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CHEMOMETRIC ASSISTED NEW STABILITY INDICATING NP-HPLC METHOD DEVELOPMENT AND VALIDATION OF CLAVULANIC ACID , AMOXCILLNE AND LACTOBACILLUS IN COMBINED DOSAGE FORM

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ABSTRACT

A new chemometric assisted by high-performance liquid chromatography (HPLC) with photodiode array (PDA) detection was implemented for the simultaneous determination of tablet dosage form. Two chemometric calibration techniques, principle component analysis (PCA) and partial least squares (PLS) were applied to the peak area at 246nm of PDA detector responses. Chromatographic separation of Amoxicillin, Clavulanic acid and Lactobacillus was achieved on Waters Alliance-e2695, by using Chiral Cell ODH 150x4.6mm, 5µ column and the mobile phase containing Hexane: THF: Acetic acid in the ratio of 96.5:3:0.5% v/v. The flow rate was 1.0 ml/min; detection was carried out by absorption at 246nm using a photodiode array detector at ambient temperature. The number of theoretical plates and tailing factor for Amoxicillin, Clavulanic acid and Lactobacillus were NLT 2000 and should not more than 2 respectively. % Relative standard deviation of peak areas of all measurements always less than 2.0. The proposed method was validated according to ICH guidelines. The method was found to be simple, economical, suitable, precise, accurate & robust method for quantitative analysis of Amoxicillin, Clavulanic acid and Lactobacillus and study of its stability. The 'UNSCRAMBLER(camo)'software was used for the numerical calculations. All of the two-chemometric analysis methods in this study can be satisfactorily applied for thequantitative analysis of Amoxicillin, Clavulanic acid and Lactobacillus in pharmaceutical tablet dosage form

Key words: HPLC Amoxicillin, Clavulanic acid and Lactobacillus

INTRODUCTION

In the assurance of the quality of the bulk drugs and Pharmaceutical preparations the role of data analysis is vital. The pharmacopoeias may not provide the standard analytical procedure for the determination of the newer drugs and formulations. Thus, it is essential to develop chemometric assisted RP-HPLC method to develop a rapid qualitative analysis Pharmaceutical properties of intermediate and finished dosage forms. [1]

The chemometric methods are one type of multivariate analysis that is considering more than one variable at the at a time. 2 Thus, it does not exist in one dimensional data.3 The science of chemometrics can be briefly described as the interaction of certain mathematical and statistical methods to chemical problems. It has developed as a consequence of a change of in the data obtained with the chemistry with the emergence of the new analytical techniques as well as microprocessors [4].

The applications of using chemometric techniques in analytical chemistry are now numerous and applications have been revealed in spectroscopy, chromatography and other disciplines of analytical chemistry [3].

Least square approach involves mathematical modelling by which the square of residual (difference between actual and predicted concentration) is minimized to lowest level. Four different Chemometric methods are used which are

1. Classical Least Squares
2. Inverse Least Squares
3. Principal Component Regression
4. Partial Least Squares or Projection to Latent Structures

These methods first calibrate the mathematical model by using absorbance data of calibration standards with known concentration and then predict the concentration of un-known samples from their absorbance data. If there are m number of calibration standards and l chemical components (drugs) and n is the number of wavelengths considered, all methods involve presentation of absorbance data as a matrix with m rows and n columns, concentration data as a matrix with m row and l columns.

Stability is defined as "An ability of pharmaceutical product to retain its physical, chemical, microbiological properties within specifications throughout its shelf life" according to ICH guidelines.The solution stability of Amoxicillin, Clavulanic acid and Lactobacillusin diluents can be determined by storing sample solution and tightly cab volumetric flask at room temperature for 24 hr.

Method validation can be defined as per ICH as "establishing evidence, which provide a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics".

