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DEVELOPMENT AND VALIDATION OF STABILITY INDICATING RP-HPLC METHOD FOR SIMULTANEOUS ESTIMATION OF AMOXICILLIN TRIHYDRATE AND CALVULANIC ACID IN PHARMACEUTICAL DOSAGE FORM

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Abstract:

A novel, precise, and stability-indicating reverse-phase high-performance liquid chromatography (RP-HPLC) method was developed and validated for the simultaneous estimation of Amoxicillin Trihydrate and Clavulanic Acid in pharmaceutical dosage forms. The chromatographic separation was performed using a C18 column (250 mm × 4.6 mm, 5 µm), with a mobile phase comprising phosphate buffer (pH 4.0) and acetonitrile in the ratio of 70:30 v/v. The flow rate was maintained at 1.0 mL/min, and detection was carried out at 220 nm using a UV detector.

Introduction:

The treatment of bacterial infections has been significantly revolutionized by the advent of β-lactam antibiotics, with amoxicillin and clavulanic acid being among the most widely used in combination therapy. Amoxicillin trihydrate, a semi-synthetic penicillin derivative, is a broad-spectrum antibiotic effective against both Gram-positive and Gram-negative organisms [1]. However, its therapeutic efficacy is compromised due to the enzymatic degradation by β-

lactamases, which are secreted by resistant bacterial strains. To overcome this limitation, amoxicillin is often co-formulated with clavulanic acid, a potent β-lactamase inhibitor. Clavulanic acid, although possessing minimal antibacterial activity on its own, protects amoxicillin from enzymatic hydrolysis, thereby extending its antimicrobial spectrum [2]. This synergistic combination is commonly found in various dosage forms and is clinically effective in treating respiratory, urinary, and skin infections [1].

Given their frequent co-administration, it is essential to develop a robust and validated analytical method for their simultaneous estimation in pharmaceutical dosage forms. Analytical methods play a crucial role in ensuring the quality, safety, and efficacy of drug products during manufacturing and throughout their shelf life. Furthermore, regulatory guidelines stress the importance of stability-indicating assays to detect any changes in drug composition under stress conditions, such as hydrolysis, oxidation, heat, and light exposure. These studies help in understanding degradation pathways and in designing appropriate formulations that maintain therapeutic effectiveness until the product's expiry [3].

Reverse-phase high-performance liquid chromatography (RP-HPLC) is one of the most preferred techniques for pharmaceutical analysis due to its sensitivity, precision, and capability to separate complex mixtures. It is particularly suitable for the development of stability-indicating methods, as it can resolve the parent drug from its degradation products. Despite several analytical techniques available for individual or combined estimation of amoxicillin and clavulanic acid, there is a need for a stability-indicating RP-HPLC method that is precise, reproducible, and suitable for routine quality control analysis [4].

The present study focuses on the development and validation of a novel RP-HPLC method for the simultaneous estimation of amoxicillin trihydrate and clavulanic acid in tablet dosage forms. The method is designed to be stability-indicating by subjecting the drugs to various ICH-recommended stress conditions and demonstrating the separation of the active ingredients from their degradation products. The chromatographic conditions, including mobile phase composition, pH, flow rate, detection wavelength, and column selection, are carefully optimized to achieve sharp, symmetrical, and well-resolved peaks. The method is validated in accordance with ICH Q2(R1) guidelines to assess its suitability for accuracy, precision, linearity, specificity, sensitivity, and robustness.

This study aims to provide a validated analytical tool that can be effectively employed in the pharmaceutical industry for the quality assessment of amoxicillin and clavulanic acid combination products. Moreover, the method is intended to be simple and cost-effective, making it suitable for use in both industrial quality control laboratories and academic research settings.

Materials and Methods:

Amoxicillin trihydrate and clavulanic acid reference standards were procured as gift samples from a certified pharmaceutical manufacturer. A commercial tablet formulation containing amoxicillin trihydrate and potassium clavulanate in the ratio of 500 mg and 125 mg respectively was purchased from a local pharmacy for analysis. All solvents and chemicals used in the study were of HPLC grade and analytical reagent (AR) grade. Acetonitrile, methanol, and water (HPLC grade) were obtained from Merck (India), and potassium dihydrogen phosphate and orthophosphoric acid were used for preparing the phosphate buffer. All solutions were filtered

through a 0.45 µm membrane filter and degassed prior to use to ensure particulate-free and bubble-free mobile phase delivery.

The RP-HPLC analysis was carried out using a Shimadzu LC-20AT system equipped with a quaternary pump, UV-Visible detector (SPD-20A), and a manual injector with a 20 µL loop. Chromatographic data were processed using LabSolutions software. The separation was achieved on a C18 column (250 mm × 4.6 mm, 5 µm particle size). The mobile phase was composed of phosphate buffer (pH adjusted to 4.0 with orthophosphoric acid) and acetonitrile in the ratio of 70:30 v/v. The prepared mobile phase was filtered and degassed before use. The flow rate was maintained at 1.0 mL/min, and the column was operated at room temperature (25 ± 2°C). Detection was carried out at 220 nm, where both amoxicillin and clavulanic acid exhibited adequate UV absorbance, allowing for simultaneous detection [5].

