recoveries in standard addition accuracy studies were 100.3 % for AMX and 96.75 % for CLA. The limit of detection (LOD) and limit of quantitation (LOQ) of the developed method are 20.3 ng/band and 61.6 ng/band for AMX and 18.5 ng/band and 56.2 ng/band for CLA, respectively. The new, novel high-performance thin layer chromatography (HPTLC) method that was successfully developed in this study was applied for the simultaneous determination of AMX and CLA in their fixed-dose tablet dosage forms obtained from retail pharmacies and offered comparable results with the official British Pharmacopoeial high-performance liquid chromatography (HPLC) method.

* Corresponding author.Department of Pharmaceutical Chemistry and Pharmacognosy, School of Pharmacy, College of Health Sciences, Addis Ababa University, P.O. Box. 1176, Addis Ababa, Ethiopia.

E-mail address: ayenew.ashenef@aau.edu.et (A. Ashenef).

https://doi.org/10.1016/j.heliyon.2023.e22891
Received 9 September 2023; Received in revised form 21 November 2023; Accepted 22 November 2023
Available online 28 November 2023

1. Introduction

Amoxicillin (AMX) is a penicillin [1] in the β-lactam class of antibiotics with an extended spectrum of antimicrobial activity and a similar structure to ampicillin [2]. It is a derivative of 6-aminopenicillanic acid (APA). AMX differs from other penicillin families by the acyl radical connected with the 6-APA amino group [1]. AMX trihydrate has a molecular formula of $C_{16}H_{19}N_3O_5S\bullet 3H_2O$ (Fig. 1) and a molecular weight of 419.45 g/mol [3]. It is acid stable and [4] exists as a highly hygroscopic white or slightly off-white powder [5].

Clavulanic acid (CLA) is a naturally occurring β-lactam ring isolated from ***Streptomyces clavuligerus***. It has a molecular formula of $C_8H_9NO_5$ and a molecular weight of 198 g/mol (Fig. 1) [3]. CLA is usually available and formulated as potassium salt ($C_8H_8KNO_5$) in pharmaceutical preparations [8,9]. It is also known as a powerful and irreversible inhibitor as a "suicide inhibitor", covalently bonding itself to a serine residue in the active site of the β-lactamase. It is used in combination with penicillins such as AMX and ampicillin to reverse resistance [10,11].

When AMX trihydrate is administered alone, it is not effective against certain bacterial genera (e.g., ***Klebsiella*** and ***Proteus***) that produce β-lactamase enzymes [1,6]. Therefore, the activity spectrum of AMX is extended by co-formulating it with CLA. It reverses resistance to AMX in β-lactamase-producing strains of species otherwise sensitive [7].

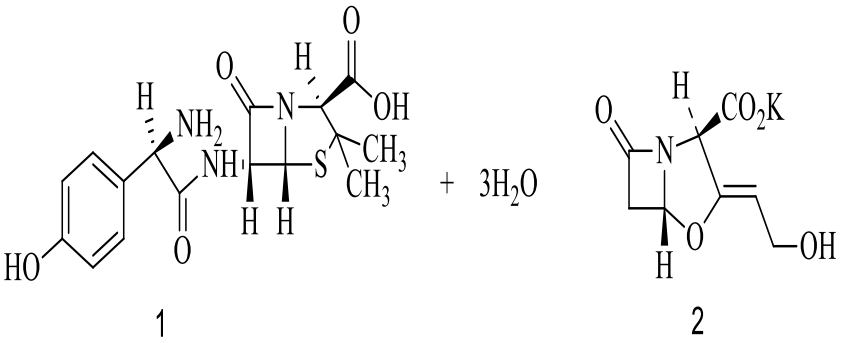
The Essential Medicine List of World Health Organization (WHO) put AMX and CLA combination as access group antibiotics [12]. The same list in Ethiopia includes 625 mg AMX trihydrate and potassium clavulanate combination to be used as an antibacterial agent. It is indicated for respiratory tract infections, genito-urinary and abdominal infections, cellulites, animal bites, severe dental infections and surgical prophylaxis [13]. There are various types of dosage forms of AMX trihydrate and potassium CLA combinations to be used for the management of the above diseases.

AMX and CLA are highly sensitive to moisture and temperature conditions. As CLA is hygroscopic and thermolabile, the storage conditions should be continuously maintained at 15–24 °C and 20 % relative humidity (RH) [14].

The synthetic production methods for AMX at industrial scale involves either chemical synthesis or enzymatic synthesis from the key intermediate 6-APA (6-aminopencillnoic acid that is obtained from Penicillin G). The conventional methods (using Dane salt for chemically obtaining AMX) typically involve more than 10 steps, require low-reaction temperatures (−30 °C), and use toxic solvents like methylene chloride and sialylation reagents. It is reported that the production of 1 kg of amoxicillin generates up to about 70 kg of non-recyclable waste. In contrast, enzymatic methods require far fewer steps, use milder reaction conditions, and generate less waste. The latter approach is being implemented for industrial production. CLA is produced by the bacterium ***Streptomyces clavuligerus***, using glyceraldehyde-3-phosphate and L-arginine as starting materials by the clavam pathway [15,16].

Slight variation in composition or in the purity of active pharmaceutical ingredient (API) content can affect the therapeutic outcome and may cause undesired effects of medicines. Therefore, there is a need to develop improved analytical methods for the pharmaceutical analysis of medicines. There are several methods for the simultaneous determination of AMX and CLA combinations, including the ultraviolet visible spectrophotometry (UV) method [17], high-performance liquid chromatography (HPLC) method with amperometric detection [18], chemiluminescence using least squares support vector regression [6] and capillary zone electrophoresis [10,19] in pharmaceutical preparations. HPLC–electrospray ionization (ESI) mass spectrometry [8], isocratic reversed-phase HPLC using UV detection [20] and HPLC with UV detection [21] are also used for the simultaneous determination of AMX and CLA combinations in human plasma for therapeutic drug monitoring and other purposes. HPLC is the official method for the determination of AMX and CLA combinations in different dosage formulations per the United States Pharmacopoeia (USP) and British Pharmacopoeia (BP) [22].

Due to low operating costs and high sample throughput, HPTLC is rapidly becoming a routine analytical technique. Using a small quantity of mobile phase, several samples can be run simultaneously [3]. The use of modern apparatuses, such as video scanners, densitometers and new chromatographic chambers, has led to more effective elution techniques and high-resolution sorbents [23]. For the sake of benefits from the abovementioned advantages, this study is aimed at developing a HPTLC-based method for the simultaneous determination of AMX and CLA combination formulations. Besides to our knowledge, a published HPTLC based method for the simultaneous determination of amoxicillin and clavulanic acid combination formulations did not exist yet. To confirm the applicability of the HPTLC methodology, it was further compared with the British Pharmacopoeia HPLC approach, which is the official method used in Ethiopia and many other countries around the globe.



1 2

Fig. 1. Chemical structure of 1-Amoxicillin trihydrate [6] and 2- Potassium Clavulanate [7].

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

Reference standards of AMX in trihydrate form and CLA lithium were from the United States Pharmacopoeia, Maryland, USA. Four brands of AMX trihydrate plus CLA potassium, Clavamyn® (Batch number K55321002, Kopran, India), Syntoclav® (Batch number N0604, Codal-synto, Cyprus), Moxiclav® (Batch number P5F015, Medochemie, Cyprus) and Klamoks® (Batch number 21181035A, Bilim, Turkey) tablets were purchased from pharmacy outlets in Addis Ababa, Ethiopia. Each of them contained 500 mg AMX trihydrate and 125 mg potassium clavulanate per the label claim.

2.1.2. Solvents and reagents

Deionized water was used as a major solvent for the preparation of standard and sample solutions in the HPTLC and HPLC methods. Analytical grade ethyl acetate (Carlo Erba, France), glacial acetic acid (Carlo Erba, France) and acetone (Mumbai-India) with water were used for the preparation of the mobile phase of the newly developed HPTLC method. Ninhydrin (GPHF ™, Germany) and ethanol (Wasse-Ethiopia) were used for the preparation of 0.018 % w/v solution employed as derivatizing reagents.

2.1.3. Instrumentation and HPTLC conditions

HPTLC operation instructions were commanded on computer winCATS version 1.4.0 software (Camag, Switzerland). Sample application was performed by Camag Linomat 5 using a 100 μL syringe (Hamilton, Switzerland). It was performed with a stream of nitrogen gas with a dosage speed of 50 nL/s and a predosage volume of 0.2 μL at a band length of 6 mm in the form of bands. The plates used were precoated with silica gel 60 F_{254} HPTLC (Merck, Germany). The plate size was 20 cm × 10 cm with 200 μm thickness (Batch No. HX71850142 and HX389048) and 20 × 20 cm (Batch No. HX99790429, HX398477 and HX86626259). A saturation pad (Camag, Switzerland) was used for saturating a 20 cm × 10 cm twin trough developing chamber (Camag, Switzerland). The thin layer chromatography (TLC) development time was 30 min.