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Amoxicillin is (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) Amoxicillin is used to treat bacterial infections in many different parts of the body. It is also used with other medicines (e.g., clarithromycin, lansoprazole) to treat H. pylori infection and duodenal ulcers[5-8]

Clavulanic acid contains a beta-lactam ring in its structure that binds in an irreversible fashion to beta-lactamases, preventing them from inactivating certain beta-lactam antibiotics, with efficacy in treating susceptible gram-positive and gram-negative infections[9-11]

Lactobacillus acidophilus has been used in alternative medicine as a likely effective aid in treating diarrhoea in children with rotavirus.Lactobacillus acidophilus has been used in alternative medicine as a possibly effective aid (in children or adults) in preventing diarrhea caused by antibiotics, travel, chemothera, or hospitalization. Lactobacillus acidophilus is also possibly effective in treating irritable bowel syndrome, bacterial vaginal infection, colic in babies, lung infections in children, skin problems in children who are allergic to milk, and other conditions.[12]

A tablet dosage from containing all the three, (amoxicillin500mg ,clavulanic acid 125mg and lactobacillus 60m.s), is commercially available andused in pain reliever and reducer for inflammation amoxicillin official in USP/NF. Clavulanic acid and lactobacillus are officially available in IP. Literature reviews revealed that UV Spectroscopy method have been reported for the determination of Amoxicillin, Clavulanic acid and Lactobacillusindividually and in pharmaceutical dosage form. Literature is also available concerning UV Spectroscopy method of with amoxicillin ,clavulanicacid and lactobacillus drug combination. .But; there is no any chemometric assisted NP-HPLC method available for Amoxicillin, Clavulanic acid and Lactobacillusin pharmaceutical dosage form. So, present study was aimed at to develop simple, accurate, precise, cost effective and reliable analytical method for estimation of Amoxicillin ,Clavulanic acid and Lactobacillus in combined dosage form.

MATERIALS AND EQUIPMENT

The developed RP-HPLC method for the estimation ofAmoxicillin, Clavulanic acid and Lactobacillus was carried out onChiral Cell ODH 150x4.6mm, 5µ column using mobile phase composition of a mixture Hexane: THF: Acetic acid in the ratio of 96.5:3:0.5% v/v with flow rate of 1.0 ml /min at 246 nm.

Materials

Instruments used-HPLC, Empower version 2.0 software, UV-Visible detector, Shimadzu Analytical balance.

Chemicals and Reagents: HPLC grade Water,Tetra hydro furan, Acetic acid and hexane

Drugs- Amoxicillin, Clavulanic acid and Lactobacillus

METHODOLOGY

METHOD DEVELOPMENT

In the present investigation, we have developed a simple and sensitive RP-HPLC method for quantitative estimation ofAmoxicillin, Clavulanic acid and Lactobacillus in bulk drug and Pharmaceutical dosage forms. These are trails performed for HPLC method development of Amoxicillin, Clavulanic acid and Lactobacillus.

1.Selection of wave length (For Detection) In setting up the conditions for development of assay method, the choice of detection wavelength was based on the scanned absorption spectrum for Amoxicillin, Clavulanic acid and Lactobacillus . The UV-Spectrum of Amoxicillin, Clavulanic acid and Lactobacillus was obtained separately by scanning the sample over the wave length range 200-400nm against blank as methanol. After thorough examination of the spectra, the wave length 226 nm was selected for further analysis.

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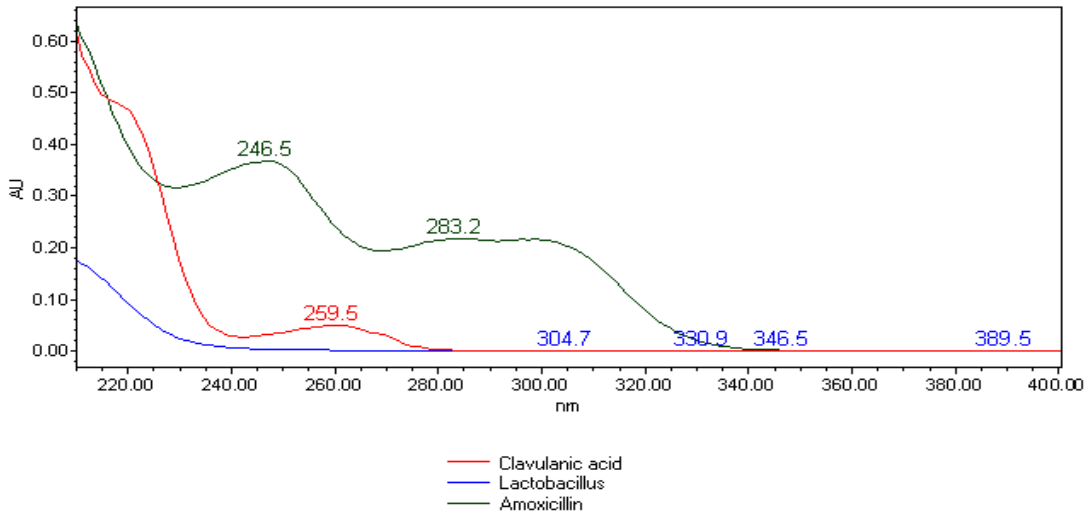

