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Development of a Validated Stability Indicating Method for Quantification of Amoxicillin, Clarithromycin and Lansoprazole in Bulk and Pharmaceutical Dosage form by RP-HPLC

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Authors' contributions

This work was carried out in collaboration among all authors. Author PS is mainly involved in this research work for compilation of her PhD thesis. Author AKMP have guided all through work. Author MD helped in the integration of the results and data compilation. The final manuscript has been read and approved by all of the contributors. All authors read and approved the final manuscript.

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ABSTRACT

Objective: The main objective of the present work is to develop an efficient, unique, reliable Reverse Phase High Performance Liquid Chromatography (RP-HPLC) method for the simultaneous quantification of Amoxicillin (AMX), Clarithromycin (CTM) and Lansoprazole (LPZ) in bulk and pharmaceutical formulations.
Methods: The chromatographic separation was achieved by using Kinetex column C18 (100 x 4.6 mm, 2.6 µm) with Buffer (2.5 g of hexane sulphonic acid and 1ml of Triethylamine which are added to 1000 ml of HPLC water and adjusted its pH at 5.0 with Ortho phosphoric acid) and acetonitrile in the ratio of 70: 30 (%v/v) as a mobile phase at flow rate of 1.0 ml/min. The column effluents were monitored by a photodiode array detector at wavelength predetermined at 240 nm.
Results: The method produced reliable results at optimized chromatographic conditions**.** The method was linear at concentration range of 15-225 µg/ml of AMX, 15-225 µg/ml of CTM and

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0.9-13.5 µg/ml of LPZ with regression coefficients of 0.9999, 0.9999, and 0.9999 respectively. The retention times of AMX, CTM, LPZ were obtained as 1.513, 3.124, 3.770 min respectively. Results obtained for system suitability, precision, LOD and LOQ were in acceptable range and were validated according to the guidelines of the International Council for Harmonization (ICH).

Conclusion: The proposed method was validated in accordance with ICH and all the obtained results were found satisfactory and were successfully applicable to the analysis of the bulk and the pharmaceutical formulations.

Keywords: Reverse phase high performance liquid chromatography (RP-HPLC); amoxicillin; clarithromycin; lansaprazole; validation.

1. INTRODUCTION

Amoxicillin is chemically (2S, 5R, 6R)-6-[(2R)-2-amino-2-(4-hydroxyphenyl) acetyl] amino]-3, 3-dimethyl-7-oxo-4-thia-1- azabicyclo [3.2.0] heptane-2-carboxylic acid with a molecular formula $C_{16}H_{19}N_3O_5S$ and molecular mass of 365.4 g mol^{-1}. It is an antibiotic [1,2] used to treat a number of bacterial infections [3] which include middle ear infection [4], strep throat [5], pneumonia [6], skin infections and urinary tract infections [7]. Common adverse effects include nausea and rash. It may also increase the risk of yeast infections [8] and, when used in combination with clavulanic acid [9], it causes adverse effects like diarrohea. It should not be used in those who are allergic to penicillin [10]. When it is to be used in patients with kidney problems, the dose may need to be decreased. Amoxicillin belongs to beta-lactam family [11] of antibiotics. It is one of the most commonly prescribed antibiotics in children.

Clarithromycin,is chemically (3R, 4S, 5S, 6R, 7R, 9R, 11S, 12R, 13S, 14R)-6-{[(2S, 3R,, 4S, 6R)-4-(dimethylamino)-3-hdroxy-6 methyloxane-2-yl]-oxy}-14-ethyl-12, 13-dihydroxy-4-{[(2R, 4R, 5S, 6S)-5-hydroxy-4-methoxy-4,6 dimethyloxan-2-yl]oxy}-7-methoxy 3, 5, 7, 9, 11, 13-hexamethyl-1-oxacyclo tetra decane-2 ,10- dione 2, 5, 7, 9, 11, 13-hexamethyl oxacyclotetradicane-2,10-dione-3,5, 7, 9, 11, 13-hexamethyloxyclotetradeclotetradecane-2, 10-dione-dione with a molecular formula $C_{38}H_{69}NO_{13}$ and molecular mass of 747.964 g mol^{-1} It is an antibiotic used to treat various bacterial infections. This includes strep throat, pneumonia, skin infections, *H. pylori* infection [12,13] and lyme disease [14]. Its common side effects include nausea, vomiting, head-aches and diarrhea [15]. The long-term medication reported liver problems and is not recommended during pregnancy. It belongs to Macrolide [16] class and exhibits its mechanism of action by decreasing protein production [17] of some bacteria.

Lansoprazole, is chemically (RS)-2-([3-methyl-4-2,2,2trifluoroethoxy) pyridin-2yl] methylsulfinyl)-1H-benzo[d]imidazole with a molecular formula $C_{16}H_{14}F_3N_3O_2S$ and molecular mass of 369.36 g mol^{-1}. It is marketed under the brand name Prevacid among others, is a medication which reduces stomach acid. It is used to treat peptic ulcer [18], gastroesophageal reflux disease [19] and Zollinger-Ellison syndrome [20,21]. Onset is over a few hours and effects last up to a couple of days. Common side effects include constipation [22,23], abdominal pain and nausea. Serious side effects may include osteoporosis [24,25] low blood magnesium, clostridium difficile infection [26] and pneumonia. It shows its mechanism of action by blocking H^+/K^+-ATPase in the parietal cells of the stomach.