Chromatographic development was performed in ascending way, and the solvent migration moved up to 7 cm in the presaturated chamber. A hair drier was used to dry the developed plate. Then, the dried chromatogram plate was immersed in derivatizing reagent (0.18 mg/mL ninhydrin solution in ethanol). After drying for 5 min, the derivatized chromatogram was heated at 110 °C for 10 min in a drying oven (Gallankamp, England). Then, densitometric scanning was adjusted with a scanning speed of 20 mm/s, and quantitative evaluation was performed. For the assay of commercial brands of AMX trihydrate and potassium clavulanate combinations, the developed HPTLC method was applied.

Other instruments and equipment used during the study included an analytical balance (Mettler Toledo-XP-205, Switzerland), mortar and pestle, pH meter (H12550 Jenway, U.K.), sonicator (PCI, Germany), micropipettes (Dragon lab, China) of 200–1000 μL and 5–50 μL, pipettes (DIN, Germany) of 1 mL (±0.01), 5 mL (±0.05), and 10 mL (±0.1), and volumetric flasks of 200 mL, 100 mL, 50 mL, 25 mL and 10 mL (class A, England). Whatman filter paper number-1 (Germany LOT No. 105065) was also used during the study.

2.1.4. Preparation of standard solution

The solutions were prepared by dissolving 25 mg AMX trihydrate reference standard (RS) and 6.25 mg clavulanate lithium RS. The reference standard was dissolved with 20 mL of water in a 50 mL volumetric flask, sonicated for 2 min and then filled to the label. The concentration prepared was 0.5 mg/mL AMX RS and 0.125 mg/mL clavulanate lithium RS stock solution. Then, 20 mL of stock solution was transferred to a 50 mL volumetric flask and diluted to 50 mL with water:ethanol (60:40 v/v). The final concentration was 0.05 mg/mL clavulanate potassium or 0.2 mg/mL AMX trihydrate.

2.2. Method development and optimization

2.2.1. Selection of the mobile phase

The selection of the mobile phase composition was based on a literature review, the chemistry of the analytes, the stationary phase and solvents, and trial and error that applies to normal phase stationary plate method development.

2.2.2. Derivatization

Derivatizing reagent was prepared by dissolving ninhydrin in ethanol (96.6 % v/v) to a 0.018 % w/v concentration. After the derivatizing reagent was added into an immersing device, the chromatogram plate was dipped into the solution [24]. Then, after derivatization was achieved, the solvent was allowed to evaporate, and the plate was heated in a dry oven at 110 °C for 10 min.

2.2.3. Selection of the scanning wavelength

Scanning wavelengths at 220 nm were reported in the USP [25] and BP [26] HPLC methods. By taking this wavelength as a starting point, spots were selected, which gave peaks. Then, the assigned spots were submitted to a full spectral scan. Finally, $\lambda_{max,}$ which gave the maximum response, was selected from the spectral pattern of spots that was used for the analysis of drugs.

2.2.4. Validation of the developed method

2.2.4.1. Specificity. The specificity of the method was determined by analyzing standard and drug samples. The spots for AMX and CLA in the samples were confirmed by comparing the R_F and spectrum of the spot with that of a standard [27]. The peak purity of AMX and CLA was determined by comparing the spectrum at three different regions of the spot, i.e., peak start, peak apex, and peak end.

2.2.4.2. Linearity. The mixed standard stock solution (1 mg/mL AMX and 0.25 mg/mL CLA) was further diluted to obtain concentrations of 0.2 mg/mL AMX and 0.05 mg/mL CLA. From diluted mixed standard solution, 2–6 μL aliquots were applied on the HPTLC plate in the form of band length at 6 mm to deposit drug amounts of 400, 600, 800, 1000 and 1200 ng/band for AMX and 100, 150, 200, 250 and 300 ng/band for CLA [28]. The peak response was plotted against the corresponding amount of AMX and CLA in ng/band to obtain the calibration plots. Then, the coefficient of determination (r^2), slope and y-intercept were determined from a linear regression equation [29].

2.2.4.3. Limit of detection and quantification. The LOD and LOQ were calculated based on the standard deviation (S.D.) of the response and the slope of the calibration curve of linearity [29,30].

$$\text{LOD} = \frac{3.3 \text{ x S. D of the response}}{\text{Slope of calibration curve}} \quad (1)$$

$$\text{LOQ} = \frac{10 \text{ x S. D of the response}}{\text{Slope of calibration curve}} \quad (2)$$

2.2.4.4. Precision. Precision is demonstrated by intraday (repeatability) and interday variation (intermediate precision) [29]. Repeatability was performed by analysis of three different levels at 80 %, 100 % and 120 % working concentrations of the two drugs three times on the same day. At similar concentration levels, i.e., at 80 %, 100 % and 120 %, intermediate precision was evaluated over a period of 3 consecutive days [27]. Sample application system precisions were performed six times in triplicate. After plate development and derivatization, densitometric scanning was performed six times at a wavelength of 428 nm at the same position to determine the scanning system precision. Relative standard deviation (RSD, %) was used to express the precision of the method.

2.2.4.5. Accuracy. The known amount of mixture of AMX trihydrate and clavulanate lithium RS related to three levels (80 %, 100 % and 120 %) of the working concentration was prepared (standard addition technique). These three levels were added to the samples. At each level of the amount, three determinations were performed, and the results obtained were compared with expected results.

2.2.4.6. Robustness. For the robustness study, experimental runs were suggested by Design-Expert software (Design-Expert (DE) version 13, Stat-Ease Inc., Minneapolis, USA). A full factorial experimental design for different factors (Table 1) and a fractional factorial experimental design for mobile phase composition (Table 2) with two central points were applied. Based on the suggested order of runs, experiments were performed, and responses were measured (Table 1). Then, mathematical models were generated by analysis of variance (ANOVA), and later, the criticality level of each of the factors was analyzed. By recording the effect of small deliberate changes in chromatographic conditions (chamber saturation time (CST) 30 ± 5 min, solvent migration distance (SMD) 70 ± 1 mm, mobile phase volume (MPV) 70 ± 1 mL and mobile phase composition (acetone, ethyl acetate, glacial acetic acid and water) ±

Table 1
Execution of the Box–Behnken experimental design and its responses for the robustness study of proposed HPTLC method for the quantitative estimation of AMX and CLA for different factors.

Std	Run	Different factors			Response			
		MPV (ml)	CST (minus)	SMD (mm)	AMX		CLA	
					R_F	Peak Area	R_F	Peak Area
8	1	21	35	7.1	0.37	2395.6	0.56	751.1
1	2	19	25	6.9	0.35	2412.5	0.57	787.8
10	*3	20	30	7	0.35	2419.2	0.54	782.4
4	4	21	35	6.9	0.36	2688.3	0.56	846.5
7	5	19	35	7.1	0.37	2669.7	0.55	862.3
3	6	19	35	6.9	0.36	2766.0	0.53	835.7
9	*7	20	30	7	0.36	2499.3	0.55	811.9
2	8	21	25	6.9	0.36	2688.3	0.52	817.9
5	9	19	25	7.1	0.36	2567.8	0.53	857.9
6	10	21	25	7.1	0.37	2669.9	0.55	831.5

NB: * At optimum level.
CST: chamber saturation time, MPV: Mobile phase volume SMD: Solvent movement distance.

Table 2
Execution of the Box–Behnken experimental design and its responses for the robustness study of proposed HPTLC method for the quantitative estimation of AMX and CLA for factor of mobile phase composition.

Std	Run	Mobile phase composition				Response			
		AC (ml)	EA (ml)	GAA (ml)	W (ml)	AMX		CLA	
						R_F	Peak Area	R_F	Peak Area
4	1	8.6	7	3	1.4	0.35	2375.8	0.54	764.1
10	*2	8.5	6.9	3.1	1.5	0.36	2359.0	0.54	791.4
5	3	8.4	6.8	3.2	1.6	0.37	2349.5	0.55	787.3
3	4	8.4	7	3	1.6	0.36	2341.0	0.54	786.6
9	*5	8.5	6.9	3.1	1.5	0.35	2399.2	0.56	758.9
1	6	8.4	6.8	3	1.4	0.36	2412.5	0.56	787.8
8	7	8.6	7	3.2	1.6	0.37	2395.6	0.56	751.1
7	8	8.4	7	3.2	1.4	0.37	2372.6	0.55	793.9
2	9	8.6	6.8	3	1.6	0.37	2379.8	0.53	789.2
6	10	8.6	6.8	3.2	1.4	0.35	2376.7	0.53	783.4

NB: * At optimum level.
AC -acetone, EA-ethyl acetate, GAA -glacial acetic acid, W-water.

