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Analytical Method Development and Validation of Aceclofenac and Serratiopeptidase by RP-HPLC

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ABSTRACT

Serratiopeptidase and Aceclofenac were measured and quantified using a quick, sensitive, and precise RP-HPLC approach that was designed and validated using the Waters HPLC System with PDA detection. Chromatography was performed using a mobile step of filtered, mixed, degassed Methanol and Buffer (80:20)on a column of Inertsil-ODS C18 (250 x 4.6 mm, 5) at a flow rate of 1.0 ml/min. In addition to being tested for quantification and detection limits, the system was also examined for linearity, precision, accuracy, and specificity.

Main words: Aceclofenac, Serratiopeptidase, Process development and validation.

INTRODUCTION

Aceclofenac is an oral non-steroidal anti-inflammatory drug (NSAID) with marked anti-inflammatory and analgesic properties used to treat osteoarthritis, rheumatoid arthritis and ankylosing spondylitis. It is reported to have a higher anti-inflammatory action or at least comparable effects than conventional NSAIDs in double-blind studies [2][3] [5] . Aceclofenac potently inhibits the cyclo-oxygenase enzyme (COX) that is involved in the synthesis of prostaglandins, which are inflammatory mediators that cause pain, swelling, inflammation, and fever. Aceclofenac belongs to BCS Class II as it possesses poor aqueous solubility [2]. It displays high permeability to penetrate into synovial joints where in patients with osteoarthritis and related conditions, the loss of articular cartilage in the area causes joint pain, tenderness, stiffness, crepitus, and local inflammation [1] . Aceclofenac is also reported to be effective in other painful conditions such as dental and gynaecological conditions [7]. In 1991, aceclofenac was developed as an analog of a commonly prescribed NSAID, Diclofenac, via chemical modification in effort to improve the gastrointestinal tolerability of the drug. It is a more commonly prescribed drug in Europe.

Serratiopeptidase (Serratia E-15 protease, also known as serralysin, serrapeptase, serratiapeptase, serratia peptidase, serratio peptidase, or serrapeptidase) is a proteolytic enzyme (protease) produced by enterobacterium *Serratia* sp. E-15, now known as *Serratia* marcescens ATCC 21074.[6] This microorganism was originally isolated in the late 1960s from silkworm (*Bombyx mori* L.) intestine.[7] Serratiopeptidase is present in the silkworm intestine and allows the emerging moth to dissolve its cocoon. Serratiopeptase is produced by purification from culture of Serratia E-15 bacteria. It is a member of the Peptidase M10B (Matrixin) family.

The chemical structures of both drugs are as shown in (Figures 1, 2).

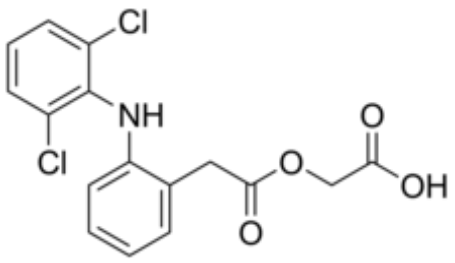

Figure 1: Chemical structure of Aceclofenac.

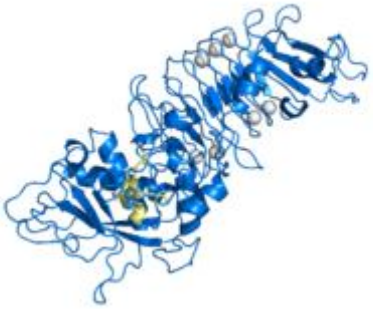

Figure 2: Chemical structure of Serratiopeptidase.

MATERIALS AND METHODS

Chemicals

Samples of Aceclofenac and Serratiopeptidase was gifted by Sun Pharmaceutical Industries Ltd, Goregaon (E), Mumbai. Merck, Schuchardt OHG, Germany provided HPLC grade Acetonitrile, KH2PO4, and phosphoric acid. Millipore Milli Q plus filtration technology was used to create ultra-pure water.

