

# Development and Validation of Stability Indicating HPLC Method for Simultaneous Estimation of Amoxicillin and Clavulanic Acid in Injection

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## Abstract

A simple, fast, precise, accurate and rugged stability indicating high performance liquid chromatography (HPLC) method has been developed for simultaneous estimation of Amoxicillin and Clavulanic acid from injectable dosage form. The stability indicating capability of the method was proven by subjecting the drugs to stress conditions as per ICH recommended test conditions such as alkaline and acid hydrolysis, oxidation, photolysis, thermal degradation and resolution of the degradation products formed therein. The separation was obtained using a mobile phase composition at a ratio of 95:5 (v/v) of pH 5.0 buffer and methanol on Inertsil C18 column (250 × 4.0 mm, 4 μm) with UV detection at 220 nm at a flow rate of 1 ml/minute. The photodiode array detector was used for stress studies. The order of elution of peaks was Clavulanic acid followed by Amoxicillin. The linear calibration range was found to be 79.51 to 315.32 μg/ml for Amoxicillin and 17.82 to 67.90 μg/ml for Clavulanic acid. The Amoxicillin and Clavulanic acid were found to be stable in solution up to 24 hours. The method validation data showed excellent results for precision, linearity, specificity, limit of detection, limit of quantification and robustness. The present method can be successfully used for routine quality control and stability studies.

**Keywords:** Amoxicillin, Clavulanic Acid, HPLC, Stability Indicating

## 1. Introduction

Clavulanic acid is a novel beta-lactam compound which was isolated from the culture fluid of *Streptomyces clavuligerus* [1]. The compound is a potent inhibitor of a large number of beta-lactamase enzymes, which are responsible for the resistance of many bacteria to beta-lactam antibiotics [2]. In the presence of clavulanic acid, beta-lactamase labile penicillins are protected from degradation by cell-free beta-lactamase preparations and by whole bacterial cultures [3].

Amoxicillin-Clavulanate potassium, a semi-synthetic penicillin beta lactamase inhibitor combination drug, is a widely used oral antibiotic [4]. Amoxicillin-Clavulanic acid has been widely used as a prophylactic antibiotic in abdominal and gynecological surgery [5]. It is effective in the prevention of wound infections in operations in which the most likely pathogens are gram-negative, anaerobic, or mixed bacteria [6]. Chemically, Amoxicillin sodium is sodium (2S,5R,6R)-6-[®(-)-2-Amino-2-(P-

hydroxyphenyl) acetamido]-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0] heptane-2-Carboxylate [7] and Clavulanate potassium is potassium (2)-(2R,5R)-3-(2-hydroxyethylidene)-7-oxo-4-1-azabicyclo[3.2.0]-heptane-2-carboxylate [8]. Both of these drugs are official in pharmacopoeias such as USP, BP etc. Several Amoxicillin and Clavulanic acid combinations are available in market in the form of tablet and suspension. In yester years injectable formulation has also been developed.

Literature survey reveals that several HPLC methods are reported for the determination of Amoxicillin [9-11] and Clavulanic acid [12,13] individually. However, no method is reported for simultaneous estimation of these two drugs in injectable formulation by HPLC.

In the present investigation, a specific stability indicating HPLC method is described for simultaneous estimation of these two drugs in injectable dosage form. Both the drugs were subjected to stress studies and the degradation products formed were separated from the main peaks. The proposed validated stability indicating

method can be used for routine quality control and stability studies of the subjected formulation.

## 2. Materials and Methods

### 2.1. Instrumentation

The high pressure liquid chromatographic (HPLC) system was used of model Agilent 1100 series equipped with binary pump (G1312A), auto sampler (G1316A), thermo stated column compartment and variable wavelength detector (G1314A) controlled by the Chem station software. A HPLC system equipped with isocratic pump (G1310a), rheodyne injector (7725i) and photodiode array detector (G1315A) was used for degradation studies. A column Inertsil C18 HPLC column (250 × 4.0 mm, 4 μm) was used as a stationary phase.