0.1 mL for each) on R_F and the peak area of the response, the robustness of the method was determined. Correlations between factors and responses were also shown for the 3D-surface response (Figs. 5 and 6).

2.2.5. Sample stability study

A stability study of the prepared sample solution was conducted for a week. Analysis began 30 min after sample preparation and then continued after 1 h, 8 h, 12 h, 24 h and 7 days.

2.2.6. Analysis of commercial tablet dosage forms

Four brands of AMX trihydrate and clavulanate potassium tablet formulations (Clavamayin®, Syntoclav®, Moxiclav® and Klamoks®) were analyzed by using the developed HPTLC method and official HPLC method based on the BP-2019 monograph. To compare the two methods, the F test for variance was calculated by using Microsoft Excel (Excel Office 2019).

3. Results and discussion

3.1. Method development and optimization

3.1.1. Solvent

Water was selected for the preparation of the sample and standard solution. However, drying of the sample band after sample application takes a long time. Gas removal from microsyringes trapped during sample filling was also difficult. This challenge was alleviated by adding ethanol [31]. This decreases the viscosity of the solvent solution and increases the evaporation speed of the solvent (decreases the sample drying time and more easily removes the gas trap from the microsyringe) from the bands. The peak response of AMX and CLA solution prepared in water: ethanol (60:40 v/v) was sharper than the peak response of AMX and CLA solution prepared by water only. Hence, the presence of ethanol decreases the migration of solvent and analyte from the center to the periphery. Thus, in this way, band broadening was reduced.

3.1.2. Stationary phase

Most thin-layer chromatography has been performed using sorbents without chemically modified surfaces. Therefore, nonmodified glass plates, i.e., normal phase precoated silica gel 60 F_{254} glass plates with a HPTLC plate layer thickness of 200 μm, were used throughout these experiments [24].

3.1.3. Mobile phase selection

In this study, several solvent compositions were tested based on a literature review, chemistry, and trial and error [32]. Selection of the optimum mobile phase that gave excellent separation between the studied drugs with sharp symmetric nontailed peaks was determined. After alternating adjustments, the mobile phase composition acetone: glacial acetic acid: ethyl acetate: water (11:4:9:2 v/v) was found to be a suitable mobile phase composition to give sharp and well-separated peaks of the two drugs (Fig. 4).

3.1.4. Post development derivatization

With postdevelopment derivatization, all standards and samples can react simultaneously under the same conditions without affecting the solutes' ability to separate [24,32]. For the mobile phase optimization, postdevelopment derivatization was performed with different concentrations. By comparing densitometric scanning results, 0.18 mg/mL ninhydrin in ethanol (0.018 % w/v) was selected as the optimized reagent concentration. The chromatogramme comparisons before and after derivatizations had been shown in Fig. 2.

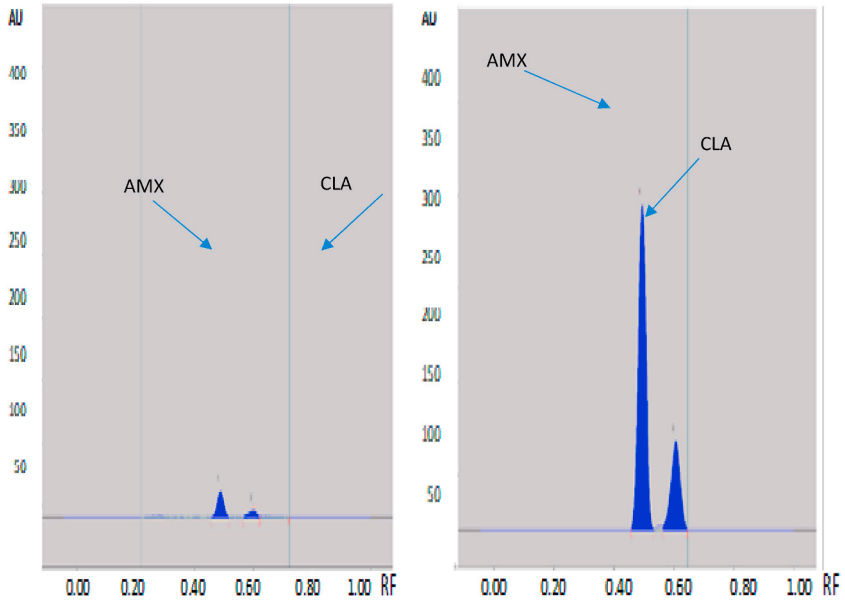


Fig. 2. Densitogram of AMX and CLA, A- At 227 nm before derivatized, B–At 428 nm after derivatized with 0.18 mg/mL ninhydrin in ethanol solution.

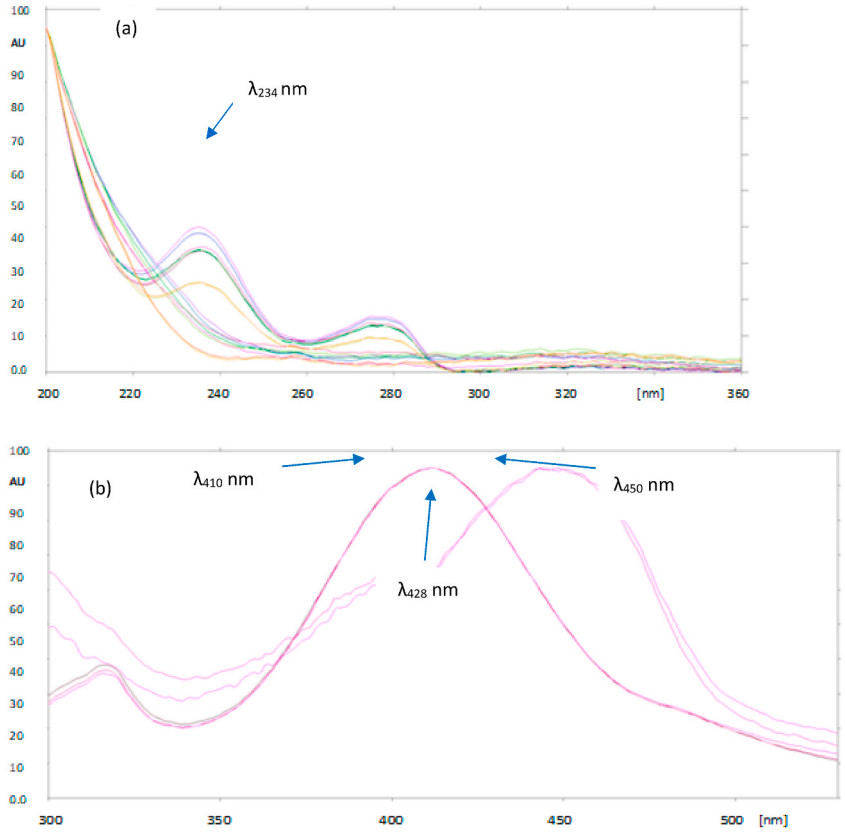


Fig. 3. Spectral scanning of developed chromatogram, Mobile phase composition(MPC)- Acetone(AC): Ethylacetate (EA): Glacial acetic acid (GAA): water (W) (11:9:4:2 v/v). (a) at λ 200 nm–360 nm before and (b) at λ 300 nm–530 nm after derivatized.

3.1.5. Densitometric scanning

Densitometric scanning was performed in the wavelength range of 200 nm–360 nm before derivatization and 300 nm–530 nm after derivatization [31]. In this study, spectral scanning before derivatization gave two patterns, one having λ_{max} at a wavelength of 234 nm for AMX and the other having λ_{max} at a wavelength difficult to determine for CLA (Fig. 3A). On the other hand, densitometric scanning of the postderivatized plate gave two patterns with λ_{max} at 410 nm for AMX and 450 nm for CLA, both intersecting at a wavelength of 428 nm (Fig. 3B). Both AMX and CLA gave a maximum response simultaneously at λ 428 nm, which was selected as the wavelength for the simultaneous determination of AMX trihydrate plus potassium clavulanate in a 625 mg tablet combination formulation.