Standard stock solutions were prepared by dissolving 100 mg of amoxicillin trihydrate and 25 mg of clavulanic acid separately in 100 mL of the mobile phase to obtain stock concentrations of 1000 µg/mL and 250 µg/mL respectively. Working standard solutions were prepared by further diluting these stock solutions with the mobile phase to obtain a final concentration range of 10–100 µg/mL for amoxicillin and 2.5–25 µg/mL for clavulanic acid. For sample preparation, 20 tablets were accurately weighed and powdered. A quantity of powder equivalent to one tablet (containing 500 mg of amoxicillin and 125 mg of clavulanic acid) was transferred into a 100 mL volumetric flask. The contents were dissolved in the mobile phase using sonication for 15 minutes, filtered through a 0.45 µm membrane filter, and diluted suitably to bring the concentrations within the linearity range.

To establish the stability-indicating nature of the method, forced degradation studies were performed by exposing the drugs to various stress conditions. Acidic degradation was carried out using 0.1 N HCl, basic degradation using 0.1 N NaOH, oxidative degradation using 3% hydrogen peroxide, thermal degradation by heating the sample at 80°C, and photolytic degradation by exposing the sample to UV light for 24 hours. The degraded samples were analyzed to confirm the separation of degradation products from the main peaks. The developed method was validated according to ICH Q2(R1) guidelines by evaluating specificity, linearity, accuracy, precision, robustness, limit of detection (LOD), limit of quantification (LOQ), and system suitability parameters.

Results:

Accurately measured volume of mixed standard stock solution was diluted with diluents to get the final concentration of standard as 25-150%. Six point linearity was determined.

The chromatographic conditions were set as per the optimized parameters and mobile phase was allowed to equilibrate with stationary phase as was indicated by the steady base line. Standard solutions of different concentration were injected separately and the chromatograms were recorded.

Peak areas were recorded for each injected concentration of drugs and the calibration curves, concentration vs. peak area were constructed for the drugs. Linearity performance parameters are depicted below. Peak areas were recorded and the graphs, concentration vs. peak area were constructed for the drugs. The statistical data's for Amoxicillin and Clavulanic acid are given below.

Table : Linearity results for Amoxicillin and Clavulanic acid

Linearity Range	Concentration (µg/ml)	Area (Amoxicillin)	Concentration (µg/ml)	Area (Clavulanic acid)
25	10	809400	1.425	447787
50	20	1620170	2.85	891560
75	30	2429868	4.275	1335531
100	40	3242888	5.7	1780200
125	50	4046675	7.125	2223369
150	60	4844410	8.55	2667786

