Analytical Method Validation Protocol for the Determination of Assay of Alpha Amylase & Papain By Microbiological Assay Method

1. PURPOSE:

This Protocol is designed to validate the analytical test procedure for the determination of Assay of Alpha Amylase & Papain Capsules as per ICH –Guidelines Q2 (R1): Validation of analytical procedure: Text and Methodology.

2. SCOPE:

This protocol is applicable to the analytical method validation study for the determination of assay of Alpha Amylase & Papain Capsules by Microbiological Assay Method.

3. DEFINITIONS/ ABBREVIATION:

RSD	:	Relative Standard Deviation.
W.R.T	:	With respect to
API	:	Active Pharmaceutical Ingredient
ICH	:	International conference on Harmonisation

4. RESPONSIBILITY:

Executive – Quality Control is responsible for execution of the protocol and compilation of the task and preparation of the report.

Manager -QC is responsible for reviewing the protocol and report.

Manager -QA is responsible for approving the protocol and reprot.

5. PROCEDURE :

5.1 VALIDATION PARAMETERS:

1. Specificity
2. Precision
    - 2.1 System precision
    - 2.2 Method precision
    - 2.3 Intermediate precision or Ruggedness
3. Accuracy as recovery
4. Linearity & Range
5. Robustness

5.2 PRE STUDY-REQUISITES:

Test procedure for assessment of the quality levels of pharmaceutical articles are subject to various requirements according to section 501 of the federal food , drug and cosmetic act , assays and specifications in monograph constitute legal standard . The current good manufacturing practice regulations [21CFR211.194 (a)] require that test methods, which are used for assessing compliance of pharmaceutical articles which established specifications, must meet proper standards of accuracy & reliability.

All validation experiments will be performed with qualified equipments and trained analysts.

According to the procedures and standards sets in QC Dept.

5.3 TEST PROCEDURE:

5.3.1 REAGENTS & PRERQUISITIES:

- Working Standard of Alpha (α) Amylase & Papain
- 90mm Petriplates
- Disodium hydrogen phosphate
- Citric acid
- Casein
- Sodium hydroxide
- Ortho phosphoric acid
- Hydrochloric acid
- Starch soluble
- Sodium chloride
- Agar
- Iodine
- Distilled water

5.3.2 EQUIPMENTS:

- Antibiotic Zone Reader
- Calibrated Weighing Balance
- Micropipette

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5.3.3 PERSONEL INVOLVED:

- Sr. Executive QC
- Executive QC
- Sr. Executive QA

Method for the determination of Assay in Alpha Amylase & Papain Capsules:

5.4 Macllvaine's citrate phosphate buffer pH 6.0 (single strength):

Dissolve 107.5 gm of Disodium hydrogen phosphate and 12.5 gm of citric acid in water and made up to 500 ml with water adjust the pH with 1N sodium hydroxide or phosphoric acid.

5.5 Macllvaine's citrate phosphate buffer pH 6.0 (Half strength):

Transfer 250 ml of double strength Macllvaine's buffer to 500 ml volumetric flask and make up volume with distilled water.

5.6 Casein solution:

Dissolve 4.0 gm of Hammerstein's Casein in 90 ml of water. Add 5.0ml of 1N sodium hydroxide solution drop by drop mix slowly with the help of glass rod or blender avoid frothing. Blend the casein solution at a very low speed and 1N HCl drop by drop avoid cluster formation, adjust the pH 7.0 with 1N HCl and add the volume 40 ml water prepare fresh solution every time.

5.7 Starch solution:

Dissolve 2.2 g of soluble starch (on dried basis) in 75 ml of boiled water and cool it to room temperature. Than add 1.8 gm of sodium Chloride.