### 2.2. Materials

Working standards of Amoxicillin trihydrate and Clavulanate potassium with lithium of potencies 86.12% (on as basis as Amoxicillin) and 42.11% (on as in basis as Clavulanic acid). Potassium dihydrogen phosphate monohydrate, sodium hydroxide and ortho-phosphoric acid were used of analytical reagent grade. HPLC grade methanol manufactured by E. Merck was procured from commercial source. HPLC grade water was obtained using milipore water purification system. Clavam 1.2 g (Amox-

icillin and Clavulanate potassium for injection) is a dry powder injection contains Amoxicillin sodium equivalent to 1 g of Amoxicillin and Clavulanate potassium equivalent to 0.2 g of Clavulanic acid.

### 2.3. Methods

#### 2.3.1. Chromatographic Conditions

The analysis was carried out on binary HPLC system using the column Inertsil C18 (250 × 4.0 mm, 4 μm) with UV-detection of 220 nm at ambient temperature using 20 μl injection volume. For degradation studies, an isocratic pump and a photodiode array detector were used.

#### 2.3.2. Selection of Wavelength

The bands of peaks are showing the maximum detection level for Amoxicillin in the range of 210 nm to 240 nm and Clavulanic acid in the range of 210 nm to 230 nm. A single wavelength has selected for estimation of Amoxicillin and Clavulanic acid as 220 nm as both the peaks have the significant response. Overlaid spectrum of Amoxicillin and Clavulanic acid is given in **Figure 1**.

#### 2.3.3. Mobile Phase

A degassed mixture of (95:5 v/v) pH 5.0 buffer (7.8 g of sodium dihydrogen phosphate monohydrate was dissolved, diluted to 1000ml with water and adjusted the pH to 5.0 ± 0.05 using dilute ortho-phosphoric acid solution) and methanol.

