ANALYTICAL METHOD VALIDATION REPORT FOR SERRATIOPEPTIDASE (ASSAY)

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1.0 Pre-Approval

This review page is the first page of the Protocol for Analytical Method Validation for Assay of Serratiopeptidase in Aceclofenac, Paracetamol & Serratiopeptidase Tablets by Colorimetric method and is a record of document approval. Signatures below indicate that this document has been reviewed and approved by a representative of various departments and ensures all relevant sections meet requirements.

APPROVED BY:

QC Manager	
MANAGER QA	

ANALYTICAL METHOD VALIDATION REPORT FOR SERRATIOPEPTIDASE (ASSAY)

2.0 Objective:

The aim of the validation of the Analytical method, for Assay of Serratiopeptidase in Aceclofenac, Paracetamol & Serratiopeptidase Tablets, as per the ICH Guidelines, is to ensure that a selected analytical procedure will give reproducible and reliable results.

3.0 Scope:

This document covers the method for Assay of Serratiopeptidase in Aceclofenac, Paracetamol & Serratiopeptidase Tablets. Equipment, location and analyst are used to validate the analytical method as per ICH guidelines.

4.0 Responsibilities:

S.No.	Department	Designation	Responsibility
1.	Quality Control	Officer QC	Preparation of Protocol and to carry out QC test Procedures
2.	Quality Control	Asst. Manager QC	Implementation and supervision of protocol
3.	Quality Assurance	Manager QA	Approval of protocol and review data with QC

5.0 Product profile:

Name of active material	Serratiopeptidase
Specifications	In-house
Label claim	Each Film coated tablet contains: Aceclofenac BP….. 100 mg Paracetamol BP ….. 325 mg Serratiopeptidase …. 15 mg (As enteric coated granules) (Eq. to 30000 Enzymatic units of Serratiopeptidase) Colour : Ferricoxide (Yellow) USP-NF

6.0 Equipment / Material needed:

For testing (Chemicals & Reagent)	Testing equipment
Disodium tetraborate buffer	Colorimeter
Casein hammerstein substrate	Cuvette
Trichloroacetic acid reagent	Weighing balance
Diluted Folins Ciocalteau Reagent	Filtration Assembly
Folin's reagent	Glass apparatus
Tyrosine Solution	

7.0 Analytical method to be used:

The tyrosine units are dissloved in sodium carbonate solution, which is alkaline in nature. The Folin's ciocalteau reagent helps in colour development where the tyrosine units bind to the copper molecule in the reagent and causes the reduction of phosphomolybdate which is present in the reagent. There is formation of tyrosine-copper molybdate coloured complex. The intensity of colour depends upon the tyrosine units present which is read at 660 nm.

Disodium tetraborate buffer. Dissolve 19.0 gm of disodium tetraborate in 900 ml of water, adjusted to pH 9.0 with 1 M hydrochloric acid and dilute to 1000 ml with water.

Casein hammerstein substrate. Dissolve 1.2 gm of Casein Hammerstein to 100 ml of the sodium tetraborate buffer. Allow the casein to disolve and form homogeneous solution. Boil the solution in boiling water-bath for 2 minutes. After removing from boiling waer-bath, cool the casein substrate immediately in ice cold water. Filter this solution through cotton plug to get clear solution and dilute to 200 ml with water.

NOTE- Always add casein to the buffer while strirring. Do not add buffer to casein as this will cause lumps and not go into solution completely and giving incorrect values.

Trichloroacetic acid reagent. Dissolve 18 gm of trichloroacetic acid, 30 gm of anhydrous sodium acetate and 20 ml of glacial acetic acid in 1000 ml of water.

NOTE-TCA should be in the crystal form and not in liquid form as this will effect the quantity weighed and thereby interfere with the complete precipitation of casein. Use sodium acetate anhysdrous because if, sodium acetate trihydrate should be used, the trihydrate forms gives lower results.

