ANALYTICAL METHOD VALIDATION REPORT FOR THE TEST OF ASSAY OF FOLIC ACID IN CARBONYL IRON, FOLIC ACID, CYANOCOBALAMIN, ASCORBIC ACID, ZINC, ALPHA TOCOPHERYL ACETATE AND SELENIUM CAPSULES

Site Address: SAFETAB LIFE SCIENCE
Plot No.A-67 to 72, PIPDIC Electronic Park,
Thirubuvanai, Puducherry-605 107

1.0 INDEX

2.0 REPORT APPROVAL SHEET

PREPARED BY		
Name	:	S. SANTHI
Designation	:	ASST. MANAGER - QC
Signature	:	
Date	:	07/02/2024
REVIEWED BY		
Name	:	M. VIJAYAKUMAR
Designation	:	GM - QC
Signature	:	
Date	:	08/02/2024
APPROVED BY		
Name	:	
Designation	:	AGM - QA
Signature	:	
Date	:	09/02/2024

Effective Date	:	10/02/2024

3.0 OBJECTIVE

To validate the method for the test of assay of Assay of Folic Acid in Carbonyl Iron, Folic acid, Cyanocobalamin, Ascorbic acid, Zinc, Alpha Tocopheryl Acetate and Selenium Capsules by HPLC.

4.0 GENERAL INFORMATION

REFERENCE : In-House

TYPE OF VALIDATION : Validation of non-pharmacopoeial method

TEST VALIDATION : Assay of Folic Acid in Carbonyl Iron, Folic acid, Cyanocobalamin, Ascorbic acid, Zinc, Alpha Tocopheryl Acetate and Selenium Capsules

COMPOSITION : Each hard gelatin capsule contains:

Content	Strength
Carbonyl Iron Equivalent to Elemental iron	100mg
Folic Acid BP	500mcg
Cyanocobalamin (Vitamin B12) (Coated)	15mcg
Ascorbic Acid (coated)	75mg
Zinc Sulphate monohydrate BP (Equivalent to elemental zinc 22.5mg)	61.8mg
Alpha Tocopheryl Acetate BP	15IU
Sodium selenate Equivalent to Selenium	65mcg

BATCH NO	:	G17231116
SPECIFICATION LIMIT	:	Not less than 95.0% of the labeled claim
VALIDATION STUDY	:	QC-Laboratory, Safetab Life Science, Puducherry-605107
VALIDATION TEAM	:	1. C.K.Saravanan 2. T.Amudha 3. E.Meena

5.0 DETAILS OF STANDARD,SAMPLES AND PLACEBO TO BE USED

Mention the name and Batch No., Potency of the reference/working std., test samples / placebo to be used during Validation.

NAME OF THE MATERIAL	ID NO/BATCH NO	POTENCY/PURITY
Sample	B.No: G17231116	Not applicable
Plain Placebo	B.No: NA	Not applicable
Working standard Folic Acid BP	WS. No: ST/WS/23/034	92.8% (As is basis)
Alpha Tocopheryl acetate BP	WS. No: WS/TC/006/23	50.27% (As is basis)
Cyanocobalamin	WS. No: SLL/RM/STD/009	97.6% (As is basis)

6.0 DETAILS OF INSTRUMENTS/EQUIPMENTS, COLUMN, SOLVENTS AND CHEMICALS TO BE USED :

INSTRUMENTS/EQUIPMENTS :

High performance liquid chromatograph with PDA detector

Make : Shimadzu, Model : LC-2050C 3D Prominence i

High performance liquid chromatograph with UV visible detector

Make : Shimadzu, Model : LC-2050C

Analytical Balance:

Make : Sartorius, Model : Quintix-125D-1OIN

COLUMN:

Inertsil ODS 3V C18, (250mmx 4.6mm) 5µ or equivalent

SOLVENTS AND CHEMICALS WITH GRADE :

Folic acid (Working standard)

Potassium dihydrogen orthophosphate (AR grade)

Dipotassium hydrogen orthophosphate (AR grade)

Purified Water (Milli-Q water (or) equivalent)

Methanol (HPLC grade)

Acetonitrile (HPLC grade)

Hydrochloric acid (AR grade)

Sodium Hydroxide (AR grade)

Hydrogen Peroxide 30% (AR grade)

7.0 DESCRIPTION OF ANALYTICAL METHOD

Chromatographic Conditions:

Column	:	Inertsil ODS 3V C18, (250mmx 4.6mm) 5µ or equivalent
Flow rate	:	0.8ml/min
Wavelength	:	280 nm
Injection Volume	:	20 µL
Column temperature	:	40°C
Run time	:	20 minutes

Diluent preparation:

A mixture of 80 volume of water and 20 volume of Acetonitrile.

Buffer preparation:

Dissolve 11.0g of Potassium dihydrogen orthophosphate and 6.0g of Dipotassium hydrogen orthophosphate in 1000mL of purified water.

Mobile phase preparation:

A mixture of 90 volume of Buffer and 10 volume of Methanol and mix well. Filter through 0.45µ nylon membrane filter and degas.

Standard preparation:

Weigh accurately and transfer about 20.0mg of Folic acid working standard into a 100mL volumetric flask. Add 5mL of 1N sodium hydroxide sonicate to dissolve and dilute to volume with diluent. Further dilute 5mL of this solution to 100mL with diluent. Filter the solution through 0.45µ nylon membrane filter. (**Concentration:** 0.01mg/ml of Folic Acid).

Test preparation:

Weigh 20 capsules and calculate the average weight and crush to fine powder. Weigh accurately and transfer powdered sample equivalent to about 1.0mg of Folic acid into a 100mL volumetric flask. Add 5mL of 1N of sodium hydroxide to disperse the sample and add 60mL of diluent, sonicate for 15 minutes with intermittent shaking (Maintain the bath temperature below 25°C) and dilute to volume with diluent. Filter the solution through 0.45µ nylon membrane filter. (**Concentration:** 0.01mg/ml of Folic Acid).

Blank preparation:

Pipette out 5mL of 1N Sodium hydroxide into 100mL volumetric flask and dilute upto volume with diluent.

Procedure:

Inject the blank (diluent) in single injection, standard preparation as five replicate injection in to the chromatogram and measure the response of the peak of Folic acid in term of standard area and test in duplicate.

The test is not valid unless it meets the system suitability parameters. Calculate the system suitability with respect to the following parameters.

System suitability:

Theoretical plate	: NLT 2000 for Folic acid peak
Tailing factor	: NMT 2.0 for Folic acid peak
Relative standard deviation	: NMT 2.0% for five replicate injections of Folic acid peak.

Inject 20µl of the above solution as per following sequence.

Injection sequence:

S. No	Sample Name	No. of injections
1	Blank (Diluent)	1
2	Standard preparation	5
3	Test preparation	2
4	Bracketing standard	1 Each after every 6 sample injection

Calculation:

Calculate the assay of Folic acid in mcg/capsule as follows:

$$= \frac{AT}{AS} \times \frac{WS}{100} \times \frac{5}{100} \times \frac{100}{WT} \times \frac{P}{100} \times 1000 \times ANW$$

Where,

AT = Average area of Folic acid peak in Test preparation

AS = Average area of Folic acid peak in standard preparation.

WS = Weight of Folic acid working standard taken in mg

WT = Weight of sample taken in mg.

P = Potency of Folic acid Working standard (% on as basis).

ANW = Average Net Weight of capsule in mg.

Calculate the assay of Folic acid in % as follows:

$$= \frac{\text{mcg/capsule}}{LC} \times 100$$

LC = Label claim of Folic acid in mcg/capsule.

8.0 VALIDATED PARAMETERS:

Following parameters shall be selected for validation	
S.No.	**VALIDATION PARAMETERS**
1	**System suitability**
2	**Specificity (Selectivity)** Interference from blank and placebo
3	**Linearity and Range**
4	**Interference and Degradants (Forced degradation)**
	i) Acid degradation ii) Alkali Degradation iii) Oxidative Degradation
5	**Accuracy**
6	**Precision** i) System Precision ii) Method Precision iii) Intermediate Precision
7	**Stability of analytical solution**
8	**Filter paper study**
9	**Robustness** i) Flow rate change ii) Wavelength change iii) Column oven temperature change

Note: More than one parameter may be performed at once with relevant sequence having common system suitability with bracketing preparation.

9.0 VALIDATION RESULTS:

9.1 SYSTEM SUITABILITY:

Study Design:

Five replicates of standard preparation are injected into HPLC and following system suitability parameters are evaluated.

1) Theoretical plate for Folic acid peaks.

2) Tailing Factor for Folic acid peaks.

3) % RSD of area of five replicate standard injections

Results are tabulated in Table 1.

Table 1: System suitability

System Suitability Parameter	Limit	Observed Result
Theoretical Plates	NLT 2000	8056
Tailing Factor	NMT 2.0	0.960
% RSD	NMT 2.0	0.295

Result and Conclusion:

The System suitability test results are well within the acceptance criteria and the study concludes the suitability of analytical system for the analysis.

9.2 SPECIFICITY (SELECTIVITY)

9.2.1 Interference from blank and placebo

Study Design:

Blank, standard, placebo and placebo spiked with analyte and sample are analyzed as per the method to examine the interference of blank and placebo with Folic acid peaks.

Peak purity of the analyte peak and the representative chromatograms of blank, standard, placebo, placebo spiked with analyte and sample are attached.

Results are tabulated in Table 2.

Acceptance criteria:

1) There should not be any interference due to blank, placebo peak with analyte.

2) Peak purity index is not less than 0.995 accordingly to lab solution software.

Table 2: Specificity

Sr.No	Sample ID	Peak Name	Retention time	Peak Purity index
1	Blank	Blank peak	No peak	Not applicable
2	Standard preparation	Folic acid	12.120	1.000
3	Folic acid working standard	Folic acid	12.167	1.000
4	Alpha tocopheryl acetate + Cyanocobalamin working standard	Blank peak	No peak	Not applicable
5	Plain placebo for Folic acid	Blank peak	No peak	Not applicable
6	Plain Placebo + Folic acid working standard	Folic acid	12.167	1.000
7	Plain Placebo + Alpha tocopheryl acetate + Cyanocobalamin working standard	Blank peak	No peak	Not applicable
8	Sample preparation	Folic acid	12.161	1.000

Results and Conclusion:

The Blank and Placebo peaks are not interfere with retention time Folic acid peak in test preparation and Peak purity passes within specified limits. Hence method is selective and specific.

9.3 LINEARITY AND RANGE:

Study Summary:

Analytical solutions for Folic acid Working standard were prepared over the range of 50% to 150% concentration with respect to target concentration (i.e. 50%, 75%, 100%, 125% and 150%). Replicate injections of these solutions are injected and checked for Linearity and Range.

The results are tabulated in Table 3 for Linearity and Table 4 for Range.

Acceptance criteria:

1) The squared correlation coefficient r^2 should not be less than 0.995.

2) To conclude the range % RSD for peak areas of linearity levels 50%, 75%, 100%, 125% & 150% should not be more than 2.0.

Table 3: Linearity Table for Folic acid

Linearity Levels (%)	Conc. in ppm (X- axis)	Avg. Area (Y- axis)
50%	5.040	139794
75%	7.560	199523
100%	10.080	259146
125%	12.600	326371
150%	15.120	391756
Slope		31083
CC		1.000
Sqaured R		1.000
Intercept		2546.8

Fig. : Liner Graph for Folic acid

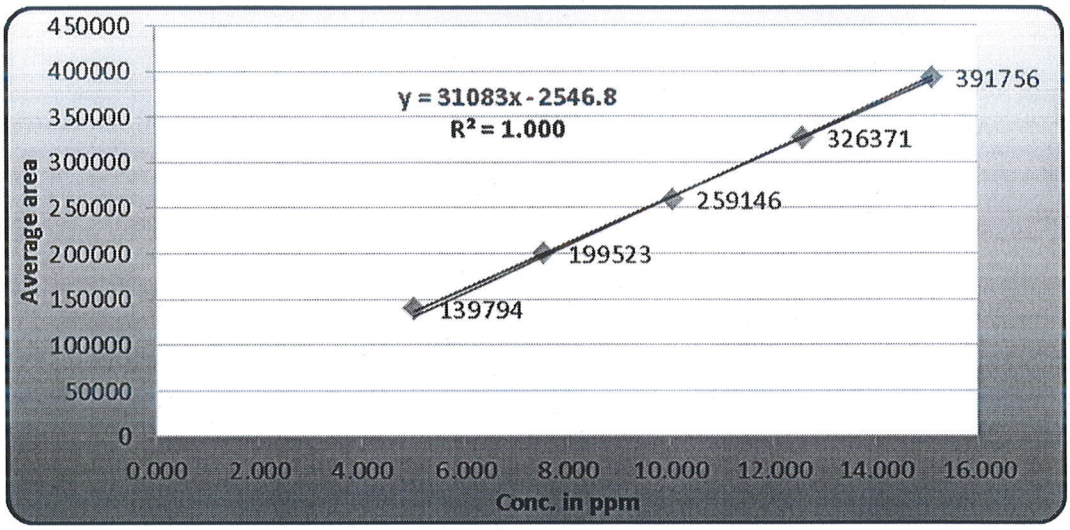

Table:4 Range for Folic acid

Linearity Levels (%)	% RSD for Folic acid
50%	0.317
75%	0.322
100%	0.116
125%	0.393
150%	0.126

Result and Conclusion:

Squared correlation coefficient and Range, %RSD of areas at 50%, 75%, 100%, 125 & 150% levels within limits.

9.4 INTERFERENCE FROM DEGRADANTS (Forced degradation)

In order to prove specificity of method, further degradation was carried out and peak purity of Folic acid peak was monitored.

9.4.1 Acid Degradation:

Weigh 20 capsules and calculate the average weight and crush to fine powder. Weigh accurately and transfer powdered sample equivalent to about 1.0mg of Folic acid into a 100mL volumetric flask. Add 5mL of 1N of sodium hydroxide to disperse the sample and add 60mL of diluent, sonicate for 15 minutes with intermittent shaking (Maintain the bath temperature below 25°C). Add 5ml of 5N Hydrochloric acid and heat on water bath at 80°C for 30minutes Cool and neutralized with 5ml of 5N Sodium hydroxide and Dilute to volume with diluent and mix. Filter the solution through 0.45µ Nylon membrane filter.

9.4.2 Alkali degradation:

Weigh 20 capsules and calculate the average weight and crush to fine powder. Weigh accurately and transfer powdered sample equivalent to about 1.0mg of Folic acid into a 100mL volumetric flask. Add 5mL of 1N of sodium hydroxide to disperse the sample and add 60mL of diluent, sonicate for 15 minutes with intermittent shaking (Maintain the bath temperature below 25°C). Add 5ml of 5N Sodium hydroxide and heat on water bath at 80°C for 30minutes Cool and neutralized with 5ml of 5N Hydrochloric acid and Dilute to volume with diluent and mix. Filter the solution through 0.45µ nylon membrane filter.

9.4.3 Oxidative Degradation:

Weigh 20 capsules and calculate the average weight and crush to fine powder. Weigh accurately and transfer powdered sample equivalent to about 1.0mg of Folic acid into a 100mL volumetric flask. Add 5mL of 1N of sodium hydroxide to disperse the sample and add 60mL of diluent, sonicate for 15 minutes with intermittent shaking (Maintain the bath temperature below 25°C). Add 5ml of 30% Hydrogen peroxide solution and heat on water bath at 80°C for 30minutes, Cool and dilute to volume with diluent and mix. Filter the solution through 0.45µ Nylon membrane filter.

Acceptance criteria:

i) There should not be any interference due to degradants with analyte in stressed sample.

ii) The desired degradation should be 10-30% in acid, alkali and oxidation degration, (if possible).

iii) If about 10% to 30% degradation is not achieved by applying above stressed condition. Same shall be documented and reported.

iv) Peak purity should not be less than 0.950 according to Lab solution software.

Table 5: Peak purity (Chemical degradation)

S.No	Sample name	Peak name	Assay in (%)	% Difference from unfiltered sample	Peak purity index
1	Sample as such	Folic acid	120.4	Not applicable	1.000
2	Acid degradation	Folic acid	96.9	24.26	1.000
3	Alkali degradation	Folic acid	111.3	8.18	1.000
4	Oxidative Degradation	Folic acid	111.6	7.89	1.000

Result and Conclusion:

There is No any interference due to degradants with analyte in stressed samples and Peak purity was passes According to Lab solution.

9.5 ACCURACY (RECOVERY)

Study Design:

Known quantity of Folic acid working standard were spiked with placebo at three different levels (at level of 50%, 100% and 150% of targeted concentration).

Prepared the recovery samples in triplicate for e ach level. The samples were analyzed as per the proposed method. The results are tabulated in Table 6 for Folic acid respectively to demonstrate the accuracy of the method.

The mean % recovery at each level for Folic acid should be 98.0 to 102.0.

Table 6: Accuracy for Folic acid

Recovery level	Sample No.	% Recovery	Mean	% RSD
50%	1	101.75	101.37	0.416
	2	101.43		
	3	100.92		
100%	1	100.84	100.76	0.242
	2	100.95		
	3	100.49		
150%	1	101.99	101.50	0.506
	2	101.53		
	3	100.97		

Result and Conclusion:

All the results are well within the acceptance criteria and results indicate that the method is accurate and precise.