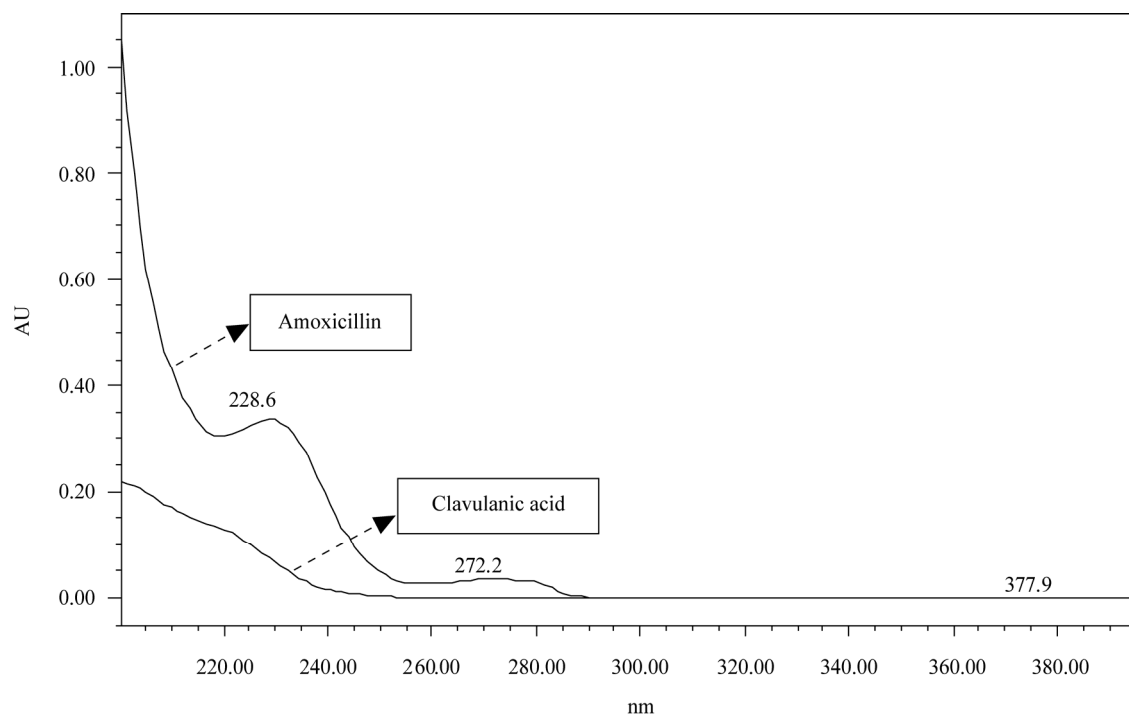


Figure 1. Overlaid spectrum of Amoxicillin and Clavulanic acid.

### 2.3.4. Standard Solution

Standard stock solution was prepared by dissolving Amoxicillin trihydrate working standard equivalent to 100 mg of Amoxicillin and Clavulanate potassium working standard equivalent to 20 mg of Clavulanic acid in 60 ml of water and then diluted to 100 ml with the same solvent. This stock solution was further diluted with water to get a solution of concentration 200 µg/ml of Amoxicillin and 40 µg/ml of Clavulanic acid. A typical chromatogram is given in **Figure 2**.

### 2.3.5. Calibration

Seven different concentration (79.51-315.32 µg/ml of Amoxicillin and 17.82 to 67.90 µg/ml of Clavulanic acid) of mixture of Amoxicillin and Clavulanic acid were prepared for linearity studies. The responses were measured as peak areas and plotted against concentration.

### 2.3.6. Estimation of Amoxicillin and Clavulanic Acid from Injectable Formulation

Ten samples of Clavam 1.2 g were emptied completely and mixed. The pooled powder sample equivalent to about 20 mg of Clavulanic acid and 100 mg of Amoxicillin was transferred to dried 100ml volumetric flask. Dissolved and diluted to 100mL with water. This stock solution was further diluted with water to get a solution of concentration 200 µg/mL of Amoxicillin and 40 µg/ml of Clavulanic acid. 20 µl of each standard and test preparations were injected into the chromatography and the responses were recorded.

## 2.4. Validation Parameters

The method validation was carried out as per ICH guidelines [14]. Various method validation parameters ascertained are as follows.

### 2.4.1. Precision

Injection repeatability (System precision):

The injection repeatability was established by six replicate injections of the standard solution containing both the analytes of interest.

Sample repeatability (Method precision):

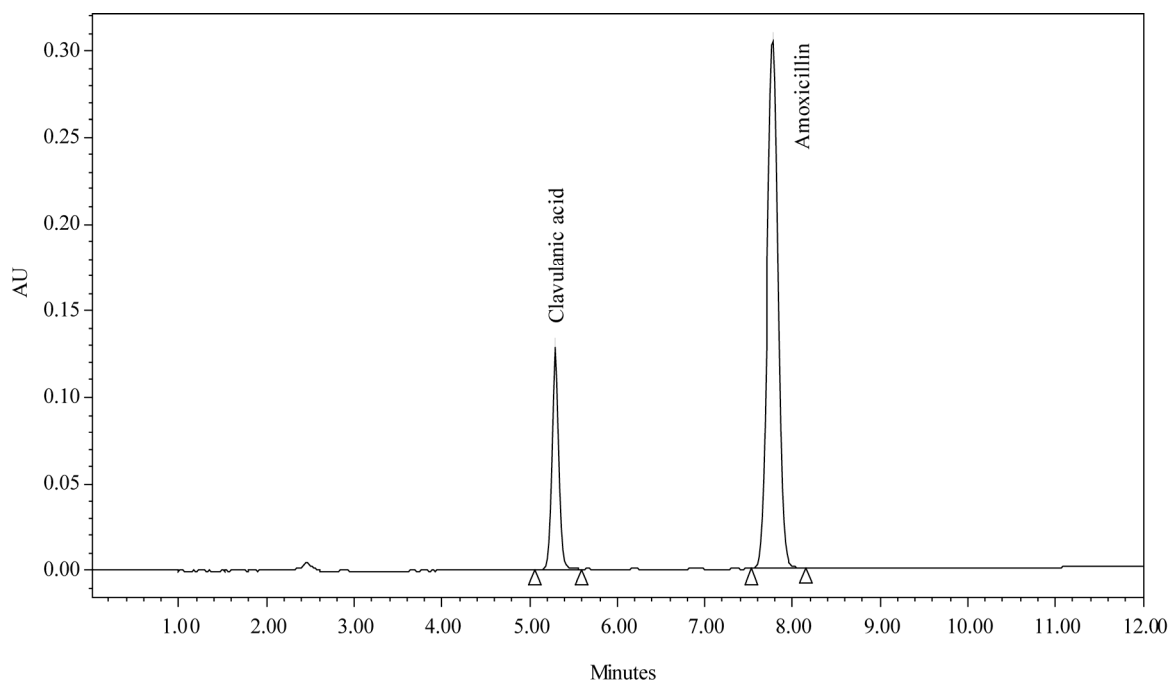
The sample repeatability was established by carrying out the analysis of the analytes six times.

