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EVALUATION AND VALIDATION OF A UPLC METHOD FOR SIMULTANEOUS ESTIMATION OF AMOXICILLIN AND POTASSIUM CLAVULANATE IN ORAL DOSAGE FORM

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ABSTRACT

The method for Reverse Phase Ultra Performance Liquid Chromatography was optimized for Amoxicillin and Potassium Clavulanate by different trials were done by using mobile phase and buffer combination. The mobile phase is a mixture of two solvent systems namely, solvent -A and solvent - B. The solvent– A, contains Trifluroacetic acid. The solvent – B, contains KH2PO4: Methanol (80:20) v/v with gradient flow programming was used as mobile phase and effluent was monitored at 248 nm. The % RSD values for assays performed in the different laboratories by two analysts were found to be on (Acquity UPLC Waters, 2695H) instrumentation - 1 (0.03% and 0.04%) on (Agilent Technologies, 1290) instrumentation – 2 (0.03% and 0.02%) did not exceed 2, indicating the ruggedness of the method. LOD and LOQ were determined by injecting progressively lower concentrations of two drugs. The LOD of Amoxicillin and Potassium Clavulanate were found to be 0.0029 µg/mL and 0.0052 µg/mL respectively. The LOQ of Amoxicillin and Potassium Clavulanate were found to be 0.0091 µg/mL and 0.0160 µg/mL respectively. Assay studies for the analysis of formulation was performed and was found to be satisfactory with 99.28% and 99.41% purity of percentage for Amoxicillin and Potassium Clavulanate.

KEYWORDS: amoxicillin, potassium clavulanate, oral dosage form, UPLC method.

INTRODUCTION

Amoxicillin (AMX), (2*S*,5*R*,6*R*)-6-{[(2*R*)-2-amino- 2-(4-hydroxyphenyl)-acetyl]amino}-3,3-dimethyl-7- oxo-4-thia-1-azabicyclo[3.2.0]heptane-24-carboxylic acid, is a β-lactam semisynthetic penicillin from the aminopenicillin class with a broad antibacterial spectrum, used to treat a large number of infections with susceptible Gram-positive and Gram-negative bacteria. It is one of the most frequently prescribed penicillin derivatives within the class because it is better absorbed, following oral administration, than other β-lactam antibiotics (Block, Beale, 2011).

AMX is susceptible to degradation by β-lactamase producing bacteria, which are resistant to a narrow spectrum of β-lactam antibiotics, such as natural penicillins. For this reason, it is often combined with clavulanic acid, a β-lactamase inhibitor.

Clavulanic acid (C L A) , (2*R*, 5*R*,*S*) - 3 - (2 - hydroxyethylidene)-7-oxo-4-oxa-1-aza-bicyclo[3.2.0] heptane-2-carboxylic acid, is an oxapenam derivative lacking the 6-acylamino side chain characteristic for penicillin derivatives, which exhibits very weak antibacterial activity, and, therefore, is not useful as an antibiotic. It is used combined with penicillin group antibiotics to overcome resistance to bacteria that secrete β-lactamase. CLA can be described as a "suicide inhibitor", covalently bonding to a serine residue in the active site of the β-lactamase (Block, Beale, 2011).

Combining these two drugs increases effectiveness by reducing susceptibility to β-lactamase resistance. Combinations of AMX trihydrate and the potassium salt of CLA are available in various fixed-doses of oral and injectable dosage forms intended for the treatment of skin, respiratory, ear, and urinary tract infections caused by β-lactamase producing bacterial strains (Block, Beale, 2011; Todd, Benfield, 1990).

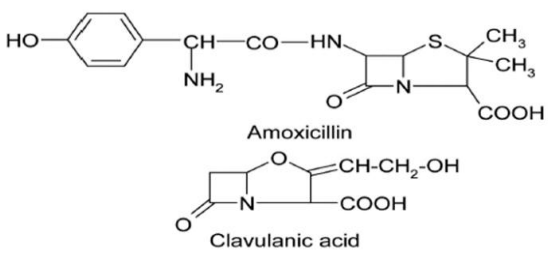


Fig.1 Chemical structure of amoxicillin and potassium clavulanate.