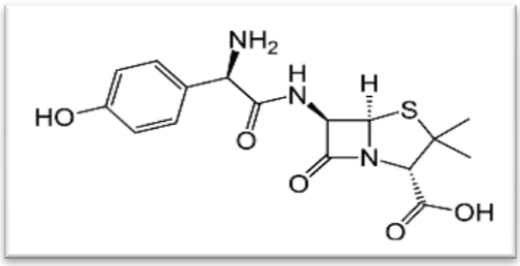

Fig. 1. Structure of Amoxicillin

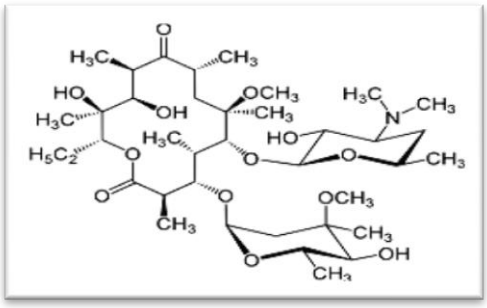

Fig. 2. Structure of Clarithromycin

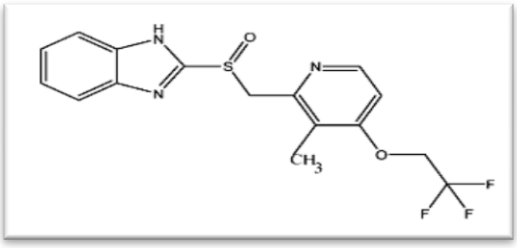

Fig.3. Structure of Lansoprazole

There were several analytical methods available in the literature for the determination of the drugs individually and simultaneously in combination with other drugs. A Liquid chromatographic – Chemometric techniques for the simultaneous determination of Lansoprazole, Amoxicillin, and Clarithromycin in commercial preparation by HPLC was reported [27]. A RP-HPLC Method for prevpac combination therapy drugs in spiked human plasma, Deplin and spasmonal in pharmaceutical dosage was reported [28]. The literature review till date for this combination of drugs reveals that there were no reported stability indicating RP-HPLC method for simultaneous determination of Amoxicillin, Clarithromycin, Lansaprazole. Hence this work aims to develop an accurate, sensitive and stability indicating method for the proposed combination of drugs in bulk and pharmaceutical dosage form.

2. MATERIALS AND METHODS

2.1 Chemicals

Analytical grade reagents like HPLC grade acetonitrile, orthophosphoric acid, hexane sulphonic acid, triethylamine and water (HPLC grade), were purchased from Merck Ltd., Mumbai, India. APIs of amoxicillin, clarithromycin and lansoprazole as reference standards were procured from Spectrum Pharma research solutions pvt. Ltd, Hyderabad.

2.2 Instrumentation

The HPLC system used for the method development and validation consisted of Waters Alliance e-2695 chromatographic system equipped with quaternary pump and waters 2996 PDA detector. Data acquisition, recording and chromatographic integration was performed by software Empower-2.0.

2.3 Chromatographic Conditions

The chromatographic separation was performed on Kinetex column C18(100 x 4.6mm, 2.6µm) with Buffer (2.5g of hexane sulphonic acid and 1ml of triethylamine were added to 1000 ml of HPLC water and adjusted its pH at 5.0 with ortho phosphoric acid) and acetonitrile in the ratio of 70:30 (%v/v) as a mobile phase at flow rate of 1.0 ml/min in an isocratic mode with injection volume of 10 µl for all samples. The buffer was filtered through 0.45µ filter paper and degassed in sonicator before its use.

2.4 Preparation of Standard Solution

Accurately weighed and transferred 150mg of AMX, 150mg of CTM and 9mg of LPZ working

standards into 100ml volumetric flask and added approximately 70ml of diluents. This solution was sonicated for 30min to dissolve it. The final volume was made up to the mark with diluents; which was used as stock solution.

The above stock solution of about 5 ml was transferred into 50ml volumetric flask and made up to the mark with diluents to obtain the solutions of concentrations respectively. (AMX-150 µg/ml, CTM-150 µg/ml and LPZ-9µg/ml).

2.5 Preparation of Sample Solution

Ten Capsules of LPZ and AMX and CTM tablets were taken into a mortar and powdered. The powdered sample equivalent to the weight of 150mg of AMX, 150mg of CTM and 9mg of LPZ were transferred into 100ml volumetric flask. 70ml of diluents was added and sonicated for 30min to dissolve it and final volume was made up to the mark with diluent. 5ml of the above sample stock solution was transferred into 50ml volumetric flask and made up to the mark with diluent. The resultant solutions were of concentrations (AMX-150 µg/ml, CTM-150 µg/ml and LPZ-9µg/ml) respectively.

2.6 Method Development and Optimization

The significant difference in the physical and chemical properties of APIs in proposed method leads the selection of a suitable mobile phase a critical step. Several ratios of components of mobile phases and columns were trailed to achieve a separation of API's with good resolution. The suitability of the column and the mobile phase used in the optimized method have been decided based upon the basis of the selectivity, sensitivity as well as acceptable chromatographic parameters of the produced peaks in terms of peak sharpness, peak symmetry, tailing factor and resolution between the two peaks. We used the mobile phase as a diluent for all samples to ensure minimum noise and to eliminate any unwanted solvent peaks.