3.2. Method validation

3.2.1. Specificity

The specificity of the method was determined by analyzing standard and drug samples on the same plate. The spots for AMX and CLA in the samples were confirmed by comparing the R_F and spectrum of the spot with that of a standard [25]. The peak purities of AMX and CLA were also assessed by comparing the spectra at the peak start (S), peak apex (M) and peak end (E) positions of the spot, i.e., r (S, M) = 0.99937 and r (M, E) = 0.9953 for AMX trihydrate and r (S,M) = 0.99914 and r (M, E) = 0.9969 for potassium clavulanate. This was correlated with standards having r (S, M) = 0.99931 and r (M, E) = 0.9962 for AMX trihydrate and r (S, M) = 0.99919 and r (M, E) = 0.9963 for clavulanic lithium. It was also demonstrated that good correlation was obtained between the standard and sample spectra of AMX and CLA. The closeness of peak purity near one tells that the spots were due to a single compound. Based on the results correlation, it can be concluded that excipients present in the formulation did not interfere with the peaks of the samples [27].

3.2.2. Linearity, range and calibration curve

Linear relationships were good in the concentration range 400–1200 ng/band for amoxicillin and 100–300 ng/band for clavulanic acid. It gave r^2 values of 0.9997 and 0.9966 for amoxicillin and clavulanic acid, respectively. r^2 should be as close to 1 as possible. Since r^2 is $\geq$ 0.997 [33], which is above the satisfactory value of 0.995, the developed method has good linearity [34]. The r^2, y-intercept and slopes of the regression line are shown in the table (Table 3). The range usually depends on the purpose of the method to use, which is derived from the linear range. When the method is used for the assay of different finished products, the range is 80 %–120 % of the test (working) concentration. Thus, the minimal range was 640–960 ng/spot for amoxicillin and 160–240 ng/spot for clavulanic acid.

3.2.3. Limit of detection and quantification

Based on the formula mentioned above (equations (1) and (2)), the LOD and LOQ were calculated from the calibration curve. The results were 20.3 ng/band and 61.6 ng/band for AMX and 18.5 ng/band and 56.2 ng/band for CLA, respectively [29,30].

3.2.4. Robustness

The relative standard deviations of retention factors and peak areas of the obtained band were used to evaluate the robustness of the method. There were no large variations in the peak area and R_F values observed through small variations in the parameters for both AMX and CLA. A deliberate change in the mobile phase composition with a small variation of 1 mL one solvent at a time, two at a time and three at a time also did not produce a significant change in the peak area or relative standard deviation (RSD <2 %). The RSD values found were 1.87 % and 1.69 % for AMX and CLA, respectively. The standard deviation of the R_F value (SD < 0.02) was ±0.01 for AMX and ±0.015 for CLA. These values were less than 2 for the peak area and less than 0.02 for R_F. This indicates that the changes had very little effect on the determination.

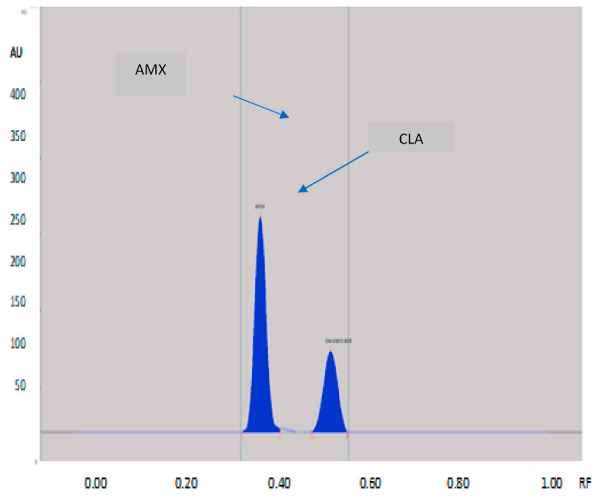


Fig. 4. Typical Densitogram of AMX and CLA bands after derivatization using the optimal conditions.

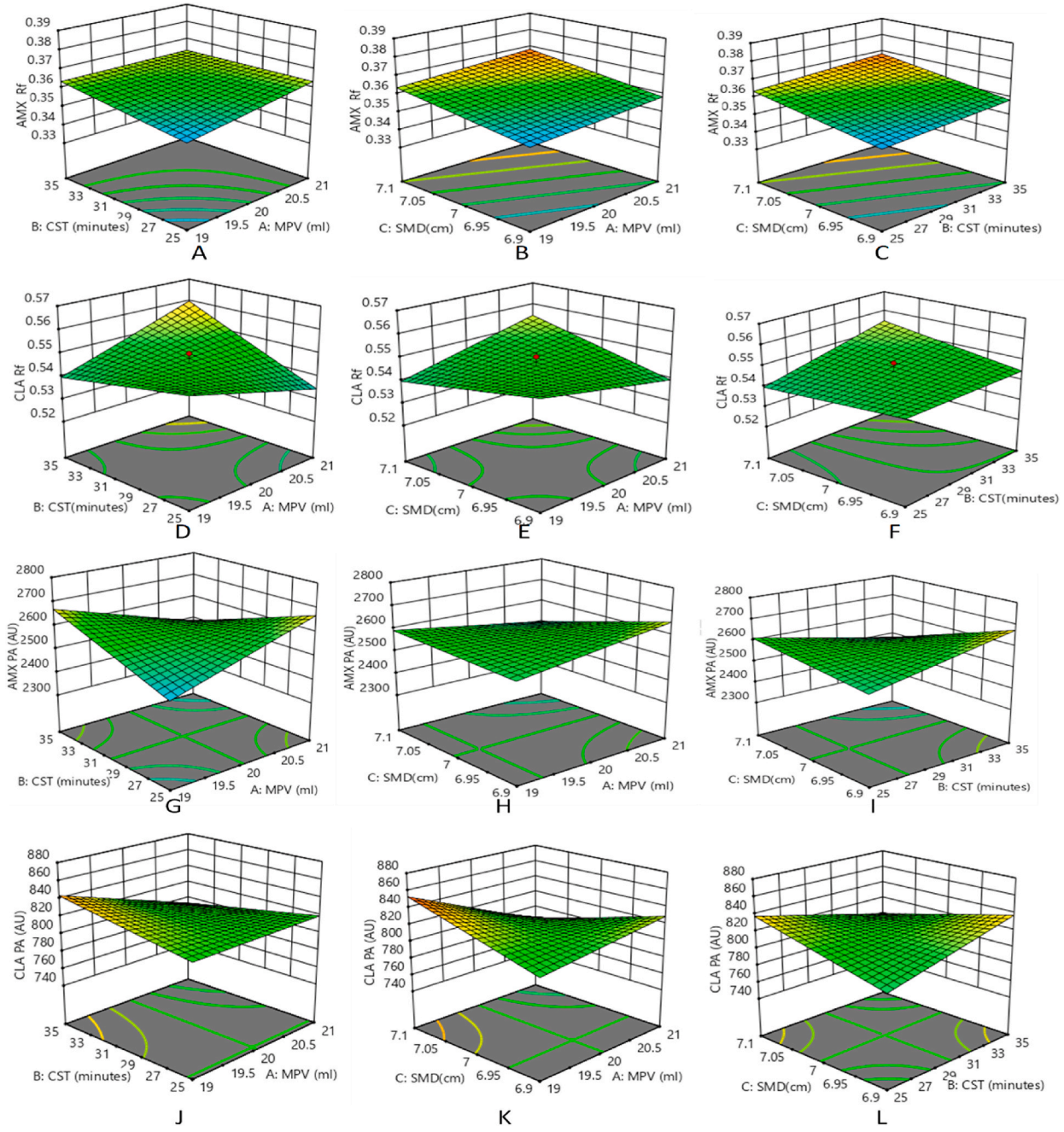


Fig. 5. 3D -response surface plot of different factors in robustness study
Where: MPV -mobile phase volume, CST-chamber saturation time, SMD-solvent migration distance, AMX R_F -retardation factor of amoxicillin, CLA R_F -retardation factor of clavulanic acid. AMX PA -peak area of amoxicillin and CLA PA -peak area of clavulanic acid.

Based on the software (DE13), an ANOVA table was generated (Table 5). This reveals the probability value for the selected model of different factors. The P values of this selected model were found to be 0.1216 and 0.0782 for R_F and 0.0915 and 0.0728 for the peak areas of AMX and CLA, respectively. Therefore, the HPTLC method developed was robust to small changes in factors because the P values for the factors analyzed were greater than 0.05, which indicates that the model is not significant for the analysis of factor criticality.