Diluted Folins Ciocalteau Reagent. Dilute 1 ml of Folin's Ciocalteau reagent with 2 ml of water to get 3 ml of the reagent.

Note- Folin's Ciocalteau reagent should be of the protein estimation grade and not the indicator grade. Folin's reagent should be diluted to just before addition to the tubes since it is sensitive to light. Do not prepare this solution at the beginning of the assay.

Tyrosine reference solution. Dissolve 160 mg of tyrosine in 100 ml of 0.2 M Hydrochloric acid.Dilute 1 ml of this solution to 100 ml with 0.2 M hydrochloric acid. Use 2 ml of this solution for colour development

Trisodium phosphate buffer pH 6.8. Weigh 19 g of trisodium phosphate and 6.4 ml of hydrochloric acid in 1000 ml of water, adjusted to pH 6.8.

Test solution. Weigh and powder 20 tablets. Disperse a quantity of powder containing about 120 mg of Serratiopeptidase with 100 ml of trisodium phosphate buffer pH 6.8. Stri on magnetic stirrer for 1 hour. Further dilute 5.0 ml to 25 ml with 5.0 % ammonium sulphate solution, stir for 5 mins. And filter. Dilute 2 ml of the filtrate to 100 ml with sodium tetraborate buffer. 1 ml of resulting solution is used for analysis. Set the water bath to 37º.

For each sample, keep four test tubes. Mark two test tubers as TEST and two as BLANK. Add 5 ml of Casein Substrate to the tubes marked as TEST and 5 ml of TCA to the test tube marked as BLANK.

Prewarm these tubes for five mins. Along with the tubes containing the sample dilutions. Add 1 ml of enzyme solution to all these tubes and vertex the tubes for exactly 10 seconds. Keep the test tubes in the water-bath at 37º for 20 mins. for the reaction. After exactly 20 mins., add 5 ml of TCA to tubes marked as TEST and 5 ml of Casein Substrate to the tube marked as BLANK. Vertex all the tubes exactly for 30 seconds. Keep the tubes in the water-bath at 37º for 30 mins. For precipitation, filter.

NOTE-For tablets, settlement may take a longer time than 30 mins. So filter the solution after the settlement is clearly seen (Vertex in between for better precipitation).

Mark tubes as TEST, BLANK, TYROSINE and TYROSINE BLANK.

Add the following respectively;

TEST – 2 ml of the TEST filtrate.

BLANK – 2 ml of the BLANK filtrate.

TYROSINE – 2 ml of TYROSINE standard solution.

TYROSINE BLANK – 0.2 M hydrochloric acid.

Add 5 ml of 6 % sodium carbonate solution to all tubes. Add 1 ml of diluted Folin;s reagent to all the tubes. Keep the tubes at 37º for 30 mins. For colour development. Measure the absorbance at about 660 nm

Serratiopeptidase Units / Tablets =

$$\frac{A_1 - A_2}{A_3 - A_4} \times 176 \times \frac{1}{20} \times \frac{100}{\text{Spl. Wt. (g)}} \times \frac{25}{5} \times \frac{100}{2} \times \text{Avg. wt. in gm}$$

$$\% \text{ of L.A.} = \frac{\text{Serratiopeptidase Units / Tablet}}{\text{L.A.}} \times 100$$

Where,
176 = Conversion Co-efficient of Tyrosine to Serratiopeptidase
20 = Reaction time (minutes)
A_1 = Absorbance of test solution
A_2 = Absorbance of blank solution
A_3 = Absorbance of tyrosine reference solution
A_4 = Absorbance of 0.2 M hydrochloric acid.
L.A. =20000 Units / tablet

Specification:

Serratiopeptidase In-House 15 mg: NLT 90.0%

Validation of used Reagent

S.No.	Blank Solution	Tyrosine Reference Solution	0.2 M Hydrochloric Acid
1	-0.459	0.719	1.04
2	-0.460	0.718	1.03
3	-0.457	0.717	1.06
4	-0.462	0.718	1.08
5	-0.461	0.721	1.05
Average	**-0.460**	**0.719**	**1.05**

8.0 The following analytical performance parameters are to be carried out.
8.1 Specificity
8.2 Precision
8.3 Intermediate precision
8.4 Accuracy
8.5 Linearity

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Serratiopeptidase:

8.1 Specificity

Interference from Placebo

Interference from blank was carried out as per procedure given in 7.0.