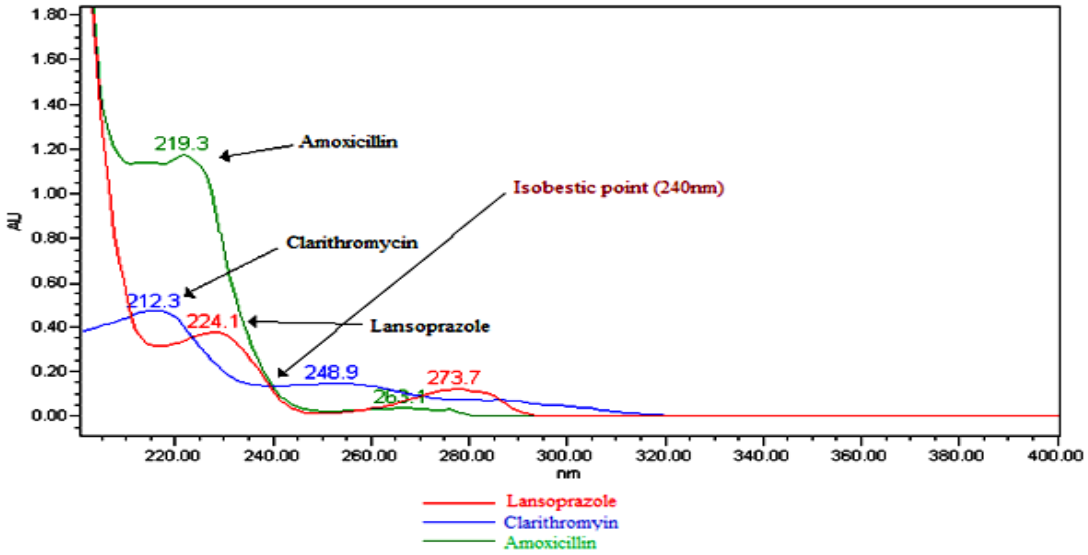

Fig.4. Overlay Spectrum of Amoxicillin, Clarithromycin and Lansoprazole

Table 1. Optimized Chromatographic Conditions

Stationary Phase	Kinetex C18 column (100x4.6mm, 2.6µm)
Mobile Phase	Buffer: Acetonitrile 70:30 (%v/v)
pH of Buffer	5.0 (adjustable with orthophophoric acid)
Injection Volume	10 µl
Flow Rate	1.0 ml/min
Column Temperature	25°C
Wave Length	240 nm
Run time	7 min.

2.7 Validation

The validation of any method demonstrates that the method is suitable for its intended purpose, as stated in ICH Q2(R1) guidelines [29]. The method was validated for linearity, precision (method precision and intermediate precision), accuracy, selectivity and specificity.

2.8 System Suitability

System suitability test is performed to ensure that the resolution and reproducibility of the chromatographic systems are adequate for the analysis to be carried out. The limits were set for No. of theoretical plates, tailing factor, and resolution. The HPLC system was stabilized for 60 min to get a stable baseline. Six replicate injections of standard solution were injected.

2.9 Specificity

The specificity of the analytical method was established by injecting the 100 µg/mL concentration solutions of diluent (blank), placebo, working standards and sample solution individually to investigate interference from the representative peaks.

2.10 Linearity

The area of the linearity peak versus various concentrations has been evaluated for AMX, CTM, LPZ. Linear regression analysis was plotted using peak area against concentration data. Correlation coefficients were calculated for the percent regression, Y-intercept and slope of the calibration curves. The linearity was observed in the concentration range of 15-225 µg/ml of amoxicillin, 15-225 µg/ml of Clarithromycin and 0.9-13.5 µg/ml of lansoprazole with the triplicate injections (n=3) for each concentration.

3. PRECISION

3.1 Method Precision

Method precision was investigated by the analysis of six separately prepared samples of the same batch. From these six separate sample solutions were injected and the peak areas obtained were used to calculate mean and percentage RSD values.

3.2 Intermediate Precision

Ruggedness of the method was studied and showed that chromatographic parameters did not significantly change when different HPLC system, analyst, column were applied.

3.3 Accuracy

Accuracy was evaluated in triplicate, at three different concentration levels equivalent to 50%, 100% and 150% of the target concentration of active ingredient, by adding a known amount of each of the Standard to a pre-analysed concentration of all drugs (AMX, CTM and LPZ) and calculating the % of recovery**.**

3.4 Limit of Detection (LOD) and Limit of Quantification (LOQ)

Limits for the identification and quantification of the drug were calculated from the calibration curves of the drugs. The standard deviation and slope were considered from the calibration plots.

3.5 Robustness

The conditions of the experiment were designed to test the robustness of established system intentionally altered, such as flow rate, organic percentage in movable phase; all these varied conditions.

3.6 Forced Degradation Studies

3.6.1 Stock solution preparation

Accurately weighed 37.4 mg of sample was taken. in 10 ml vacuum flask and added 7 ml of diluents. This solution is sonicated for 30 minutes and diluted to the final volume with diluents.

3.6.2 Acid degradation

The above sample stock solution of 1ml was transferred to a 10ml volumetric flask. To the flask 1 ml of 1N HCl was added and kept for 15 minutes. After 15 minutes, 1ml of 1N NaOH was added and the final volume was made up to the mark with diluents.

3.6.3 Alkali degradation

The above sample stock solution of 1ml was transferred to a 10ml volumetric flask. To the

flask 1 ml of 0.1N NaOH was added and kept for 15 minutes. After 15 minutes 1ml of the 1N HCl was added and the final volume was made up to the mark with diluents.