9.6 PRECISION:

9.6.1 SYSTEM PRECISION

Study design:

Five replicate injections of standard preparation are injected into the HPLC system. The area response for Folic acid Peaks along with % RSD are tabulated in Table 7.

Acceptance criteria:

% RSD of area of analyte peak in five replicate standard injections should not be more than 2.0.

Table 7: System precision

Injection No.	Folic acid Area
1	257858
2	258958
3	258554
4	258607
5	257045
Mean	258204
% RSD	0.295

Results and Conclusion:

The results are well within the acceptance criteria and the % RSD observed for the replicate injections indicates the system precision of HPLC system used.

9.6.2 Method Precision:

Study Design:

Six Assay preparations of test are analyzed as per the method. The Assay of Folic acid is calculated. The results are tabulated in Table 8.

Acceptance criteria:

% RSD for Assay of six test preparations should not be more than 5.0.

Table 8: Method precision for Folic acid

No. of Preparation	Assay of Folic acid
1	120.0
2	116.0
3	115.3
4	116.3
5	118.1
6	119.6
Mean	117.6
% RSD	1.68

Results and Conclusion:

The results are well within the acceptance criteria and the % RSD observed for assay values indicates the precision of the analytical method.

9.6.3 Intermediate Precision:

Study summary:

Six Assay preparations of test are analyzed as per the method by different analyst using different instrument and different column on different day. The assay of Folic acid is calculated. The results are tabulated in Table 9 and cumulative results are tabulated in Table 10.

Acceptance criteria:

1) % RSD for Assay of six test preparations should not be more than 5.0.

2) Cumulative % RSD for Assay of twelve test preparations (of method and intermediate precision) should not be more than 5.0.

Table 9: Intermediate precision for Folic acid

No. of Preparation	Assay of Folic acid
1	116.1
2	117.3
3	118.0
4	115.5
5	114.4
6	110.5
Mean	115.3
% RSD	2.34

The Cumulative results of Method Precision and Intermediate Precision are tabulated in Table 10.

Table 10: Cumulative % RSD for Folic acid

Parameter	Assay of Folic acid %
Method Precision	120.0
	116.0
	115.3
	116.3
	118.1
	119.6
Intermediate Precision	116.1
	117.3
	118.0
	115.5
	114.4
	110.5
Mean	116.4
% RSD	2.19

Result and Conclusion:

The results are well within the acceptance criteria and the % RSD observed for drug release indicates the precision of the method.

9.7 STABILITY OF ANALYTICAL SOLUTION:

Study design:

Sample solution:

Test preparation are prepared as per the proposed method and injected into the system initially and at various time intervals and data tabulated in Table 11.

Table 11: Stability of test solution for Folic acid

Time in hours	Area of Sample solution	Absolute % Difference
Initial	343835	Not applicable
7	343575	0.08
14	340376	1.02
18	337708	1.81
21	336394	2.21
25	332672	3.36
29	330115	4.16
Mean	337811	2.10
% RSD	1.546	Not applicable

Acceptance criteria:

The test solution shall be considered stable for the final period till which the area difference between initial and next periodic interval should be not more than ±2%.

Standard solution:

Standard preparation are prepared as per the proposed method and injected into the system initially and at various time intervals and data tabulated in Table 12.

Table 12: Stability of standard solution for Folic acid

Time in hours	Area of Standard solution	Absolute % Difference
Initial	258204	Not applicable
7	257830	0.15
14	259169	-0.37
18	260789	-0.99
21	264592	-2.41
25	269939	-4.35
29	272985	-5.41
Mean	263358	-2.23
% RSD	2.293	Not applicable

Results and conclusions:

The Standard solution and test solution was stable upto 18 hours at room temperature.

9.8 FILTER PAPER STUDY:

Study design:

The filter paper study of analytical method was performed by filtering test solution through 0.45µ Nylon and 0.45µ PVDF membrane filter against that of unfiltered test. The results were tabulated in Table 13.

Table 13: Filter paper study for test solution of Folic acid

Filter study	Assay in %	% difference from unfiltered sample
Unfiltered sample (Centrifuged)	114.8	Not applicable
Filter Set-1 (0.45µ Nylon membrane)	117.6	-2.38
Filter Set-1 (0.45µ PVDF membrane)	115.6	-0.69

Acceptance criteria:

The % difference on filter solution should not differ ±5.0 against that of unfiltered.

Results and conclusions:

The % difference on filtered sample (0.45µ Nylon and PVDF) membrane within limit against that of unfiltered.

9.9 ROBUSTNESS:

Study Design:

Five replicate injections of standard preparation and duplicate injections of test preparation were injected varying different chromatographic conditions as per protocol. System suitability parameters and mean assay difference with respect to assay value in method precision were calculated. The results are tabulated in table 14 Folic acid peaks respectively.

Table 14: Robustness of analytical method for Folic acid

Parameter	Theoretical Plates (NLT 2000)	Tailing Factor (NMT 2.0)	% RSD (NMT 2.0)	Mean Assay value (method precision)	Folic acid Assay	Absolute % Difference
Low wavelength (277nm)	8054.0	0.93	0.164	117.0	117.8	-0.75
High wavelength (283nm)	8074.0	0.93	0.402		115.9	1.05
Low flow rate (0.7ml/minute)	7878.0	0.94	0.110		120.3	-3.27
High flow rate (0.9ml/minute)	8148.0	0.94	0.052		115.0	2.05
Low column Temperature (35^{0}C)	8861.0	0.95	0.178		120.5	-3.55
High column Temperature (45^{0}C)	7462.0	0.93	0.283		121.1	-4.10

Acceptance criteria:

1) System Suitability Should Comply for Each Variable.

2) % of drug not differ ±5% from mean assay value of method precision.

Result and Conclusion:

Each chromatographic variation System suitability parameters are within limits. % Difference of assay within limits at each variation.

10.0 SUMMARY:

S.No	Validation parameter	Acceptance criteria	Results
1	System suitability	1) % RSD of area of analyte in Five replicate standard injections should not be more than 2.0.	0.295
		2) Theoretical plate should be not less than 2000.	8056
		3) Tailing factor should not be more than 2.0.	0.960
2	Specificity Interference from blank, placebo and placebo spiked with analyte.	1) There should not be any interference due to blank and placebo with analyte. 2) Peak purity of analyte should pass	Blank and Placebo peaks are not interference with retention time of Folic acid peak in test preparation and Peak purity passes within specified limits.
3	Linearity and Range	1) R^2 Should be NLT 0.995	Squared correlation coefficient for 1.000

<table>
<tr><th>S.No</th><th>Validation parameter</th><th>Acceptance criteria</th><th>Results</th></tr>
<tr><td></td><td></td><td>2) To conclude the range, %RSD for peak area of linearity level-50%, 75%, 100%, 125% and 150% should be not more than 2.0.</td><td><table><tr><th>Level</th><th>%RSD</th></tr><tr><td>50%</td><td>0.317</td></tr><tr><td>75%</td><td>0.322</td></tr><tr><td>100%</td><td>0.116</td></tr><tr><td>125%</td><td>0.393</td></tr><tr><td>150%</td><td>0.126</td></tr></table></td></tr>
<tr><td>4</td><td>Interference from degradants (Forced degradation)</td><td>1) There should not be any interference due to degradants with analyte in stressed samples.

2) The desired degradation should be 10-30% in acid, alkali and oxidation degradation, (if possible).

3) If about 10% to 30% degradation is not achieved by applying above stressed condition, same shall be documented and reported.

4) Peak purity should not be less than 0.950 according to Lab solution.</td><td>There is no any interference due to degradants with analyst in stressed sample and peak purity was passes according to lab solution.</td></tr>
</table>

S.No	Validation parameter	Acceptance criteria	Results
5	Accuracy (Recovery)	The mean % recovery at each level should be 98.0 to 102.0.	Level %Recovery **Folic acid:** 50% : 101.37 100% : 100.76 150% : 101.50
6	**Precision** 1) System Precision	%RSD of area of analyte peaks in five replicate standard injections should not be more than 2.0.	0.295
	2) Method Precision	%RSD of Assay of six test preparations should not be more than 5.0.	1.68
	3)Intermediate Precision	1) % RSD for assay of six test preparations should not be more than 5.0. 2) Cumulative %RSD for assay of twelve test preparations (of method and intermediate precision) should not be more than 5.0.	2.34 2.19

S.No	Validation parameter	Acceptance criteria	Results
7	Stability for analytical solution	The sample and standard solution shall be considered stable for the final period till which the area difference between initial and next periodic interval should not be more than ±2%.	The Standard and test solution was stable upto 18 hours at room temperature.
8	Filter paper study (0.45µ Nylon and PVDF)	The % difference on filter solution should not differ ±5.0 against that of unfiltered.	The % difference on filtered sample 0.45µ Nylon and PVDF within limit against that of unfiltered.
9	Robustness (i) Flow rate change (ii) Wavelength change (iii) Column oven Temperature Change	System suitability parameters should comply.	Each chromatographic variation System suitability parameters are within limits. % Difference of assay within limits at each variation.

11.0 CONCLUSION:

Validation studies have been conducted for Assay of Folic acid in Carbonyl Iron, Folic acid, Cyanocobalamin, Ascorbic acid, Zinc, Alpha Tocopheryl Acetate and Selenium Capsules for the parameters of system suitability, Specificity, Degradation, System Precision, Method precision, Intermediate precision, Linearity and range, Accuracy, Filter paper study, Solution stability and Robustness by using the proposed method. The data is complied and found satisfactory with the analytical method for all the parameters analysed. Hence it is concluded that the method can be used for regular analysis.

12.0 ABBREVIATION:

mg	:	Milligram
S.No	:	Number
ml	:	Milliliter
%	:	Percentage
ID	:	Identification
API	:	Active pharmaceutical ingredient
HPLC	:	High performance liquid chromatography
B.NO	:	Batch number
WS.NO	:	Working standard number
mm	:	Millimeter
µm	:	Micrometer
min	:	Minutes
°C	:	Degree centigrade
nm	:	Nanometer
RSD	:	Relative standard deviation
µl	:	Micro litre

13.0 REVISION HISTORY:

Report No.	Effective date	Reason for Review
ST/AMVAFR/23/033	10/02/2024	New Report prepared.

ANALYTICAL METHOD VALIDATION PROTOCOL FOR THE TEST OF ASSAY OF FOLIC ACID IN CARBONYL IRON, FOLIC ACID, CYANOCOBALAMIN, ASCORBIC ACID, ZINC, ALPHA TOCOPHERYL ACETATE AND SELENIUM CAPSULES

Site Address: SAFETAB LIFE SCIENCE
Plot No.A-67 to 72, PIPDIC Electronic Park,
Thirubuvanai, Puducherry-605 107

1.0 INDEX

2.0 PROTOCOL APPROVAL SHEET

PREPARED BY		
Name	:	S. SANTHI
Designation	:	ASST. MANAGER - QC
Signature	:	
Date	:	18/01/24
REVIEWED BY		
Name	:	M. VIJAYAKUMAR
Designation	:	GM - QC
Signature	:	
Date	:	19/01/2024
APPROVED BY		
Name	:	S. Mani
Designation	:	AGM - QA
Signature	:	
Date	:	20/01/2024

Effective Date	:	22/01/2024

3.0 OBJECTIVE

To validate the method for the test of assay of Folic Acid in Carbonyl Iron, Folic acid, Cyanocobalamin, Ascorbic acid, Zinc, Alpha Tocopheryl Acetate and Selenium Capsules by HPLC.

4.0 GENERAL INFORMATION:

REFERENCE : In-House

TYPE OF VALIDATION : Validation of non-pharmacopoeial method

TEST TO BE VALIDATED : Assay of Folic Acid in Carbonyl Iron, Folic acid, Cyanocobalamin, Ascorbic acid, Zinc, Alpha Tocopheryl Acetate and Selenium Capsules

COMPOSITION : Each hard gelatin capsule contains:

Content	Strength
Carbonyl Iron Equivalent to Elemental iron	100mg
Folic Acid BP	500mcg
Cyanocobalamin (Vitamin B12) (Coated)	15mcg
Ascorbic Acid (coated)	75mg
Zinc Sulphate monohydrate BP Equivalent to elemental zinc 22.5mg	61.8mg
Alpha Tocopheryl Acetate BP	15IU
Sodium selenate Equivalent to Selenium	65mcg

BATCH NO	:	G17231116
SPECIFICATION LIMIT	:	Not less than 95.0% of the labeled claim
VALIDATION STUDY	:	QC-Laboratory, Safetab Life Science, Puducherry-605107
VALIDATION TEAM	:	1. C.K.Saravanan 2. T.Amudha 3. E.Meena

5.0 DETAILS OF STANDARD, SAMPLES AND PLACEBO TO BE USED

Mention the name and Batch No., Potency of the reference/working std, test samples / placebo to be used during Validation.

NAME OF THE MATERIAL	ID NO/BATCH NO	POTENCY/PURITY
Sample	G17231116	COA attached
Plain Placebo	Not Applicable	Not Applicable
Working standard Folic Acid BP	To be mentioned in report	To be mentioned in report
Alpha Tocopheryl acetate BP	To be mentioned in report	To be mentioned in report
Cyanocobalamin	To be mentioned in report	To be mentioned in report

6.0 DETAILS OF INSTRUMENTS/EQUIPMENTS, COLUMN, SOLVENTS AND CHEMICALS TO BE USED:

INSTRUMENTS/EQUIPMENTS :

High performance liquid chromatograph with PDA detector

Make : Shimadzu, Model : LC-2050C 3D Prominence i

High performance liquid chromatograph with UV detector

Make : Shimadzu, Model : LC-2050C Prominence i

Analytical Balance:

Make : Sartorius, Model : Quintix-125D-1OIN

COLUMN:

Inertsil ODS 3V C18, (250mmx 4.6mm) 5µ or equivalent

SOLVENTS AND CHEMICALS WITH GRADE :

Folic acid (Working standard)

Potassium dihydrogen orthophosphate (AR grade)

Dipotassium hydrogen orthophosphate (AR grade)

Purified Water (Milli-Q water (or) equivalent)

Methanol (HPLC grade)

Acetonitrile (HPLC grade)

Hydrochloric acid (AR grade)

Sodium Hydroxide (AR grade)

Hydrogen Peroxide 30% (AR grade)

7.0 DESCRIPTION OF ANALYTICAL METHOD

Chromatographic Conditions:

Column	:	Inertsil ODS 3V C18, (250mmx 4.6mm) 5µ or equivalent
Flow rate	:	0.8ml/min
Wavelength	:	280 nm
Injection Volume	:	20 µL
Column temperature	:	40°C
Run time	:	20 minutes

Diluent preparation:

A mixture of 80 volume of water and 20 volume of Acetonitrile.

Buffer preparation:

Dissolve 11.0g of Potassium dihydrogen orthophosphate and 6.0g of Dipotassium hydrogen orthophosphate in 1000mL of purified water.

Mobile phase preparation:

A mixture of 90 volume of Buffer and 10 volume of Methanol and mix well. Filter through 0.45µ nylon membrane filter and degas.

Standard preparation:

Weigh accurately and transfer about 20.0mg of Folic acid working standard into a 100mL volumetric flask. Add 5mL of 1N sodium hydroxide sonicate to dissolve and dilute to volume with diluent. Further dilute 5mL of this solution to 100mL with diluent. Filter the solution through 0.45µ nylon membrane filter. (**Concentration:** 0.01mg/ml of Folic Acid).

Test preparation:

Weigh 20 capsules and calculate the average weight and crush to fine powder. Weigh accurately and transfer powdered sample equivalent to about 1.0mg of Folic acid into a 100mL volumetric flask. Add 5mL of 1N of sodium hydroxide to disperse the sample and add 60mL of diluent, sonicate for 15 minutes with intermittent shaking (Maintain the bath temperature below 25°C) and dilute to volume with diluent. Filter the solution through 0.45µ nylon membrane filter. (**Concentration:** 0.01mg/ml of Folic Acid).

Blank preparation:

Pipette out 5mL of 1N Sodium hydroxide into 100mL volumetric flask and dilute upto volume with diluent.

Procedure:

Inject the blank (diluent) in single injection, standard preparation as five replicate injection in to the chromatogram and measure the response of the peak of Folic acid in term of standard area and test in duplicate.

The test is not valid unless it meets the system suitability parameters. Calculate the system suitability with respect to the following parameters.

System suitability:

Theoretical plate	: NLT 2000 for Folic acid peak
Tailing factor	: NMT 2.0 for Folic acid peak
Relative standard deviation	: NMT 2.0% for five replicate injections of Folic acid peak.

Inject 20µl of the above solution as per following sequence.

Injection sequence:

S. No	Sample Name	No. of injections
1	Blank (Diluent)	1
2	Standard preparation	5
3	Test preparation	2
4	Bracketing standard	1 Each after every 6 sample injection

Calculation:

Calculate the assay of Folic acid in mcg/capsule as follows:

$$= \frac{AT}{AS} \times \frac{WS}{100} \times \frac{5}{100} \times \frac{100}{WT} \times \frac{P}{100} \times 1000 \times ANW$$

Where,

AT = Average area of Folic acid peak in Test preparation

AS = Average area of Folic acid peak in standard preparation.

