METHOD DEVELOPMENT AND VALIDATION FOR AMOXICILLIN AND CLAVULANIC ACID BY RP – HPLC METHOD

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ABSTRACT

A new, simple, precise, rapid and accurate RP-HPLC method has been developed for the simultaneous estimation of amoxicillin and clavulanic acid from pharmaceutical dosage forms. 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.

Key Words: Stability, Validation, HPLC, Amoxicillin, Clavulanic acid.

INTRODUCTION

Amoxicillin is a widely-used antibiotic drug. It belongs to the penicillin group of drugs and is prescribed to treat certain infections that are caused by bacteria [1]. Amoxicillin is chemically 7-[2-amino-2-(4-hydroxyphenyl)-acetyl] amino-3,3 dimethyl-6-oxo-2-thia-5-azabicyclo[3.2.0] heptanes-4-carboxylic acid. Clavulanic acid is a novel beta-lactam compound which was isolated from the culture fluid of Streptomyces clavuligerus [2]. 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 [3]. Chemically clavulanate potassium is potassium (2)-(2R,5R)-3-(2-hydroxyethylidene)-7-oxo-4-1-azabicyclo[3.2.0]-heptane-2-carboxylate [4]. In the presence of clavulanic acid beta-lactamase labile penicillin’s are protected from degradation by cell-free beta-lactamase preparations and by whole bacterial cultures [5]. Amoxicillin-Clavulanate potassium, a semi-synthetic penicillin beta lactamase inhibitor combination drug, is a widely used oral antibiotic [6]. Amoxicillin-Clavulanic acid has been widely used as a prophylactic antibiotic in abdominal and gynecological surgery [7]. It is effective in the prevention of wound infections in operations in which the most likely pathogens are gram-negative, anaerobic, or mixed bacteria [8]. Literature survey reveals that several HPLC methods are reported for the determination of Amoxicillin [9-11] and Clavulanic
acid 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 [12, 13].

MATERIALS AND METHODS:
Amoxicillin and Clavulanic Acids were procured from Ranbaxy Laboratories Ltd. (Bollarum, Hyderabad, A.P, India).

Simultaneous Estimation of Amoxicillin and Clavulanic Acid in Pharmaceutical Dosage Form by RP-HPLC Method:
The developed RP-HPLC method for the estimation of Amoxicillin and Clavulanic Acid was carried out on ZORBAX C18 250×4.6mm, 5μm HPLC column in isocratic mode using mobile phase composition of Buffer (PH 3 adjusted with ortho phosphoric acid): Methanol [70 : 30, v / v] with flow rate of 1.0 ml / min at 220 nm. The Asymmetric factor was found to be 1.67.

PREPARATIVE STEPS FOR THE ASSAY METHOD DEVELOPMENT:
Preparation of Buffer:
Weigh accurately 7.8 g of Sodium dihydrogen phosphate (NaH₂PO₄) and dissolve it in 1000ml of Milli-Q water. Adjust the pH to 3 with ortho-phosphoric acid, filter through 0.45µm nylon membrane filter and degas.

Preparation of Mobile Phase:
Buffer and Methanol were mixed in the ratio of 70:30 and degassed by sonication.

Preparation of Blank (Diluent):
Mixed MilliQ water and methanol in the ratio of 50:50 v/v and degassed by sonication.

Preparation of Standard stock Solution:
Accurately weighed and transferred about 500 mg of Amoxicillin working standard and 125mg of Clavulanic Acid working standard into a 50 ml volumetric flask, added to it about 35 ml of diluent and sonicated to dissolve, diluted up to the mark with diluent and mixed well. Further diluted 1.0 ml of the above solution to 50 ml with diluent and mixed. Filtered through 0.45μ nylon membrane filter discarded first few ml of filtrate.

Preparation of Test solution of Amoxicillin and Clavulanic Acid (500/125mg) Tablets
Randomly selected 20 tablets and determined the average weight and crush the tablets into fine powder. Weigh and transfer tablet powders of 710.50mg into a 50ml of volumetric flask add 10ml of water and sonicate for 10 minutes with intermittent shaking. Allowed the solution to cool at room temperature and diluted to volume with diluents and mixed well. Further diluted 1.0 ml of the above solution to 50 ml with diluents. Filtered through 0.45μ nylon membrane filter discarded first few ml of filtrate.