3.6.4 Peroxide degradation

The above sample stock solution of 1ml was transferred to a 10ml volumetric flask. To the flask 0.3 ml 30 percent hydrogen peroxide was added and the final volume was made up to the mark with diluents.

3.6.5 Reduction degradation

The above sample stock solution of 1ml was transferred to a 10 ml volumetric flask. To the flask 1 ml of 30 percent sodium bisulphate solution was added and the final volume was made up to the mark with diluents.

3.6.6 Thermal degradation

The sample solution was set in an oven at 105º for 6 hours.

3.6.7 Hydrolysis degradation

The above sample stock solution of 1ml was transferred to a 10 ml volumetric flask. To the flask 1ml of water was added and made up to the mark with diluents.

All the above final solutions were injected into system and % degradation was observed. Forced degradation studies were conducted on the basis of ICH requirements including acid, base, oxidation, reduction, thermal and hydrolysis degradation.

4. RESULTS AND DISCUSSION

4.1 System Suitability

The system suitability was estimated by the mentioned parameters and all the obtained values were within the specified limits in accordance with ICH guidelines. The results were summarized in below Table 2.

4.2 Specificity

The obtained chromatograms in Figs. 5 to 11 it can be inferred that there were no co-eluting peaks at the retention time of AMX, CTM, LPZ which shows that peak of analyte was pure and the excipients in the formulation did not interfere with the analyte of interest.

Table 2. System Suitability Results

Parameter	Amoxicillin	Clarithromycin	Lansoprazole
No of Theoretical plates	4154	4256	5666
Tailing factor	1.04	1.07	1.09
Resolution	-	8.77	3.26
%RSD	0.58	0.69	0.33