WS = Weight of Folic acid working standard taken in mg

WT = Weight of sample taken in mg.

P = Potency of Folic acid Working standard (% on as basis).

ANW = Average Net Weight of capsule in mg.

Calculate the assay of Folic acid in % as follows:

$$= \frac{\text{mcg/capsule}}{LC} \times 100$$

LC = Label claim of Folic acid in mcg/capsule.

8.0 PARAMETERS TO BE VALIDATED:

Following parameters shall be selected for Validation	
S.No.	**VALIDATION PARAMETERS**
1	**System suitability**
2	**Specificity (Selectivity)** i) Interference from blank and placebo
3	**Linearity and Range**
4	**Interference and Degradants (Forced degradation)**
	(i) Acid degradation (ii) Alkali Degradation (iii) Oxidative Degradation
5	**Accuracy**
6	**Precision** i) System precision ii) Method precision iii) Intermediate Precision
7	**Stability of analytical solution**
8	**Filter paper study**
9	**Robustness** i) Wavelength change ii) Flow rate change iii) Column oven temperature change

Note: More than one parameter may be performed at once with relevant sequence having common system suitability with bracketing preparation.

9.0 DETAILS OF VALIDATION PARAMETERS:

9.1 SYSTEM SUITABILITY:

Purpose:

To establish system suitability as per methodology.

Study Design:

Sequence shall be in following provisional manner.

S.No.	Description of solution	No. of Injections
1	Blank (Diluent)	1
2	Standard preparation	5

Evaluate the following system suitability parameters:

1) % RSD of area of Folic acid peak in five replicate standard injections.

2) Theoretical plates for Folic acid peak in standard injection.

3) Tailing factor for Folic acid peak in standard injection.

Acceptance Criteria:

1) % RSD of area for Folic acid peak in five replicate standard injections should not more than 2.0%.

2) Theoretical plates for Folic acid peak in standard injection should not less than 2000.

3) Tailing factor for Folic acid peak in standard injection should not more than 2.0.

9.2 SPECIFICITY (SELECTIVITY)

9.2.1 Interference from blank and placebo

"The specificity is the ability of an analytical procedure to measure accurately an analyte in presence of componenets that may be expected present in sample matrix".

Purpose:

To demonstrate that the placebo not interfering with the analyte peak.

Study Design:

Sequence shall be in following provisional manner.

S.No.	Description of solution	No. of injections
1	Blank (Diluent)	1
2	Standard preparation	5
3	Folic acid Working standard	1
4	Alpha tocopheryl acetate + Cyanocobalamin working standard	1
5	Plain placebo for Folic acid	1
6	Plain Placebo + Folic acid working standard	1
7	Plain Placebo + Alpha tocopheryl acetate + Cyanocobalamin working standard	1
8	Sample preparation	1

Acceptance Criteria:

i) There should not be any interference due to blank, Placebo peak with analyte.

ii) Peak purity index Not less than 0.995 according to Lab solution software.

9.3 LINEARITY AND RANGE:

"The linearity of the analytical method is it's ability to elecit test results data directly proportional to the concentration of the analyte in samples within given range".

Purpose:

To Establish the linearity of analyte within the specified range.

Study Design:

To demonstrate the linearity and range of analytical method over the range of 10% to 150% of targeted concentration.

Linearity stock solution, linearity level, expected concentration, linearity stock dilution and calculated concentration are tabulated below.

Linearity Stock solution	50	1	1	1	1	500 ppm (con. ppm)
	100	1	1	1	1	

Lin level	Exp conc (ppm)	Lin Stock Vol (ml)	Dil to (ml)	Calc conc (ppm)
50%	5.0	1	100	5.0
75%	7.5	3	200	7.5
100%	10.0	2	100	10.0
125%	12.5	5	200	12.5
150%	15.0	3	100	15.0

Sequence shall be in following provisional manner.

S.No.	Description of solution	No. of Injections
1	Blank (Diluent)	1
2	Level – 1 (50%)	3
3	Blank (Diluent)	1
4	Level – 2 (75%)	3
5	Blank (Diluent)	1
6	Level – 3 (100%)	3
7	Blank (Diluent)	1
8	Level – 4 (125%)	3
9	Blank (Diluent)	1
10	Level – 5 (150%)	3

Acceptance criteria:

(i) To conclude the linearity, the squared correlation coefficient r^2 should not be less than 0.995.

(ii) To conclude the range, % RSD for peak area of linearity level of 50%, 75%, 100%, 125% and 150% should be not more than 2.0.

9.4 INTERFERENCE FROM DEGRADANT (forced degradation)

Study design:

To evaluate the interference from degradants, carry out a forced degradation study by stressing the test preparation under the following maximum stress conditions.

Degradation	Stress Condition
Acid degradation	Exposure to 5ml of 5N HCL and heat on water bath at 80°C for 30minutes
Alkali degradation	Exposure to 5ml of 5N NaOH and heat on water bath at 80°C for 30minutes
Oxidative degradation	Exposure to 5ml of 30% H_2O_2 and heat on water bath at 80°C for 30minutes

Sequence shall be in following provisional manner, For forced chemical degradation:

S.No.	Description of solution	No. of Injections
1	Blank (Diluent)	1
2	Standard preparation	5
3	Test preparation (As such)	2
4	Test preparation (Acid degradation)	2
5	Test preparation (Alkali degradation)	2
6	Test preparation (Oxidative degradation)	2
7	Standard preparation (Bracketing)	1

Chromatograph the samples of chemical and physical forced degradation into HPLC system equipped with diode array detector and evaluate the peak purity for the analytes in stressed samples and the degradation profiles under each stressed condition.

Acceptance Criteria:

1) There should not be any interference due to degradants with analyte in stressed samples.

2) The desired degradation should be 10-30% in acid, alkali and oxidative degradation, (if possible).

3) If about 10% to 30% degradation is not achieved by applying above stressed condition, same shall be documented and reported.

9.5 ACCURACY (RECOVERY):

"The accuracy of an analytical method is the closeness of results obtained by that method to the true value. Accuracy may often be expressed as present recovery by the assay of known, add amount of analyte".

Purpose: To establish the accuracy of the analytical method in the specified range.

Sequence shall be in following provisional manner

S.No.	Description of solution	No. of Injections
1	Blank (Diluent)	1
2	Standard preparation	5
3	Blank (Diluent)	1
4	Level – 1 Set – 1 (50%)	1
5	Level – 1 Set – 2 (50%)	1
6	Level – 1 Set – 3 (50%)	1
7	Blank (Diluent)	1
8	Level – 2 Set – 1 (100%)	1
9	Level – 2 Set – 2 (100%)	1
10	Level – 2 Set – 3 (100%)	1
11	Blank (Diluent)	1
12	Level – 3 Set – 1 (150%)	1
13	Level – 3 Set – 2 (150%)	1
14	Level – 3 Set – 3 (150%)	1
15	Standard preparation (Bkt)	1

Study design:

To demonstrate the accuracy of the analytical method, prepare recovery samples by spiking known quantities of drug (at level 50%, 100% and 150% of targeted concentration) to placebo. Prepare the recovery samples in triplicate for each level and inject only one injection for each samples.

Acceptance criteria: The mean % recovery at each level should be 98.0 to 102.0.

9.6 PRECISION

9.6.1 SYSTEM PRECISION

Purpose:

To establish the precision of the HPLC system being used for the analysis.

Study Design:

Sequence shall be in following provisional manner.

S.No.	Description of solution	No. of Injections
1	Blank (Diluent)	1
2	Standard preparation	5

Acceptance Criteria:

% RSD of area of analyte peak in five replicate standard injections should not be more than 2.0.

9.6.2 METHOD PRECISION

Purpose:

To establish the repeatability of test results obtained by the analytical method.

Study design:

To demonstrate the method precision, analyze six sample preparations as per the methodology representing a single batch and determine the assay for the same. Evaluate the method precision by computing the percentage and relative standard deviation of the assay results.

.No.	Description of solution	No. of Injections
1	Blank (Diluent)	1
2	Standard preparation	5
3	Test preparation-1	2
4	Test preparation-2	2
5	Test preparation-3	2
6	Standard preparation (BKT)	1
7	Test preparation-4	2
8	Test preparation-5	2
9	Test preparation-6	2
10	Standard preparation (BKT)	1

Acceptance Criteria:

% RSD for assay of six preparations should not be more than 5.0.

9.6.3 INTERMEDIATE PRECISION

Purpose:

To demonstrate the reproducibility of test results obtained by the analytical method for the variability of instrument, column (different lot no) analyst and day. Analyse six sample preparations as per the methodology representing a single batch and determine the assay for the same. Evaluate the intermediate precision by computing the percentage and relative standard deviation of the assay results.

S.No.	Description of solution	No. of Injections
1	Blank (Diluent)	1
2	Standard preparation	5
3	Test preparation-1	2
4	Test preparation-2	2
5	Test preparation-3	2
6	Standard preparation (BKT)	1
7	Test preparation-4	2
8	Test preparation-5	2
9	Test preparation-6	2
10	Standard preparation (BKT)	1

Acceptance criteria:

1) % RSD for assay of six preparations should not be more than 5.0.

2) Cumulative % RSD for assay of twelve preparations (i.e. method precision and intermediate precision) should not be more than 5.0.

9.7 STABILITY OF ANALYTICAL SOLUTION:

Study design:

Prepare Standard and Test solution as per the methodology and store at room temperature. Chromatograph this solution at regular intervals by using same diluent. Calculate the % difference of analyte peak area for standard and sample preparations with that of initial. The study may be stopped if 2 consecutive failure of sample solution.

Sequence shall be in following provisional

S.No.	Description of solution	No. of Injections
1	Blank (Mobile phase)	1
2	Standard preparation	5
3	Standard preparation (Initial)	1
4	Test preparation (Initial)	1
5	Standard preparation (Time interval)	1
6	Test preparation (Time interval)	1

Acceptance criteria:

The Test and standard solution shall be considered stable for the final period till which the area difference between initial and next periodic interval should not be more than ±2%.

9.8 FILTER PAPER STUDY:

Study design:

The filter paper study of the analytical method shall perform by filtering Test solution through 0.45µ Nylon and 0.45µ PVDF filter against that of unfiltered.

Sequence shall be in following provisional manner.

S.No.	Description of solution	No. of Injections
1	Blank (Diluent)	1
2	Standard preparation	5
3	Test preparation –Unfiltered sample (Centrifuged)	1
4	Test preparation –Filter Set 1 (0.45µ Nylon membrane filter)	1
5	Test preparation –Filter Set 1 (0.45µ PVDFmembrane filter)	1
6	Standard preparation (BKT)	1

Acceptance criteria:

The % area difference of filter solution should not differ ±5.0 against that of unfiltered Centrifuged.

9.9 ROBUSTNESS:

Purpose:

To establish the robustness of the analytical method.

Study Design:

The robustness of the analytical method can be established by demonstrating its reliability against deliberate changes in chromatographic conditions.

Sequence shall be in following provisional manner.

As such		
S.No.	**Description of solution**	**No. of Injections**
1	Blank (Diluent)	1
2	Standard preparation	5
3	Test preparation	2
4	Bracketing standard	1
According to each variable		
S.No.	**Description of solution**	**No. of Injections**
1	Blank (Diluent)	1
2	Standard preparation	5
3	Test preparation	2
4	Bracketing standard	1

Following variable shall be done according to deliberate changes in chromatographic parameters.

a) Flow rate change by ±10% mean (i.e 0.7 ml/min and 0.9 ml/minute)

b) Wave length change by ± 3nm (i.e. 277nm and 283nm)

c) Column oven Temperature change by ± 5.0°C (i.e. 35°C and 45°C)

Acceptance criteria:

System suitability should comply for each variable and % of drug not differ ±5% from mean assay value of method precision.

10.0 ABBREVIATION:

mg	:	Milligram
S.No	:	Serial Number
ml	:	Milliliter
%	:	Percentage
ID	:	Identification
API	:	Active pharmaceutical ingredient
HPLC	:	High performance liquid chromatography
B.No	:	Batch number
mm	:	Millimeter
µm	:	Micrometer
min	:	Minutes
°C	:	Degree centigrade
nm	:	Nanometer
RSD	:	Relative standard deviation
µl	:	Micro litre
HCL	:	Hydrochloric acid
NaOH	:	Sodium Hydroxide
H_2O_2	:	Hydrogen Peroxide

11.0 REVISION HISTORY:

Protocol No.	Effective date	Reason for Review
ST/AMVAFP/23/033	22/01/2024	New Protocol prepared.

ANALYTICAL METHOD VALIDATION PROTOCOL FOR THE TEST OF ASSAY OF ASCORBIC ACID IN CARBONYL IRON, FOLIC ACID, CYANOCOBALAMIN, ASCORBIC ACID, ZINC, ALPHA TOCOPHERYL ACETATE AND SELENIUM CAPSULES

Site Address: SAFETAB LIFE SCIENCE
Plot No.A-67 to 72, PIPDIC Electronic Park,
Thirubuvanai, Puducherry-605 107

1.0 INDEX

2.0 PROTOCOL APPROVAL SHEET

PREPARED BY		
Name	:	K. SARAVANAN
Designation	:	Dy. Manager - QC
Signature	:	
Date	:	11/01/2024
REVIEWED BY		
Name	:	M. VIJAYAKUMAR
Designation	:	GM - QC
Signature	:	
Date	:	12/01/2024
APPROVED BY		
Name	:	S. Yaran
Designation	:	AGM - QA
Signature	:	
Date	:	13/01/2024

Effective Date	:	18/01/2024

3.0 OBJECTIVE

To verify the method for the test of assay of Ascorbic Acid in Carbonyl Iron, Folic acid, Cyanocobalamin, Ascorbic acid, Zinc, Alpha Tocopheryl Acetate and Selenium Capsules.

4.0 GENERAL INFORMATION

REFERENCE : In-House

TYPE OF VALIDATION : Validation of pharmacopoeial method

TEST TO BE VALIDATED : Assay of Ascorbic Acid in Carbonyl Iron, Folic acid, Cyanocobalamin, Ascorbic acid, Zinc, Alpha Tocopheryl Acetate and Selenium Capsules

COMPOSITION : Each hard gelatin capsule contains:

Content	Strength
Carbonyl Iron Equivalent to Elemental iron	100mg
Folic Acid BP	500mcg
Cyanocobalamin (Vitamin B12) (Coated)	15mcg
Ascorbic Acid (coated)	75mg
Zinc Sulphate monohydrate BP (Equivalent to elemental zinc 22.5mg)	61.8mg
Alpha Tocopheryl Acetate BP	15IU
Sodium selenate Equivalent to Selenium	65mcg

BATCH NO : G17231116

SPECIFICATION LIMIT	:	Not less than 71.25mg (Not less than 95.0% of the labeled claim)
VALIDATION STUDY	:	QC-Laboratory, Safetab Life science, Puducherry-605107
VALIDATION TEAM	:	1. V.VIGNESH

5.0 DETAILS OF STANDARD, SAMPLES AND PLACEBO TO BE USED

Mention the name and Batch No., Potency of the reference/working std., Impurities standard, test samples/placebo to be used during Validation.

NAME OF THE MATERIAL	ID NO / BATCH NO	POTENCY / PURITY
Sample	G17231116	Not Applicable
Plain Placebo	Not Applicable	Not Applicable
Working standard Ascorbic Acid BP	To be mentioned in report	To be mentioned in report

6.0 DETAILS OF SOLVENTS AND CHEMICALS TO BE USED:

Analytical Balance

Make : Sartorius, Model : BSA224S-CW

Chemicals with grade:

Concentrated Sulphuric acid (AR grade)

Iodine (AR grade)

Starch (AR grade)

Purified Water (Milli-Q water (or) equivalent)

7.0 DESCRIPTION OF ANALYTICAL METHOD

Reference: In-House

Procedure: By Titration

Procedure: (Capsules)

Weigh 20 capsules and calculate the average weight and crush to fine powder. Weigh accurately about 150 mg equivalent of Ascorbic acid and dissolve as completely as possible, add 15mL of 1M sulphuric acid sonicate for 10minutes to dissolve and add 15 mL of water. Add 1 ml of starch solution. Titrate with 0.05 M iodine until a persistent violet-blue colour is obtained. Carryout a blank titration

1 ml of 0.05M Iodine is equivalent to 8.806 mg of Ascorbic acid.

Calculate the content of Ascorbic acid in mg/capsule as follows:

$$= \frac{\text{(Titer value - Blank) x Molarity of 0.05M Iodine x 8.806 x Avg.net weight}}{\text{Sample weight (mg) X 0.05}}$$

Calculate the content of Ascorbic acid in % as follows:

$$= \frac{\text{mg/capsule}}{\text{LC}} \times 100$$

LC = Label claim of Ascorbic acid in (mg/capsule).

8.0 PARAMETERS TO BE VALIDATED:

Following parameters shall be selected for validation.	
S.No.	**VALIDATION PARAMETER**
1	**Specificity (Selectivity)** i) Interference from blank and placebo
2	**Linearity and Range**
3	**Accuracy**
4	**Precision** i) Method precision ii) Intermediate Precision

Note: More than one parameter may be performed at once with relevant sequence having common system suitability with bracketing preparation.