Figure 01: Overlay spectrum of Amoxicillin, Clavulanic acid and Lactobacillus

1.2 **OPTIMIZED METHOD :**

1.2.1 Preparation of Buffer solution: Mix 1ml Ortho Tri ethyl amine in 1litre water, filtered through 0.45µm nylon membrane filter.

1.2.2 Mobile Phase A mixture of buffer and Acetonitrile in the ratio of 10:90%v/v was sonicated to degas and filtered through 0.45µm nylon membrane filter.

1.2.3. Chromatographic conditions

Column : Chiral Cell ODH 150x4.6mm, 5µ
Mobile phase ratio : Hexane, THF and Acetic acid 96.5+3+0.5
Detection wavelength : 246 nm
Flow rate : 1ml/min
Injection volume : 10µl
Run time : 6min

Retention time of Amoxicillinis about 2.969 min.
Retention time of Clavulanic acid is about 4.022 min.
Retention time of Lactobacillus is about 5.010 min
is about 13.452 min.

1.3 **Preparation of standard stock solution**: Accurately weigh and transfer 5 mg of Amoxicillin, 5mg of Clavulanic acid and 5mg of Lactobacillus working standards into a three different 10ml clean dry volumetric flask add Diluent and sonicate to dissolve it completely and make volume up to the mark with the same solvent. Further pipette 2.5ml of the above clavulanic acid solution and 0.12ml of the lactobacillus solution into a separate 10ml volumetric flasks and make up to the mark with diluents (Stock solution)

Further pipette 1 ml of the above stock solutions into a 10 ml volumetric flask and dilute up to the mark with diluent. (50ppm of Amoxicillin, 12.5ppm of Clavulanic acid and 0.6ppm of Lactobacillus)

1.4 **Preparation of Sample solution**: Accurately weighed and transfer equivalent to 9.65 mg of Amoxicillin , Clavulanic acid and Lactobacillus sample into a 10mL clean dry volumetric flask add Diluent and sonicate it up to 30 mins to dissolve, and centrifuge for 30min. to dissolve it completely and make volume up to the mark with the same solvent. Then it is filtered through 0.45 micron Injection filter. (Stock solution). Further pipette 1 ml of the above stock solutions into a 10ml volumetric flask and dilute up to the mark with diluent. (50ppm of Amoxicillin, 12.5ppm of Clavulanic acid and 0.6ppm of Lactobacillus)

Table:1 Assay Calculations

Drug	**Avg sample area (n=5)**	**Std. wt (µg/ml)**	**Sample wt. (µg/ml)**	**Label amount (mg)**	**Std purity**	**Amount found (µg/ml)**	**% assay**
Amoxicillin	2713710	5	9.65	500	99.9	4.76	98.1
Clavulanic acid	371109	1.25	9.65	125	99.7	1.24	99.1

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Lactobacillus	261552	0.06	9.65	6	99.8	0.05	98.9