Procedure:
Equilibrated the column with mobile phase for sufficient time until stable baseline is obtained. Injected blank (diluent) in single, standard solution in five replicates, and each test solution in duplicate into the
chromatographic system and recorded the chromatograms. Injected above samples as per following sequence:

Table 1: Sequence of injected samples

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Sample Name</th>
<th>No. of injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Blank (diluent)</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Standard solution</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>Test solution-1</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>Test solution-2</td>
<td>2</td>
</tr>
<tr>
<td>5</td>
<td>Test solution-3</td>
<td>2</td>
</tr>
<tr>
<td>6</td>
<td>Standard solution (as bracketing)</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>Further test solution (if any up to 6 injections)</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>Standard solution (as bracketing)</td>
<td>1</td>
</tr>
</tbody>
</table>

METHOD VALIDATION PARAMETERS:
Method validation can be defined as (ICH) “Establishing documented evidence, which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its predetermined specifications and quality characteristics”. Method validation is an integral part of the method development; it is the process by which a method is tested by the developer or user for reliability, accuracy and preciseness of its intended purpose and demonstrating that analytical procedures are suitable for their intended use that they support the identity, quality, purity, and potency of the drug substances and drug products. Simply, method validation is the process of proving that an analytical method is acceptable for its intended purpose. Methods should be reproducible when used by other analysts, on other equivalent equipment, on other days or locations, and throughout the life of the drug product. Data that are generated for acceptance, release, stability, or pharmacokinetic will only be trustworthy if the methods used to generate the data are reliable. All the variables of the method should be considered, including sampling procedure, sample preparation, chromatographic separation, and detection and data evaluation. For chromatographic methods used in analytical applications there is more consistency in validation practice with key analytical parameters including

1. System suitability
2. Specificity
3. Accuracy
4. Precision
5. Linearity
6. Limit of Detection
7. Limit of Quantitation and
8. Robustness

1. SYSTEM SUITABILITY

Stock solution was prepared using Amoxicillin And Clavulanic Acid working standards as per test method and was injected six times into HPLC system. The system suitability parameters were evaluated from
standard Chromatograms obtained, by calculating the % RSD of retention times, tailing factor, theoretical plates and peak areas from six replicate injections.

**Acceptance criteria**

1. The % RSD for the retention times of principal peak from 6 replicate injections of each Standard solution should be not more than 2.0 %
2. The number of theoretical plates (N) for the amoxicillin and clavulanic acid peaks should be not less than 2000.
3. The Tailing factor (T) for the amoxicillin and clavulanic acid peaks should be not more than 2.0.

From the system suitability studies it was observed that all the parameters were within limit. Hence it was concluded that the Instrument, Reagents and Column were suitable to perform the Assay.

**2. LINEARITY**

A series of standard solutions of Amoxicillin and Clavulanic acid were prepared in the range of about 50-150% of test concentration and injected into the HPLC system as per the rest method and labeled as solution 1, 2, 3, 4 and 5 respectively. Concentration ranging from 100µg/ml to 300µg/ml for Amoxicillin and 25µg/ml to 75µg/ml for Clavulanic Acid. The solutions were injected in to HPLC system as per test procedure.

**Acceptance criteria**

1. Correlation Coefficient should be not less than 0.9990.
2. % RSD of peak area’s for Solution 1, 2, 3, 4 and 5 should be not more than 2.0 %.

**3. PRECISION**

Prepare six replicate injection of the standard solution of the same concentration and inject six times one after the other as per test method. The %RSD for the area of six replicate injections was found to be within the specified limits.

**Acceptance criteria**

The % RSD for the area of five standard injections results should not be more than 2%.

**4. ACCURACY**

A study of accuracy was conducted by preparing three different concentrations of the sample solutions of Amoxicillin and Clavulanic acid i.e. 50%, 100% and 150%. Inject them into the HPLC as per test method. The Individual % recovery, mean % recovery, % RSD for recovery at each level was calculated and the results were found to be within the acceptable limits.

**Acceptance criteria**

The mean % recovery of the amoxicillin and clavulanic acid at each spike level should be not less than 98.0 % and not more than 102.0 %.

**5. ROBUSTNESS**

The robustness of the proposed method was determined by analysis of aliquots from homogenous lots by differing physical parameters like flow rate, Temperature and mobile phase composition which may differ but the responses were still within the specified limits of the assay.
a) Effect of variation of flow rate:
A study was conducted to determine the effect of variation in flow rate. Standard solution was prepared and injected into the HPLC system by keeping the flow rate varied from 0.8 to 1.2ml.