Instruments

We used a Waters 2695 HPLC system with a PAD detector (Waters Corporation, Milford, USA) that could detect wavelengths from 200nm to 400 nm. We also used it to test the method. Empower software was used to look at and process the outgoing signal on a Pentium computer that had a hard drive (Digital Equipment Co). Hydrolysis experiments were conducted using a Cintex computerized water bath. In a photo stability chamber, tests were conducted to determine the shelf life of various items (Sanyo, Leicestershire, UK).

Selection of wave length (λ max)

A solution of 100 µg/ml of Aceclofenac and Serratiopeptidase were prepared in Mobile phase. The resulting solutions were scanned individually on HPLC PDA detector from 200nm to 400 nm. The optimal response for both of them were obtained at 270nm. Hence the complete method was processed at the wave length of 270nm.

Preparation of stock and sample solutions

In order to make a stock solution of Aceclofenac and Serratiopeptidase (1000 mg/ml), 20 mg of the Aceclofenac and 25 mg of the Serratiopeptidase of the drug ingredient were dissolved in a 100ml volumetric flask of the diluent (mobile phase) for 30 minutes and sonicated for 30 minutes. All of these solutions were pipetted into a 100-ml volumetric flask and mixed with the correct amount of diluent. Then, for 10 minutes, they were sonicated at a high speed. Then prepare 20, 30, 40, 50, 60,70 and 80 ppm solutions were prepared by utilizing the above stock solution.

METHOD OF VALIDATION

Method validation can be defined as International Council for Harmonisation (ICH) “establishing documented evidence which provides a high degree of assurance that 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 of demonstrating that analytical procedures are suitable for their intended use and that they support the identity, quality, purity, an and drug products. Simply, method validation is the process of proving that and potency of the drug substances analytical method is acceptable for its intended purpose. For chromatographic methods used in analytical applications there is more consistency in validation practice with key analytical parameters.

Recovery:

The absolute recovery of analytical method is measured as the response of a processed spiked matrix standard expressed as a percentage of the response of pure standard which has not been subjected to sample pre treatment and indicates whether the method provides a response for the entire amount of analyte that is present in the sample.

Sensitivity

The method is said to be sensitive if small changes in concentration cause large changes in response function. The sensitivity of an analytical method is determined from the slope of the calibration line. The limits of quantification (LOQ) or working dynamic range of bio analytical method are defined as the highest and lowest concentrations, which can determined with acceptable accuracy. It is suggested that, this be set at 15% for both the upper and lower limit of quantitation respectively. Any sample concentration that falls outside the calibration range cannot be

interpolated from the calibration line and extrapolation of the calibration curve is discouraged. If the concentration is over range, the sample should be diluted in drug-free matrix and re-assayed.

Precision

The purpose of carrying out a determination is to obtain a valid estimate of a 'true' value. When one considers the criteria according to which an analytical procedure is selected, precision and accuracy are usually the first time to come to mind. Precision and accuracy together determine the error of an individual determination. They are among the most important criteria for judging analytical procedures by their results.

Accuracy

Accuracy normally refers to the difference between the mean x****, of the set of results and the true or correct value for the quantity measured. According to IUPAC accuracy relates to the difference between results (or mean) and the true value. For analytical methods, there are two possible ways of determining the accuracy, absolute method and comparative method.

Accuracy is best reported as percentage bias, which is calculated from the expression

$$\%\text{Recovery} = \frac{\text{Amount found}}{\text{Amount added}} \times 100$$

The accuracy of analytical method is then determined at each concentration by assessing the agreement between the measured and nominal concentrations of the analytes in the spiked drug – free matrix sampler.