5.8 Standard preparation of Papain and alpha amylase:

Weigh accurately and transfer about 250 mg or Papain working std and about 90 mg of alpha amylase working std in a 25 ml Volumetric flask add 15 ml of half strength Macllvaine's buffer pH 6.0 allow and shake well and make up the volume up to the mark. With half strength Macllvaine's buffer. This std preparation (solution A) contain about 10.00 mg/ml of Papain and 3.6 mg/ml of alpha amylase. Mark this solution as **S_H for Papain**. Transfer 5ml aliquot dilutions (SH) in test tube add 5ml of half strength of Macllvaine's buffer. Mark this as **S_L for Papain**

5.9 Standard preparation for Alpha amylase:

Transfer from solution a 5ml aliquot in 100 ml volumetric flask and make up the volume with half strength Macllvaine's buffer. Mark this solution as S_H for Alpha amylase transfer 5ml aliquots of the dilution in the test tube add 5 ml of half strength of Macllvaine's buffer. Mark this solution as S_L for Alpha amylase.

5.10 Test preparation:

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Select 20 Capsule and Find the average fill and collect the powder. Weigh the capsule powder equivalent to 250 mg of papain and make the dilution as per standard dilution and 90 mg of alpha amylase in a 25 ml volumetric flask. Add 15 ml of half strength Macllvaine's buffer pH 6.0 and shake gently and make up the volume up to the mark with half strength Macllvaine's buffer mark as solution B and proceed further as given for solution A in the std preparation of Papain and alpha amylase and mark T_H & T_L.

5.11 Procedure:

Prepare a medium: Weigh the 3 gm agar powder in a 500 conical flask and add 120 ml purified water autoclave for sterilization at 121 ^{0}C for 15 minute. After sterilization remove the media from autoclave when media temperature is aprox 85^{0}C. add slowly – slowly, 25 ml single strength of Macllvaine's buffer pH 6.0, 22.5ml of casein solution and 22.5 ml of starch solution than mix gently pour the equally 25 ml portion of the molten medium while hot in seven Petri dishes kept at flat surface and allow to solidify at room temperature for 15 minute and then keep the Petri dishes in a refrigerator (8 ^{0}C) for 30 -60 minute use 2 Petri dishes for analysis of Papain and keep two for alpha amylase make four cup of 8.0 mm diameter with card borer on each plate. In each Petri dish pour 100µl each of std solution (S_H & S_L) and test solution (T_H & T_L). Keep the plate 1 hour for diffusion of solution. Transfer the plate carefully in to incubator set at 37 ^{0}C. So that there is no spill of dilution filled into each cup. Incubate Petri dishes at 37 ^{0}C for 24 hours.

Measure the diameter white zone produced by Papain after incubation and the reading flood the alpha amylase plates with 0.1 % w/v Iodine solution to estimate amylase activity of amylase by measuring the clear zone diameters on the blue background.

Calculation:

% if potency = Antilog 2 + a log I

Where, $$a = \frac{(T_H + T_L) - (S_H + S_L)}{(T_H - T_L) + (S_H - S_L)}$$

log I = Ratio of dilution

Papain = $\frac{\text{Antilog}}{100}$ X $\frac{WS_1}{25}$ X $\frac{25}{WT}$ X Potancy X Av wt.

Alpha amylase = $\frac{\text{Antilog}}{100}$ X $\frac{WS_2}{25}$ X $\frac{25}{WT}$ X Potancy X Av wt.

6.0 SPECIFICITY:

Specificity provides the information about the suitability of the diluent in the sample and or the standard preparation at room temperature .

Specificity of the analytical method shall be demonstrated by the following experiment.

A) Alpha Amylase : -

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Placebo preparation:

Prepare the placebo of the sample by adding the all contents of the Capsules except the active component Alpha amylase.

Prepare the Standard as in the 5.8 & 5.9 under the assay method.

From the prepared Placebo take 925 mg of Placebo of capsule and proceed the test preparation procedure as in the 5.10 under the assay method.

Perform the test Procedure with the Placebo sample against the standard as in the 5.11 under the assay method and observe the results.

B) Papain:-

Placebo preparation:

Prepare the placebo of the sample by adding the all contents of the Capsules except the active component Papain

Prepare the Standard as in the 5.8 under the assay method.

From the prepared Placebo take 1110 mg Placebo and proceed the test preparation procedure as in the 5.10 under the assay method.

Perform the test Procedure with the Placebo sample against the standard as in the 5.11 under the assay method and observe the results.