### 2.4.2. Specificity/Selectivity

Specificity of the method was evaluated by injecting the blank, individual Amoxicillin, Clavulanic acid and sample solution prepared by spiking known impurities of Amoxicillin and Clavulanic acid (*i.e.*, 6-Aminopenicillanic acid and p-Hydroxyphenyl glycine) in sample at 1% level of test concentration and injected into HPLC system to check the co-elution, if any, at the retention time of Amoxicillin peak and Clavulanic acid peak.

### 2.4.3. Stress Testing (Forced Degradation)

To further confirm the stability indicating nature of the analytical method, Amoxicillin and Clavulanate potassium were subjected to stress testing as per ICH recommended test conditions [15,16]. The drugs were subjected



**Figure 2.** A typical chromatogram of standard.

to acid hydrolysis by using 0.5M hydrochloric acid and base hydrolysis by using 0.1N sodium hydroxide solution; oxidation by using 0.1%v/v solution of hydrogen peroxide; thermal and photolysis.

The objective of stress study was to generate the degradation products under various stress conditions. The stress conditions varied both in terms of temperature and time from moderate to extreme to achieve the appropriate degradation. The spectral purity of the main peaks was evaluated using photodiode array detector and chemstation to verify that the degradation peaks are well resolved from the main peaks.

#### 2.4.4. Limit of Detection and Limit of Quantitation

The limit of detection (LOD) and the limit of quantitation (LOQ) for Amoxicillin and Clavulanic acid were determined.

#### 2.4.5. Ruggedness (Intermediate Precision)

The ruggedness of the method was demonstrated by analysis of the sample by using other column of same brand with same specification and other instrument of same brand and same configuration (Waters alliance HPLC with Empower software). The percent relative standard deviation (% RSD) of two sets of data indicates the ruggedness of the method and t-test was performed.

#### 2.4.6. Robustness

The robustness of the method was determined as a measure of the analytical method capability to be unaffected by small variations in method parameters. The different variations such as variation in flow rate by  $\pm 0.2$  ml/minute; variation in wavelength by  $\pm 2$  nm, variation in temperature by  $\pm 5^\circ\text{C}$ , variation in composition of mobile phase  $\pm 5\%$  absolute ( in terms of organic component) and variation in pH of buffer by  $\pm 0.2$  units. At these changed conditions, the standard and sample solutions were injected. The system suitability was evaluated in each varied condition. The amounts of Amoxicillin and Clavulanic acid were calculated from sample solution in each varied condition.

#### 2.4.7. Stability of the Solution

A sample solution of Amoxicillin and Clavulanate potassium for injection was prepared and analyzed initially and also at different time intervals by keeping the solution at room temperature.