Limit of detection (LOD)

The limit of detection (LOD) of an analytical method may be defined as the concentration, which gives rise to an instrument signal that is significantly different from the blank. For spectroscopic techniques or other methods that rely upon a calibration curve for quantitative measurements, the IUPAC approach employs the standard deviation of the intercept (σ), which may be related to LOD and the slope of the calibration curve, S, by

$$\textbf{LOD} = 3\sigma / S$$

Limit of quantification (LOQ)

The LOQ is the concentration that can be quantitate reliably with a specified level of accuracy and precision. The LOQ represent the concentration of analyte that would yield a signal-to-noise ratio of 10.

$$\textbf{LOQ} = 10\sigma / S$$

Where, σ - the estimate is the standard deviation of the peak area ratio of analyte to IS (5 injections) of the drugs. S -is slope of the corresponding calibration curve.

Ruggedness

Method Ruggedness is defined as the reproducibility of results when the method is performed under actual use conditions. This includes different analysts, laboratories, columns, instruments. Method ruggedness may not be known when a method is first developed, but insight is obtained during subsequent use of that method.

Robustness

The concept of robustness of an analytical procedure has been defined by the ICH as "a measure of its capacity to remain unaffected by small but deliberate variations in method parameters". The robustness of a method is the ability to remain unaffected by small changes in parameters such as Robustness studies are performed by introducing deliberately small changes in the flow rate (±0.2 mL/min).

System suitability

System suitability experiments can be defined as tests to ensure that the method can generate results of acceptable accuracy and precision. The requirements for system suitability are usually developed after method development and validation have been completed. (or) The USP (2000) defines parameters that can be used to determine system suitability prior to analysis.

The criteria selected will be based on the actual performance of the method as determined during its validation. For example, if sample retention times form part of the system suitability criteria, their variation (SD) during validation can be determined system suitability might then require that retention times fall within a 3 SD range during routine performance of the method.

RESULTS AND DISCUSSION

Validation of the Developed Method

The process of ensuring that the HPLC strategy utilized to perform a given test is appropriate for its intended use is known as HPLC method validation. The planned and refined HPLC technology was put to the test. The validation is carried out according to ICH guidelines.

Move Mobile: 80:20 V/V-ratio degassed Methanol and Buffer.

Preparation of Buffer(KH2PO4 0.1 M): Weight In a beaker filled with 1000 ml of clear water, totally dissolve 3.8954 g of di-sodium hydrogen phosphate and 3.4023 g of potassium dihydrogen phosphate. Orthophosphoric acid is used to bring the pH level down to 2.5 before the solution is run through a 0.45-micron membrane filter.

Table No 1: Optimized Chromatographic Condation:

Parameters	Method
Stationary phase (column)	Inertsil -ODS C_{18}(250 x 4.6 mm, 5 µ)
Mobile Phase	Methanol and Buffer (80:20)
Flow rate (ml/min)	1.0 ml/min
Run time (minutes)	8 min
Column temperature (°C)	Ambient
Volume of injection loop (µl)	20
Detection wavelength (nm)	254nm
Drug RT (min)	3.049min for Aceclofenac and 4.317 for Serratiopeptidase.

Based on the system suitability investigations, it was discovered that the optimized standard chromatogram had a peak at 3.049 & 4.317 min and that the mobile phase was Methanol and Buffer (80:20) at 254nm. For Aceclofenac and Serratiopeptidase, it was discovered that the technique exhibited linearity in the concentration ranges of 20 ppm to 80ppm based on linearity data. A linearity graph was constructed for the peak area of Aceclofenac and Serratiopeptidase versus concentration. The coefficient was found to be within the range of 0.999 for both of these compounds. It was necessary to inject the standard solution five times to determine the compatibility of the system, and the area of each injection was measured using an HPLC. Observations were made to ensure that the % RSD and MEAN remained within the prescribed parameters. Precision was determined to be within the acceptable System Precision, Method Precision, Intermediate Precision and it was computed as percent assay for and percent RSD for assay calculation, respectively. Different concentration levels, such as 50 percent, 100 percent, and 150 percent, were developed in order to improve the accuracy of computations. We discovered that recovery is possible within the parameters. This study calculated robustness for different flow rates of mobile phase, such as 0.8ml/min, 1.0ml/min, and 1.2ml/min, and calculated %RSD We measured ruggedness and found that system to system variability was 100.35, indicating that the approach was accurate. We also measured precision and found that the method was 100.08. Results reveal that RSD of retention time and accuracy, ruggedness, robustness linearity and precision are all within the acceptable ranges of results.