9.0 DETAILS OF VALIDATION PARAMETERS:

9.1 SPECIFICITY (SELECTIVITY)

9.1.1 Interference from blank and placebo

"The specificity is the ability of an analytical procedure to measure accurately an analyte in presence of componenets that may be expected present in sample matrix".

Purpose:

To demonstrate that the placebo not interfering with the analyte peak.

Study Design :

Sequence shall be in following provisional manner.

S.No.	Description of solution	No. of Titration
1	Blank	1
2	Ascorbic acid API preparation	1
3	Plain placebo	1
4	Plain placebo with Ascorbic acid API	1
5	Plain placebo with Carbonyl iron API	1
6	Plain placebo with Folic acid API	1
7	Plain placebo with Cyanocobalamin API	1
8	Plain placebo with Zinc sulphate API	1
9	Plain placebo with Vitamin E API	1
10	Plain placebo with Selenium sulphate API	1
11	Sample preparation Ascorbic acid capsules (G17231116)	1

Acceptance Criteria :

i) There should not be any interference due to blank and Placebo with analyte.

9.2 LINEARITY AND RANGE:

"The linearity of the analytical method is it's ability to elecit test results data directly proportional to the concentration of the analyte in samples within give range".

Purpose:

To Establish the linearity of analyte within the specified range.

Study Design :

To demonstrate the linearity and range of analytical method over the range of 10% to 150% of targeted concentration.

Sequence shall be in following provisional manner.

S.No.	Description of solution	No. of Titration
1	Level – 1 (10%)	2
2	Level – 2 (50%)	2
3	Level – 3 (75%)	2
4	Level – 4 (100%)	2
5	Level – 5 (125%)	2
6	Level – 6 (150%)	2

Acceptance criteria:

(i) To conclude the linearity, the squared correlation coefficient should not be less than 0.995

(ii) To conclude the range, % RSD for peak area of linearity level of 10%, 50%, 75%, 100% 125% and 150% should be not more than 2.0.

9.3 ACCURACY STUDY (RECOVERY STUDY)

"The accuracy of an analytical method is the closeness of results obtained by that method to the true value. Accuracy may often be expressed as present recovery by the assay of known, add amount of analyte".

Purpose:

To establish the accuracy of the analytical method in the specified range.

Sequence shall be in following provisional manner

S.No.	Description of solution	No. of Titration
1	Level – 1 Set – 1 (50%)	1
2	Level – 1 Set – 2 (50%)	1
3	Level – 1 Set – 3 (50%)	1
4	Level – 2 Set – 1 (100%)	1
5	Level – 2 Set – 2 (100%)	1
6	Level – 2 Set – 3 (100%)	1
7	Level – 3 Set – 1 (150%)	1
8	Level – 3 Set – 2 (150%)	1
9	Level – 3 Set – 3 (150%)	1

Study design:

To demonstrate the accuracy of the analytical method, prepare recovery samples by spiking known quantities of drug (at level 50%, 100% and 150% of targeted concentration) to placebo. Prepare the recovery samples in triplicate for each level and inject only one injection for each samples.

Acceptance criteria:

The mean % recovery at each level should be 98.0 to 102.0.

9.4 PRECISION

9.4.1 METHOD PRECISION

Purpose:

To establish the repeatability of test results obtained by the analytical method.

Study design:

To demonstrate the method precision, analyze six sample preparations as per the methodology representing a single batch and determine the assay for the same. Evaluate the method precision by computing the percentage and relative standard deviation of the assay results.

S.No.	Description of solution	No. of Titration
1	Sample solution-1	1
2	Sample solution-2	1
3	Sample solution-3	1
4	Sample solution-4	1
5	Sample solution-5	1
6	Sample solution-6	1

Acceptance Criteria :

% RSD for assay of six preparations should not be more than 2 .0.

9.4.2 INTERMEDIATE PRECISION

Purpose:

To demonstrate the method precision, analyze six test preparations as per the methodology representing a single batch and determine the assay for the same. Evaluate the intermediate precision by computing the percentage and relative standard deviation of the assay results.

S.No.	Description of solution	No. of Titration
1	Sample solution-1	1
2	Sample solution-2	1
3	Sample solution-3	1
4	Sample solution-4	1
5	Sample solution-5	1
6	Sample solution-6	1

Acceptance criteria:

1) % RSD for assay of six preparations should not be more than 2.0.

2) Cumulative % RSD for assay of twelve preparations (i.e. method precision and intermediate precision) should not be more than 2.0.

10.0 ABBREVIATION:

mg	:	Milligram
S.No	:	Serial Number
ml	:	Milliliter
%	:	Percentage
API	:	Active pharmaceutical ingredient
B.NO	:	Batch number
RSD	:	Relative standard deviation

11.0 REVISION HISTORY:

Specification No.	Effective date	Reason for Review
ST/AMVAAP/23/033	18/01/2024	New Protocol prepared.

ANALYTICAL METHOD VALIDATION REPORT FOR THE TEST OF ASSAY OF ASCORBIC ACID IN CARBONYL IRON, FOLIC ACID, CYANOCOBALAMIN, ASCORBIC ACID, ZINC, ALPHA TOCOPHERYL ACETATE AND SELENIUM CAPSULES

Site Address: SAFETAB LIFE SCIENCE
Plot No.A-67 to 72, PIPDIC Electronic Park,
Thirubuvanai, Puducherry-605 107

1.0 INDEX

2.0 REPORT APPROVAL SHEET

PREPARED BY		
Name	:	K. SARAVANAN
Designation	:	Dy. Manager-QC
Signature	:	
Date	:	12/02/2024
REVIEWED BY		
Name	:	M. VIJAYAKUMAR
Designation	:	GM-QC
Signature	:	
Date	:	13/02/2024
APPROVED BY		
Name	:	S. Malar
Designation	:	AGM-QA
Signature	:	
Date	:	14/02/2024

Effective Date	:	15/02/2024

3.0 OBJECTIVE

To verify the method for the test of assay of Ascorbic Acid in Carbonyl Iron, Folic acid, Cyanocobalamin, Ascorbic acid, Zinc, Alpha Tocopheryl Acetate and Selenium Capsules.

4.0 GENERAL INFORMATION

REFERENCE : In-House

TYPE OF VERIFICATION : Verification of Pharmacopoeial method.

TEST TO BE VERIFIED : Assay of Ascorbic Acid in Carbonyl Iron, Folic acid, Cyanocobalamin, Ascorbic acid, Zinc, Alpha Tocopheryl Acetate and Selenium Capsules

COMPOSITION : Each hard gelatin capsule contains:

Content	Strength
Carbonyl Iron Equivalent to Elemental iron	100mg
Folic Acid BP	500mcg
Cyanocobalamin (Vitamin B12) (Coated)	15mcg
Ascorbic Acid (coated)	75mg
Zinc Sulphate monohydrate BP (Equivalent to elemental zinc 22.5mg)	61.8mg
Alpha Tocopheryl Acetate BP	15IU
Sodium selenate Equivalent to Selenium	65mcg

BATCH NO : G17231116

SPECIFICATION LIMIT	:	Not less than 71.25mg (Not less than 95.0% of the labeled claim)
VALIDATION STUDY	:	QC-Laboratory, Safetab Life science, Puducherry-605107
VALIDATION TEAM	:	1. V.VIGNESH 2. N.NAVEEN KUMAR

5.0 DETAILS OF STANDARD,SAMPLES AND PLACEBO TO BE USED.

Mention the name and Batch No., Potency of the reference/working std., Impurities standard, test samples/placebo to be used during validation.

NAME OF THE MATERIAL	ID NO/BATCH NO	POTENCY/PURITY
Sample	G17231116	Not Applicable
Plain Placebo	Not Applicable	Not Applicable
Working standard Ascorbic Acid BP	B.No:ST/WS/23/042	99.9% (As such basis)

6.0 DETAILS OF SOLVENTS AND CHEMICALS TO BE USED :

Analytical Balance

Make : Sartorius, Model : BSA224S-CW

Chemicals with grade:

Concentrated Sulphuric acid (AR grade)

Iodine (AR grade)

Starch (AR grade)

Purified Water (Milli-Q water (or) equivalent)

7.0 DESCRIPTION OF ANALYTICAL METHOD

Reference: In-House

Procedure: By Titration

Procedure: (Capsules)

Weigh 20 capsules and calculate the average weight and crush to fine powder. Weigh accurately about 150 mg equivalent of Ascorbic acid and dissolve as completely as possible, add 15mL of 1M sulphuric acid sonicate for 10minutes to dissolve and add 15 mL of water. Add 1 ml of starch solution. Titrate with 0.05 M iodine until a persistent violet-blue colour is obtained. Carryout a blank titration

1 ml of 0.05M Iodine is equivalent to 8.806 mg of Ascorbic acid.

Calculate the content of Ascorbic acid in mg/capsule as follows:

$$= \frac{\text{(Titer value - Blank) x Molarity of 0.05M Iodine x 8.806 x Avg.net weight}}{\text{Sample weight (mg) X 0.05}}$$

Calculate the content of Ascorbic acid in % as follows:

$$= \frac{\text{mg/capsule}}{\text{LC}} \times 100$$

LC = Label claim of Ascorbic acid in (mg/capsule).

8.0 VERIFIED PARAMETERS :

Following parameters shall be selected for validation.	
S.No.	**VALIDATION PARAMETERS**
1	**Specificity (Selectivity)** i) Interference from blank and placebo
2	**Linearity and Range**
3	**Accuracy**
4	**Precision** i) Method precision ii) Intermediate Precision

Note: More than one parameter may be performed at once with relevant sequence having common system suitability with bracketing preparation.

9.0 VERIFICATION RESULTS :

9.1 SPECIFICITY (SELECTIVITY)

9.1.1 Interference from Blank and Placebo

Placebo solutions were prepared by using equivalent weight of placebo present in portion of test preparation as per test method and titrated as per methodology.

Results are tabulated in Table 1.

Acceptance criteria:

There should not be any interference due to blank, placebo, placebo with analyte.

Table 1: Specificity

Sr.No	Sample ID	Volume of 0.05M Iodine consumed
1	Blank	0
2	Ascorbic acid API preparation	17.8
3	Plain placebo	NA
4	Plain placebo with Ascorbic acid API	17.9
5	Plain placebo with Carbonyl iron API	0.00
6	Plain placebo with Folic acid API	0.00
7	Plain placebo with Cyanocobalamin API	0.00
8	Plain placebo with Zinc sulphate API	0.00
9	Plain placebo with Vitamin E API	0.00
10	Plain placebo with Selenium sulphate API	0.00
11	Sample preparation Ascorbic acid capsules (G17231116)	18.0

Results and Conclusion:

From the Blank and placebo with analyte and sample are not interfere with test preparation.

9.2 LINEARITY AND RANGE:

Study Summary:

Analytical solutions for sample were prepared over the range of 10% to 150% concentration with respect to target concentration (i.e. 10%, 50%, 75%, 100%, 125% and 150%). The sample were analyst as per proposed method.

The results are tabulated in Table 2 for Linearity and Table 3 for Range.

Acceptance criteria:

1) The squared correlation coefficient should not be less than 0.995.

2) To conclude the range % RSD for peak areas of linearity levels 10%, 50%, 75%, 100%, 125% and 150% should not be more than 2.0.

Table 2: Linearity for Ascorbic acid

Linearity Levels (%)	Weight taken in mg (X- axis)	Titer value (Y- axis)
10%	0.0153	1.8
50%	0.0754	8.9
75%	0.1123	13.4
100%	0.1502	17.8
125%	0.1877	22.3
150%	0.2253	26.7
Slope (Y)		118.52
CC		1.000
Sqaured R		1.000
Intercept		0.0169

Fig. : Linearity Graph for Ascorbic acid

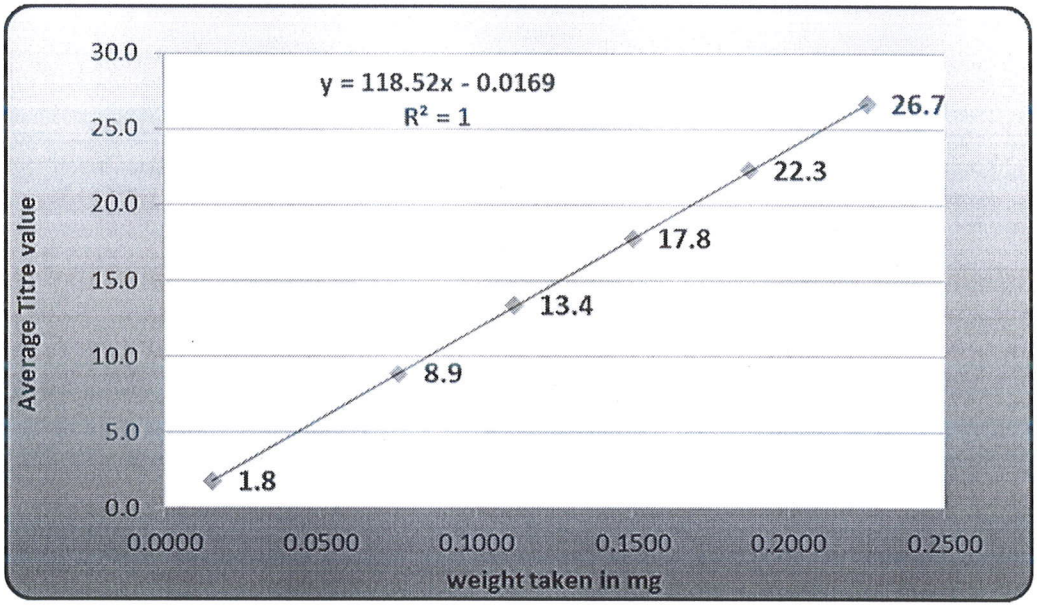

Table: 3 Range for Ascorbic acid

Linearity Levels (%)	%RSD
10%	0.000
50%	0.799
75%	0.530
100%	0.000
125%	0.318
150%	0.265

Result and Conclusion:

Squared correlation coefficient and Range, %RSD of areas at 10%, 50%, 75%, 100%, 125 & 150% levels within limits.

9.3 ACCURACY (RECOVERY)

Study Design:

Known quantity of Ascorbic acid RM were spiked with placebo at three different levels (at level of 50%, 100% and 150% of targeted concentration).

Prepared the recovery samples in triplicate for each level. The samples were analyzed as per the proposed method. The results are tabulated in Table 4 Ascorbic acid respectively to demonstrate the accuracy of the method.

The mean % recovery at each level for Ascorbic acid should be 98.0 to 102.0.

Table 4: Accuracy for Ascorbic acid

Recovery level	Sample No.	% Recovery	Mean %	% RSD
50%	1	100.67	100.9	0.56
	2	101.60		
	3	100.57		
100%	1	100.77	101.0	0.315
	2	100.80		
	3	101.33		
150%	1	100.78	100.9	0.214
	2	101.18		
	3	100.84		

Result and Conclusion:

All the results are well within the acceptance criteria and results indicate that the method is accurate and precise.

9.4 PRECISION:

9.4.1 Method Precision:

Study Design:

Six Assay preparations of sample were analyzed as per the method. The Assay of Ascorbic acid is calculated. The results are tabulated in Table 5.

Acceptance criteria:

% RSD for Assay of six sample preparations should not be more than 2.0.

Table 5: Method precision

No. of Preparation	% of Ascorbic acid
1	101.0
2	100.5
3	99.9
4	99.3
5	100.5
6	99.9
Mean	100.2
% RSD	0.59

Results and Conclusion:

The results are well within the acceptance criteria and the % RSD observed for assay values indicates the precision of the analytical method.

9.4.2 Intermediate Precision (Ruggedness):

Study summary:

Six Assay preparations of sample were analyzed as per the method by different analyst and on different day. The assay of Ascorbic acid is calculated. The results are tabulated in Table 6 and cumulative results are tabulated in Table 7.

Acceptance criteria:

1) % RSD for Assay of six sample preparations should not be more than 2.0.

2) Cumulative % RSD for Assay of twelve sample preparations (of method and intermediate precision) should not be more than 2.0.

Table 6: Intermediate precision

No. of Preparation	% of Ascorbic acid
1	99.7
2	100.9
3	100.9
4	99.9
5	100.5
6	99.4
Mean	100.2
% RSD	0.65

The Cumulative results of Method Precision and Intermediate Precision are tabulated in Table 7.

Table 7: Cumulative % RSD

Parameter	% of Ascorbic acid
Method Precision	101.0
	100.5
	99.9
	99.3
	100.5
	99.9
Intermediate Precision	99.7
	100.9
	100.9
	99.9
	100.5
	99.4
Mean	100.2
% RSD	0.59

Result and Conclusion:

The results are well within the acceptance criteria and the % RSD observed for drug release indicates the precision of the method.