The above sample were recorded as titration reading and observed for any interference from blank in the titration.

Acceptance criteria

There should not be any interference from blank.

Conclusion

Since no interference of blank was observed, so the method is specific for Serratiopeptidase.

2 Precision

Precision of analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.

Sample preparation –

Six samples of quantity of powder containing about 120 mg of Serratiopeptidase with 100 ml of trisodium phosphate buffer pH 6.8. Stri on magnetic stirrer for 1 hour. Further dilute 5.0 ml to 25 ml with 5.0 % ammonium sulphate solution, stir for 5 mins. And filter. Dilute 2 ml of the filtrate to 100 ml with sodium tetraborate buffer. 1 ml of resulting solution is used for analysis. Set the water bath to 37º.

Acceptance Criteria: Relative Standard Deviation (RSD): NMT 2.0 %.

Observation:

Sample No.	Test Absorbance	% Assay
1	-0.698	99.41
2	-0.700	100.25
3	-0.702	101.08
4	-0.699	99.83
5	-0.701	100.67
6	-0.697	99.00

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Sample No.	Test Absorbance	% Assay
Ave	-	**100.04**
STD	-	**0.780**
% RSD	-	**0.780**

$$\frac{A_1 - A_2}{A_3 - A_4} \times 176 \times \frac{1}{20} \times \frac{100}{\text{Spl. Wt. (g)}} \times \frac{25}{5} \times \frac{100}{2} \times \text{Avg. wt. in gm}$$

$$\% \text{ of L.A.} = \frac{\text{Serratiopeptidase Units / Tablet}}{\text{L.A.}} \times 100$$

Result: The relative standard deviation of the above results is 0.780%.

Conclusion

The RSD results are well within limits (< 2%) so indicates that method is precise.

3 Intermediate precision (Ruggedness):

Ruggedness was carried out using 2 different person analyst 1 and analyst 2 perform test on different days.

Acceptance criteria: RSD NMT 2.0%

Observation: No significant change observed**.**

A. Assay :
Day-1, - Analyst 1.

S.No.	Test Absorbance	Batch 1	Batch 2	Batch 3
1	Analyst 1	-0.698	-0.698	-0.670
2		-0.699	-0.698	-0.698
3		-0.701	-0.698	-0.699

B. Assay.
Day - 2 , Analyst 2.

S.No.	Test Absorbance	Batch 1	Batch 2	Batch 3
1.	Analyst 2	-0.697	-0.697	-0.697
2.		-0.699	-0.697	-0.700
3.		-0.698	-0.701	-0.700

RSD between Day – 1 and Day – 2

Batch Numbers	% RSD
Batch 1	-0.196 %
Batch 2	-0.211 %
Batch 3	-1.702 %

Result : No significant change observed and the results are consistent.