Acceptance Criteria:

There shall be no zone of inhibition in the sample solution against a clear zone of inhibition of standard solution.

7. PRECISION:

Precision Provides the degree of agreement among the individual test results when the procedure is applied repeatedly to multiple analysis of a homogenous sample.

Prepared the standard and sample as mentioned in the assay method and perform the Alpha amylase & Papain assay in Six replicates.

Prepare sample solution six times as per methodology.

Calculate the Average and %RSD of six assay values.

7.1 Intermediate precision (Ruggedness):

This study shall be carried out as per method precision by a different analyst, different day by using different set of standard solution and sample solution.

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ACCEPTANCE CRITERIA:

% RSD Should be Not More Than 10.

Intermediate precision: The overall RSD of twelve assay value should not be more than 10.0 %.

8. ACCURACY:

The accuracy (trueness) of an 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 known as recovery as % recovery. Prepare the recovery solutions at 80 %, 100%, and 120 % level as given below:

A) Alpha Amylase:

Preparation of standard solution:

Preparation of standard Solution as as in the 5.8 & 5.9 under the assay method .

Preparation of recovery solutions:

Sample-1 at 80 % :-

Accurately weigh about 975 mg of Placebo and add accurately about 200 mg of Papain working standard in 25 ml volumetric flask (Solution A). Accurately weigh about 1128mg of Placebo and add 72 mg of Alpha amylase and dissolve and Sonicate then make up with Distilled water to 25 ml (Solution B).

Papain:-

Take above solution (TH).

Take 5ml from Solution A and add 5ml of half strength of Macllvaine's buffer(TL).

Alpha Amylase:-

Take 5ml from Solution B and make up the volume upto 100ml with half strength of Macllvaine's buffer (TH).

Take 5ml from TH and add 5ml of half strength of Macllvaine's buffer(TL).

Sample-1 at 100 % :-

Accurately weigh about 925 mg of Placebo and add accurately about 250 mg of Papain working standard in 25 ml volumetric flask(Solution A). Accurately weigh about 1110mg of Placebo and add 90 mg of Alpha Amylase and dissolve and Sonicate then make up with Distilled water to 25 ml (Solution B).

Papain:-

Take above solution (TH).

Take 5ml from Solution A and add 5ml of half strength of Macllvaine's buffer(TL).

Alpha Amylase:-

Take 5ml from Solution B and make up the volume upto 100ml with half strength of Macllvaine's buffer (TH).

Take 5ml from TH and add 5ml of half strength of Macllvaine's buffer(TL).

Sample-1 at 120 % :-

Accurately weigh about 875 mg of Placebo and add accurately about 300 mg of Papain working standard in 25 ml volumetric flask(Solution A). Accurately weigh about 1092 mg of Placebo and add 108 mg of Alpha Amylase and dissolve and Sonicate then make up with Distilled water to 25 ml (Solution B).

Papain:-

Take above solution (TH).

Take 5ml from Solution A and add 5ml of half strength of Macllvaine's buffer(TL).

Alpha Amylase:-

Take 5ml from Solution B and make up the volume upto 100ml with half strength of Macllvaine's buffer (TH).

Take 5ml from TH and add 5ml of half strength of Macllvaine's buffer(TL).

Acceptance Criteria

Recovery for assay from the sample obtained with triplicate test preparations at each level

(I.e. about 80%, 100%, 120 % of specification level) should be between 98 and 102 %. 90 and 110%.

% RSD should be within 10.0.

9. LINEARITY:

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

Or Linearity provides the ability of the method to obtain the results, which are directly or after the mathematical transformation proportional to the concentration of the analyte within a given range.

Establish linearity over a range of five different concentrations of the analyte from 80 % to at least 120% of specification level (minimum five points), test each concentration in triplicate.

Standard-1 at 80 % :-

Accurately weigh about 200 mg of Papain working standard in 25 ml volumetric flask(Solution A). Accurately weigh about 72 mg of Alpha Amylase and dissolve and Sonicate then make up with Distilled water to 25 ml (Solution B).

Papain:-

Take above solution (SH).

Take 4ml from Solution A and add 6ml of half strength of Macllvaine's buffer(SL).