10.0 SUMMARY:

S.No	Validation parameter	Acceptance criteria	Results
1	Specificity Interference from blank, placebo and placebo spiked with analyte.	There should not be any interference due to blank and placebo with analyte.	Blank and placebo spiked with analyte are not interfere with Ascorbic acid in test preparation.
2	Linearity and Range	1) R^2 Should be NLT 0.995 2) To conclude the range, %RSD for peak area of linearity level-10%, 50%, 75%, 100%, 125% and 150% should be not more than 2.0.	**Squared correlation coefficient for Ascorbic acid:** 1.000 Level / %RSD 10% / 0.00 50% / 0.799 75% / 0.530 100% / 0.000 125% / 0.318 150% / 0.265
3	Accuracy (Recovery)	The mean % recovery at each level should be 98.0 to 102.0.	**Level %Recovery Ascorbic acid:** 50% : 100.9 100% : 101.0 150% : 100.9

S.No	Validation parameter	Acceptance criteria	Results
4	**Precision** 1) Method Precision	%RSD of Assay of six sample preparations should not be more than 2.0.	0.59
	2)Intermediate Precision	1) % RSD for assay of sample six preparations should not be more than 2.0.	0.65
		2) Cumulative %RSD for assay of twelve preparations (of method and intermediate precision) should not be more than 2.0.	0.59

11.0 CONCLUSION:

Verification studies have been conducted for assay of Ascorbic acid in Carbonyl Iron, Folic acid, Cyanocobalamin, Ascorbic acid, Zinc, Alpha Tocopheryl Acetate and Selenium Capsules for the parameters of specificity, method precision, Intermediate precision, Linearity and range and accuracy by using the proposed method. The data is complied and found satisfactory with the analytical method for all the parameters analysed. Hence it is concluded that the method can be used for regular analysis.

12.0 ABBREVIATION:

mg	:	Milligram
No	:	Number
ml	:	Milliliter
%	:	Percentage
ID	:	Identification
B.NO	:	Batch number
WS.NO	:	Working standard number
RSD	:	Relative standard deviation

13.0 REVISION HISTORY:

Report No.	Effective date	Reason for Review
ST/AMVAAR/23/033	15/02/2024	New Report prepared.

ANALYTICAL METHOD VALIDATION PROTOCOL FOR THE TEST OF ASSAY OF IRON CONTENT IN CARBONYL IRON, FOLIC ACID, CYANOCOBALAMIN, ASCORBIC ACID, ZINC, ALPHA TOCOPHERYL ACETATE AND SELENIUM CAPSULES

Site Address: SAFETAB LIFE SCIENCE
Plot No.A-67 to 72, PIPDIC Electronic Park,
Thirubuvanai, Puducherry-605 107

1.0 INDEX

2.0 PROTOCOL APPROVAL SHEET

PREPARED BY		
Name	:	K-SARAVANAN
Designation	:	Dy. Manager-QC
Signature	:	
Date	:	18/01/2024
REVIEWED BY		
Name	:	M. VIJAYAKUMAR
Designation	:	GM-QC
Signature	:	
Date	:	19/01/2024
APPROVED BY		
Name	:	S. Maran
Designation	:	AGM-QA
Signature	:	
Date	:	20/01/2024

Effective Date	:	22/01/2024

3.0 OBJECTIVE

To verify the method for the test of assay of Iron Content in Carbonyl Iron, Folic acid, Cyanocobalamin, Ascorbic acid, Zinc, Alpha Tocopheryl Acetate and Selenium Capsules.

4.0 GENERAL INFORMATION

REFERENCE : In-House

TYPE OF VALIDATION : Validation of pharmacopoeial method

TEST TO BE VALIDATED : Assay of Iron Content in Carbonyl Iron, Folic acid, Cyanocobalamin, Ascorbic acid, Zinc, Alpha Tocopheryl Acetate and Selenium Capsules

COMPOSITION : Each hard gelatin capsule contains:

Content	Strength
Carbonyl Iron Equivalent to Elemental iron	100mg
Folic Acid BP	500mcg
Cyanocobalamin (Vitamin B12) (Coated)	15mcg
Ascorbic Acid (coated)	75mg
Zinc Sulphate monohydrate BP (Equivalent to elemental zinc 22.5mg)	61.8mg
Alpha Tocopheryl Acetate BP	15IU
Sodium selenate Equivalent to Selenium	65mcg

BATCH NO : G17231116

SPECIFICATION LIMIT : 95.0% to 110.0% of the labeled claim

VALIDATION STUDY : QC-Laboratory, Safetab Life science, Puducherry-605107

VALIDATION TEAM : 1. V.VIGNESH

5.0 DETAILS OF STANDARD, SAMPLES AND PLACEBO TO BE USED

Mention the name and Batch No., Potency of the reference/working std., Impurities standard, test samples/placebo to be used during Validation.

NAME OF THE MATERIAL	ID NO / BATCH NO	POTENCY / PURITY
Sample	G17231116	Not Applicable
Plain Placebo	Not Applicable	Not Applicable
Working standard Ascorbic Acid BP	To be mentioned in report	To be mentioned in report

6.0 DETAILS OF SOLVENTS AND CHEMICALS TO BE USED:

UV-Visible Spectrophotometer

Make : Shimadzu, Model : UV-1700

Analytical Balance

Make : Sartorius, Model : BSA224S-CW

pH meter:

Make : Eutech, Model No : PC700

Chemicals with grade:

Ferrous ammonium sulphate hexahydrate (AR grade)

Sulphuric acid (AR grade)

Water (Purified)

Sodium metabisulphite (AR grade)

2-2, Bipridyl dye (AR grade)

Glacial acetic acid (AR grade)

Sodium acetate (AR grade)

Acetic acid (AR grade)

7.0 DESCRIPTION OF ANALYTICAL METHOD

Reference: In-House

Procedure: By UV

Acetate buffer pH 4.7:

To 0.3mL glacial acetic acid, add 0.681gms of sodium acetate and add 90ml of water. Adjust the pH to 4.7 with sodium acetate solution or acetic acid as required.

Sodium metabisulphite solution:

5.0g of Sodium metabisulphite in 100ml of water (5.0% w/v)

2-2, Bipridyl Solution:

300mg of 2-2, Bipridyl in 100ml of water. (0.3% w/v)

Sulphuric acid solution:

Add 10.0ml of sulphuric acid to about 80ml of water, mix well, cool and make up the volume to 100ml with water (10% v/v).

Standard preparation:

Weigh accurately and transfer about 175mg of Ferrous ammonium sulphate hexahydrate AR in to 100ml volumetric flask. Add 20ml of Sulphuric acid solution heat on a water bath for about 30 minutes, cool and dilute to 100ml with water. Dilute 5ml of this solution to 100ml with water. (**Concentration:** 0.0125 mg/ml of Iron)

Sample preparation:

Weigh 20 capsules and calculate the average weight and crush to fine powder. Weigh accurately and transfer about equivalent to 25mg of Iron in to 100ml standard flask. Add 20ml Sulphuric acid solution and heat on water bath for about 30 minutes, cool and dilute to 100ml with water and filter. Dilute 5ml of this solution 100ml with water. (**Concentration:** 0.0125 mg/ml of Iron)

Procedure:

Take three 25ml volumetric volumetric flask and mark as standard, sample and reagent blank and add the solutions as given the table.

SOLUTIONS	STANDARD	SAMPLE	REAGENT BLANK
Standard solution	5.0mL	----	----
Sample solution	----	5.0mL	----
Water	----	----	5.0mL
Acetate buffer pH 4.7	5.0mL	5.0mL	5.0mL
Sodium metabisulphite solution	2.5mL	2.5mL	2.5mL
Allow to stand at room temperature for 15 minutes and add the following solution			
2-2, Bipridyl solution	2.5mL	2.5mL	2.5mL

Shake well, and make up the volume to 25.0mL with water. Measure the absorbance at about 510 nm using reagent blank as blank solution.

Calculate the assay of Carbonyl Iron Equivalent to Elemental Iron in mg/capsule as follows:

$$\frac{TAB}{SAB} \times \frac{WS}{100} \times \frac{5}{100} \times \frac{100}{WT} \times \frac{100}{5} \times \frac{P}{100} \times \frac{55.845}{392.14} \times ANW$$

Where,

TAB = Absorbance of Sample preparation.
SAB = Absorbance of Standard preparation.
WS = Weight of Ferrous ammonium sulphate hexahydrate.
WT = Weight of sample taken in mg.
55.845 = Molecular weight of Elemental Iron
392.14 = Molecular weight of Ferrous Ammonium sulphate hexahydrate.
ANW = Average net weight of the capsule in mg.

Calculate the assay of Elemental Iron in % as follows:

$$= \frac{\text{mg/capsule}}{LC} \times 100$$

LC = Label claim of Carbonyl Iron equivalent to elemental iron in mg/capsule.

8.0 PARAMETERS TO BE VALIDATED:

Following parameters shall be selected for validation.	
S.No.	**VALIDATION PARAMETER**
1	**System suitability**
2	**Specificity (Selectivity)** i) Interference from blank and placebo
3	**Linearity and Range**
4	**Accuracy**
5	**Precision** i) Method precision ii) Intermediate Precision
6	**Stability of analytical solution**
7	**Filter paper study**

Note: More than one parameter may be performed at once with relevant sequence having common system suitability with bracketing preparation.

9.0 DETAILS OF VALIDATION PARAMETERS:

9.1 SYSTEM SUITABILITY:

Purpose:

To establish system suitability as per methodology.

Study Design:

Sequence shall be in following provisional manner.

S.No.	Description of solution	No. of Absorption
1	Blank (Reagent blank)	1
2	Standard preparation	5

Evaluate the following system suitability parameters:

1) % RSD of Absorbance of Carbonyl Iron in five replicate standard.

Acceptance Criteria:

1) % RSD of Absorbance of Carbonyl Iron in five replicate standard should not more than 2.0%.

9.2 SPECIFICITY (SELECTIVITY)

9.2.1 Interference from blank and placebo

"The specificity is the ability of an analytical procedure to measure accurately an analyte in presence of componenets that may be expected present in sample matrix".

Purpose:

To demonstrate that the placebo not interfering with the analyte peak.

Study Design :

Sequence shall be in following provisional manner.

S.No.	Description of solution	No. of Absorption
1	Blank	1
2	Ferrous Ammonium sulphate	1
3	Plain placebo	1
4	Plain placebo with Ferrous Ammonium sulphate	1
5	Plain placebo with Ascorbic acid WS	1
6	Plain placebo with Folic acid WS	1
7	Plain placebo with Cyanocobalamin WS	1
8	Plain placebo with Zinc sulphate WS	1
9	Plain placebo with Vitamin E WS	1
10	Plain placebo with Sodium selenite API	1
11	Sample preparation (G17231116)	1

Acceptance Criteria :

i) There should not be any interference due to blank and Placebo with analyte.

9.3 LINEARITY AND RANGE:

"The linearity of the analytical method is it's ability to elecit test results data directly proportional to the concentration of the analyte in samples within give range".

Purpose:

To Establish the linearity of analyte within the specified range.

Study Design :

To demonstrate the linearity and range of analytical method over the range of 50% to 150% of targeted concentration.

Linearity Stock solution	273.5	10	1	1	1	1094.0 ppm (con. ppm)
	50	50	1	1	1	

Lin level	Exp conc (ppm)	Lin Stock Vol (ml)	Dil to (ml)	Calc conc (ppm)
50%	43.76	2	50	43.76
75%	65.64	3	50	65.64
100%	87.52	4	50	87.52
125%	109.40	5	50	109.40
150%	131.28	6	50	131.28

Sequence shall be in following provisional manner.

S.No.	Description of solution	No. of Absorption
1	Level – 1 (50%)	3
2	Level – 2 (75%)	3
3	Level – 3 (100%)	3
4	Level – 4 (125%)	3
5	Level – 5 (150%)	3

Acceptance criteria:

(i) To conclude the linearity, the squared correlation coefficient should not be less than 0.995

(ii) To conclude the range, % RSD for peak area of linearity level of 50%, 75%, 100% 125% and 150% should be not more than 2.0.

9.4 ACCURACY STUDY (RECOVERY STUDY)

"The accuracy of an analytical method is the closeness of results obtained by that method to the true value. Accuracy may often be expressed as present recovery by the assay of known, add amount of analyte".

Purpose:

To establish the accuracy of the analytical method in the specified range.

Sequence shall be in following provisional manner

S.No.	Description of solution	No. of Absorption
1	Level – 1 Set – 1 (50%)	1
2	Level – 1 Set – 2 (50%)	1
3	Level – 1 Set – 3 (50%)	1
4	Level – 2 Set – 1 (100%)	1
5	Level – 2 Set – 2 (100%)	1
6	Level – 2 Set – 3 (100%)	1
7	Level – 3 Set – 1 (150%)	1
8	Level – 3 Set – 2 (150%)	1
9	Level – 3 Set – 3 (150%)	1

Study design:

To demonstrate the accuracy of the analytical method, prepare recovery samples by spiking known quantities of drug (at level 50%, 100% and 150% of targeted concentration) to placebo. Prepare the recovery samples in triplicate for each level and inject only one injection for each samples.

Acceptance criteria:

The mean % recovery at each level should be 98.0 to 102.0.

9.5 PRECISION

9.5.1 METHOD PRECISION

Purpose:

To establish the repeatability of test results obtained by the analytical method.

Study design:

To demonstrate the method precision, analyze six sample preparations as per the methodology representing a single batch and determine the assay for the same. Evaluate the method precision by computing the percentage and relative standard deviation of the assay results.

S.No.	Description of solution	No. of Absorption
1	Blank	1
2	Sample solution-1	1
3	Sample solution-2	1
4	Sample solution-3	1
5	Sample solution-4	1
6	Sample solution-5	1
7	Sample solution-6	1

Acceptance Criteria :

% RSD for assay of six sample preparations should not be more than 2 .0.

9.5.2 INTERMEDIATE PRECISION

Purpose:

To demonstrate the method precision, analyze six test preparations as per the methodology representing a single batch and determine the assay for the same. Evaluate the intermediate precision by computing the percentage and relative standard deviation of the assay results.

S.No.	Description of solution	No. of Absorption
1	Blank	1
2	Sample solution-1	1
3	Sample solution-2	1
4	Sample solution-3	1
5	Sample solution-4	1
6	Sample solution-5	1
7	Sample solution-6	1

Acceptance criteria:

1) % RSD for assay of six sample preparations should not be more than 2.0.

2) Cumulative % RSD for assay of twelve preparations (i.e. method precision and intermediate precision) should not be more than 2.0.

9.6 STABILITY OF ANALYTICAL SOLUTION:

Study design:

Prepare Standard and sample solution as per the methodology and store at room temperature. Absorbance of this solution at regular intervals by using same diluent. Calculate the % difference of analyte peak area for standard and sample preparations with that of initial. The study may be stopped if 2 consecutive failure of sample solution.

Sequence shall be in following provisional

S.No.	Description of solution	No. of Absorption
1	Blank (Water)	1
2	Standard preparation	1
3	Standard preparation (Initial)	1
4	Sample preparation (Initial)	1
5	Standard preparation (Time interval)	1
6	Sample preparation (Time interval)	1

Acceptance criteria:

The sample and standard solution shall be considered stable for the final period till which the absorbance difference between initial and next periodic interval should not be more than ±2%.

9.7 FILTER PAPER STUDY:

Study design:

The filter paper study of the analytical method shall perform by filtering sample solution through 0.45µ Nylon, PVDF membrane and Whatman filter against that of unfiltered.

Sequence shall be in following provisional manner.

S.No.	Description of solution	No. of Absorption
1	Blank (Water)	1
2	Standard preparation	1
3	Sample preparation –Unfiltered sample (Centrifuged)	1
4	Sample preparation –Filter Set 1 (0.45µ Nylon membrane filter)	3
5	Sample preparation –Filter Set 1 (0.45µ PVDF membrane filter)	3
6	Sample preparation –Filter Set 1 (Whatman filter)	3
7	Standard preparation	1

Acceptance criteria:

The % area difference of filter solution should not differ ±2.0 against that of unfiltered (Centrifuged).

10.0 ABBREVIATION:

mg	:	Milligram
S.No	:	Serial Number
ml	:	Milliliter
%	:	Percentage
API	:	Active pharmaceutical ingredient
B.NO	:	Batch number
RSD	:	Relative standard deviation
BKT	:	Bracketing standard

11.0 REVISION HISTORY:

Specification No.	Effective date	Reason for Review
ST/AMVACP/23/033	22/01/2024	New Protocol prepared.

ANALYTICAL METHOD VALIDATION REPORT FOR THE TEST OF ASSAY OF IRON CONTENT IN CARBONYL IRON, FOLIC ACID, CYANOCOBALAMIN, ASCORBIC ACID, ZINC, ALPHA TOCOPHERYL ACETATE AND SELENIUM CAPSULES

Site Address: SAFETAB LIFE SCIENCE
Plot No.A-67 to 72, PIPDIC Electronic Park,
Thirubuvanai, Puducherry-605 107

1.0 INDEX

2.0 REPORT APPROVAL SHEET

PREPARED BY		
Name	:	K. SARAVANAN
Designation	:	Dy. Manager-QC
Signature	:	
Date	:	06/02/2024
REVIEWED BY		
Name	:	M. VIJAYAKUMAR
Designation	:	GM-QC
Signature	:	
Date	:	07/02/2024
APPROVED BY		
Name	:	S. Mani
Designation	:	AGM-QA
Signature	:	
Date	:	08/02/2024

Effective Date	:	09/02/2024

3.0 OBJECTIVE

To verify the method for the test of assay of Iron Content in Carbonyl Iron, Folic acid, Cyanocobalamin, Ascorbic acid, Zinc, Alpha Tocopheryl Acetate and Selenium Capsules.