The ANOVA table also gave 0.1450 and 0.2920 for R_F and 0.2862 and 0.721 for the peak areas of amoxicillin and clavulanic acid, respectively, for the probability value for the selected model of mobile phase composition. These values were also greater than 0.05, which also indicates that the P value was not significant for factor criticality analysis in the developed method. Therefore, the

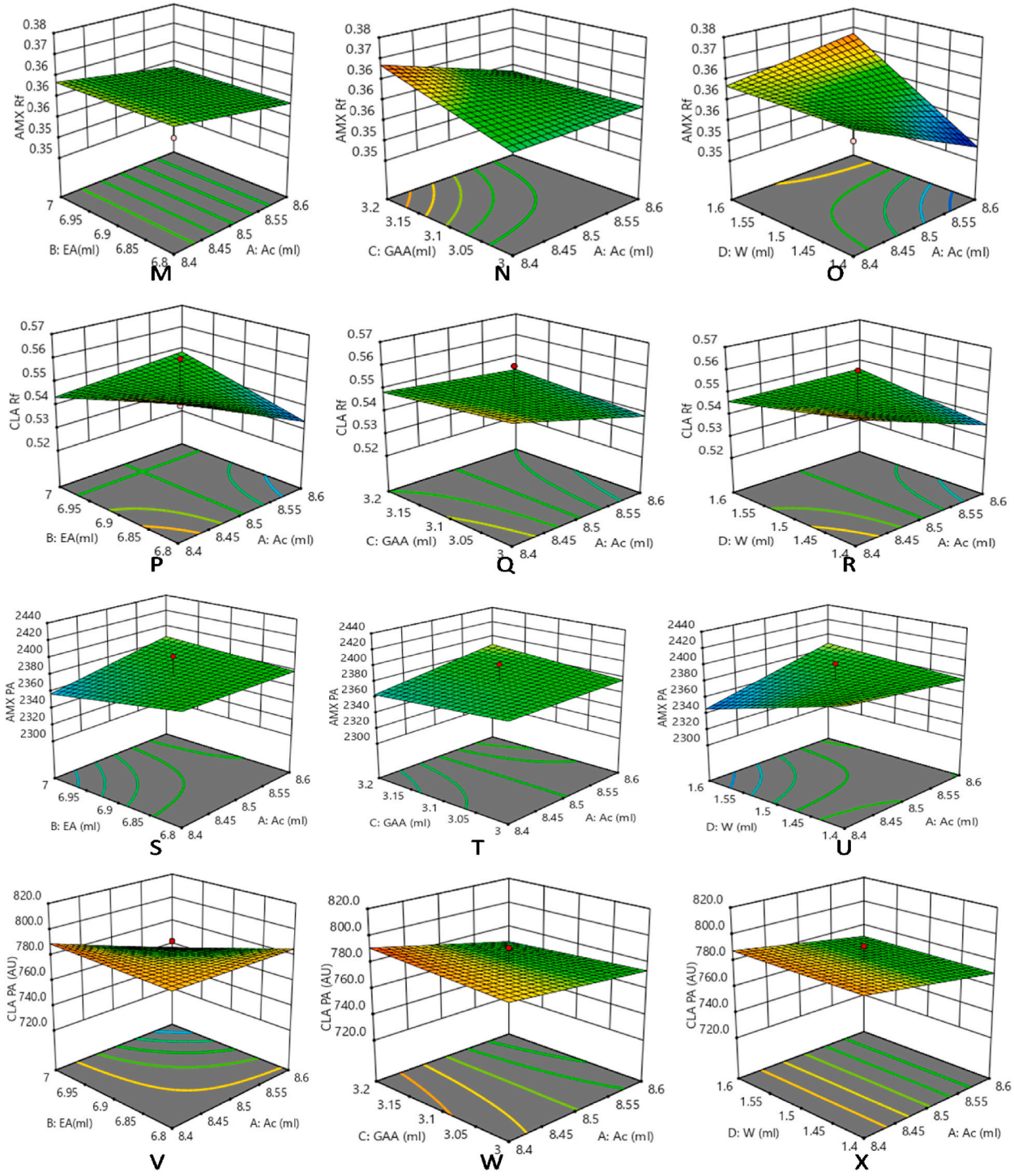


Fig. 6. 3D-response surface plot of mobile phase composition in robustness study
Where: AC -acetone, EA-ethyl acetate, GAA -glacial acetic acid, W-water, AMX R_F -retardation factor of amoxicillin, CLA R_F -retardation factor of clavulanic acid. AMX PA -peak area of amoxicillin and CLA PA -peak area of clavulanic acid.

developed HPTLC method was robust to mobile phase composition variation.

From ANOVA, the equation of prediction for R_F and peak area for AMX and CLA was developed in terms of codded factors that were used to predict the response of the developed HPTLC method toward the suspected level respective factors. By comparing the factors, the coefficient model equation also helps to identify the relative impact of the factors.

Table 3
Coefficient of determination, y-intercept and slope of the regression line for calibration.

Parameters	Values for	
	Amoxicillin	Clavulanic Acid
Linearity rang(ng/spot)	400–1200	100–300
Slope	2.1945	4.26
y-intercept	+640.85	−24.333
Linear regression equation	Y = 2.1945X + 640.85	Y = 4.26X −24.333
Coefficient of determination (r^2)	0.9997	0.9966

Adequate precision, which measures the signal-to-noise ratio of the model, is required to be greater than four. As shown in the table (Table 4), adequate precision for all retention factors and peak areas were in the range of 4.36–5.78 for AMX and 4.01–11.01 for CLA. These values indicate that the model proposed in this method was able to produce an adequate signal, but some values obtained near the lowest recommended value of 4.0 means that the different noises that may be generated in the experiments should be carefully watched. In addition, given that the models were used primarily to identify significant factors, the specific values of the R^2 parameters were not of great importance. The significance of the factors can be determined independently of the shortcomings of the models. The coefficient of variance (% CV) (measure of reproducibility) of the model selected in the developed method was calculated by DE13 software. The coefficient of variance (% CV) of R_F and peak area were in the range of 0.71–3.57 for AMX and 0.24–2.45 for CLA. For all responses, % CV values were less than the needed value (10 %), which indicates that the model was highly reproducible.

The other values to be considered in robustness are adjusted R^2 and predicted R^2. For the method to be robust, the difference between the adjusted and predicted $R^{2\ values}$ should be less than 0.2. In the study of robustness of the developed HPTLC method, the difference in adjusted R^2 and predicted R^2 for all $_{RFs}$ and the peak area response of both amoxicillin and clavulanic acid was less than 0.2. Therefore, the models' R squared values, i.e., adjusted R^2 and predicted $R^{2,}$ were in reasonable agreement, indicating good prediction power of both selected models (Table 4).

However, calculations of SD and RSD the extent of effect due to factor variation is not clearly depicted in Tables 3 and 4. The result of factor interaction cannot be interpreted clearly. Therefore, 3D surface plots generated by DE 13 software were used to visually observe the effect of factors and their interaction on the robustness of the developed method. In the robustness study, the mobile phase volume (A-factor), chamber saturation time (B-factor) and solvent migration distance (C-factor) variation were tested, and the result was revealed in the form of a 3D-response surface (Fig. 5). Based on the DE 13 software 3D-response surface analysis, two factors were studied by keeping the remaining factors constant. The R_F value of AMX was affected negatively when both chamber saturation time and mobile phase volume had gone low (Fig. 5A). When either of two or both factors (A and B) reach a high level, the R_F value of AMX remains constant. A pair of factors (A and C) and (B and C) gave high R_F values at their high level (+1) and low R_F values of AMX at their low level (−1) (Fig. 5 B and C).

The resistance of the developed method to small variations in different factors that give a consistent R_F value of CLA was also studied and provided as a 3D-response surface result. The pair of factors A and B, A and C as well as B and C gave low R_F values for CLA in combination with their low level (−1). They also resulted in a high retention factor for CLA when the two factors of the pair reached high levels (+1) (Fig. 5 D, E and F).

The AMX peak area was also studied and provided as a 3D-response surface result during a robustness study of the method for different factors. The pair of factors A with B, A with C and B with C gave a low peak area response for AMX in combination with their high level (+1) or low level (−1). They also resulted in a high peak area of AMX when one factor of the pair became high or low (Fig. 5 G, H and I).

This 3D-response surface indicates that the peak area of CLA reached a maximum response when the combination of factors B, C and C at high levels (+1) corresponded to low levels (−1) of A, A and B, respectively (Fig. 5 J, K and L). However, the peak area of CLA becomes relatively low when the pair of factors goes to a low level or high level at the same time (Fig. 5 J, K and L).