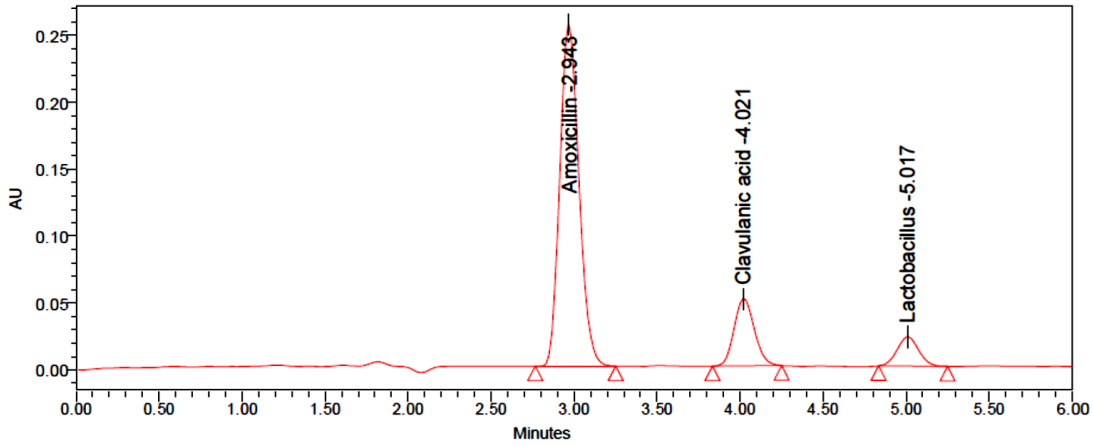

Figure02: A Representative chromatograph of Standard

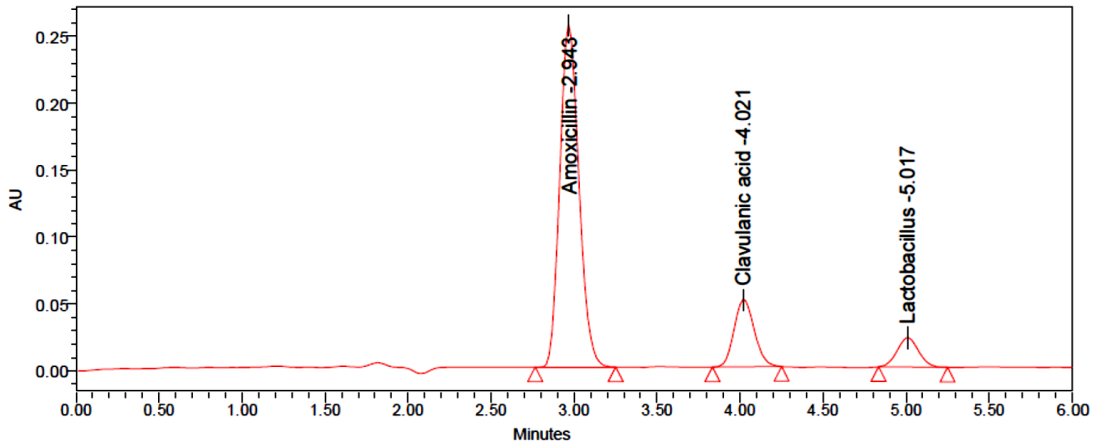

Figure03: A Representative chromatograph of sample

METHOD VALIDATION

Analytical method validation is a process of performing several tests designed to verify that an analytical test method is suitable for its intended purpose and is capable of providing useful and valid analytical data. A validation study involves testing multiple attributes of a method to determine that it can provide useful and valid data when used routinely. There are several parameters that are considered in the method validation process as per International Conference of Harmonization (ICH) guidelines and the values for these parameters are asfollows.

Table 2: Results of linearity for Amoxicillin and Clavulanic acid and Lactobacillus

S.NO	Amoxicillin		Clavulanic acid		Lactobacillus	
	Conc.(µg/ml)	Peak area	Conc.(µg/ml)	Peak area	Conc.(µg/ml)	Peak area
1	12.50	711715	3.13	104301	0.15	59337
2	25.00	1463987	6.25	189653	0.30	132045
3	37.50	2077973	9.38	295299	0.45	190437
4	50.00	2764341	12.50	378191	0.60	242224
5	62.50	3457487	15.63	488539	0.75	305632
6	75.00	4132107	18.75	570639	0.90	365632

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Regression equation	y = 54823.48x +30920.82	y = 30502.07x +3560.54	y = 404682.14x +2936.89
Slope	54823.48	30502.07	404682.14
Intercept	30920.82	3560.54	2936.89
R^2	0.9998	0.9995	0.9994

Table3: Accuracy results of Amoxicillin by RP-HPLC method

%Concentration(at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	1393139	2.5	2.52	100.8	100.6
100%	2794035	5	5.05	101.0	
150%	4145321	7.5	7.49	99.9	

Table 4: The Accuracy results for Clavulanic acid by RP-HPLC method

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	189441	0.63	0.63	100.8	101.0
100%	376021	1.25	1.26	100.8	
150%	569308	1.88	1.90	101.3	