4.0 GENERAL INFORMATION

REFERENCE : In-House

TYPE OF VERIFICATION : Verification of Pharmacopoeial method.

TEST TO BE VERIFIED : Assay of Iron Content in Carbonyl Iron, Folic acid, Cyanocobalamin, Ascorbic acid, Zinc, Alpha Tocopheryl Acetate and Selenium Capsules

COMPOSITION : Each hard gelatin capsule contains:

Content	Strength
Carbonyl Iron Equivalent to Elemental iron	100mg
Folic Acid BP	500mcg
Cyanocobalamin (Vitamin B12) (Coated)	15mcg
Ascorbic Acid (coated)	75mg
Zinc Sulphate monohydrate BP (Equivalent to elemental zinc 22.5mg)	61.8mg
Alpha Tocopheryl Acetate BP	15IU
Sodium selenate Equivalent to Selenium	65mcg

BATCH NO : G17231116

SPECIFICATION LIMIT : 95.0% to 110.0% of the labeled claim

VALIDATION STUDY : QC-Laboratory, Generic Healthcare Private Limited, Puducherry-605107

VALIDATION TEAM : 1. V.VIGNESH

5.0 DETAILS OF STANDARD,SAMPLES AND PLACEBO TO BE USED.

Mention the name and Batch No., Potency of the reference/working std., Impurities standard, test samples/placebo to be used during validation.

NAME OF THE MATERIAL	ID NO/BATCH NO	POTENCY/PURITY
Sample	G17231116	Not Applicable
Plain Placebo	Not Applicable	Not Applicable
Working standard Ferrous ammonium sulphate API	B.No: G060C21	Not Applicable

6.0 DETAILS OF SOLVENTS AND CHEMICALS TO BE USED :

UV-Visible Spectrophotometer

Make : Shimadzu, Model : UV-1700

Analytical Balance

Make : Sartorius, Model : BSA224S-CW

pH meter:

Make : Eutech, Model No : PC700

Chemicals with grade:

Ferrous ammonium sulphate hexahydrate (AR grade)

Sulphuric acid (AR grade)

Water (Purified)

Sodium metabisulphite (AR grade)

2-2, Bipridyl dye (AR grade)

Glacial acetic acid (AR grade)

Sodium acetate (AR grade)

Acetic acid (AR grade)

7.0 DESCRIPTION OF ANALYTICAL METHOD

Reference: In-House

Procedure: By UV

Acetate buffer pH 4.7:

To 0.3mL glacial acetic acid, add 0.681gms of sodium acetate and add 90ml of water. Adjust the pH to 4.7 with sodium acetate solution or acetic acid as required.

Sodium metabisulphite solution:

5.0g of Sodium metabisulphite in 100ml of water (5.0% w/v)

2-2, Bipridyl Solution:

300mg of 2-2, Bipridyl in 100ml of water. (0.3% w/v)

Sulphuric acid solution:

Add 10.0ml of sulphuric acid to about 80ml of water, mix well, cool and make up the volume to 100ml with water (10% v/v).

Standard preparation:

Weigh accurately and transfer about 175mg of Ferrous ammonium sulphate hexahydrate AR in to 100ml volumetric flask. Add 20ml of Sulphuric acid solution heat on a water bath for about 30 minutes, cool and dilute to 100ml with water. Dilute 5ml of this solution to 100ml with water. (**Concentration:** 0.0125 mg/ml of Iron)

Sample preparation:

Weigh 20 capsules and calculate the average weight and crush to fine powder. Weigh accurately and transfer about equivalent to 25mg of Iron in to 100ml standard flask. Add 20ml Sulphuric acid solution and heat on water bath for about 30 minutes, cool and dilute to 100ml with water and filter. Dilute 5ml of this solution 100ml with water. (**Concentration:** 0.0125 mg/ml of Iron)

Procedure:

Take three 25ml volumetric volumetric flask and mark as standard, sample and reagent blank and add the solutions as given the table.

SOLUTIONS	STANDARD	SAMPLE	REAGENT BLANK
Standard solution	5.0mL	----	----
Sample solution	----	5.0mL	----
Water	----	----	5.0mL
Acetate buffer pH 4.7	5.0mL	5.0mL	5.0mL
Sodium metabisulphite solution	2.5mL	2.5mL	2.5mL
Allow to stand at room temperature for 15 minutes and add the following solution			
2-2, Bipridyl solution	2.5mL	2.5mL	2.5mL

Shake well, and make up the volume to 25.0mL with water. Measure the absorbance at about 510 nm using reagent blank as blank solution.

Calculate the assay of Carbonyl Iron Equivalent to Elemental Iron in mg/capsule as follows:

$$\frac{TAB}{SAB} \times \frac{WS}{100} \times \frac{5}{100} \times \frac{100}{WT} \times \frac{100}{5} \times \frac{P}{100} \times \frac{55.845}{392.14} \times ANW$$

Where,

TAB = Absorbance of Sample preparation.
SAB = Absorbance of Standard preparation.
WS = Weight of Ferrous ammonium sulphate hexahydrate.
WT = Weight of sample taken in mg.
55.845 = Molecular weight of Elemental Iron
392.14 = Molecular weight of Ferrous Ammonium sulphate hexahydrate.
ANW = Average net weight of the capsule in mg.

Calculate the assay of Elemental Iron in % as follows:

$$= \frac{\text{mg/capsule}}{LC} \times 100$$

LC = Label claim of Carbonyl Iron equivalent to elemental iron in mg/capsule.

8.0 VERIFIED PARAMETERS :

Following parameters shall be selected for validation.

S.No.	VALIDATION PARAMETERS
1	**System suitability**
2	**Specificity (Selectivity)** i) Interference from blank and placebo
3	**Linearity and Range**
4	**Accuracy**
5	**Precision** i) Method precision ii) Intermediate Precision
6	**Stability of analytical solution**
7	**Filter paper study**

Note: More than one parameter may be performed at once with relevant sequence having common system suitability with bracketing preparation.

9.0 VERIFICATION RESULTS :

9.1 SYSTEM SUITABILITY:

Study Design:

Five replicates of standard preparation are absorbed into UV spectrophotometer and following system suitability parameters are evaluated.

1) % RSD of Absorbance of Carbonyl Iron in five replicate standard.

Results are tabulated in Table 1.

Table 1: System suitability

System Suitability Parameter	Limit	Observed Result
% RSD	NMT 2.0	0.150

Result and Conclusion:

The System suitability test results are well within the acceptance criteria and the study concludes the suitability of analytical system for the analysis.

9.2 SPECIFICITY (SELECTIVITY)

9.2.1 Interference from Blank and Placebo

Placebo solutions were prepared by using equivalent weight of placebo present in portion of test preparation as per test method and absorbance as per methodology.

Results are tabulated in Table 2.

Acceptance criteria:

There should not be any interference due to blank, placebo, placebo with analyte.

Table 2: Specificity

Sr.No	Sample ID	Absorption
1	Blank	0
2	Ferrous Ammonium sulphate	0.367
3	Plain placebo	NA
4	Plain placebo with Ferrous Ammonium sulphate	0.366
5	Plain placebo with Ascorbic acid WS	0.000
6	Plain placebo with Folic acid WS	0.001
7	Plain placebo with Cyanocobalamin WS	0.001
8	Plain placebo with Zinc sulphate WS	0.000
9	Plain placebo with Vitamin E WS	0.000
10	Plain placebo with Sodium selenite API	0.000
11	Sample preparation (G17231116)	0.358

Results and Conclusion:

From the Blank and placebo with analyte are not interfere with test preparation.

9.3 LINEARITY AND RANGE:

Study Summary:

Analytical solutions for sample were prepared over the range of 50% to 150% concentration with respect to target concentration (i.e. 50%, 75%, 100%, 125% and 150%). The sample were analyst as per proposed method.

The results are tabulated in Table 3 for Linearity and Table 4 for Range.

Acceptance criteria:

1) The squared correlation coefficient should not be less than 0.995.

2) To conclude the range % RSD for peak areas of linearity levels 50%, 75%, 100%, 125% and 150% should not be more than 2.0.

Table 3: Linearity for Carbonyl Iron

Linearity Levels (%)	Conc. in ppm (X- axis)	Absorbance (Y- axis)
50%	43.814	0.183
75%	65.722	0.278
100%	87.629	0.366
125%	109.536	0.455
150%	131.443	0.554
Slope (Y)		0.0042
CC		0.999
Sqaured R		0.9996
Intercept		0.0007

Fig. : Linearity Graph for Carbonyl Iron

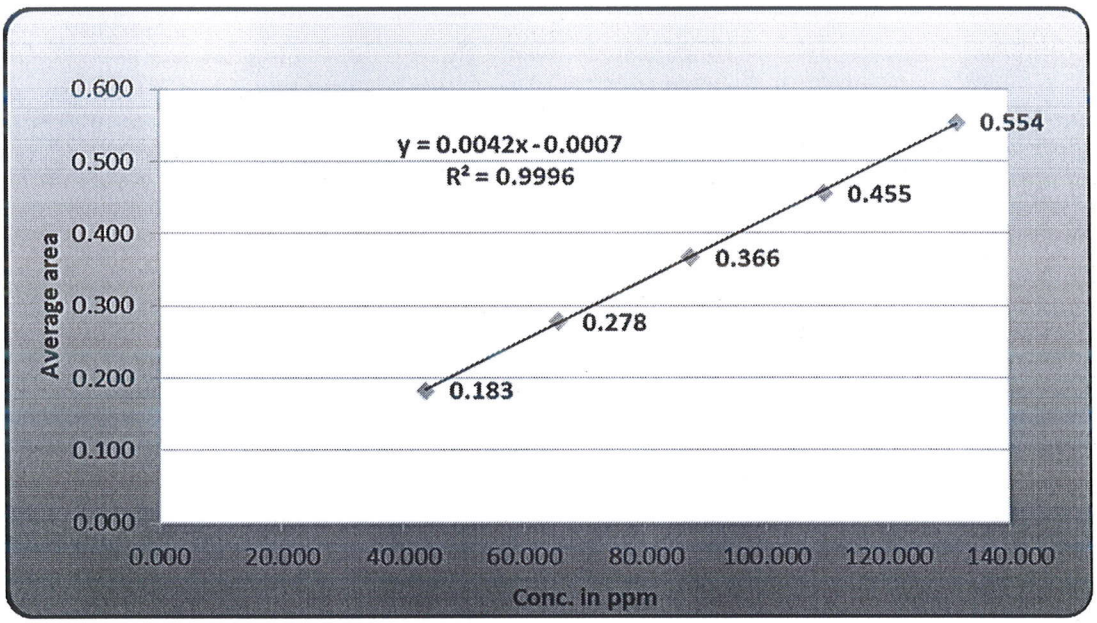

Table: 4 Range for Carbonyl Iron

Linearity Levels (%)	%RSD
50%	0.316
75%	0.750
100%	0.273
125%	0.220
150%	0.276

Result and Conclusion:

Squared correlation coefficient and Range, %RSD of areas at 50%, 75%, 100%, 125 & 150% levels within limits.

9.4 ACCURACY (RECOVERY)

Study Design:

Known quantity of Carbonyl Iron RM were spiked with placebo at three different levels (at level of 50%, 100% and 150% of targeted concentration).

Prepared the recovery samples in triplicate for each level. The samples were analyzed as per the proposed method. The results are tabulated in Table 4 Carbonyl Iron respectively to demonstrate the accuracy of the method.

The mean % recovery at each level for Carbonyl Iron should be 98.0 to 102.0.

Table 5: Accuracy for Carbonyl Iron

Recovery level	Sample No.	% Recovery	Mean %	% RSD
50%	1	100.16	101.07	0.827
	2	101.80		
	3	101.25		
100%	1	101.53	101.07	0.564
	2	101.25		
	3	100.43		
150%	1	100.89	101.07	0.181
	2	101.25		
	3	101.07		

Result and Conclusion:

All the results are well within the acceptance criteria and results indicate that the method is accurate and precise.

9.5 PRECISION:

9.5.1 Method Precision:

Study Design:

Six Assay preparations of sample were analyzed as per the method. The Assay of Carbonyl Iron is calculated. The results are tabulated in Table 6.

Acceptance criteria:

% RSD for Assay of six sample preparations should not be more than 2.0.

Table 6: Method precision

No. of Preparation	% of Carbonyl Iron
1	97.0
2	97.3
3	98.6
4	99.4
5	97.5
6	98.2
Mean	98.0
% RSD	0.93

Results and Conclusion:

The results are well within the acceptance criteria and the % RSD observed for assay values indicates the precision of the analytical method.

9.5.2 Intermediate Precision:

Study summary:

Six Assay preparations of sample were analyzed as per the method by different analyst and on different day. The assay of Carbonyl Iron is calculated. The results are tabulated in Table 7 and cumulative results are tabulated in Table 8.

Acceptance criteria:

1) % RSD for Assay of six sample preparations should not be more than 2.0.

2) Cumulative % RSD for Assay of twelve sample preparations (of method and intermediate precision) should not be more than 2.0.

Table 7: Intermediate precision

No. of Preparation	% of Ascorbic acid
1	94.7
2	95.7
3	96.5
4	97.1
5	96.2
6	96.7
Mean	96.2
% RSD	0.89

The Cumulative results of Method Precision and Intermediate Precision are tabulated in Table 8.

Table 8: Cumulative % RSD

Parameter	% of Carbonyl Iron
Method Precision	97.0
	97.3
	98.6
	99.4
	97.5
	98.2
Intermediate Precision	94.7
	95.7
	96.5
	97.1
	96.2
	96.7
Mean	97.1
% RSD	1.32

Result and Conclusion:

The results are well within the acceptance criteria and the % RSD observed for drug release indicates the precision of the method.

9.6 STABILITY OF ANALYTICAL SOLUTION:

Study design:

Sample solution:

Sample preparation were prepared as per the proposed method and absorbance into the system initially and at various time intervals and data tabulated in Table 9.

Table 9: Stability of sample solution for Carbonyl Iron

Time in hours	Absorbance of Sample solution	Absolute % Difference
Initial	0.361	Not applicable
2	0.359	0.56
4	0.358	0.84
6	0.355	1.69
8	0.358	0.84
10	0.358	0.84
12	0.353	2.27
14	0.353	2.27
Mean	0.357	1.33
% RSD	0.813	Not applicable

Acceptance criteria:

The sample solution shall be considered stable for the final period till which the absorbance difference between initial and next periodic interval should be not more than ±2%.

Standard solution:

Standard preparation were prepared as per the proposed method and absorbance into the system initially and at various time intervals and data tabulated in Table 10.

Table 10: Stability of standard solution for Carbonyl Iron

Time in hours	Absorbance of Standard solution	Absolute % Difference
Initial	0.368	Not applicable
2	0.363	1.38
4	0.364	1.10
6	0.363	1.38
8	0.363	1.38
10	0.362	1.66
12	0.360	2.22
14	0.358	2.79
Mean	0.363	1.70
% RSD	0.807	Not applicable

Results and conclusions:

The Standard and Sample solution was stable upto 10hours at room temperature.

9.7 FILTER PAPER STUDY:

Study design:

The filter paper study of analytical method was performed by filtering sample solution through 0.45µ Nylon, 0.45µ PVDF membrane and Whatman filter against that of unfiltered sample. The results were tabulated in Table 11.

Table 11: Filter paper study for Sample solution of Carbonyl Iron

Filter study	Assay in %	% difference from unfiltered sample
Unfiltered sample (Centrifuged)	97.4	Not applicable
Filter Set-1 (0.45µ Nylon membrane)	97.7	-0.28
Filter Set-2 (0.45µ Nylon membrane)	97.9	-0.55
Filter Set-3 (0.45µ Nylon membrane)	96.8	0.56
Filter Set-1 (0.45µ PVDF membrane)	97.7	-0.28
Filter Set-2 (0.45µ PVDF membrane)	97.7	-0.28
Filter Set-3 (0.45µ PVDF membrane)	98.2	-0.83
Filter Set-1 (Whatman filter)	103.3	-5.77
Filter Set-2 (Whatman filter)	103.3	-5.77
Filter Set-3 (Whatman filter)	104.4	-6.75

Acceptance criteria:

The % difference on filter solution should not differ ±2.0 against that of unfiltered.

Results and conclusions:

The % difference on filtered sample (0.45µ Nylon and PVDF) membrane and Whatman filter within limit against that of unfiltered.