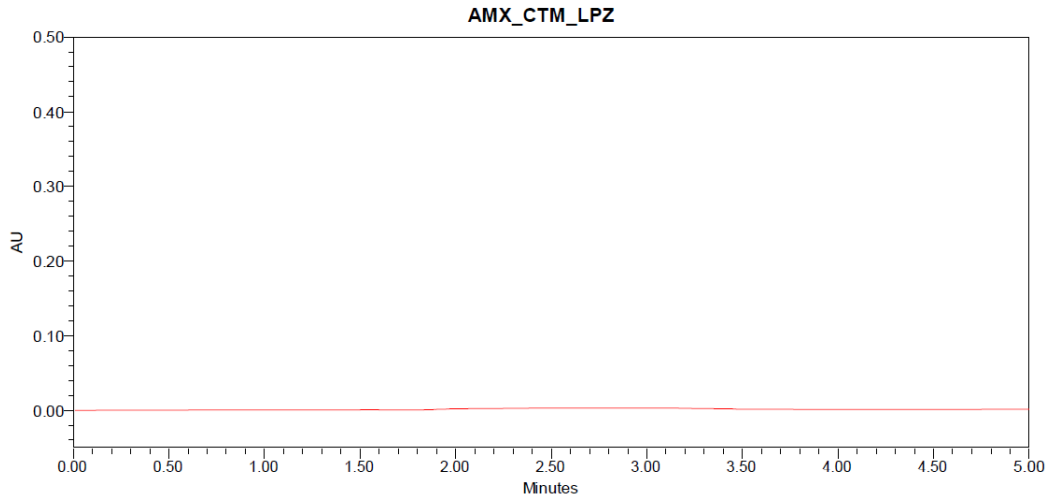

Fig.5. Chromatogram of Blank

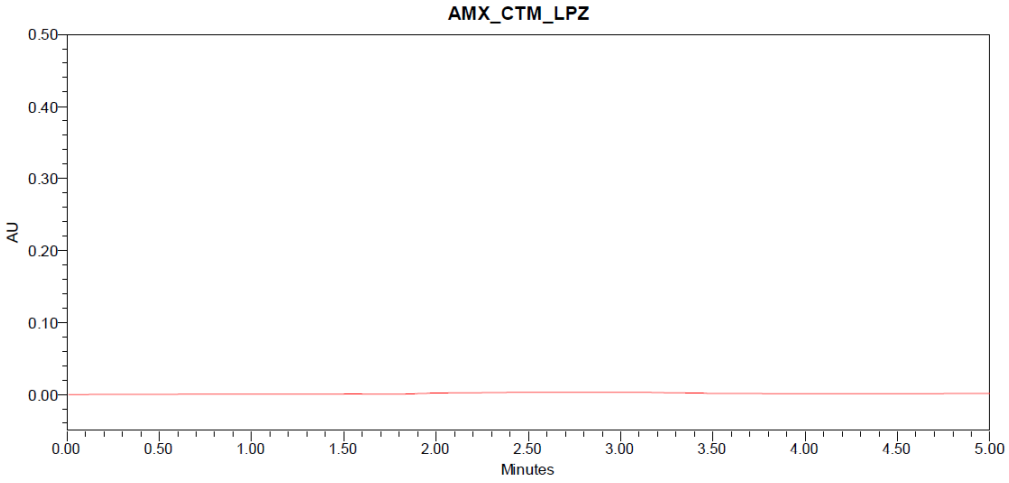

Fig.6. Chromatogram of Placebo

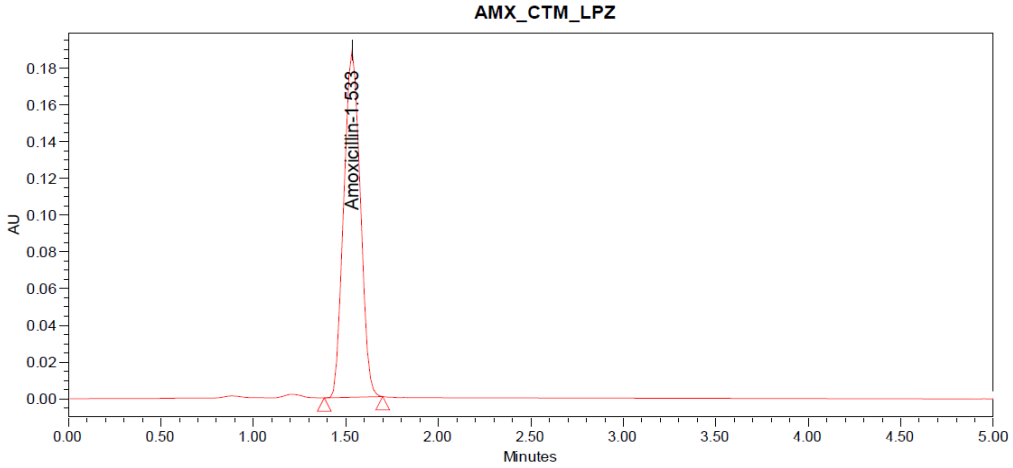

Fig.7. Chromatogram of Amoxicillin

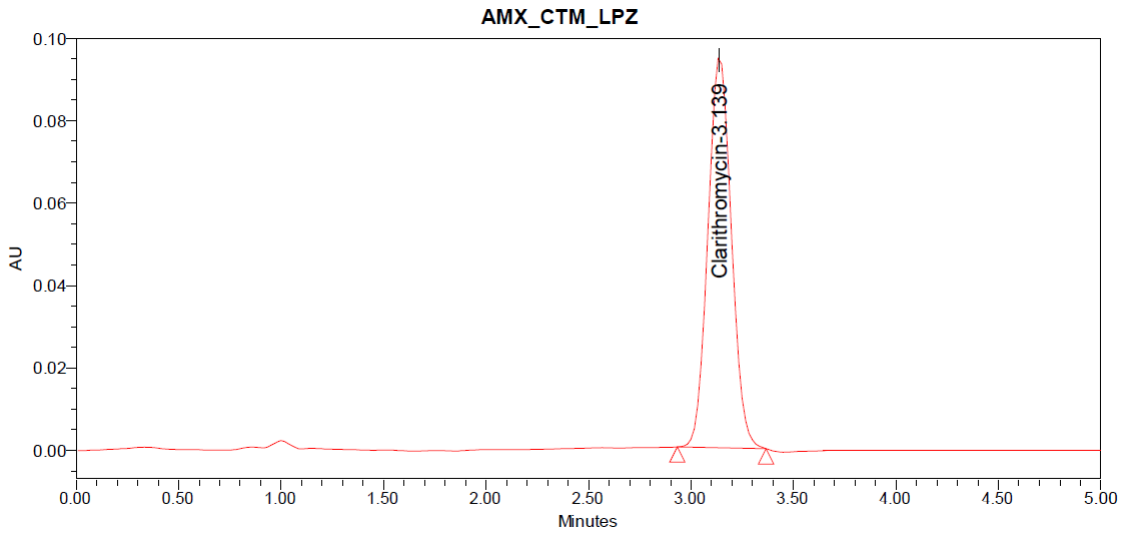

Fig.8. Chromatogram of Clarithromycin

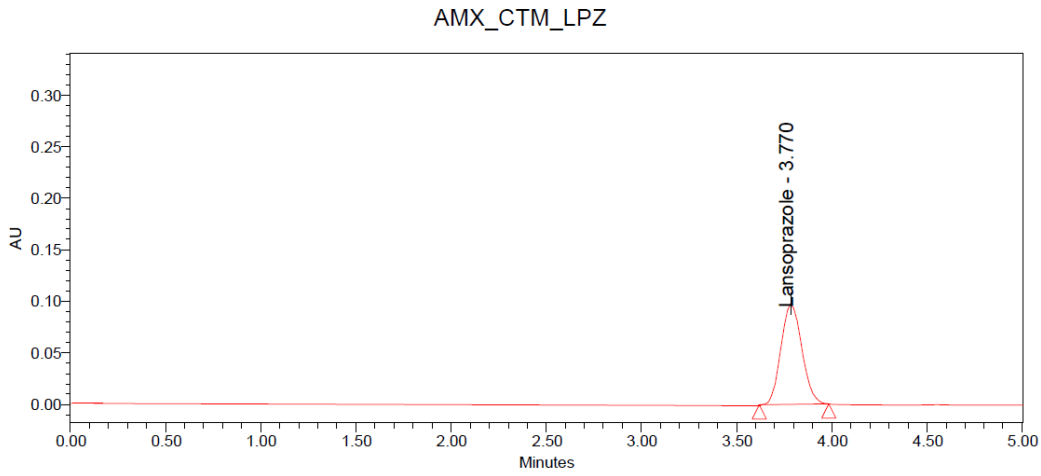

Fig.9. Chromatogram of Lansoprazole

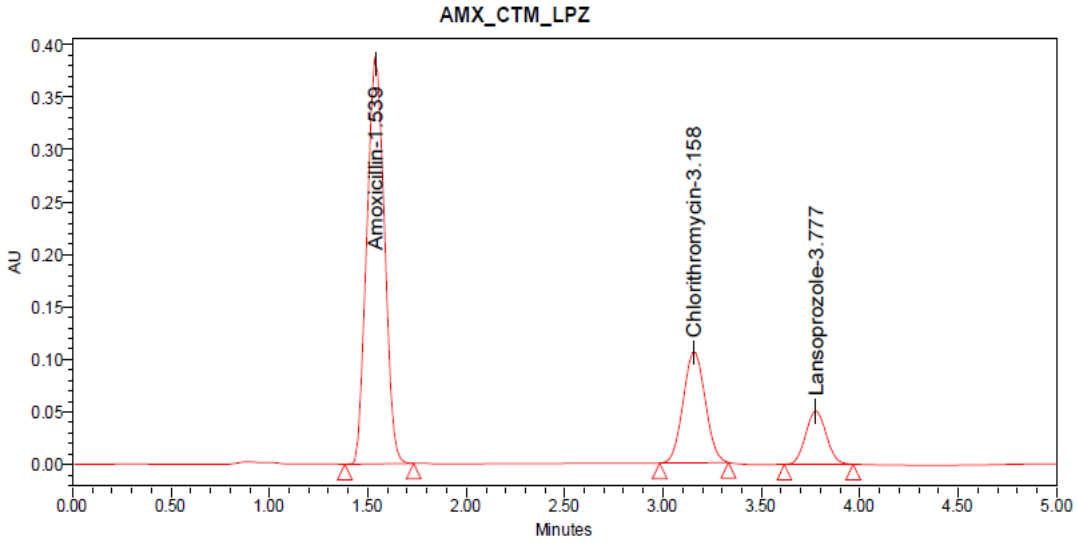

Fig.10. Chromatogram of working standard solution

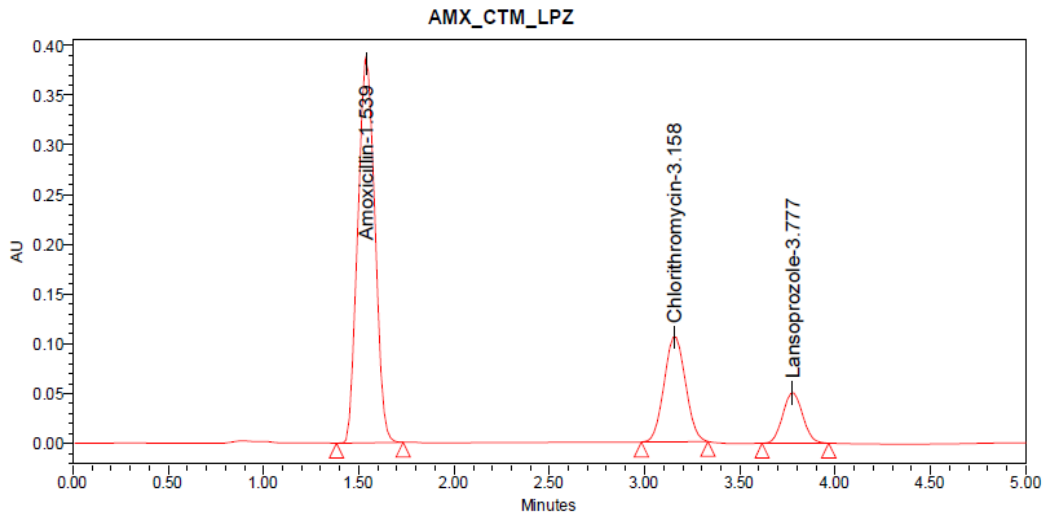

Fig.11. Chromatogram of sample solution

Table 3. Results of Linearity

S. No.	Amoxicillin		Clarithromycin		Lansoprazole	
	Concentration (µg/ml)	Area	Concentration (µg/ml)	Area	Concentration (µg/ml)	Area
1	15	214642	15	180489	0.9	76689
2	37.5	532381	37.5	386475	2.25	176734
3	75	1054869	75	774292	4.5	376658
4	150	2053049	150	1525405	9	748573
5	187.5	2610129	187.5	1915511	11.25	912695
6	225	3110621	225	2279474	13.5	1111233
Slope	13771		10060		82061	
Intercept	12275		20082		879.2	
CC	0.9999		0.9999		0.9999	