Validation of analytical methods (USP/ICH)

Method validation, according to the United States Pharmacopeia (USP), is performed to ensure that an analytical methodology is accurate, specific, reproducible, and rugged over the specified range that an analyte will be analyzed. Regulated laboratories must perform method validation in order to be in compliance with FDA regulations. In a 1987 guideline (Guideline for Submitting Samples and Analytical Data for Methods Validation), the FDA designated the specifications in the current edition of the USP as those legally recognized when determining compliance with the Federal Food, Drug and Cosmetic Act can be referred to as the "eight steps of method validation."

Experimental

MATERIALS

EQUIPMENTS	SOURCE
Ultra Pressure Liquid Chromatography (UPLC)	Acquity UPLC Systems, Waters Laboratories
Chromatographic data software	Empower
Column	Xterra RP-8 (150mm x 4.6 mm i.d., particle size 5 µm)
Detector	PDA
Injector	Automated
Electronic Balance	Eagle
Sonicator	Band Line Sonerex
p^H Meter	Lab India p^H meter

METHODOLOGY

Method Validation

The analytical procedure refers to the way of performing the analysis. It should describe in detail the steps necessary to perform each analytical test. This may include but is not limited to: the sample, the reference standard and the reagents preparations, use of the apparatus, generation of the calibration curve, use of the formulae for the calculation, etc. The described method extensively validated in terms of specificity, system suitability, linearity, accuracy, precision, limit of detection, limit of quantification and robustness.

RESULTS

Preparation of Standard Stock Solution

The pure drug of Amoxicillin trihydrate and Potassium Clavulanate were injected into the UPLC system and run in different solvent systems. Different mobile phases like acetonitrile and water; methanol and water; methanol and buffer were tried in order to find the best conditions for the separation of Amoxicillin trihydrate and Potassium Clavulanate. It was found that Methanol and Potassium dihydrogen phosphate gives satisfactory results as compared to other mobile phases. This mobile phase system was tried with different proportions and using different flow rates. A mixture of Buffer and Methanol in the ratio of 80:20 was prepared and pH = 3.0 was maintained, at which they showed better separation. Hence 80:20 v/v ratio of mobile phase was considered to be the optimal composition and pH of mobile phase is maintained to 3.0.

1. Preparation of mobile phase

Mobile phase: KH2PO4: Methanol (80:20)

2. Preparation of standard stock solution

A standard stock solution was prepared by accurately weighing about 100 mg of Amoxicillin trihydrate and 25 mg of Potassium Clavulanate standard and is transferred into 20 ml volumetric flask; add 5ml of methanol and sonicate for 10min, make up the volume to 20 with methanol (stock solution-I). From this, transfer 5ml of above solution to 50ml volumetric flask and make up the volume with methanol labeled as stock solution-II.

1. Selection of analytical wavelength

By appropriate dilution of standard stock solution with mobile phase, various concentrations of Amoxicillin trihydrate and Potassium Clavulanate were prepared separately. The solution was scanned using double beam UV-visible spectrophotometer 1700 in the "Spectrum mode" between the range of 400 to 200 nm and their spectra was overlaid. From the overlaid spectra of Amoxicillin trihydrate and Potassium Clavulanate, 248 nm was selected as analytical wavelength for multi component analysis using UPLC method.

2. Chromatographic conditions

The mobile phase containing mixture of Methanol and Potassium dihydrogen phosphate buffer whose ratio was 20:80 was selected as the optimum composition of mobile phase, of which pH was maintained to 3.0, because it was found that this solvent system resolved both the components ideally. The flow rate was set to 0.5 ml/min and UV detection was carried out at 248 nm. The mobile phase and samples were degassed by ultrasonication for 20 min and filtered through 0.4 µm membrane filter paper.

3. Selection of analytical concentration range and preparation of calibration curve for Amoxicillin trihydrate and potassium clavulanate

Amoxicillin trihydrate

Appropriate aliquots were pipetted out from the standard stock solution (500 µg/ml) in to a series of 50 ml volumetric flasks. The volume was made up to the mark with the mobile phase to get a set of solutions having the

concentration range, ranging from 250, 375, 500, 625 and 750 µg/ml of Amoxicillin trihydrate.

Triplicate dilutions of each of the above mentioned concentrations were prepared separately and from these triplicate solutions, 20 µl of each concentration of the drug were injected into the UPLC system two times separately and their chromatograms were recorded under the same chromatographic conditions as described above. Peak areas were recorded for all the peaks and a standard calibration curve of Area against concentration was plotted.