#### 2.4.8. System Suitability Tests

System suitability was verified by injecting standard solution on various days of the validation. The percent RSD was verified from the replicate injections of standard solution. Various parameters such as tailing factor

and resolution between the peaks of Amoxicillin and Clavulanic acid were obtained.

### 3. Results and Discussion

#### 3.1. Precision

The values of the relative standard deviation of six replicate injections of the standard solution containing both the analytes of interest lie well within the limits (% RSD  $\leq 2.0$ ), indicating the injection repeatability of the method. The values of the percent relative standard deviation for sample repeatability also lie well within the limits (% RSD  $\leq 2.0$ ) indicating the sample repeatability of the method. The results are given in **Table 1**.

#### 3.2. Specificity/Selectivity

There was no interference from the blank and also from the impurities (*i.e.*, 6-Aminopenicillanic acid and p-Hydroxyphenyl glycine) at the retention time of Amoxicillin and Clavulanic acid peaks. The peak purity data shows the peaks are pure and there was no co-eluting peak at the retention time of Amoxicillin peak and Clavulanic acid peaks.

**Table 1. Compiled data of method precision and ruggedness.**

S. No.	% Assay of Amoxicillin		% Assay of Clavulanic acid	
	I	II	I	II
1.	99.25	99.10	103.41	103.72
2.	98.81	99.50	106.94	103.41
3.	99.11	99.29	102.71	102.81
4.	100.21	100.10	102.41	102.79
5.	98.71	101.57	106.14	100.39
6.	101.72	101.65	102.01	99.97
Mean.	99.85	99.41	104.19	101.94
SD	1.851	1.751	1.944	1.740
% RSD	1.87	1.75	1.85	1.69
Overall Mean.	99.49		104.015	
Overall SD	1.801		1.842	
Overall % RSD	1.81		1.77	

I-Method Precision, II-Ruggedness.

### 3.3. Stress Testing (Forced Degradation)

The % degradation of Amoxicillin in acid hydrolysis, base hydrolysis, oxidation, thermal and photolytic was 6.65, 6.21, 5.01, 13.12 and 8.59 respectively. The % degradation of Clavulanic acid in acid hydrolysis, base hydrolysis, oxidation, thermal and photolytic was 23.21, 14.41, 0.00, 17.80 and 10.21 respectively. Peak purity was also found to be passed. The peaks of the degradation products were well resolved from the peaks of the main component. The results of the stress studies are tabulated in **Tables 2(a)** and **2(b)**.

### 3.4. LOD and LOQ

The LOD and LOQ for Amoxicillin and Clavulanic acid were found to be 3.98 µg/ml and 12.06 µg/ml for Amoxicillin and 7.13 µg/ml and 21.62 µg/ml for Clavulanic acid, respectively.

### 3.5. Ruggedness (Intermediate Precision) & Robustness

The percent relative standard deviation (% RSD) of two sets of data indicates the ruggedness of the method. Further, the t-test was performed on the data and the differ-

ence was found to be not significant. The compiled data of method precision and ruggedness are given in **Table 1**. The method is not affected by the deliberate variations such as flow rate, wavelength, mobile phase composition, temperature and pH.

### 3.6. Linearity

Calibration curve obtained by the least square regression analysis between average peak area and concentration showed linear relationship with a correlation coefficient of 0.9998 0.9994 for Amoxicillin and Clavulanic acid respectively over the calibration ranges tested.

The linear calibration range was found to be 79.51 to 315.32 µg/ml for Amoxicillin and 17.82 to 67.90 µg/ml for Clavulanic acid. The linearity of the calibration curves indicated the suitability of the method over a wide range of concentration of both the actives.

Linear regression least square fit data obtained from the calibration curves are given in **Tables 3(a)** and **3(b)**.