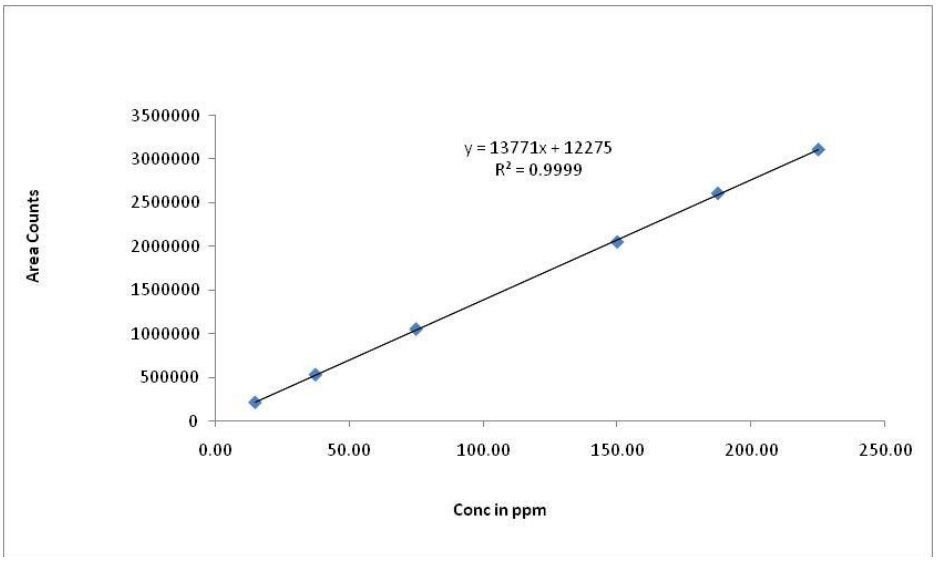

Fig.12. Calibration Curve of Amoxicillin

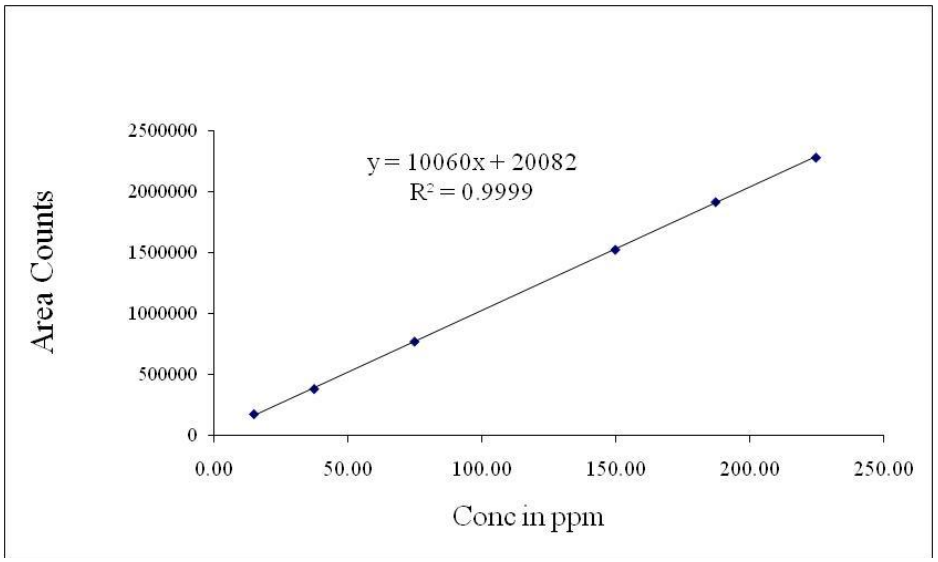

Fig.13. Calibration Curve of Clarithromycin

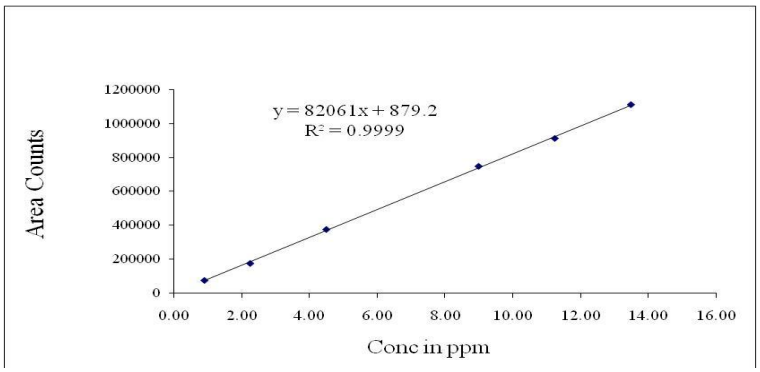

Fig.14. Calibration Curve of Lansoprazole

Table 4. Results of Method Precision and Intermediate Precision

S.NO	Amoxicillin Peak area	Lansoprazole Peak area	Clarithromycin Peak area
1	2043598	742735	1949640
2	2011696	743895	1927598
3	2032323	748878	1952467
4	2022357	748481	1957796
5	2021758	741365	1939246
6	2012665	743815	1919763
Mean	2024066	744862	1941058
Std dev	12173.121	3098.705	14979.347
% RSD	0.60	0.42	0.77

S.NO	Amoxicillin		Lansoprazole		Clarithromycin	
	Day-1 Peak area	Day-2 Peak area	Day-1 Peak area	Day-2 Peak area	Day-1 Peak area	Day-2 Peak area
1	2057759	2054786	744471	745674	1927241	1955473
2	2041695	2048617	743722	745682	1925517	1922465
3	2040724	2057143	745628	743125	1952717	194731
4	2031596	2041673	744699	748012	1932355	1956482
5	2041691	2052437	744816	743710	1918983	1914735
6	2023213	2014357	749074	744158	1913152	1930245
Mean	2039446	2044836	745402	745060	1928328	1937787
Std dev	11595.08	15884.01	1900.601	1780.927	13694.98	17755.19
% RSD	0.57	0.78	0.25	0.24	0.71	0.92

Table 5. Accuracy Results

Accuracy results of Amoxicillin					
Percentage Concentration (at the level of specification)	Region	Amount added(mg)	Amount found(mg)	Percentage recovery	Average recovery
50	958255	75	75.11	98.5	99.2
100	1941184	150	150.8	100.7	
150	2863133	225	225.6	98.5	
Accuracy results of Clarithromycin					
50	958255	75	75.11	98.5	99.2
100	1941184	150	150.8	100.7	
150	2863133	225	225.6	98.5	
Accuracy results of Lansoprazole					
50	371223	4.5	4.5	99.7	100.1
100	745471	9	9	100.1	
150	1114192	13.5	13.5	100.6	

Table 6. Results of LOD & LOQ

Drug name	LOD (µg / ml)	LOQ (µg / ml)
Amoxicillin	0.15	1.5
Lansoprazole	0.009	0.09
clarithromycin	0.15	1.5