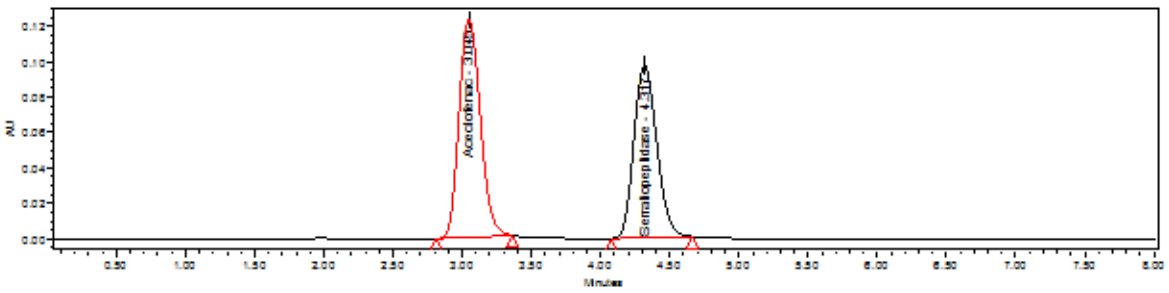

Figure – 1: Standard Chromatography

Inference: The chromatogram was obtained at RTs of 3.049min for Aceclofenac and 4.317min for Serratiopeptidase.

Test for System Suitability

Chromatography is a way to make sure that the system is going to work. It is used to check how well the system works for its intended use. System suitability's major purpose is to ensure that the entire testing process, including the instrument and the analyst, is appropriate for the task at hand.

Table - 1: Data of SST for Aceclofenac

	RT	Peak Area	USP Plate count	USP Tailing
Mean	3.0468	1414906	10336.825471	1.144
SD	0.000775	2796.31	-------	-------
% RSD	0.091075	0.197	-------	-------

Table - 2: Data of SST for Serratiopeptidase

	RT	Peak Area	USP Plate count	USP Tailing
Mean	4.3144	204138	8358.875421	1.047
SD	0.003782	185.59	-------	-------
% RSD	0.087649	0.090	-------	-------

Precision:

Precision refers to how well a set of measurements that were taken from the same homogenous sample under the same conditions line up. There are three types of parameters system precision, method precision and intermediate precision all of which are important for getting the job done.

Table No 3: Aceclofenac Precision Data:

	System Precise %Assay	Precision method %Assay	Intermediate Precision Information %Assay
Mean	100.36	100.32	100.21
SD	100.26	0.258	0.346
% RSD	0.103	0.257	0.345

Table No 4: Serratiopeptidase Precision Data:

	System Precise %Assay	Precision method %Assay	Intermediate Precision Information%Assay
Mean	100.78	100.69	100.54
SD	100.67	0.127	0.091
% RSD	0.167	0.126	0.091

Linearity

Aceclofenac & Serratiopeptidase assay concentrations 20ppm-80ppm were used to test the method's linearity. The LC system was injected with each solution. Using a correlation coefficients greater than 0.999, the calibration curve between the average peak area and the concentration was found to be linear.

Table No 5: Aceclofenac Linearity Data:

Statistical Analysis	
Slope	35296
y-Intercept	-1504
Correlation Coefficient	0.999

Table No 6: Serratiopeptidase Linearity Data:

Statistical Analysis	
Slope	5099
y-Intercept	-489.1
Correlation Coefficient	0.999

Ruggedness:

Method Ruggedness is defined as the reproducibility of results when the method is performed under actual use conditions. This includes different analysts, laboratories, columns, instruments. Method ruggedness may not be known when a method is first developed, but insight is obtained during subsequent use of that method.