As shown in Fig. 6 N, while glacial acetic acid volume was increased at low levels of acetone, the R_F of AMX had increased. However, there was no variation in R_F at the other three levels of combination GAA and AC (low: high, low: low, high: high). This indicates that the R_F value of AMX was affected by only glacial acetic acid volume. The B-factor (ethyl acetate) volume and A-factor (acetone) volume variation did not cause AMX R_F changes (Fig. 6 M). At a high level of the A-factor (acetone), an increase in the D-factor (water) volume increased the R_F of AMX. However, at a low level of water, an increase in acetone volume decreased the R_F of AMX (Fig. 6 O). The R_F of clavulanic acid was affected negatively at high levels of acetone, while ethyl acetate and water were affected at low levels (Fig. 6 P, Q and R). However, the R_F value of CLA increased when ethyl acetate and water decreased alternatively, while acetone was at a low level.

Fig. 6 (S, T and U) shows that at low levels of each of the three factors (ethyl acetate, glacial acetic acid, water), the increment of acetone did not cause a change in the AMX peak area response. These 3D results indicated that the peak area response of AMX increased at high levels of the former three factors and increased the acetone volume to a high level. Specifically, at a low level of acetone, the increase in water volume decreased the peak area response of AMX (Fig. 6 U). The highest peak area response of CLA was observed at the combination of a low level of acetone with any level of the other factor (EA, GAA and W) (Fig. 6 V, W and X).

3.2.5. Precision

As shown in Table 5, the % RSD of the intraday precision of the three determinations at the three concentration levels were 2.11,

Table 4
ANOVA result of robustness study of developed HPTLC method for different parameters.

	Drug		S. S	DF	M. S	F-val	P-val	Std. Dev	M	C.V. %	R^2	Adj-R^2	Pre- R^2	Ade-Pre
For mobile phase volume, chamber saturation time and solvent migration distance.														
R_f	AMX	Md	0.0003	4	0.0001	3.12	0.12	0.0053	0.3610	1.47	0.7143	0.4857	0.463	5.3452
		LF	0.0001	4	0.0000	0.45	0.79							
	CLA	Md	0.0074	7	0.0011	12.14	0.08	0.009	0.546	1.54	0.9770	0.8965	0.7344	11.006
		LF	$5.6*10^{-6}$	1	$5.6*10^{-06}$	0.03	0.89							
PA	AMX	Md	$1.3*10^{5}$	4	31548.79	3.71	0.09	92.21	2577.66	3.58	0.7480	0.5364	0.3542	5.7811
		LF	39301.53	4	9825.38	3.06	0.40							
	CLA	Md	9949.56	5	1989.91	4.97	0.07	20.02	818.5	2.45	0.8613	0.6879	0.5634	7.1724
		LF	1167.34	3	389.11	0.89	0.63							
For mobile phase composition (acetone, ethyl acetate, glacial acetic acid and water) variation.														
R_f	AMX	Md	0.0006	5	0.0001	3.14	0.15	0.005	0.3610	1.64	0.7971	0.5435	4368	4.3644
		LF	0.0001	3	0.0000	0.60	0.71							
	CLA	Md	0.0010	6	0.0002	2.08	0.29	0.008	0.5460	1.64	0.8065	0.4194	0.4823	4.0089
		LF	0.0000	2	0.0000	0.10	0.91							
PA	AMX	Md	3640.65	6	606.77	2.13	0.29	16.90	2376.17	0.7111	0.8095	0.4286	0.4641	5.0577
		LF	48.47	2	24.24	0.03	0.97							
	CLA	Md	0.0025	5	0.0005	1.93	0.2721	0.02	779.37	0.2415	0.7069	0.3404	0.3152	4.0550
		LF	0.0002	3	0.0001	0.06	0.9742							

PA: Peak area; SS = sum of squares; DF = degree of freedom; F-f ratio; and P- probability; Md = model; LF = lack of fit; MS = mean square F-Val: F-Value P Val: P-value; M = mean; Adj-R^2 adjusted r^2;Pre-R^2 = predicted r^2; and Ade-Pre = Adequate precision.

Table 5
Repeatability and intermediate precision study.

Concentration ng/band	Repeatability (n = 3)			Intermediate precision (n = 3)		
	Mean	SD	RSD, %	Mean	SD	RSD, %
Amoxicillin						
600	1623.83	34.39	2.11	1990.93	60.64	3.04
800	1872.64	51.76	2.76	2387.1	27.66	1.15
1000	2071.53	35.02	1.69	2870.46	41.75	1.45
Clavulanic lithium						
150	781.51	14.84	1.89	610.72	13.78	2.25
200	923.65	4.13	0.44	796.23	15.08	1.89
250	1108.9	12.56	1.13	994.53	17.270	1.74

2.76 and 1.69 for AMX and 1.89, 0.44 and 1.13 for CLA. The interday precisions of the developed HPTLC method were 3.04, 1.15 and 1.45 for AMX and 2.25, 1.89 and 1.74 for CLA at the corresponding three concentration levels. It is recommended that the RSD should not exceed 2 % for repeatability and 3 % for intermediate precision. Except for the first concentration level of AMX and CLA, the % RSD was found to be 3.04 %; for all others, the % RSD values of intraday and interday precision were less than 2 %, which indicated that the developed HPTLC method for the simultaneous determination of AMX and CLA combinations was precise enough.

The % RSD of the mean area of the response for sample application precision was 1.61 % for AMX and 1.64 % for CLA. It also gave % RSD results of scanning system precision of 1.87 % and 1.24 % for AMX and CLA, respectively (Table 6). These % RSD values were less than 2, and the system precision passed the requirements. Therefore, the method developed is precise in sample application and scanning systems.

3.2.6. Accuracy

By dividing the response value obtained from the spiked assay by the response value obtained from the nonspiked assay, the result of the recovery was calculated. The average recovery result was 100.3 % for AMX (Table 7). This indicates that the newly developed HPTLC method is accurate enough for AMX in simultaneous quantitative determination of the tablet dosage form of amoxicillin trihydrate plus potassium clavulanate with the acceptance range. The recovery study also gave a value of 96.75 % for CLA. This is slightly lower than the expected 98.00 %–102.00 %, which may be due to the stability issue of CLA. Thus, it is imperative that future studies identify an appropriate approach to address the stability issues related to CLA.

3.3. Sample solution stability

As shown in Table 8, the percentage reduction of the sample solution was less than 10 % (5.08 %) for AMX and more than 10 % (10.89) for CLA within 8 h. This may be because clavulanic acid is more unstable than amoxicillin. After 8 h, a significant reduction greater than 10 % for both drugs was observed. The content reduction of the solution was approximately 21.92 % and 16.60 % for AMX and CLA, respectively, after 12 h. Thus, analysis of the drug should be performed within 8 h after solution preparation to minimize systemic error due to sample solution degradation.

3.4. Commercial dosage forms analysis

The four brands (Moxiclav®, Clavamayin®, Syntoclav® and Klamoks®) were analyzed by using the developed HPTLC and official HPLC methods. These two methods gave the assay results of the four brands, as shown in Table 9, in the average ranges of 95.90 ± 0.30–98.65 ± 0.30 and 96.16 ± 0.20–98.82 ± 0.16 for AMX and 90.63 ± 0.62–99.75 ± 1.36 and 91.05 ± 0.11–101.87 ± 0.09 for CLA, respectively. This is in the range of acceptance of official BP specifications that should be in the range of 90 %–105 % of the label claim [22]. The CLA % content of the brands decreased from the 1st trial to the 3rd trial on the HPTLC method, which may be due to the stability issue of clavulanic acid, as it takes some time gap from the first run to the second and third runs. However, the average content

Table 6
System precision study.

Trial	**Sample application precision**		**Scanning precision**	
	AMX Peak Area	CLA Peak Area	AMX Peak Area	CLA Peak Area
T1	3428.4	841.6	3442.4	841.6
T2	3367.85	826.95	3385.5	826.95
T3	3334	821.65	3348.6	841.65
T4	3329.05	846.15	3315.9	846.15
T5	3447.55	843.85	3289.45	843.85
T6	3442.4	859.6	3278.3	859.6
Mean	3391.54	839.96	3343.36	843.3
SD	54.51	13.76	62.46	10.46
%RSD	1.61	1.64	1.87	1.24

Table 7
Accuracy (recovery) study of the developed HPTLC method (n = 3).

Name of the drug	Label claim (ng/band)	Std added (ng/band)	Std added (%)	Total amount (ng/band)	Amount recovered (ng/band)	% Recovery
AMX	400	0	0	400	393.3	98.33
	400	320	80	720	710.21	98.64
	400	400	100	800	808.16	101.02
	400	480	120	880	890.91	101.24
Average recovery (%)						100.3
CLA	100	0	0	100	95.85	95.85
	100	80	80	180	173.12	96.17
	100	100	100	200	194.49	97.24
	100	120	120	220	213.08	96.85
Average recovery (%)						96.75

Table 8
Sample stability study.