10.0 SUMMARY:

S.No	Validation parameter	Acceptance criteria	Results
1	System suitability	1) % RSD of absorbance of analyte in Five replicate standard absorbance should not be more than 2.0.	0.150
2	Specificity Interference from blank, placebo and placebo spiked with analyte.	There should not be any interference due to blank and placebo with analyte.	Blank and placebo spiked with analyte are not interfere with Carbonyl Iron in test preparation.
3	Linearity and Range	1) R^2 Should be NLT 0.995 2) To conclude the range, %RSD for peak area of linearity level-50%, 75%, 100%, 125% and 150% should be not more than 2.0.	**Squared correlation coefficient for Carbonyl Iron:** 0.9996 **Level** / **%RSD** 50% / 0.316 75% / 0.750 100% / 0.273 125% / 0.220 150% / 0.276

S.No	Validation parameter	Acceptance criteria	Results
4	Accuracy (Recovery)	The mean % recovery at each level should be 98.0 to 102.0.	**Level %Recovery** **Carbonyl Iron:** 50% : 101.07 100% : 101.07 150% : 101.07
5	**Precision** 1) Method Precision	%RSD of Assay of six sample preparations should not be more than 2.0.	0.93
	2) Intermediate Precision	1) % RSD for assay of sample six preparations should not be more than 2.0.	0.89
		2) Cumulative %RSD for assay of twelve preparations (of method and intermediate precision) should not be more than 2.0.	1.32

S.No	Validation parameter	Acceptance criteria	Results
6	Stability for analytical solution	The sample and standard solution shall be considered stable for the final period till which the absorbance difference between initial and next periodic interval should not be more than ±2%.	The Standard and Sample solution was stable upto 10hours at room temperature.
7	Filter paper study (0.45µ Nylon, PVDF and Whatman filter)	The % difference on filter solution should not differ ±2.0 against that of unfiltered.	The % difference on filtered sample (0.45µ Nylon and PVDF) membrane and Whatman filter within limit against that of unfiltered.

11.0 CONCLUSION:

Verification studies have been conducted for assay of Iron Content in Carbonyl Iron, Folic acid, Cyanocobalamin, Ascorbic acid, Zinc, Alpha Tocopheryl Acetate and Selenium Capsules for the parameters of specificity, method precision, Intermediate precision, Linearity and range and accuracy by using the proposed method. The data is complied and found satisfactory with the analytical method for all the parameters analysed. Hence it is concluded that the method can be used for regular analysis.

12.0 ABBREVIATION:

mg	:	Milligram
No	:	Number
ml	:	Milliliter
%	:	Percentage
ID	:	Identification
B.NO	:	Batch number
WS.NO	:	Working standard number
RSD	:	Relative standard deviation

13.0 REVISION HISTORY:

Report No.	Effective date	Reason for Review
ST/AMVACR/23/033	09/02/2024	New Report prepared.

ANALYTICAL METHOD VALIDATION REPORT FOR THE TEST OF ASSAY OF ALPHA TOCOPHERYL ACETATE IN CARBONYL IRON, FOLIC ACID, CYANOCOBALAMIN, ASCORBIC ACID, ZINC, ALPHA TOCOPHERYL ACETATE AND SELENIUM CAPSULES

Site Address: SAFETAB LIFE SCIENCE
Plot No.A-67 to 72, PIPDIC Electronic Park,
Thirubuvanai, Puducherry-605 107

1.0 INDEX

2.0 REPORT APPROVAL SHEET

PREPARED BY		
Name	:	S. SANTHI
Designation	:	ASST. MANAGER- QC
Signature	:	
Date	:	25/01/24
REVIEWED BY		
Name	:	M. VIJAYAKUMAR
Designation	:	GM-QC
Signature	:	
Date	:	27/01/2024
APPROVED BY		
Name	:	J. Maran
Designation	:	AGM-QA
Signature	:	
Date	:	29/01/2024

Effective Date	:	30/01/2024

3.0 OBJECTIVE

To validate the method for the test of assay of Alpha Tocopheryl Acetate in Carbonyl Iron, Folic acid, Cyanocobalamin, Ascorbic acid, Zinc, Alpha Tocopheryl Acetate and Selenium Capsules by HPLC.

4.0 GENERAL INFORMATION

REFERENCE : In-House

TYPE OF VALIDATION : Validation of non-pharmacopoeial method

TEST VALIDATION : Assay of Alpha Tocopheryl Acetate in Carbonyl Iron, Folic acid, Cyanocobalamin, Ascorbic acid, Zinc, Alpha Tocopheryl Acetate and Selenium Capsules

COMPOSITION : Each hard gelatin capsule contains:

Content	Strength
Carbonyl Iron Equivalent to Elemental iron	100mg
Folic Acid BP	500mcg
Cyanocobalamin (Vitamin B12) (Coated)	15mcg
Ascorbic Acid (coated)	75mg
Zinc Sulphate monohydrate BP (Equivalent to elemental zinc 22.5mg)	61.8mg
Alpha Tocopheryl Acetate BP	15IU
Sodium selenate Equivalent to Selenium	65mcg

BATCH NO	:	G17231116
SPECIFICATION LIMIT	:	Not less than 95.0% of the labeled claim
VALIDATION STUDY	:	QC-Laboratory, Safetab Life Science, Puducherry-605107
VALIDATION TEAM	:	1. C.K.Saravanan 2. S.Priyadarsini 3. S.Gomathi

5.0 DETAILS OF STANDARD,SAMPLES AND PLACEBO TO BE USED.

Mention the name and Batch No., Potency of the reference/working std., test samples / placebo to be used during Validation.

NAME OF THE MATERIAL	ID NO/BATCH NO	POTENCY/PURITY
Sample	B.No: G17231116	Not applicable
Plain Placebo	B.No: Not applicable	Not applicable
Working standard Alpha Tocopheryl Acetate BP	WS. No: WS/TC/006/23	50.27% (As is basis)

6.0 DETAILS OF INSTRUMENTS/EQUIPMENTS, COLUMN, SOLVENTS AND CHEMICALS TO BE USED:

INSTRUMENTS/EQUIPMENTS:

High performance liquid chromatograph with PDA detector

Make : Shimadzu, Model : LC-2050C 3D Prominence i

High performance liquid chromatograph with UV visible detector

Make : Shimadzu, Model : LC-2050C

Analytical Balance:

Make : Sartorius, Model : Quintix-125D-1OIN

pH:

Make: Eutech instruments, Model No: PH 700

COLUMN:

Water Xterra, 150mmx 4.6mm, 5µ or equivalent

SOLVENTS AND CHEMICALS WITH GRADE:

Vitamin E 50% powder (Alpha tocopheryl acetate) (Working standard)

Isopropyl alcohol (AR grade)

Purified Water (Milli-Q water (or) equivalent)

Methanol (HPLC grade)

Acetonitrile (HPLC grade)

Hydrochloric acid (AR grade)

Sodium Hydroxide (AR grade)

Hydrogen Peroxide 30% (AR grade)

7.0 DESCRIPTION OF ANALYTICAL METHOD

Chromatographic Conditions:

Column	:	Water Xterra, 150mmx 4.6mm, 5µ or equivalent
Flow rate	:	1.0ml/min
Wavelength	:	268 nm
Injection Volume	:	20 µL
Temperature	:	30°C
Retention time	:	About 15 minutes

Diluent preparation:

A mixture of 25 volume of methanol, 50 volume of Isopropyl alcohol and 25 volume of Acetonitrile.

Mobile phase preparation:

A mixture of 98 volume of Methanol and 2 volume of water and mix well. Filter through 0.45µ nylon membrane filter and degas.

Standard preparation:

Weigh accurately and transfer about 40.0mg of Vitamin E 50% powder working standard into a 100mL volumetric flask. Add 70mL of diluent, sonicate to dissolve and dilute to volume with diluent. Filter through 0.45µ nylon membrane filter and degas. (**Concentration:** 0.2IU/ml of Alpha tocopheryl acetate).

Test preparation:

Weigh 20 capsules and calculate the average net weight and crush to fine powder. Weigh accurately and transfer powdered of sample equivalent to about 20IU of Vitamin E into a 100mL volumetric flask. Add 60mL of diluent to disperse the sample, sonicate for 20 minutes with intermittent shaking (Maintain the bath temperature below 25°C) and dilute to volume with diluent. Filter through 0.45µ nylon membrane filter. (**Concentration:** 0.2IU/ml of Alpha tocopheryl acetate).

Procedure:

Inject the blank (Diluent) in single injection, standard preparation as five replicate injection in to the chromatogram and measure the response of the peak of Alpha tocopheryl acetate in term of standard area and test in duplicate. The test is not valid unless it meets the system suitability parameters. Calculate the system suitability with respect to the following parameters.

System suitability:

Theoretical plate	: NLT 2000 for Alpha tocopheryl acetate peak.
Tailing factor	: NMT 2.0 for Alpha tocopheryl acetate peak.
Relative standard deviation	: NMT 2.0% for five replicate injections of Alpha tocopheryl acetate peak.

Inject 20µl of the above solution as per following sequence.

Injection sequence:

S. No	Sample Name	No. of injections
1	Blank (Diluent)	1
2	Standard preparation	5
3	Test preparation	2
4	Standard Preparation (Bracketing standard)	1 Each after every 6 sample injection

Calculation:

Calculate the assay of Vitamin E acetate in IU/capsule as follows:

$$= \frac{AT}{AS} \times \frac{WS}{100} \times \frac{100}{WT} \times \frac{P}{100} \times ANW$$

Where,

AT = Average area of Alpha tocopheryl acetate peak in Test preparation

AS = Average area of Alpha tocopheryl acetate peak in standard preparation.

WS = Weight of Vitamin E 50% powder working standard taken in mg

WT = Weight of sample taken in mg.

P = Potency of Vitamin E 50% powder Working standard.

ANW = Average net weight of capsule in mg.

Calculate the assay of Vitamin E acetate in IU as follows:

$$= \frac{\text{IU/capsule}}{\text{LC}} \times 100$$

LC = Label claim of Vitamin E acetate in IU.

8.0 VALIDATED PARAMETERS:

Following parameters shall be selected for validation	
S.No.	**VALIDATION PARAMETERS**
1	**System suitability**
2	**Specificity (Selectivity)** Interference from blank and placebo
3	**Linearity and Range**
4	**Interference from Degradants (Forced Degradation)**
	i) Acid degradation ii) Alkali Degradation iii) Oxidative Degradation
5	**Accuracy (Recovery)**
6	**Precision** i) System Precision ii) Method precision iii) Intermediate Precision
7	**Stability of analytical solution**
8	**Filter paper study**
9	**Robustness** i) Flow rate change ii) Wavelength change iii) Oven temperature change

Note: More than one parameter may be performed at once with relevant sequence having common system suitability with bracketing preparation.

9.0 VALIDATION RESULTS:

9.1 SYSTEM SUITABILITY:

Study Design:

Five replicates of standard preparation are injected into HPLC and following system suitability parameters are evaluated.

1) Theoretical plate for Alpha Tocopheryl Acetate peaks.

2) Tailing Factor for Alpha Tocopheryl Acetate peaks.

3) % RSD of area of five replicate standard injections of Alpha Tocopheryl Acetate peaks.

Results are tabulated in Table 1.

Table 1: System suitability

System Suitability Parameter	Limit	Observed Result
Theoretical Plates	NLT 2000	3018
Tailing Factor	NMT 2.0	0.970
% RSD	NMT 2.0	0.064

Result and Conclusion:

The System suitability test results are well within the acceptance criteria and the study concludes the suitability of analytical system for the analysis.

9.2 SPECIFICITY (SELECTIVITY)

9.2.1 Interference from blank and placebo

Study Design:

Blank, standard, placebo and placebo spiked with analyte and sample are analyzed as per the method to examine the interference of blank and placebo with Alpha Tocopheryl Acetate peaks.

Peak purity of the analyte peak and the representative chromatograms of blank, standard, placebo, placebo spiked with analyte and sample are attached.

Results are tabulated in Table 2.

Acceptance criteria:

1) There should not be any interference due to blank, placebo peak with analyte.

2) Peak purity index is not less than 0.995 accordingly to lab solution software.

Table 2: Specificity

Sr.No	Sample ID	Peak Name	Retention time	Peak Purity index
1	Blank	Blank peak	Nil	Not applicable
2	Standard preparation	Vitamin-E	4.29	1.000
3	Alpha Tocopheryl Acetate Working standard	Vitamin-E	4.28	1.000
4	Plain placebo	Placebo peaks	Nil	Not applicable
5	Working standard for Ascorbic acid, Sodium selenate, Cyanocobalamin, Folic acid, carbonyl iron, Zinc sulphate monohydrate	Blank peak	Nil	Not applicable
6	Placebo + Working standard for Ascorbic acid, Sodium selenate, Cyanocobalamin, Folic acid, carbonyl iron, Zinc sulphate monohydrate	Placebo peaks	Nil	Not applicable
7	Plain Placebo + Alpha Tocopheryl Acetate Working standard	Vitamin-E	4.28	1.000
8	Sample preparation (Richfer)	Vitamin-E	4.33	1.000

Results and Conclusion:

From the Blank and Placebo peaks are not interfere with Alpha Tocopheryl Acetate peak in sample preparation and Peak purity passes within specified limits. Hence method is selective and specific.

9.3 LINEARITY AND RANGE:

Study Summary:

Analytical solutions for Alpha Tocopheryl Acetate Working standard are prepared over the range of 50% to 150% concentration with respect to target concentration (i.e. 50%, 75%, 100%, 125% and 150%). Replicate injections of these solutions are injected and checked for Linearity and Range.

The results are tabulated in Table 3 for Linearity and Table 4 for Range.

Acceptance criteria:

1) The squared correlation coefficient r^2 should not be less than 0.995.

2) To conclude the range % RSD for peak areas of linearity levels 50%, 75%, 100%, 125% & 150% should not be more than 2.0.

Table 3: Linearity Table for Alpha Tocopheryl Acetate

Linearity Levels (%)	Conc. in ppm (X- axis)	Avg. Area (Y- axis)
50%	100.842	260277
75%	151.262	390825
100%	201.683	519201
125%	252.104	656657
150%	302.525	776295
Slope		2574.1
CC		0.9997
Sqaured R		0.9997
Intercept		1503.7

Fig. : Liner Graph for Alpha Tocopheryl Acetate

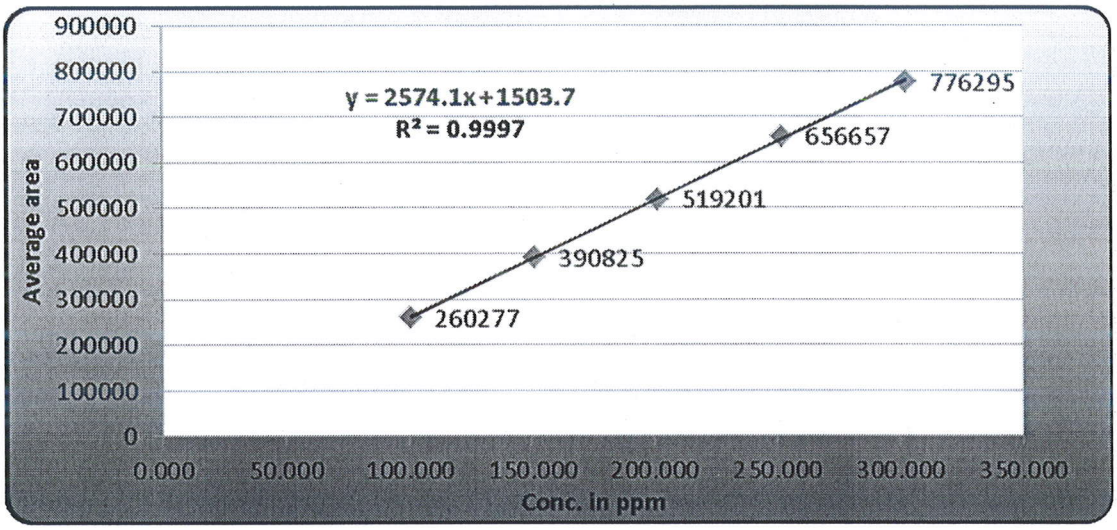

Table:4 Range for Alpha Tocopheryl Acetate

Linearity Levels (%)	% RSD for Alpha Tocopheryl Acetate
50%	0.011
75%	0.040
100%	0.086
125%	0.003
150%	0.007

Result and Conclusion:

Squared correlation coefficient and Range, %RSD of areas at 50%, 75%, 100%, 125 & 150% levels within limits.

9.4 INTERFERENCE FROM DEGRADANTS (Forced degradation)

In order to prove specificity of method, further degradation is carried out and peak purity of Alpha Tocopheryl Acetate peak is monitored.

9.4.1 Acid Degradation:

Weigh 20 capsules and calculate the average net weight and crush to fine powder. Weigh accurately and transfer powdered of sample equivalent to about 20IU of Vitamin E into a 100mL volumetric flask. Add 60mL of diluent to disperse the sample, sonicate for 20 minutes with intermittent shaking (Maintain the bath temperature below 25°C). Add 5ml of 5N Hydrochloric acid and heat on water bath at 80°C for 30minutes Cool and neutralized with 5ml of 5N Sodium hydroxide and Dilute to volume with diluent and mix. Filter the solution through 0.45µ Nylon membrane filter.

9.4.2 Alkali degradation:

Weigh 20 capsules and calculate the average net weight and crush to fine powder. Weigh accurately and transfer powdered of sample equivalent to about 20IU of Vitamin E into a 100mL volumetric flask. Add 60mL of diluent to disperse the sample, sonicate for 20 minutes with intermittent shaking (Maintain the bath temperature below 25°C). Add 5ml of 5N Sodium hydroxide and heat on water bath at 80°C for 30minutes Cool and neutralized with 5ml of 5N Hydrochloric acid and Dilute to volume with diluent and mix. Filter the solution through 0.45µ Nylon membrane filter.