Table 7. Results of Robustness

Parameter	Amoxicillin						
	Condition	RT (min)	Peak area	Resolution	Tailing	Plate count	% RSD
Flow rate	Less flow (0.8 ml / min)	1.887	2250290	-	1.06	4158	0.53
	More flow (1.2 ml / min)	1.272	1456263	-	1.04	4175	0.80
Organic phase	Less organic (73:27)	1.290	2583632	-	1.01	4136	0.36
	More organic (67:33)	1.507	1813880	-	1.07	4140	0.10
Clarithromycin							
Flow rate	Less flow (0.8 ml / min)	3.897	2181142	8.54	1.05	4212	0.87
	More flow (1.2 ml / min)	2.603	1416494	8.67	1.08	4256	0.26
Organic phase	Less organic (73:27)	3.239	2240010	8.57	1.07	4164	0.75
	More organic (67:33)	2.458	1747974	8.50	1.04	4185	1.25
Lansoprazole							
Flow rate	Less flow (0.8 ml / min)	4.699	885479	3.24	1.07	5654	0.15
	More flow (1.2 ml / min)	3.132	544217	3.22	1.05	5687	0.15
Organic phase	Less organic (73:27)	3.877	836479	3.14	1.04	5512	0.21
	More organic (67:33)	2.955	714788	3.27	1.08	5423	0.10

Table 8. Results of Forced Degradation Studies

Degradation condition	Peak area	Percent degradation	Purity Angle	Purity Threshold	Pass/Fail
Amoxicillin					
control	2035823	-0.1	0.134	1.228	Pass
Acid	1396487	31.5	0.146	1.276	Pass
Alkali	1386524	32	0.177	1.282	Pass
Peroxide	1395502	31.5	0.114	1.429	Pass
Reduction	1402156	31.2	0.146	1.135	Pass
Hydrolysis	1412363	30.7	0.183	1.264	Pass
Thermal	1381502	32.2	0.106	1.412	Pass
Photolytic	1405975	31	0.158	1.043	Pass
Clarithromycin					
control	1934217	0.1	0.952	3.331	Pass
Acid	1315024	32	1.354	3.721	Pass
Alkali	1320659	31.7	1.363	3.717	Pass

Degradation condition	Peak area	Percent degradation	Purity Angle	Purity Threshold	Pass/Fail
Peroxide	1325478	31.4	7.074	9.391	Pass
Reduction	1302547	32.6	0.449	1.432	Pass
Hydrolysis	1326985	31.4	1.379	3.708	Pass
Thermal	1356555	29.8	7.088	9.384	Pass
Photolytic	1365784	29.4	0.489	1.434	Pass
Lansoprazole					
control	744218	-0.1	1.215	8.044	pass
Acid	523644	29.6	1.036	9.084	pass
Alkali	512045	31.2	1.129	9.136	pass
Peroxide	502478	32.5	4.168	23.229	pass
Reduction	523697	29.6	1.104	3.491	pass
Hydrolysis	526346	29.3	1.112	9.145	pass
Thermal	526847	29.2	4.189	23.214	pass
Photolytic	513695	31	1.204	3.508	pass

4.3 Linearity

The Linearity plots of the drugs executed a honest linearity which were confirmed by the correlation coefficients for all drugs achieved are greater than 0.999. The slope, Y-intercept values were presented in Table 3.

4.4 Precision

4.4.1 Method precision (or) repeatability

The calculated mean and percentage RSD values of the three drugs obtained have inferred that the method was precise.

4.4.2 Intermediate precision

The value of percentage RSD was below 2% exhibits the ruggedness of the developed method. The results of both method precision and intermediate precision were present in Table 4.

4.4.3 Accuracy

Accuracy was evaluated in triplicate, at three different concentration levels equivalent to 50, 100 and 150% of the target.

concentration of active ingredient, by adding a known amount of each of the Standard to a pre-analysed concentration of all drugs (AMX, CTM and LPZ) and calculating the % of recovery**.** The recovery results should be not less than 98% and not more than 102%.

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

The calculation of LOD and LOQ were proceeded using the following equation in compliance with the ICH guidelines.

4.5 Robustness

The resolution between active ingredients from impurities was not significantly affected and there was no significant influence on the time of retention, plate count and tailing factor. Hence this method was robust. Robustness of the method was found to be % RSD should be less than 2%. Slightly variations were done in the optimized method parameters like flow rate (±20%), organic content in mobile phase (±10%). The results are present in Table 7.

4.6 Forced Degradation Studies

It is evident from the obtained data that the selected drugs were stable under the applied stress conditions although the degraded peaks were observed the percentage degradation was with in the acceptable limits.

5. CONCLUSION

In this study a novel, simple, rapid, economical, sensitive and easily available HPLC method was developed for the simultaneous determination of AMX, CTM and LPZ in bulk and pharmaceutical dosage form. The shorter run time, low price, accessibility, sensitivity, reliability and reproducibility of the method proves in applicability in rapid quantification of many samples in routine and quality control analysis of tablets. The validation of all the parameters like linearity, accuracy, precision, robustness was performed and found to be within the acceptance criteria. The RSD values for all parameters were found to be less than 2%, which indicates the validity of method and results obtained by this method are in fair agreement. So, the proposed method could be easily applied for the routine

analysis and pharmaceutical formulations of amoxicillin, clarithromycin and lansoprazole in quality control laboratories without any preliminary separation.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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