Potassium clavulanate

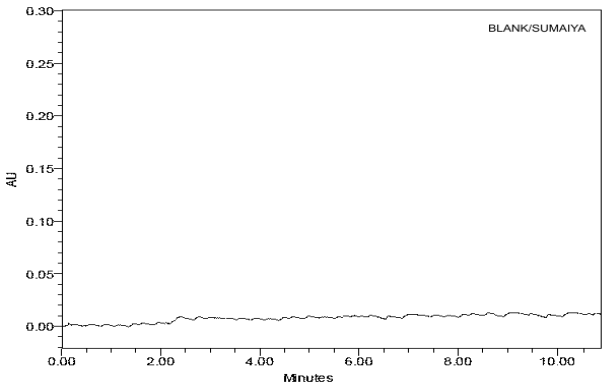
Appropriate aliquots were pipetted out from the standard stock solution (125 µg/ml) in to a series of 50 ml volumetric flasks. The volume was made up to the mark with the mobile phase to get a set of solutions having the concentration range, ranging from 62.5, 93.75, 125, 156.25 and 187.5 µg/ml of Potassium Clavulanate.

Triplicate dilutions of each of the above mentioned concentrations were prepared separately and from these triplicate solutions, 20 µl of each concentration of the drug were injected into the UPLC system two times separately and their chromatograms were recorded under the same chromatographic conditions as described above. Peak areas were recorded for all the peaks and a standard calibration curve of Area against concentration was plotted.

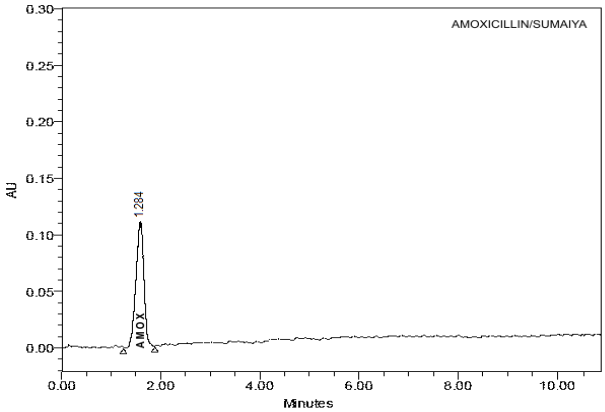
Both the drugs follow the Beer's & Lambert's law in the concentration range of 250 to 750µg/ml for Amoxicillin trihydrate and 62.5 to 187.5 µg/ml for Potassium Clavulanate. The linearity of calibration curves and adherence of the system to Beer's & Lambert's law was validated by high value of correlation coefficient and less than 2% relative standard deviation (R.S.D.) for the intercept value.

Gradient composition of amoxicillin and potassium clavulanate:

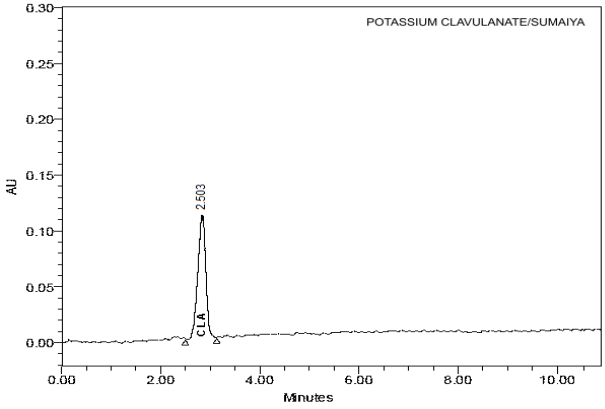
Time Interval (Mins.)	Solvent – A% (Trifluroacetic acid)	Solvent – B% (Mobile Phase)
0-2	0	100
2-4	25	75
4-8	15	85
8-10	0	100



Chromatogram of blank chromatogram.



Chromatogram of amoxicillin.



Chromatogram of potassium clavulanate.

Accuracy study.

Drug	Level %	Amount Added (µg/ml)	Amount found(µg/ml)	% Recovery	Mean recovery(%)	Std. Dev	% RSD
Amoxicillin	50	5.27	5.22	99.05	98.69%	0.306	0.31%
	100	10.02	9.87	98.50			
	150	15.12	14.90	98.54			
Potassium Clavulanate	50	1.25	1.24	99.20	98.98%	0.194	0.20%
	100	2.5	2.47	98.82			
	150	3.78	3.74	98.94			
Note: Each value corresponds to the mean of three replicates.							