Table No 7 : Aceclofenac Ruggedness Data:

	Peak area	Assay % of Aceclofenac
Mean	1415339	100.35
%RSD	0.147	0.147

Table No 8 : Serratiopeptidase Ruggedness Data:

	Peak area	Assay % of Serratiopeptidase
Mean	204350	100.59
%RSD	0.100	0.100

Robustness:

The concept of robustness of an analytical procedure has been defined by the ICH as "a measure of its capacity to remain unaffected by small but deliberate variations in method parameters". The robustness of a method is the ability to remain unaffected by small changes in parameters such as Robustness studies are performed by introducing deliberately small changes in the flow rate (±0.2 mL/min).

Table No 9 : Aceclofenac Robustness Data:

Flow 0.8 ml	Tailing factor	Flow 1 ml	Tailing factor	Flow 1.2 ml	Tailing factor
Avg	1.010	**Avg**	1.035	**Avg**	1.086
SD	1.011	**SD**	1.052	**SD**	1.069
%RSD	0.003	**%RSD**	0.018	**%RSD**	0.019

Table No 10 : Serratiopeptidase Robustness Data:

Flow 0.8 ml	Tailing factor	Flow 1 ml	Tailing factor	Flow 1.2 ml	Tailing factor
Avg	1.048	**Avg**	1.069	**Avg**	1.032
SD	1.060	**SD**	1.083	**SD**	1.033
%RSD	0.015	**%RSD**	0.009	**%RSD**	0.014

Detection and quantification limits (LOD and LOQ)

Aceclofenac and Serratiopeptidase of LOD and LOQ were caluculated by S/N ratio Ex:To determine LOD and LOQ, we must first determine how low a concentration must be and how many samples must be obtained to achieve that concentration and quantity. Both 3:1 and 10:1 signal-to-noise ratios are regarded to be correct. In other method The LOD and LOQ are calculated using the linearity plot.By using the following equation we can calculate LOD value of Aceclofenac , and Serratiopeptidase Here can be calculated by using average area of system suitability data, and slope can be calculated by using linearity data.

Aceclofenac :

The LOD and LOQ are computed from the linearity plot:

$$LOD = \frac{3.3\,\sigma}{S}$$

$$= \frac{3.3 \times 2796.31}{35296} = 0.261$$

$$LOQ = \frac{10\,\sigma}{S}$$

$$= \frac{10 \times 2796.31}{35296} = 0.792$$

Serratiopeptidase;

$$LOD = \frac{3.3\ \sigma}{S}$$

$$= \frac{3.3 \times 185.59}{5099} = 0.120$$

$$LOQ = \frac{10\ \sigma}{S}$$

$$= \frac{10 \times 185.59}{5099} = 0.363$$

CONCLUSION

The development of the analytical method involved researching several parameters. First off, it was discovered that the maximal absorbance for Serratiopeptidase was at 237nm and 275nm, respectively. The peaks purity was outstanding, and the typical wavelength will be 252 nm. The 20 injection volume chosen provided a suitable peak area. Inertsil C18 was the study's chosen column, and ODS selected it for its good peak form. Ambient temperature was found to be adequate for the nature of medication solution. Because of the good peak area, satisfactory retention time, and good resolution, the flow rate was set at 1.0 ml/min. Different mobile phase ratios were investigated, however the ratio of Methanol and Buffer (80:20)was ultimately chosen because to its good peak symmetry and resolution. Thus, the suggested study made use of this mobile phase.

The precision of the system and the procedure were both confirmed to be precise and within bounds. Curve fitting, correlation coefficient, and a linearity investigation were all successful. For both medicines, it was discovered that the analytical method was linear over the range of 20-80 ppm of the target concentration. The analysis passed the tests for ruggedness and robustness. The relative standard deviation in both circumstances was very acceptable.

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