Acceptance criteria
The tailing factor of amoxicillin and clavulanic acid standard should be not more than 2.0 for Variation in flow.
1. The % RSD of Asymmetry and retention time of amoxicillin and clavulanic acid standard should be not more than 2.0 % for variation in flow.

b) Effect of variation of mobile phase composition:
A study was conducted to determine the effect of variation in mobile phase ratio by changing the ratio of mobile phase i.e. methanol: Buffer of change Mobile ±10%.

Acceptance criteria
1. Tailing Factor of amoxicillin and clavulanic acid standard should not be more than 2.0 for Variation in composition of mobile phase.
2. The % RSD of tailing factor and retention times of amoxicillin and clavulanic acid standard should be not more than 2.0 for Variation in composition of mobile phase.

6. LIMIT OF DETECTION (LOD):
Based on Signal-to-Noise: This approach can only be applied to analytical procedures which exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

7. LIMIT OF QUANTIFICATION (LOQ)
Based on Signal-to-Noise: This approach can only be applied to analytical procedures that exhibit baseline noise. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.

RESULTS AND DISCUSSION

Fig 1: Chromatogram
By the chromatogram analyzed, identified, purified and quantify the compounds.

Table 2: Analytical Method Validation Reports For Amoxicillin And Clavulanic Acid By RP-HPLC

<table>
<thead>
<tr>
<th>S.No</th>
<th>PARAMETERS</th>
<th>LIMIT</th>
<th>OBSERVATIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>System suitability</td>
<td>%RSD - NMT 2 TF - NMT 2 TP - NLT 2000</td>
<td>Amoxicillin : TF-1.1 Clavulanic acid : TF-1.1 TP-9305</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Resolution : 9.6</td>
</tr>
<tr>
<td>2</td>
<td>Specificity</td>
<td>No Interferences at retention time of the analyte peak.</td>
<td>Interference at retention time of the analyte peak not observed</td>
</tr>
<tr>
<td>3</td>
<td>Precision</td>
<td>RSD NMT 2.0%</td>
<td>Amoxicillin : %RSD- 0.10% Clavulanic acid : %RSD- 0.12%</td>
</tr>
<tr>
<td>4</td>
<td>Linearity of detector response</td>
<td>Correlation co-efficient NLT 0.999</td>
<td>Amoxicillin : 0.9999 Clavulanic acid : 0.9999</td>
</tr>
<tr>
<td>5</td>
<td>Range</td>
<td>----</td>
<td>Amoxicillin : 100 - 300µg/mL Clavulanic acid : 25 -75µg/ml</td>
</tr>
<tr>
<td>5</td>
<td>Accuracy</td>
<td>% Recovery range 98-102%</td>
<td>Amoxicillin : 99.4-101.05% Clavulanic acid : 98.4-101%</td>
</tr>
<tr>
<td>6</td>
<td>Robustness</td>
<td>Should comply with system suitability parameters</td>
<td>Within limits</td>
</tr>
<tr>
<td>7</td>
<td>Assay</td>
<td>%Assay 100%±2%</td>
<td>Amoxicillin : 98.00% Clavulanic acid : 98.05%</td>
</tr>
</tbody>
</table>

CONCLUSION

It is evident that the responses for Amoxicillin and Clavulanic Acid were found to be linear in the studied concentration ranges from 100µg/ml to 300µg/ml and 25µg/ml to 75µg/ml respectively and the correlation coefficient were found to be $r^2 = 0.9999$ and $r^2 = 0.9999$ for Amoxicillin and Clavulanic Acid respectively. The recovery studies were also carried out at 3 levels 50%, 100% and 150% to ensure the accuracy of the method. The average percentage recovery was found to be in the range of 100.3% for Amoxicillin and 99.7% for Clavulanic Acid respectively. In this nearly 100% recovery showed that the method was free from the interference of the excipients used in the formulation. The low %RSD values (NMT 2%) indicated that the developed method was sufficiently precise. The Robustness was performed by making deliberate changes in flow rate and column temperature. It shows that there is no change in the retention time even after making deliberate change in the analytical procedure. Then the method was found to be robust.
All the above method has shown good precision and accuracy. Hence it was concluded that the developed method was found to be Simple, Precise, Accurate, Linear and Rapid method for the analysis of Amoxicillin and Clavulanic Acid in combined dosage form.

REFERENCES
3. Foulstone M and Reading C. Assay of Amoxicillin and Clavulanic Acid, the Component of Augmentin, in Biological Fluids with High Performance Liquid Chromatography. Antimicrobial Agents and Chemotherapy. 1982; 22(5); 753-762.