System precision

Procedure

"The parameters, retention time (RT), theoretical plates (N), tailing factor (T), peak asymmetry (As) and repeatability were evaluated at a concentration of 10 µg/mL (*Amoxicillin and Potassium Clavulanate)*."

Results of system precision/system suitability parameters

Table: Results of system suitability for amoxicillin.

S.no	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Amoxicillin	1.324	795554	143273	5827	1.4
2	Amoxicillin	1.315	795638	143456	5973	1.3
3	Amoxicillin	1.324	795473	143377	5745	1.5
4	Amoxicillin	1.311	795832	143483	5886	1.3
5	Amoxicillin	1.184	795589	143439	5824	1.5
Mean			795617.2			
Std. Dev			134.30			
% RSD			0.02%			

Table: Results of system suitability for potassium clavulanate.

S.no	Name	Rt	Area	Height	USP plate count	USP Tailing
1	Clavulanate	2.496	538947	143284	5958	1.4
2	Clavulanate	2.493	539072	143472	6021	1.3
3	Clavulanate	2.623	539245	143394	5964	1.5
4	Clavulanate	2.508	539367	143498	5932	1.3
5	Clavulanate	2.316	539182	143474	6088	1.5
Mean			539162.6			
Std. Dev			160.96			
% RSD			0.03%			

Precision

Results of method Precision

Replicate	***Amoxicillin***		**Clavulanate**
S.No.	**Injection volume (µl)**	**Area**	**Area**
1	10 ul	795554	538990
2		795353	538952
3		795356	538947
4		795452	538964
5		795455	538873
6		795236	538748
Average	795401		538912
Std.Dev	109.98		89.47
% RSD	0.01%		0.02%
Standard weight	500 mg		125 mg
Standard potency	99.98%		99.98%

Linearity

Linearity level	***Amoxicillin***		***Potassium Clavulanate***	
Level	**Concentration (µg/ml)**	**Area**	**Concentration (µg/ml)**	**Area**
1	250	273880	62.5	140028
2	375	524320	93.75	266542
3	500	745761	125	400057
4	625	992201	156.25	527571
5	750	1258641	187.5	645085
Correlation co-efficient	0.9992		0.9994	
Slope	247703		129188	
Intercept	234494		122276	

For Amoxicillin

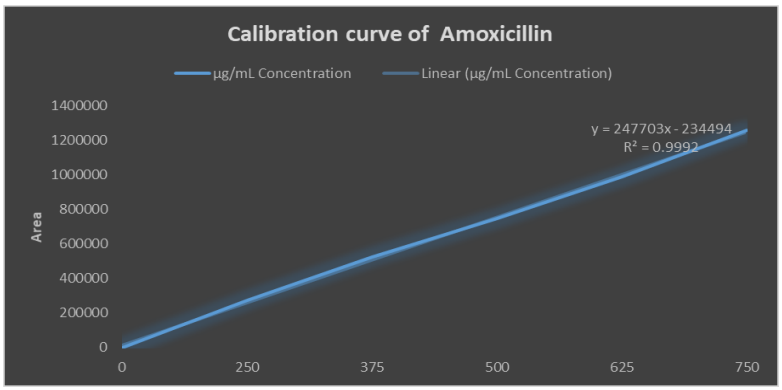


Figure : Calibration curve of Amoxicillin.

For potassium clavulanate

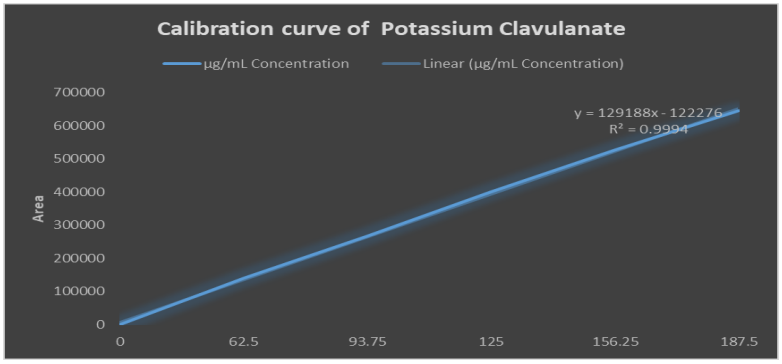


Figure : Calibration curve of potassium clavulanate.