Table 5: The Accuracy results for Lactobacillus by RP-HPLC Method

%Concentration (at specification Level)	Area	Amount Added (mg)	Amount Found (mg)	% Recovery	Mean Recovery
50%	137538	0.03	0.03	100.0	99.8
100%	267768	0.061	0.06	98.4	
150%	399137	0.089	0.09	101.1	

Precision:

Table6 : 6system Precision for Amoxicillin, Clavulanic acid and Lactobacillus by RP-HPLC method

Injection	Area for Amoxicillin	Area for Clavulanic acid	Area for Lactobacillus
Injection-1	2769274	375679	262319
Injection-2	2799553	372432	263247
Injection-3	2794189	373387	263054
Injection-4	2785510	377485	265138
Injection-5	2764115	372066	262055
Injection-6	2783067	371679	261359
Average	2782618	373788	262862
Standard Deviation	13781.66	2308.96	1309.23
%RSD	0.50	0.62	0.50

Table7 : Intermediate Precision for Amoxicillin, Clavulanic acid and Lactobacillus by RP-HPLC method

Injection	Area for Amoxicillin	Area for Clavulanic acid	Area for Lactobacillus
Injection-1	2791234	374786	263430

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Injection-2	2750328	372367	265320
Injection-3	2781384	371542	263461
Injection-4	2723784	375143	264312
Injection-5	2773128	376357	265761
Injection-6	2713354	372687	266124
Average	2755535	373813	264734
Standard Deviation	31832.62	1881.69	1168.70
%RSD	1.16	0.50	0.44

Table 8: Robustness results of Amoxicillin by RP-HPLC

Parameter	**Amoxicillin**					
	Condition	**Retention time(min)**	**Peak area**	**Resolution**	**Tailing**	**Plate count**
Flow rate Change(mL/min)	Less flow(0.8ml)	3.252	3052641		1.15	3507
	Actual(1ml)	2.969	2794621		1.15	3097
	More flow(1.2ml)	2.673	2456982		1.14	3056
Organic Phase change	Less Org	3.295	2994138		1.11	3123
	Actual	2.965	2752861		1.11	3054
	More Org	2.727	2604138		1.11	3225

Table 9: Robustness results of Clavulanic acid by RP-HPLC

Parameter	**Clavulanic acid**					
	Condition	**Retention time(min)**	**Peak area**	**Resolution**	**Tailing**	**Plate count**
Flow rate Change(mL/min)	Less flow(0.8ml)	4.408	395623	4.85	1.03	5345
	Actual(1ml)	4.022	375038	4.74	1.12	5095
	More flow(1.2ml)	3.600	356298	4.21	1.11	5311
Organic Phase change	Less Org	4.422	387542	4.60	1.16	5342
	Actual	4.024	374948	4.51	1.18	5036
	More Org	3.633	348502	4.59	1.03	5362

Table10 : Robustness results of Lactobacillus by RP-HPLC

Parameter	**Lactobacillus**					
	Condition	**Retention time(min)**	**Peak area**	**Resolution**	**Tailing**	**Plate count**
Flow rate Change(mL/min)	Less flow(0.8ml)	5.487	304269	4.40	1.11	7001
	Actual(1ml)	5.010	265354	4.18	1.06	6857

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	More flow(1.2ml)	4.508	198524	3.89	1.04	7862
Organic Phase change	Less Org	5.916	294268	5.25	1.04	7012
	Actual	5.015	262874	4.26	1.02	6827
	More Org	4.270	208257	2.33	1.17	7021

Table11: Sensitivity parameters (LOD & LOQ) by RP-HPLC

Name of drug	LOD(µg/ml)	LOQ(µg/ml)
Amoxicillin	1.5	5
Clavulanic acid	0.375	1.25
Lactobacillus	0.018	0.06

STABILITY STUDIES

Table12 : Forced Degradation results for Amoxicillin and Clavulanic acid and Lactobacillus

Results: % Degradation results	Amoxicillin		Clavulanic acid		Lactobacillus	
	Area	% Degradation	Area	% Degradation	Area	% Degradation
Control	2765672	0	374245	0	264469	0
Acid	2413597	12.7	330194	11.8	235857	10.8
Base	2404741	13	321480	14.1	228347	13.6
Thermal	2454475	11.2	335901	10.3	232204	12.2
Photo	2476812	10.4	330478	11.7	233236	11.8
Peroxide	2447412	11.5	328039	12.4	231924	12.3
Hydrolysis	2756412	0.3	372287	0.5	262749	0.6
Reduction	2352412	14.9	315548	15.7	220901	16.4

CHEMOMETRIC ANALYSIS

In this chemometrics assisted HPLC study ,PCA,PLS calibrations were used to analyse the drugs of Amoxicillin, Clavulanic acid and Lactobacillus at 246 nm by using PDA detector. The data obtained from analysed drugs were stored in computer having required software to perform chemometric analysis.