Drug name	Analysis time	Average peak area	SD	%RSD	Gained result	%Reduction from initial content
AMX	30 min	2812.50	105.86	3.76	–	–
	1hr	2809.90	85.99	2.99	99.91	0.09
	8hr	2769.70	77.89	2.92	94.92	5.08
	12hr	2195.67	61.32	2.79	78.08	21.92
	24hr	1872.60	51.76	2.76	66.58	33.42
	7days	1774.73	116.02	4.54	63.10	36.90
CLA	30 min	924.50	23.57	2.55	–	–
	1hr	911.53	20.35	2.23	98.60	1.40
	8hr	823.90	4.36	0.47	89.11	10.89
	12hr	771.10	7.26	1.08	83.40	16.60
	24hr	672.03	28.64	4.26	72.69	27.31
	7days	470.67	9.11	1.94	50.91	49.09

Table 9
Assay result of commercial combination tablets by developed HPTLC and official HPLC method.

No. of assay	Content (%) of amoxicillin and clavulanic acid in four different brands							
HPTLC	Clavamyn®		Syntoclav®		Moxiclav®		Klamoks®	
	AMX	CLA	AMX	CLA	AMX	CLA	AMX	CLA
1	95.77	93.77	95.65	91.35	97.61	93.84	98.41	101.31
2	96.19	90.36	95.82	90.26	97.46	92.51	98.57	98.78
3	96.23	88.73	96.24	90.28	97.89	92.23	98.99	99.17
Average amount	96.06	90.95	95.90	90.63	97.65	92.86	98.65	99.75
SD	0.25	2.57	0.30	0.62	0.21	0.86	0.30	1.36
RSD	0.27	2.83	0.32	0.69	0.22	0.93	0.30	1.36
Mean ± SD	96.06 ± 0.25	90.95 ± 2.57	95.90 ± 0.30	90.63 ± 0.62	97.65 ± 0.21	92.86 ± 0.86	98.65 ± 0.30	99.75 ± 1.36
HPLC								
1	97.43	92.51	96.3	91.05	96.39	94.79	98.9	101.76
2	98.22	92.34	96.03	90.94	96.11	94.73	98.64	101.9
3	98.04	92.44	96.18	91.16	95.98	90.33	98.94	101.95
Average amount	97.89	92.43	96.17	91.05	96.16	93.28	98.82	101.87
SD	0.41	0.08	0.13	0.11	0.20	2.55	0.16	0.09
RSD	0.42	0.09	0.14	0.12	0.21	2.74	0.16	0.09
Mean ± SD	97.89 ± 0.41	92.43 ± 0.08	96.17 ± 0.13	91.05 ± 0.11	96.16 ± 0.20	93.28 ± 2.55	98.82 ± 0.16	101.87 ± 0.09

of different brands analyzed on the developed HPTLC method and official HPLC method passed the specification in the limit range.

The results of amoxicillin and clavulanic acid analysis using the newly developed HPTLC method and the official BP HPLC methods were compared statistically by F-ratio (Table 10). The F values calculated for amoxicillin (1.02) and clavulanic acid (1.33) at the 95 % confidence level and 3 degrees of freedom were less than the F-tabulated values of 9.28 and 9.28, respectively. Therefore, the newly developed HPTLC method and the official HPLC method gave comparable results without statistically significant differences.

3.5. Transferability of the developed method

To check the applicability of the developed method for fully automated HPTLC (a new model instrument with many improvements) in another laboratory set up, the sharpness of peaks and resolution of chromatograms with the optimized method were assessed. By

Table 10
F-test two-sample -variances of developed HPTLC and official HPLC method.

	AMX		CLA	
	HPTLC	HPLC	HPTLC	HPLC
Mean	97.26	97.07	94.66	93.54
Variance	1.77	1.74	23.95	17.99
Observations	4	4	4	4
Df	3	3	3	3
F	1.02		1.33	
P(F< = f) one-tail	0.49		0.41	
F Critical one-tail	9.28		9.28	

NB. DF-degree of freedom, F-f ratio and P- probability.

using VisionCats version 3.0 software (Camag, Switzerland), commands were preset for sample application, chamber saturation development (ADC 2 (Automated Development Chamber) (Camag, Switzerland)) and the scanner (Camag, Switzerland) of the plate before and after derivatization.

Both HPTLC methods gave pure, sharp and well-resolved peaks of AMX and CLA (Fig. 7 A and B). It has been confirmed that the two different model types of HPTLC equipment in different laboratories offer comparable results based on this new method.

4. Conclusion

The developed HPTLC method was found to be suitable for the determination of amoxicillin plus clavulanic acid combination dosage formulations. The developed method was comparable with the official HPLC method, as demonstrated by statistical analysis (F test). Validation of the analytical method also demonstrated that the current method is specific, precise, accurate and robust. It is also simple, economical and time saving for the analysis of amoxicillin plus clavulanic acid combined dosage forms. Therefore, the developed method can be used for quality control of amoxicillin and clavulanic acid combined drugs in pharmaceutical industries and other quality control laboratories. But we also suggest:

1. The developed method can be further utilized in post market surveillance for quality control of amoxicillin and clavulanic acid combined drugs of different brands. It can be utilized in pharmaceutical industries as well as in quality control laboratories.
2. Further works about stability study for forced degradation can be done. Nevertheless, clavulanic acid had a known stability issue.

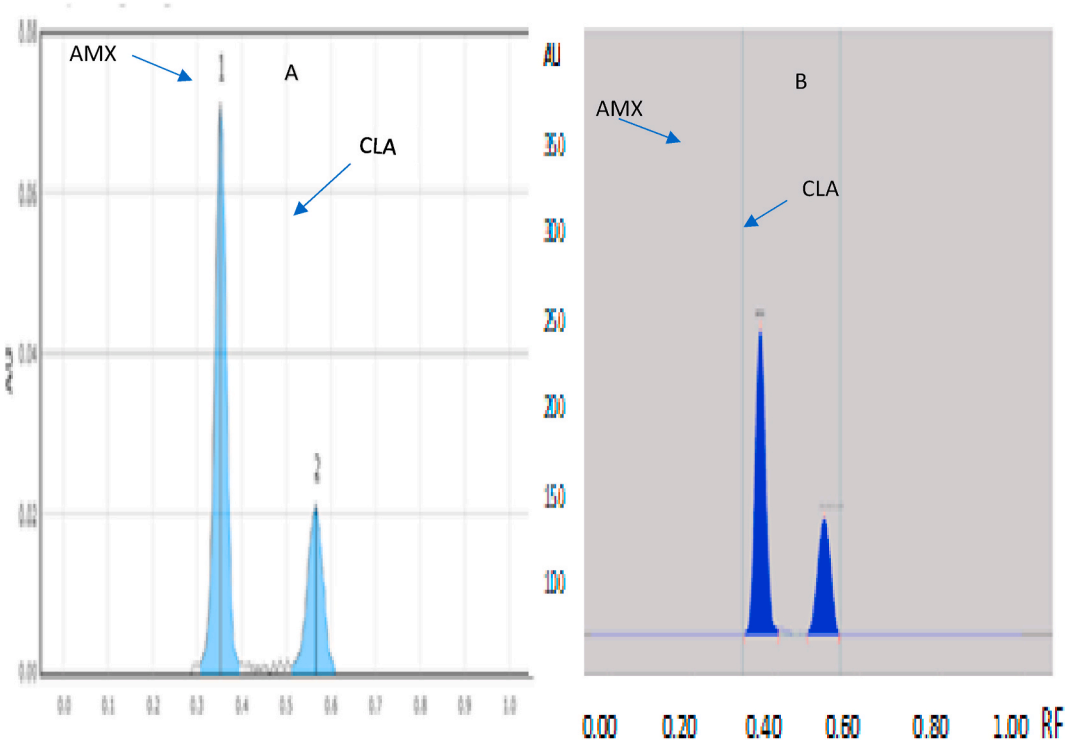


Fig. 7. Densitogram of A-full automated HPTLC and B-semi-automated HPTLC at λ 428 nm, MPC AC: EA: GAA: W (11: 9: 4: 2 (v/v)).

3. Proficiency tests can also be carried out to further check the accuracy of the HPTLC method developed.

Funding

This work was supported by Addis Ababa University graduate studies research support and Ministry of innovation and technology, Government of Ethiopia grants. Great thanks is forwarded to Mizan Tepi University for being sponsorship to MSc study leave of Esubalew Asres. However, our thanks go to the Ethiopian Food and Drug Authority (EFDA) for its permission to perform laboratory work in its quality control unit. The United States Pharmacopoeia provided reference standards for this project. The Ministry of Health, Government of Ethiopia, pharmaceutical and medical equipment directorate kindly donated us the HPTLC stationary plates used in this study.