Robustness

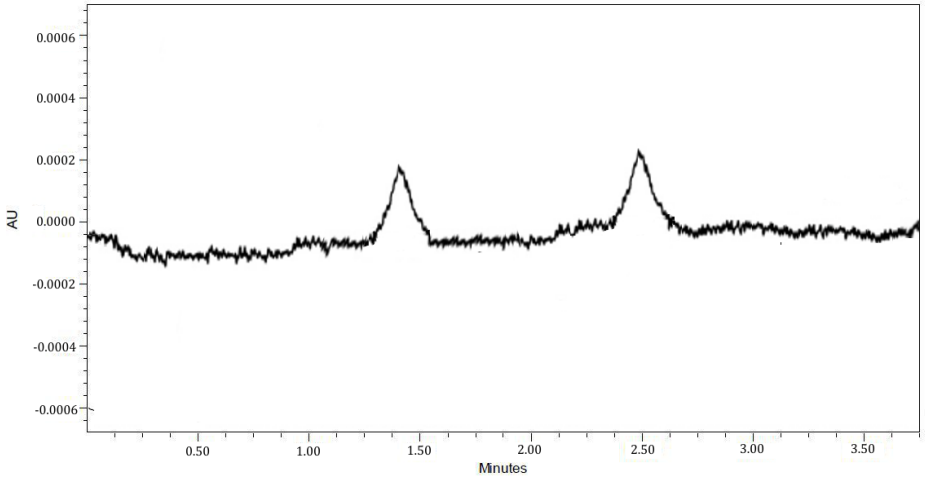
Parameter	*Amoxicillin*		*Potassium Clavulanate*	
	Peak Area	% RSD	Peak Area	%RSD
Low	794891	0.03%	538633	0.04%
	794773		538248	
	794425		538384	
Actual	795386	0.02%	538647	0.02%
	795621		538912	
	795432		538738	
High	796288	0.02%	539339	0.03%
	796492		539421	
	796631		539672	

Ruggedness

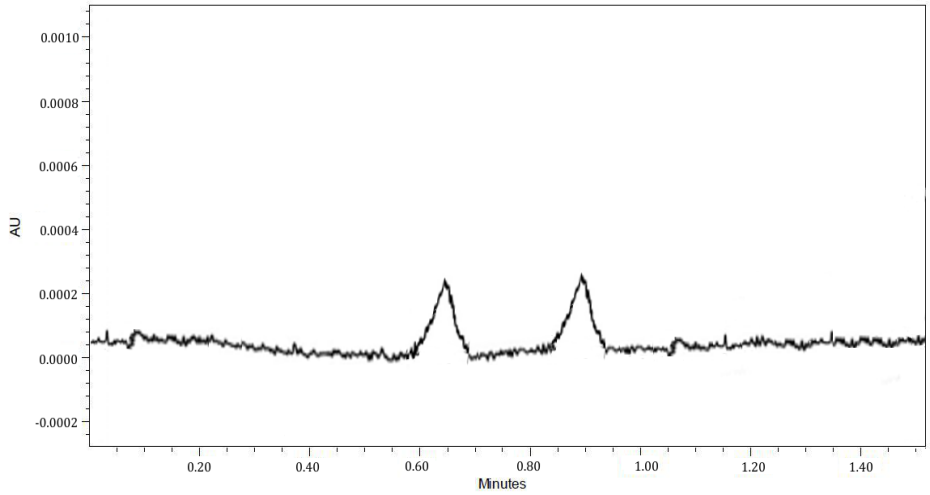
Parameter	Amoxicillin		Potassium Clavulanate	
	Peak Area	% RSD	Peak Area	%RSD
Intraday precision	795341	0.02%	538734	0.04%
	795689		538281	
	795537		538598	
Inter day precision	795834	0.01%	538943	0.03%
	795728		538654	
	795684		538589	
Instrument:1 Acquity UPLC Waters,2695H	796132	0.03%	538514	0.04%
	796552		538403	
	796476		538117	
Instrument:2 Agilent Technologies,1290	798158	0.03%	539524	0.02%
	798562		539454	
	798464		539628	

The formula for finding is LOD and LOQ
LOD=3(SD/S)
LOQ=10(SD/S)

Procedure
The limit of detection and limit of quantification were evaluated by serial dilutions of of Amoxicillin and Potassium Clavulanate stock solution in order to obtain signal to noise ratio of 3:1 for LOD and 10:1 for LOQ as per ICH guidelines.