Acquisition software: In present study we are using following chemometric techniques.

- Principal component analysis(PCA)
- Partial least squares technique(PLS)

We are download the unscrambler (camo software),it facilitates the PCA,PLS analysis more robust, accessible.

3.1 PLS Approach:

PLS calibration using the orthogonalized PLS algorithm involves, simultaneously, independent and dependent variables on the data compression and decomposition operations. In the HPLC data analysis, HPLC-PLS calibration was obtained by decomposition of both the drugs of concentration , peak area matrix into latent variables. PLS calibration was obtained using the relationship between the decomposed peak area data and concentration set.

Table13: PLS Accuracynumericaldata of Clavulanic acid, Amoxcyclline and lactobacillus.

S.No	REFERENCE	PREDICTED	PREDICTED	PREDICTED
		Lactobacillus	Clavulanic acid	Amoxcyclline
1	1	2	2	
2	50.000	50.3499	50.9637	49.9831

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3	50.000	49.2014	49.6786	49.2661
4	50.000	50.9691	50.5670	50.3692
5	100.000	101.1921	99.8295	100.4074
6	100.000	97.9189	98.5205	100.0139
7	100.000	100..1743	99.3285	100.4194
8	150.000	152.4789	150.4518	149.0461
9	150.000	148.6635	149.8998	151.2026
10	150.000	149.0519	150.7807	149.2922