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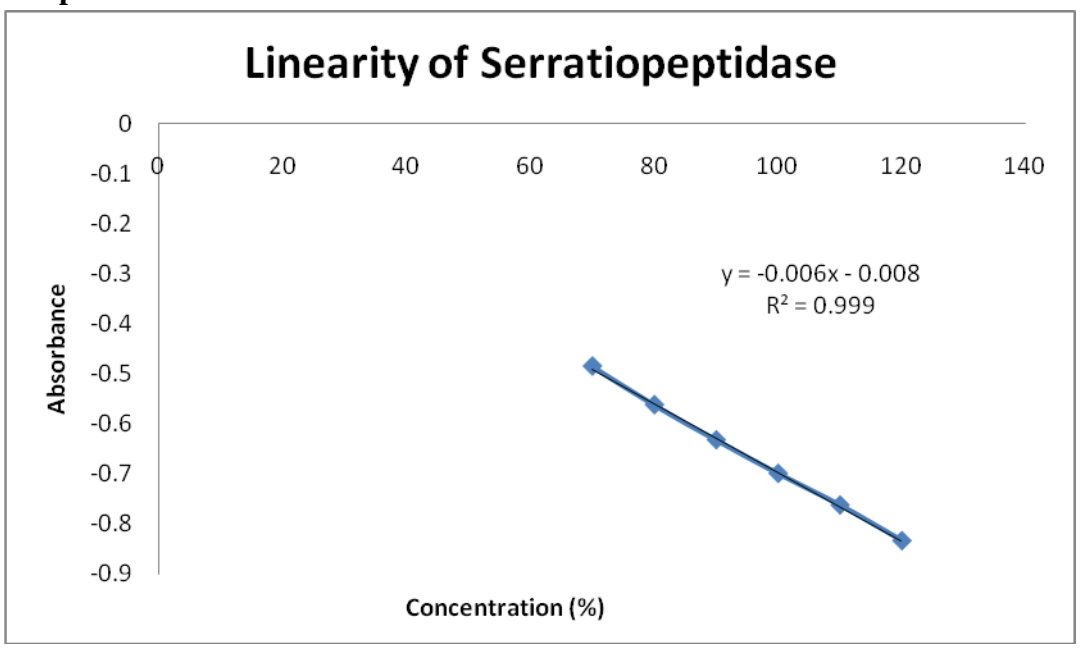
4. Linearity

84 mg, 96 mg, 108 mg, 120 mg, 132 mg and 144 mg samples were taken and analysed as in 7.0. Absorbance of Test was noted. A graph of weight of sample in mg on the X-axis is plotted against reading in ml on Y-axis.

Acceptance criteria: A straight line indicates linearity and the regression coefficient 'r' is NLT 0.999.

Sample No.	Concentration(%)	Test Absorbance
1	70	-0.485
2	80	-0.562
3	90	-0.632
4	100	-0.699
5	110	-0.762
6	120	-0.833

Graph :



Results : Correlation Coefficient 'r' : 0.999.

5 Accuracy

96 mg, 120 mg and 144 mg sample were taken & analysed as per method 7.0 and calculated the recovery of assay.

Acceptance criteria: % Recovery between 98.00% to 102.0%.

Sample	Wt. of sample in mg	Test Absorbance	% recovery
1	96	-0.564	100.67 %
2	120	-0.699	100.57 %
3	132	-0.837	99.88 %

Results : % recovery is between 99.88 % to 100.67%.

9.0 Overall Conclusion:

Specificity :

In specificity it was found that none of the excipients used in the placebo are having a retention time identical with that of Serratiopeptidase.

Precision :

%RSD for peak areas of Serratiopeptidase is found to be 0.780 %

Intermediate precision-Ruggedness :

When a batch of Tablets was analysed on two different days by two different chemists using two different columns, no appreciable difference in the contents of the product was observed.

RSD between Day 1 & Day 2**.**

Batch 1 -0.196 %
Batch 2 -0.211 %
Batch 3 -1.702 %

Accuracy was observed :

The percentage recovery for Serratiopeptidase is between 99.88 % to 100.67%.

Linearity & Range :

A plot of concentration v/s area indicates linearity.

The range of analytical method is between 70 % to 120 % R= 0.999 (NLT 0.99)

Based on the above data it can be concluded that the method used for the Assay of **Serratiopeptidase in Aceclofenac, Paracetamol & Serratiopeptidase Tablets** as described in the method of analysis under 7.0 is Specific, Precise, Rugged, Accurate and Linear.

10.0 Approval:

Analysed by **:**

Checked by **:**

Approved by **:**