Chromatogram no: chromatogram of LOD study.



Chromatogram no: chromatogram of LOQ study.

Calculations of LOD and LOQ

LOD for *amoxicillin*
LOD=3.3(SD/S)
SD= 223.797
S= 247703

$$\text{LOD: } 3.3\ \frac{223.797}{247703}$$

LOD= 0.0029 (µg/ml)

LOQ for *amoxicillin*
LOQ=10.1(SD/S)
SD= 223.797
S= 247703

$$\text{LOQ} = 10.1\ \frac{223.797}{247703}$$

LOQ= 0.0091 (µg/ml)

LOD for potassium clavulanate
LOD=3.3(SD/S)
SD= 204.82
S= 129188

$$\text{LOD} = 3.3\ \frac{204.82}{129188}$$

LOD= 0.0052 (µg/ml)

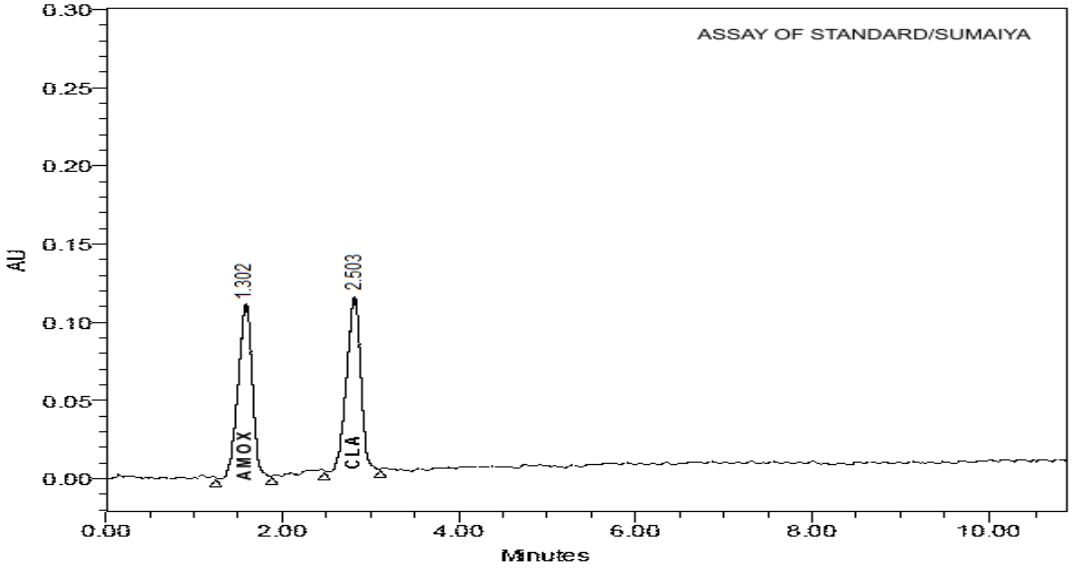
LOQ for potassium clavulanate
LOQ=10.1(SD/S)
SD= 204.82
S= 129188

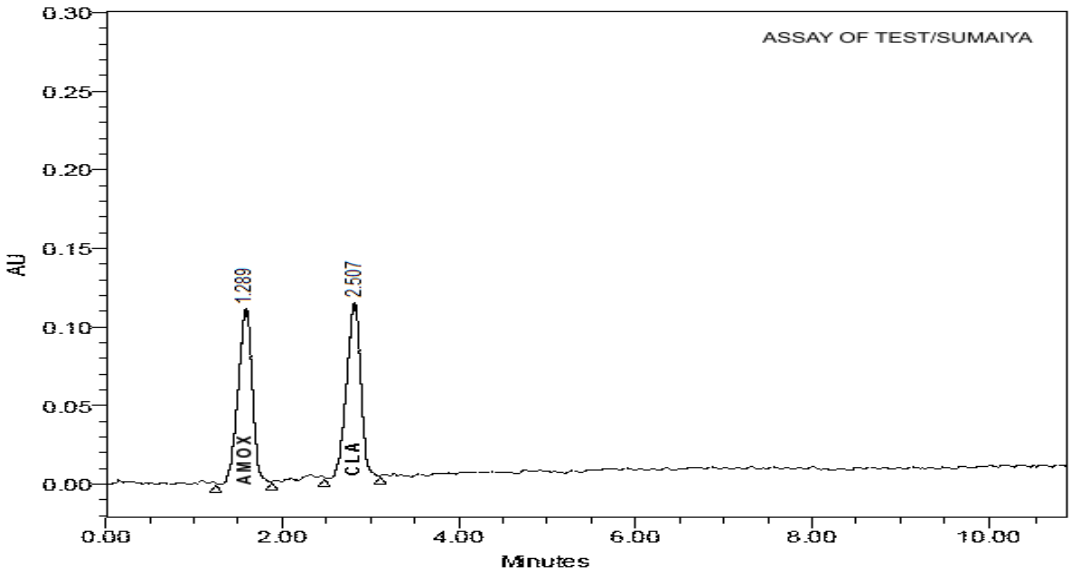
$$\text{LOQ= } 10.1\ \frac{204.82}{129188}$$