Figure04: PLSofaccuracy spectraldataoflactobacillus

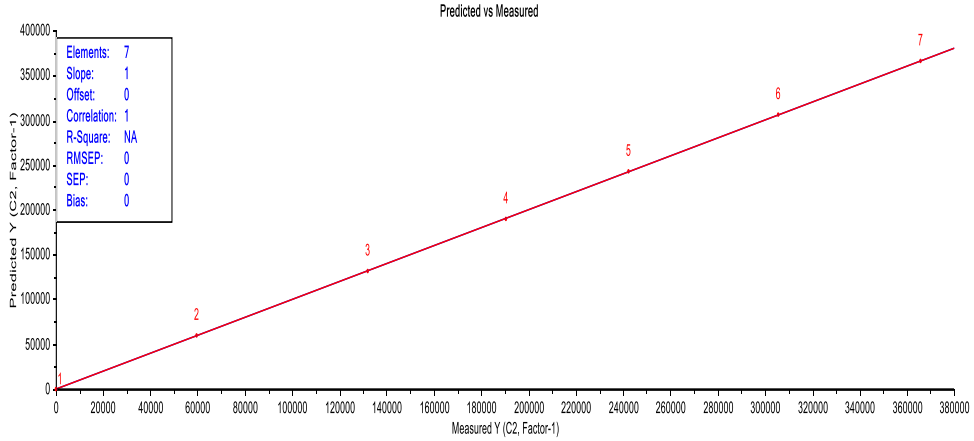

Figure05: PLSofaccuracy spectraldataof Clavulanic acid

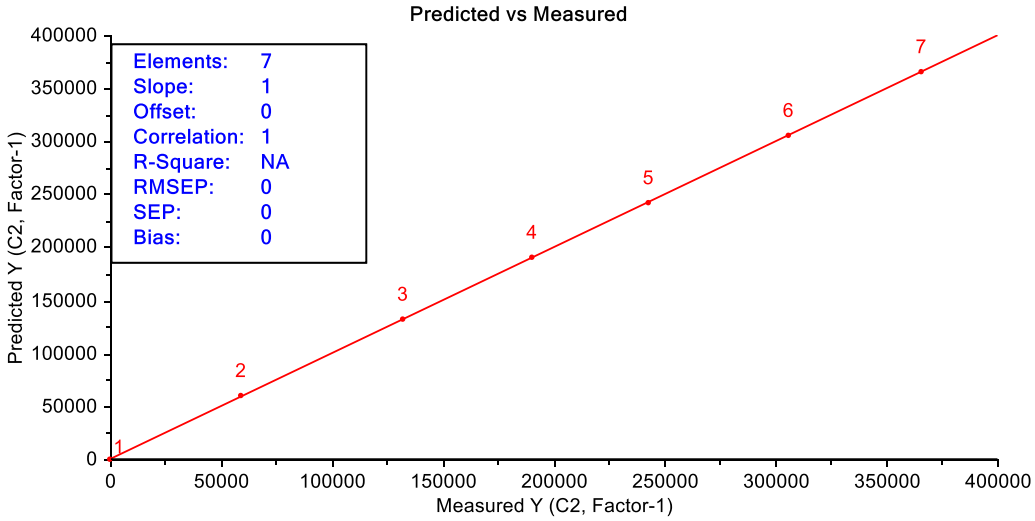

Figure06 : PLSofaccuracy spectraldataof Amoxicillin

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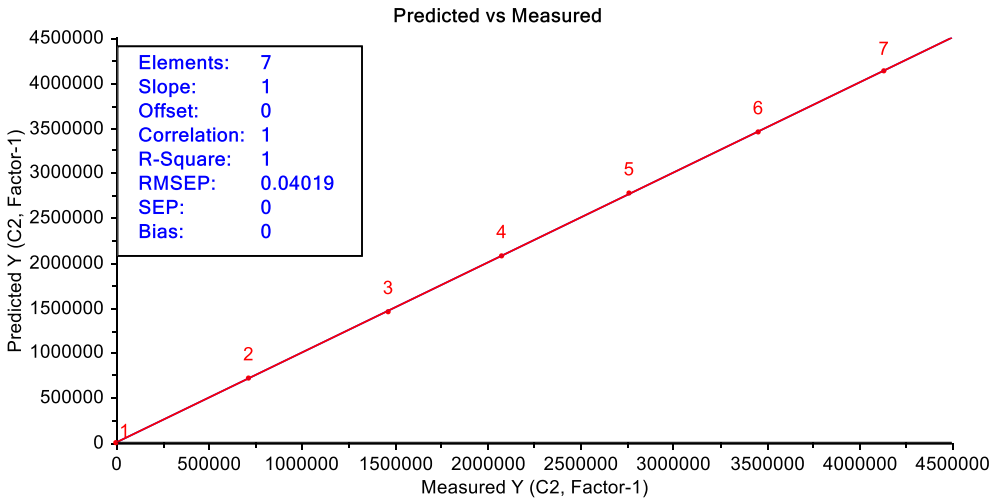

Table14: PLS linearityynumericaldata of Clavulanic acid, Amoxcyclline and lactobacillus.

S.No	REFERENCE	PREDICTED	PREDICTED	PREDICTED
		Lactobacillus	Clavulanic acid	Amoxcyclline
	1	2	2	
1	0.000	-0.0556	-0.0111	-0.0110
2	1.2500	1.1647	0.2329	0.2496
3	2.5000	2.6600	0.5320	0.5229
4	3.7500	3.8609	0.7722	0.7468
5	5.000	4.9260	0.9852	0.9971
6	6.2500	6.2300	1.2460	1.2498
7	7.5000	7.4640	1.4928	1.4959

3.2 PCA approach:

In PCA technique it gives relevant information from data set, and it can be used express the data on the basis of their similarity and differences. It is used to developcorrelation structure between variables, and examine the changes. In PCA data transferred to describe the amount of same variability. In these HPLC data analysis the data of both drugs of hydrochlorothiazide and triameterene peak area we get the Bio-plot.

Table15:PCAAccuracyNumericalDataofAmoxicillin and Clavulanic acid and Lactobacillus

PC-1	PC-2
-1407885	3110.561
-1428468	823.4608
-1397362	1445.735
11356.46	-131.5851
-868.1843	-7725.252
11187.86	-3340.379
1382665	10040.06

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1440591	-7907.113
1388782	3684.38
-1407885	3110.561

Figure 06: PCA accuracy spectral data of Amoxicillin, Clavulanic acid and Lactobacillus