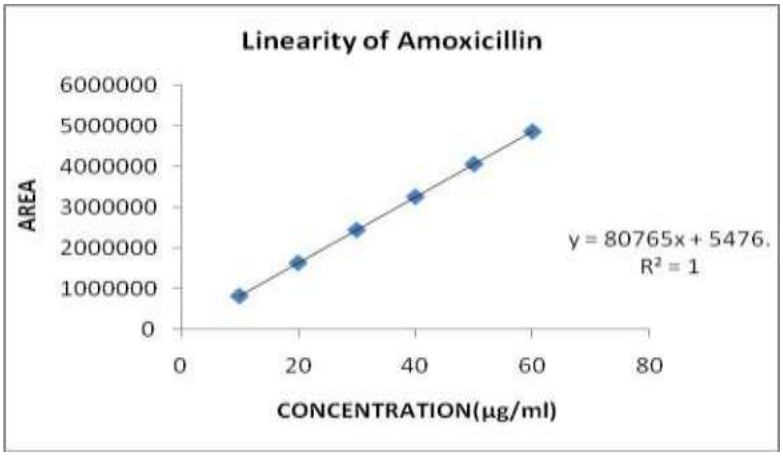

Fig. : Linearity curve of Amoxicillin

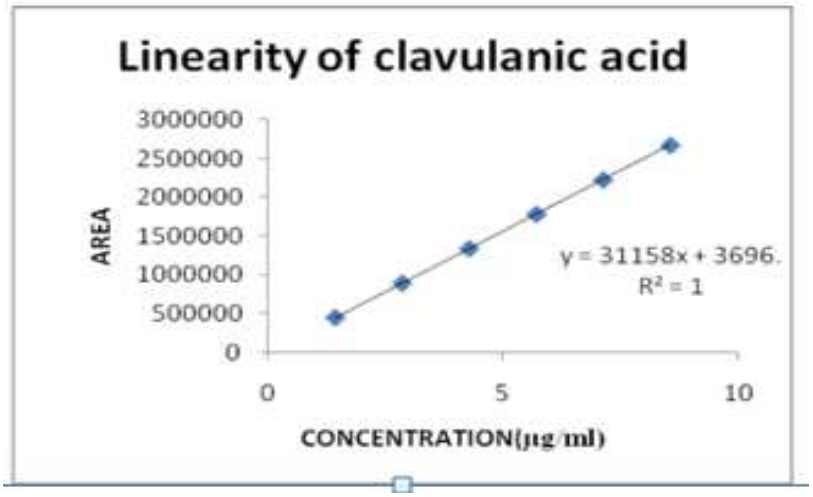

Fig. : Linearity curve of Clavulanic acid

Precision is the measurement of the degree of repeatability of analytical method under normal operation and is normally expressed as the percent relative standard deviation for statistically significant number of samples. According to ICH and FDA guideline, precision should be performed at three different levels: System precision (Repeatability), Method precision (Reproducibility), and Intermediate precision (Ruggedness).

System Precision (Repeatability): System precision (Repeatability) is the result of the method operating over a short time interval under the same conditions (Inter-assay precision). Six injections of standard preparation were injected and compliance for system suitability test was checked. Six sets of sample preparation were prepared and 2µl of each sample were injected induplicate and chromate grams were recorded. The mean area and %assay for those injections were calculated. RSD of six sets should not be more than 2.0%.

Table : System Precision results for Amoxicillin and Clavulanic acid

S. No.	Rt	Area	Rt	Area

	Amoxicillin	Clavulanic acid	Amoxicillin	Clavulanic acid
1.	5.155	3230958	2.142	1775538
2.	5.132	3241061	2.139	1782171
3.	5.199	3252137	2.136	1787435
4.	5.109	3257553	2.137	1789011
5.	5.099	3245072	2.132	1784159
6.	5.089	3245079	2.138	1784002
MEAN	5.117	3245536	2.134	1783719
STD	5.167	9181.975	2.136	
% RSD		0.28		0.26

Accuracy:

The accuracy of the method was performed. The known amount of standard drug was spiked in triplicate to the placebo and the recovery of the drug was calculated. Accuracy was performed at 3 levels: 50%, 100%, 150% of sample concentration, in triplicate at each level, using the placebo spiked with drug. Samples were prepared by adding corresponding weight of Amoxicillin and Clavulanic acid in Placebo and processes as per sample preparation.

Standard Solution: Weighed accurately and transferred about 20 mg of Amoxicillin working standard, 3 mg of Clavulanic acid working standard was taken in to 50 ml volumetric flask separately. Added about 50 ml of mobile phase, sonicated to dissolve. Further dilute10 ml of

the Amoxicillin and 10 ml of Clavulanic acid and taken in to100 ml volumetric flask. Filter the solution through 0.45 µm nylon filter or 0.45 µm PVDF membrane filter.

Sample Solution: Accurately weighted quantity of placebo was taken in five different 100.0 ml volumetric flasks. To these flask accurately weighed quantities of API equivalent to 50, 100, 150, percent of Amoxicillin and Clavulanic acid are added to their respective flasks. About 80 ml of mobile phase was added to at the flasks and then sonicated for 30 min with intermediate shaking. The volume was made up to mark with mobile phase. Further filtered the solutions through 0.45 µm PVDF filter.

Conclusion :

Drug combinations are commonly used clinically and an analysis required developing suitable methods of their analysis. Numbers of technique are available for simultaneous estimation of active ingredient in combined dosage formulation.

The literature survey revealed that few methods are available for simultaneous estimation of Amoxicillin and Clavulanic acid in combined dosage form but there is a need of a simple, economical and proper method for estimation of above combination in combined dosage form.

Hence, an attempt has been made to develop the method using RP-HPLC method for simultaneous estimation of Amoxicillin and Clavulanic acid in combined dosage form.

Waters Aliance 2690 Separations module with PDA detector with Universal C18, 250 * 4.6 mm, 5 µm with an injection volume of 20 µl is injected and eluted with the mobile phase of 0.02M Sodium dihydrogen phosphate dehydrate (pH 6.5):ACN which is pumped at a flow rate of 1.0 ml/min and detected by UV detector at 245 nm. The peaks of Amoxicillin and Clavulanic acid are found well separated at 5.069 and 2.131 respectively.

The proposed method was found to be rapid, accurate, precise, specific, robust and economical. The mobile phase is simple to prepare and economical. The sample recoveries in all formulations were in good agreement with the irrespective label claims and they suggested non-interference of formulation excipients in the estimation. This method is also having an advantage than reported method that the retention time of both the drugs is below 8 min and both the drugs can be assayed with the short time. Thus the method is not time consuming and can be used in laboratories for the routine analysis of combination drugs.

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