LOQ = 0.0160 (µg/ml)

Analysis of formulation

Assay studies for the analysis of formulation of Amoxicillin and Potassium Clavulanate. Fixed chromatographic conditions were made use for the analysis of formulation.





Calculation formula for Amoxicillin

$$\%\ Assay = \frac{AT}{AS} \times \frac{W1}{100} \times \frac{1}{25} \times \frac{100}{W2} \times \frac{25}{1} \times \frac{AW}{LC} \times P$$

Whereas,"

"AT = Average area of test preparation, 789694"

"AS = Average area of standard preparation, 795839"

"W1 = Weight taken of reference standard (µg), 10.32"

"W2 = Weight taken of test sample (µg), 10.32"

"AW = Average weight (mg), 500.40"

"LC = Label claim (mg), 500"

"P = Potency of reference standard (%), 99.98%"

Amoxicillin

$$\%\ Assay = \frac{789694}{795839} \times \frac{10.32}{100} \times \frac{1}{25} \times \frac{100}{10.32} \times \frac{25}{1} \times \frac{500.40}{500} \times 99.98 = 99.28\%$$

Calculation formula for potassium clavulanate.

$$\%\ Assay = \frac{AT}{AS} \times \frac{W1}{100} \times \frac{1}{25} \times \frac{100}{W2} \times \frac{25}{1} \times \frac{AW}{LC} \times P$$

Whereas,"
"AT = Average area of test preparation, 542403"
"AS = Average area of standard preparation, 548321"
"W1 = Weight taken of reference standard (µg), 2.5"
"W2 = Weight taken of test sample (µg), 2.5"
"AW = Average weight (mg), 125.65"
"LC = Label claim (mg), 125"
"P = Potency of reference standard (%), 99.98%"

Potassium clavulanate

$$\%\ Assay = \frac{542403}{548321} \times \frac{2.5}{100} \times \frac{1}{25} \times \frac{100}{2.5} \times \frac{25}{1} \times \frac{125.65}{125} \times 99.98 \quad = 99.41\%$$

CONCLUSION

A specific, precise, accurate ultra pressure liquid chromatography (UPLC) method is developed for estimation of Amoxicillin and Potassium Clavulanate 500+125MG in bulk dosage form. The method employed, with Xterra RP-8 (150mm x 4.6 mm i.d., particle size 5 µm) in a gradient mode, with mobile phase of KH2PO4: Methanol (80:20) and effluent was monitored at 248 nm. The method was validated in terms of linearity, accuracy, precision, limit of detection (LOD), limit of quantification (LOQ) etc. in accordance with ICH guidelines. Linear regression analysis data for the calibration plot showed that there was good linear relationship between response and concentration in the range of 250-750 µg/ml amoxicillin and 62.5-187.5 µg/ml for potassium clavulanate respectively. The LOD and LOQ values for were found to be 0.0029 (µg/ml) and 0.0091 (µg/ml) for amoxicillin and 0.0052 (µg/ml) and 0.0160 (µg/ml) for potassium clavulanate respectively.

The method provides selective quantification of Amoxicillin and Potassium Clavulanate 500+125MG in bulk dosage form without interference from blank affirming precise method. The proposed method is highly sensitive, reproducible, specific and rapid. The method was completely validated showing satisfactory data for all the method validation parameters.

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