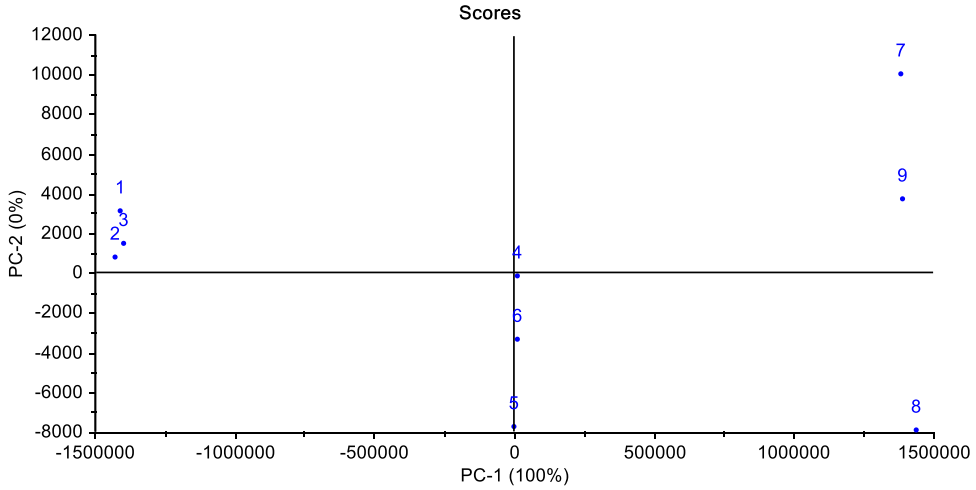

Table16:PCALinearity NumericalDataofAmoxicillin and Clavulanic acid and Lactobacillus

PC-1	PC-2
-1407885	3110.561
-1428468	823.4608
-1397362	1445.735
11356.46	-131.5851
-868.1843	-7725.252
11187.86	-3340.379
1382665	10040.06
1440591	-7907.113
1388782	3684.38
-1407885	3110.561

Figure 07: PCA Linearity spectral data of Amoxicillin, Clavulanic acid and Lactobacillus

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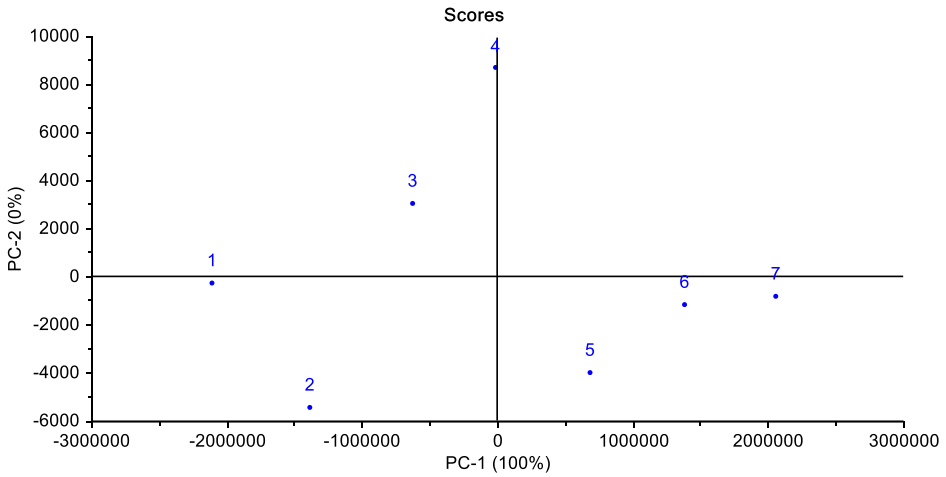

CONCLUSION

In the present investigation new analytical methods have been developed for the estimation of the potent drug Amoxicillin, Clavulanic acid and Lactobacillus. This study contains evaluation of HPLC data for the chemometric techniques of PCA and PLS. These chemometric methods could be applied with great success for the simultaneous determination of Amoxicillin, Clavulanic acid and Lactobacillusin the pharmaceutical formulation without the interference of each other.

The two chemometric method that i.e. PCA and PLS are found to be simple, precise, accurate, rapid and economical method for their simultaneous determination. The methods were successfully validated and found suitable for quality control laboratories.

It conclude that novel stability indicating method for the determination of drugs in combined dosage form for Amoxicillin, Clavulanic acid and Lactobacillusin according to ICH guidelines and it can be used for meeting the regulatory guidelines for above drugs

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