### 3.7. System Suitability and Stability of Analytical Solution

The tailing of Amoxicillin and Clavulanic acid were in the range of 0.96-0.99 and 1.07-1.14 respectively. The %

**Table 2. (a) Forced degradation for Amoxicillin.**

Mode of Degradation	Condition	% Assay	% Degradation w. r. t control	Peak purity data
Control	No treatment	101.80	-	998.31
Acid	0.5 N HCl (2 hrs)	97.10	6.65	998.64
Base	0.1 N NaOH (0 hrs)	96.42	6.21	992.71
Oxidation	0.1% v/v H <sub>2</sub> O <sub>2</sub> (0 hrs)	98.21	5.01	998.63
Thermal	50°C (3 hrs)	88.92	13.12	998.39
Photolytic	UV-light (3 hrs)	95.21	8.59	998.78

**Table 2. (b) Forced degradation for Clavulanic acid.**

Mode of Degradation	Condition	% Assay	% Degradation w. r. t control	Peak purity data
Control	No treatment	101.27	-	999.21
Acid	0.5 N HCl (2 hrs)	78.91	23.21	999.90
Base	0.1 N NaOH (0hrs)	87.72	14.41	999.43
Oxidation	0.1% v/v H <sub>2</sub> O <sub>2</sub> (0 hrs)	108.44	-	998.40
Thermal	50°C (3 hrs)	84.21	17.80	999.10
Photolytic	UV-light(3 hrs)	91.39	10.21	999.11

**Table 3. (a) Linearity of response for Amoxicillin.**

S. No.	Conc. ( $\mu\text{g/ml}$ )	Peak area
1.	79.51	2322.80
2.	118.70	3458.52
3.	158.21	4674.12
4.	197.67	5767.28
5.	236.81	6972.31
6.	276.21	8067.69
7.	315.32	9187.91
Slope.		29.18
Intercept		43.34
Correlation Coefficient		0.9998

**Table 3. (b) Linearity of response for Clavulanic acid.**

S. No.	Conc. ( $\mu\text{g/ml}$ )	Peak area
1.	17.82	610.35
2.	26.11	708.51
3.	34.38	1226.31
4.	42.80	1509.10
5.	51.09	1820.12
6.	59.58	2095.61
7.	67.90	2485.09
Slope.		35.44
Intercept		26.14
Correlation Coefficient		0.9994

RSD for five replicate injections of standard was found in the range of 0.03-0.05 for Amoxicillin peak and 0.13-0.31 for Clavulanic acid peak. Resolutions between the peaks were found in the range of 12.01-13.21. The resolution, tailing factor and % RSD values are given in **Table 4**.

The peaks of Amoxicillin and Clavulanic acid were found to be symmetrical and well defined, free from tailing. The elution order was Clavulanic acid first at retention time of about 5.3 minutes followed by Amoxicillin at retention time of about 7.5 minutes. The sensitivity of the method using the said method was found to be good. The total run time of each analysis is about 12 minutes, which is rapid and economical considering the

**Table 4. System suitability.**

Experiment	R	% RSD		Tailing factor	
		Amoxicillin peak	Clavulanic acid peak	Amoxicillin peak	Clavulanic acid peak
Method precision	12.01	0.04	0.13	0.99	1.14
Stress study	13.21	0.05	0.29	0.96	1.12
Linearity	12.32	0.03	0.14	0.99	1.14
Ruggedness	12.60	0.03	0.31	0.96	1.07

R-Resolution between Amoxicillin and Clavulanic acid peaks.

stability indicating nature of the method. The sample solutions were found to be stable up to 24 hours.

## 4. Conclusions

The analytical method is simple, specific, rugged and stability indicating. Hence it can be used for routine quality control analysis as well as stability studies of injection and might be useful for other pharmaceutical dosage forms. The developed method is stability indicating and it is LOD and LOQ values are in the range of 3.98  $\mu\text{g/ml}$  and 12.06  $\mu\text{g/ml}$  for Amoxicillin and 7.13  $\mu\text{g/ml}$  and 21.62  $\mu\text{g/ml}$  for Clavulanic acid. This method is sensitive and superior to the published methods since those are neither stability indicating nor LOD and LOQ values are estimated at low level.

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