We are also very much indebted to USAID for providing the HPTLC instrumentation and capacity in our laboratory through the Preventative Technologies Agreement managed by the Supply Chain Management System (SCMS) project of the Management Sciences for Health (MSH).

Data availability

Almost all of the data generated in this study are included in this manuscript. If any additional details are needed, the corresponding author is keen to be contacted.

Additional information

No additional information is available for this paper.

CRediT authorship contribution statement

Esubalew Asres: Writing - original draft, Validation, Investigation, Formal analysis, Data curation. **Thomas Layloff:** Writing - review & editing, Resources, Funding acquisition. **Ayenew Ashenef:** Writing - review & editing, Writing - original draft, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

References

[1] K. Svetlana, Quantitative determination of amoxicillin trihydrate in medical forms using kinetic method, J. Chem. Pharmaceut. Res. 6 (4) (2014) 1120–1125.
[2] R. Kaur, J.P. Kushwaha, N. Singh, Amoxicillin electrocatalytic oxidation using Ti/RuO2 anode: mechanism, oxidation products and degradation pathway, Electrochim. Acta 296 (2019) 856–866.
[3] United States Pharmacopeia 38, National formulary 33. The United States Pharmacopoeial Convention, Rockville 36 (4) (2015) 899.
[4] S.V. Gandhi, M.R. Mahajan, Method development, validation and comparative study of generic Vs. branded generic formulations of amoxicillin trihydrate in capsule dosage form, J. Drug Deliv. Therapeut. 9 (3) (2019) 186–192.
[5] D. Thambavita, P. Galappatthy, U. Mannapperuma, L. Jayakody, R. Cristofoletti, B. Abrahamsson, J. Dressman, Biowaiver monograph for immediate-release solid oral dosage forms of amoxicillin trihydrate, J. Pharmaceut. Sci. 106 (10) (2017) 2930–2945.
[6] F. Hasanpour, A.A. Ensafi, T. Khayamian, Simultaneous chemiluminescence determination of amoxicillin and clavulanic acid using least squares support vector regression, Anal. Chim. Acta 670 (1–2) (2010) 44–50.
[7] V.T. Huong, V.D. Hoang, Simultaneous determination of amoxicillin and clavulanate in combined tablets by nonderivative and derivative UV spectrophotometric techniques, Int. J. PharmTech Res. 1 (2009) 1173–1181.
[8] K.H. Yoon, S.Y. Lee, W. Kim, J.S. Park, H.J. Kim, Simultaneous determination of amoxicillin and clavulanic acid in human plasma by HPLC–ESI mass spectrometry, Journal of chromatography B 813 (1–2) (2004) 121–127.
[9] A.M. Idris, R.E. Elgorashe, Sequential injection chromatography against HPLC and CE: application to separation and quantification of amoxicillin and clavulanic acid, Microchem. J. 99 (2) (2011) 174–179.
[10] G. Hancu, A. Neacşu, L.A. Papp, A. Ciurba, Simultaneous determination of amoxicillin and clavulanic acid in pharmaceutical preparations by capillary zone electrophoresis, Brazilian Journal of Pharmaceutical Sciences 52 (7) (2016) 281–286.
[11] S. Mondal, Clavulanic Acid, 2018. https://www.researchgate.net/publication/325067652. accessed on April 2021).
[12] World Health Organization, WHO model list of essential medicines Technical document, Available at: https://www.who.int/publications/i/item/WHO-MHP-HPS-EML-2021.02, 2021. July 2022).
[13] EFMHACA, List of essential medicines for Ethiopia, in: Addis Ababa, Ethiopia, fourth ed., 2010, p. 15.
[14] EFMHACA, Ethiopian *medicines formulary*, in: Addis Ababa, Ethiopia, second ed., 2013, pp. 381–382.
[15] USP/PQM. https://www.usp-pqm.org/sites/default/files/pqms/article/amoxicillin-pir-jul2018.pdf, 2023. October.
[16] V.A. López-Agudelo, D. Gómez-Ríos, H. Ramirez-Malule, Clavulanic acid production by *Streptomyces clavuligerus*: insights from systems biology, strain engineering, and downstream processing, Antibiotics (Basel) 10 (1) (2021 Jan 18) 84, https://doi.org/10.3390/antibiotics10010084.
[17] E.M. Abdel-Moety, M. Abounassifm, O. Moi-Iamed, N.A. Khattab, Spectrophotometric determination of amoxycillin and clavulanic acid in pharmaceutical preparations, Talanta 36 (6) (1989) 683–685.
[18] A. Aghazadeh, G. Kazemifard, Determination of amoxycyllin and clavulanic acid in pharmaceutical dosage forms by HPLC with amperometric detection, J. Sci. I. R. Iran. 12 (2) (2001) 127–231.
[19] G. Pajchel, K. Pawłowski, S. Tyski, CE versus LC for simultaneous determination of amoxicillin/clavulanic acid and ampicillin/sulbactam in pharmaceutical formulations for injections, J. Pharmaceut. Biomed. Anal. 29 (2002) 75–81.

[20] S.M. Foroutan, A. Zarghi, A. Shafaati, A. Khoddam, H. Movahed, Simultaneous determination of amoxicillin and clavulanic acid in human plasma by isocratic reversed-phase HPLC using UV detection, J. Pharmaceut. Biomed. Anal. 45 (2) (2007) 531–534.
[21] G. Hoizey, D. Lamiable, C. Frances, T. Trenque, M. Kaltenbach, J. Denis, H. Millart, Simultaneous determination of amoxicillin and clavulanic acid in human plasma by HPLC with UV detection, J. Pharmaceut. Biomed. Anal. 30 (2002) 661–666.
[22] S. Kathirvel, K.R. Prasad, K.M. Babu, Development and validation of HPTLC method for the determination of mycophenolate mofetil in bulk and pharmaceutical formulation, Journal of Pharmaceutical Methods 3 (2) (2012) 90–93.
[23] V. Habyalimana, K.J. Mbinze, L.A. Yemoa, N.J. Kadima, P. Hubert, D.A. Marini, Simple LC isocratic methods development, validation, and application in the analysis of poor-quality antimalarial medicines, Am. J. Anal. Chem. 8 (2017) 582–603.
[24] J. Sherma, B. Fried, Handbook of Thin-Layer Chromatography, 89, third ed., Marcel Dekker, Inc., New York, 2003, p. 486.
[25] United States Pharmacopeia 43, National Formulary 38, vol. 1, The United States Pharmacopoeial forumm, Rockville, 2020, pp. 305–308.
[26] British Pharmacopoeia 9th ed., vol. 3. The British Pharmacopoeia Secretariat London, the United Kingdom. 2019, pp 409-410.
[27] M.V. Dhoka, V.T. Gawande, P.P. Joshi, HPTLC determination of amoxicillin trihydrate and bromhexine hydrochloride in oral solid dosage forms, J. Pharmaceut. Sci. Res. 2 (8) (2010) 477–483.
[28] L.D. Khata, A.Y. Kamble, M.V. Mahadik, S.R. Dhaneshwar, Validated HPTLC method for simultaneous quantitation of paracetamol, diclofenac potassium, and famotidine in tablet formulation, J. AOAC Int. 93 (3) (2010) 765–770.
[29] ICH Q2(R1), Harmonized Tripartite Guideline. Validation of Analytical Procedures: Text and Methodology, Geneva, 2005. Available at: https://somatek.com/wp-content/uploads/2014/06/sk140605h.pdf. April 2021).
[30] M.E. Swartz, I.S. Krull, Analytical Method Development and Validation, CRC press, 2018. Available at: https://book4you.org/book/6026353/6691ee. August 2021.
[31] R.E. Kaiser, Dosage techniques in HPTLC, J. Chromatogr. Libr. 9 (1977) 85–94.
[32] S.Z. Hussain, K. Maqbool, B. Naseer, High performance thin layer chromatography: principle, working and applications, Int J Res Pharm Pharm Sci 4 (2019) 83–88.
[33] C.C. Chan, H. Lam, Y.C. Lee, X.M. Zhang, Analytical Method Validation and Instrument Performance Verification, vol. 18, John Wiley & Sons, New Jersey New Jersey, 2004, p. 67.
[34] J. Peris-Vicente, J. Esteve-Romero, S. Carda-Broch, Validation of analytical methods based on chromatographic techniques, Analytical separation science 5 (2015) 1757–1808.