Alpha Amylase:-

Take 5ml from Solution B and make up the volume upto 100ml with half strength of Macllvaine's buffer (SH).

Take 4ml from SH and add 6ml of half strength of Macllvaine's buffer(SL).

Standard-2 at 90 % :-

Accurately weigh about 225 mg of Papain working standard in 25 ml volumetric flask(Solution A). Accurately weigh about 81 mg of Alpha Amylase and dissolve and Sonicate then make up with Distilled water to 25 ml (Solution B).

Papain:-

Take above solution (SH).

Take 4.5ml from Solution A and add 5.5ml of half strength of Macllvaine's buffer(SL).

Alpha Amylase:-

Take 5ml from Solution B and make up the volume upto 100ml with half strength of Macllvaine's buffer (SH).

Take 4.5ml from SH and add 5.5ml of half strength of Macllvaine's buffer(SL).

Standard-3 at 100 % :-

Accurately weigh about 250 mg of Papain working standard in 25 ml volumetric flask(Solution A). Accurately weigh about 90 mg of Alpha Amylase and dissolve and Sonicate then make up with Distilled water to 25 ml (Solution B).

Papain:-

Take above solution (SH).

Take 5ml from Solution A and add 5ml of half strength of Macllvaine's buffer(SL).

Alpha Amylase:-

Take 5ml from Solution B and make up the volume upto 100ml with half strength of Macllvaine's buffer (SH).

Take 5ml from SH and add 5ml of half strength of Macllvaine's buffer(SL).

Standard-4 at 110 % :-

Accurately weigh about 275 mg of Papain working standard in 25 ml volumetric flask(Solution A). Accurately weigh about 99 mg of Alpha Amylase and dissolve and Sonicate then make up with Distilled water to 25 ml (Solution B).

Papain:-

Take above solution (SH).

Take 5.5ml from Solution A and add 4.5ml of half strength of Macllvaine's buffer(SL).

Alpha Amylase:-

Take 5ml from Solution B and make up the volume upto 100ml with half strength of Macllvaine's buffer (SH).

Take 5.5ml from SH and add 4.5ml of half strength of Macllvaine's buffer(SL).

Standard-5 at 120 % :-

Accurately weigh about 300 mg of Papain working standard in 25 ml volumetric flask(Solution A). Accurately weigh about 108 mg of Alpha Amylase and dissolve and Sonicate then make up with Distilled water to 25 ml (Solution B).

Papain:-

Take above solution (SH).

Take 6ml from Solution A and add 4ml of half strength of Macllvaine's buffer(SL).

Alpha Amylase:-

Take 5ml from Solution B and make up the volume upto 100 ml with half strength of Macllvaine's buffer (SH).

Take 6ml from SH and add 4ml of half strength of Macllvaine's buffer(SL).

For Above all standard preparation in Linearity test, test preparation as following:

Test Preparation :-

Accurately weigh about 250 mg of Papain working standard in 25 ml volumetric flask(Solution A). Accurately weigh about 90 mg of Alpha Amylase and dissolve and Sonicate then make up with Distilled water to 25 ml (Solution B).

Papain:-

Take above solution (SH).

Take 5ml from Solution A and add 5ml of half strength of Macllvaine's buffer(SL).

Alpha Amylase:-

Take 5ml from Solution B and make up the volume upto 100ml with half strength of Macllvaine's buffer (SH).

Take 5ml from SH and add 5ml of half strength of Macllvaine's buffer(SL).

Note:For Linearity Test, test each concentration in triplicate.

Plot a graph with concentration against content and calculate the correlation coefficient (R^2 value).

Acceptance Criteria

The correlation coefficient (R^2 value) is not less than 0.90

Slope: Report value

The Y intercept: within the +/- 5.0% of the analyte response at the nominal concentration.

10. ROBUSTNESS STUDY:-

The robustness of an analytical method is a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters and provides an indication of its reliability during normal usage.

11. Reporting and evaluation of data

All the raw data and printout of the chromatograms shall be taken in the Final report and will be part of the validation exercise. On completion of the validation study, a detailed validation report shall be prepared as a separate document.