9.4.3 Oxidative Degradation:

Weigh 20 capsules and calculate the average net weight and crush to fine powder. Weigh accurately and transfer powdered of sample equivalent to about 20IU of Vitamin E into a 100mL volumetric flask. Add 60mL of diluent to disperse the sample, sonicate for 20 minutes with intermittent shaking (Maintain the bath temperature below 25°C). Add 5ml of 30% Hydrogen peroxide solution and heat on water bath at 80°C for 30minutes Cool and dilute to volume with diluent and mix. Filter the solution through 0.45µ Nylon membrane filter.

Acceptance criteria:

i) There should not be any interference due to degradants with analyte in stressed sample.

ii) The desired degradation should be 10-30% in acid, alkali and oxidation degration, (if possible).

iii) If about 10% to 30% degradation is not achieved by applying above stressed condition. Same shall be documented and reported.

iv) Peak purity should not be less than 0.950 according to Lab solution software.

Table 5: Peak purity (Chemical degradation)

S.No	Sample name	Peak name	Assay in (%)	Degradation in %	Peak purity index
1	Sample as such	Alpha Tocopheryl Acetate (Method Precision)	108.3	Not Applicable	0.999
2	Acid degradation	Alpha Tocopheryl Acetate	107.2	1.10	0.996
3	Alkali degradation	Alpha Tocopheryl Acetate	89.6	18.70	0.993
4	Oxidative Degradation	Alpha Tocopheryl Acetate	110.2	-1.90	0.997

Result and Conclusion:

There is No any interference due to degradants with analyte in stressed samples and Peak purity was passes According to Lab solution.

9.5 ACCURACY (RECOVERY)

Study Design:

Known quantity of Alpha Tocopheryl Acetate working standard is spiked with placebo at three different levels (at level of 50%, 100% and 150% of targeted concentration).

Prepared the recovery samples in triplicate for each level and inject only one injection for each level. The samples are analyzed as per the proposed method. The results are tabulated in Table 5 for Alpha Tocopheryl acetate respectively to demonstrate the accuracy of the method.

The mean % recovery at each level for Alpha Tocopheryl Acetate should be 98.0 to 102.0.

Table 5: Accuracy for Alpha Tocopheryl Acetate

Recovery level	Sample No.	% Recovery	Mean	% RSD
50%	1	99.53	99.34	0.245
	2	99.42		
	3	99.07		
100%	1	101.68	101.63	0.111
	2	101.72		
	3	101.51		
150%	1	101.49	101.64	0.148
	2	101.79		
	3	101.64		

Result and Conclusion:

All the results are well within the acceptance criteria and results indicate that the method is accurate and precise.

9.6 PRECISION:

9.6.1 SYSTEM PRECISION

Study design:

Five replicate injections of standard preparation are injected into the HPLC system. The area response for Alpha tocopheryl acetate Peaks along with % RSD are tabulated in Table 6.

Acceptance criteria:

% RSD of area of analyte peak in five replicate standard injections should not be more than 2.0.

Table 6: System precision

Injection No.	Alpha tocopheryl acetate Area
1	497594
2	497846
3	497877
4	498177
5	498412
Mean	497981
% RSD	0.064

Results and Conclusion:

The results are well within the acceptance criteria and the % RSD observed for the replicate injections indicates the system precision of HPLC system used.

9.6.2 Method Precision:

Study Design:

Six Assay preparations of sample are analyzed as per the method. The Assay of Alpha tocopheryl acetate is calculated. The results are tabulated in Table 7.

Acceptance criteria:

% RSD for Assay of six sample preparations should not be more than 2.0.

Table 7: Method precision for Alpha tocopheryl acetate

No. of Preparation	Alpha tocopheryl acetate Assay in %
1	107.6
2	107.3
3	108.4
4	107.7
5	108.2
6	107.8
Mean	107.8
% RSD	0.37

Results and Conclusion:

The results are well within the acceptance criteria and the % RSD observed for assay values indicates the precision of the analytical method.

9.6.3 Intermediate Precision:

Study summary:

Six Assay preparations of sample are analyzed as per the method by different analyst using different instrument and different column on different day. The assay of Alpha tocopheryl acetate is calculated. The results are tabulated in Table 8 and cumulative results are tabulated in Table 9.

Acceptance criteria:

1) % RSD for Assay of six sample preparations should not be more than 2.0.

2) Cumulative % RSD for Assay of twelve sample preparations (from method and intermediate precision) should not be more than 2.0.

Table 8: Intermediate precision for Alpha tocopheryl acetate

No. of Preparation	Alpha tocopheryl acetate Assay in %
1	106.0
2	107.5
3	106.7
4	106.8
5	105.8
6	107.7
Mean	106.8
% RSD	0.72

The Cumulative results of Method Precision and Intermediate Precision are tabulated in Table 9

Table 9: Cumulative % RSD for Alpha tocopheryl acetate

Parameter	Alpha tocopheryl acetate Assay in %
Method Precision	107.6
	107.3
	108.4
	107.7
	108.2
	107.8
Intermediate Precision	106.0
	107.5
	106.7
	106.8
	105.8
	107.7
Mean	107.29
% RSD	0.75

Result and Conclusion:

The results are well within the acceptance criteria and the % RSD observed for drug release indicates the precision of the method.

9.7 STABILITY OF ANALYTICAL SOLUTION:

Study design:

Sample solution:

Sample preparation is prepared as per the proposed method and injected into the system initially and at various time intervals and data tabulated in Table 10.

Table 10: Stability of sample solution for Alpha tocopheryl acetate

Time in hours	Area of Sample solution	Absolute % Difference
Initial	550558	Not applicable
5	551490	-0.17
8	550628	-0.01
17	549189	0.25
21	548599	0.36
24	547809	0.50
27	547491	0.56
Mean	549395	0.25
% RSD	0.279	Not applicable

Acceptance criteria:

The sample solution shall be considered stable for the final period till which the area difference between initial and next periodic interval should be not more than ±2%.

Standard solution:

Standard preparation is prepared as per the proposed method and injected into the system initially and at various time intervals and data tabulated in Table 11.

Table 11: Stability of standard solution for Alpha tocopheryl acetate

Time in hours	Area of Standard solution	Absolute % Difference
Initial	521141	Not applicable
5	521715	-0.11
8	522284	-0.22
17	526557	-1.03
21	531339	-1.92
24	532231	-2.08
27	533953	-2.40
Mean	527031	-1.29
% RSD	1.037	Not applicable

Results and conclusions:

The Standard solution and Sample solution was stable upto 21 hours at room temperature.

9.8 FILTER PAPER STUDY:

Study design:

The filter paper study of analytical method was performed by filtering sample solution through 0.45µ Nylon and 0.45µ PVDF membrane filter against that of unfiltered centrifuged sample. The results are tabulated in Table 12.

Table 12: Filter paper study for Sample solution of Alpha tocopheryl acetate

Filter study	Area of sample solution	Assay in %	% difference from unfiltered sample
Unfiltered sample (Centrifuged)	552498	107.8	Not applicable
Filter Set-1 (0.45µ Nylon membrane)	552357	107.7	0.03
Filter Set-1 (0.45µ PVDF membrane)	552985	107.9	-0.09

Acceptance criteria:

The % difference on filter solution should not differ ±2.0 against that of unfiltered (centrifuged).

Results and conclusions:

The % difference on filtered sample (0.45µ Nylon and PVDF) membrane within limit against that of unfiltered (Centrifuged). Hence both filters are suitable for filtration.

9.9 ROBUSTNESS:

Study Design:

Five replicate injections of standard preparation and duplicate injections of sample preparation are injected varying different chromatographic conditions as per protocol. System suitability parameters and mean assay difference with respect to assay value in method precision are calculated. The results are tabulated in table 13 for Alpha tocopheryl acetate peak respectively.

Table 13: Robustness of analytical method for Alpha tocopheryl acetate

Parameter	Theoretical Plates (NLT 2000)	Tailing Factor (NMT 2.0)	% RSD (NMT 2.0)	Assay % (Method precision)	Alpha tocopheryl acetate Assay	Absolute % Difference
Low wavelength (265nm)	2933	0.85	0.038	107.8	106.2	1.65
High wavelength (271nm)	2937	0.85	0.051		106.3	1.50
Low flow rate (0.9ml/minute)	3103	0.85	0.087		107.4	0.40
High flow rate (1.1ml/minute)	2788	0.86	0.056		107.0	0.80
Low Temperature (25^{0}C)	2972	0.83	0.035		106.7	1.10
High Temperature (35^{0}C)	2890	0.87	0.028		106.6	1.20

Acceptance criteria:

1) Theoretical plates for Alpha tocopheryl acetate peaks should be NLT 2000
2) Tailing Factor for Alpha tocopheryl acetate peaks should be NMT 2.0.
3) % RSD of area of analyte in replicate standard injections should be NMT 2.0.
4) % Assay of analyte should not differ by ±2.0 to that of method precision.

Result and Conclusion:

Each chromatographic variation System suitability parameters are within limits. % Difference of assay within limits at each variation.

10.0 SUMMARY:

S.No	Validation parameter	Acceptance criteria	Results
1	System suitability	1) % RSD of area of analyte in Five replicate standard injections should not be more than 2.0.	0.064
		2) Theoretical plate should be not less than 2000.	3018
		3) Tailing factor should not be more than 3.0.	0.970
2	Specificity Interference from blank, placebo and placebo spiked with analyte.	1) There should not be any interference due to blank and placebo with analyte. 2) Peak purity of analyte should pass	Blank and Placebo peaks are not interference with Alpha tocopheryl acetate peak in sample preparation and Peak purity passes within specified limits.
3	Linearity and Range	1) R^2 Should be NLT 0.995	Squared correlation coefficient 0.9997

<table>
<tr><th>S.No</th><th>Validation parameter</th><th>Acceptance criteria</th><th>Results</th></tr>
<tr><td></td><td></td><td>2) To conclude the range, %RSD for peak area of linearity level-50%, 75%, 100%, 125% and 150% should be not more than 2.0.</td><td><table><tr><th>Level</th><th>%RSD</th></tr><tr><td>50%</td><td>0.011</td></tr><tr><td>75%</td><td>0.040</td></tr><tr><td>100%</td><td>0.086</td></tr><tr><td>125%</td><td>0.003</td></tr><tr><td>150%</td><td>0.007</td></tr></table></td></tr>
<tr><td>4</td><td>Interference from degradants (Forced degradation)</td><td>1) There should not be any interference due to degradants with analyte and impurity in stressed samples.

2) The desired degradation should be 10-30% in acid, alkali and oxidation degradation, (if possible).

3) If about 10% to 30% degradation is not achieved by applying above stressed condition, same shall be documented and reported.</td><td>There is no any interference due to degradants with analyst in stressed sample and peak purity was passes according to lab solution software.</td></tr>
</table>

S.No	Validation parameter	Acceptance criteria	Results
		4) Peak purity of analyte peak each impurity peak should be pass (Peak purity should not be less than 0.950 according to Lab solution software.	
5	Accuracy (Recovery)	The mean % recovery at each level should be 98.0 to 102.0.	Level %Recovery 50% : 99.34 100% : 101.63 150% : 101.64
6	**Precision** 1) System Precision	%RSD of area of analyte peaks in five replicate standard injections should not be more than 2.0.	0.064
	2) Method Precision	%RSD of Assay of six sample preparations should not be more than 2.0.	0.37
	3)Intermediate Precision	1) % RSD for assay of six sample preparations should not be more than 2.0.	0.72

S.No	Validation parameter	Acceptance criteria	Results
		2) Cumulative %RSD for assay of twelve preparations (of method and intermediate precision) should not be more than 2.0.	0.75
7	Stability for analytical solution	The sample and standard solution shall be considered stable for the final period till which the area difference between initial and next periodic interval should not be more than ±2%.	The Standard and Sample solution was stable upto 21hours at room temperature.
8	Filter paper study (0.45µ Nylon and PVDF membrane filter)	The % difference on filter solution should not differ ±2.0 against that of unfiltered (Centrifuged).	The % difference on filtered sample 0.45µ Nylon and PVDF within limit against that of unfiltered (Centrifuged).
9	Robustness (i) Flow rate change (ii) Wavelength change (iii) Column oven Temperature Change	System suitability parameters should comply.	Each chromatographic variation System suitability parameters are within limits. % Difference of assay within limits at each variation.

MASTER COPY

11.0 CONCLUSION:

Validation studies have been conducted for Assay of Alpha Tocopheryl Acetate in Carbonyl Iron, Folic acid, Cyanocobalamin, Ascorbic acid, Zinc, Alpha Tocopheryl Acetate and Selenium Capsules for the parameters of system suitability, Specificity, Degradation, System Precision, Method precision, Intermediate precision, Linearity and range, Accuracy, Filter paper study, Solution stability and Robustness by using the proposed method. The data is complied and found satisfactory with the analytical method for all the parameters analysed. Hence it is concluded that the method can be used for regular analysis.

12.0 ABBREVIATION:

mg	:	Milligram
No	:	Number
ml	:	Milliliter
%	:	Percentage
ID	:	Identification
API	:	Active pharmaceutical ingredient
HPLC	:	High performance liquid chromatography
B.NO	:	Batch number
WS.NO	:	Working standard number
mm	:	Millimeter
µm	:	Micrometer
min	:	Minutes
°C	:	Degree centigrade
nm	:	Nanometer
RSD	:	Relative standard deviation
µl	:	Micro litre
Hcl	:	Hydrochloric acid
NaOH	:	Sodium Hydroxide
H_2O_2	:	Hydrogen peroxide

13.0 REVISION HISTORY:

Report No.	Effective date	Reason for Review
ST/AMVAER/23/033	30/01/2024	New Report prepared.

ANALYTICAL METHOD VALIDATION PROTOCOL FOR THE TEST OF ASSAY OF ALPHA TOCOPHERYL ACETATE IN CARBONYL IRON, FOLIC ACID, CYANOCOBALAMIN, ASCORBIC ACID, ZINC, ALPHA TOCOPHERYL ACETATE AND SELENIUM CAPSULES

Site Address: SAFETAB LIFE SCIENCE
Plot No.A-67 to 72, PIPDIC Electronic Park,
Thirubuvanai, Puducherry-605 107

1.0 INDEX

2.0 PROTOCOL APPROVAL SHEET

PREPARED BY		
Name	:	S. SANTHI
Designation	:	ASST. MANAGER - QC
Signature	:	
Date	:	10/01/24
REVIEWED BY		
Name	:	M. VIJAYAKUMAR
Designation	:	GM - QC
Signature	:	
Date	:	11/01/2024
APPROVED BY		
Name	:	S. Yaran
Designation	:	AGM - QA
Signature	:	
Date	:	12/01/2024

Effective Date	:	13/01/2024

3.0 OBJECTIVE

To validate the method for the test of assay of Alpha Tocopheryl Acetate in Carbonyl Iron, Folic acid, Cyanocobalamin, Ascorbic acid, Zinc, Alpha Tocopheryl Acetate and Selenium Capsules by HPLC.

4.0 GENERAL INFORMATION:

REFERENCE : In-House

TYPE OF VALIDATION : Validation of non-pharmacopoeial method

TEST TO BE VALIDATED : Assay of Alpha Tocopheryl Acetate in Carbonyl Iron, Folic acid, Cyanocobalamin, Ascorbic acid, Zinc, Alpha Tocopheryl Acetate and Selenium Capsules

COMPOSITION : Each hard gelatin capsule contains:

Content	Strength
Carbonyl Iron Equivalent to Elemental iron	100mg
Folic Acid BP	500mcg
Cyanocobalamin (Vitamin B12) (Coated)	15mcg
Ascorbic Acid (coated)	75mg
Zinc Sulphate monohydrate BP (Equivalent to elemental zinc 22.5mg)	61.8mg
Alpha Tocopheryl Acetate BP	15IU
Sodium selenate Equivalent to Selenium	65mcg

BATCH NO	:	G17231116
SPECIFICATION LIMIT	:	Not less than 95.0% of the labeled claim
VALIDATION STUDY	:	QC-Laboratory, Safetab Life Science, Puducherry-605107
VALIDATION TEAM	:	1. C.K.Saravanan 2. S.Priyadarsini 3. S.Gomathi

5.0 DETAILS OF STANDARD, SAMPLES AND PLACEBO TO BE USED:

Mention the name and Batch No., Potency of the reference/working std, test samples/ placebo to be used during Validation.

NAME OF THE MATERIAL	ID NO/BATCH NO	POTENCY/PURITY
Sample	G17231116	Not Applicable
Plain Placebo	Not Applicable	Not Applicable
Working standard Alpha Tocopheryl Acetate BP	To be mentioned in report	To be mentioned in report

6.0 DETAILS OF INSTRUMENTS/EQUIPMENTS, COLUMN, SOLVENTS AND CHEMICALS TO BE USED:

INSTRUMENTS/EQUIPMENTS :

High performance liquid chromatograph with PDA detector

Make : Shimadzu, Model : LC-2050C 3D Prominence i

High performance liquid chromatograph with UV detector

Make : Shimadzu, Model : LC-2050C Prominence i

Analytical Balance:

Make : Sartorius, Model : Quintix-125D-1OIN

pH:

Make: Eutech instruments, Model No: pH 700

COLUMN:

Water Xterra, 150mmx 4.6mm, 5µ or equivalent

SOLVENTS AND CHEMICALS WITH GRADE :

Vitamin E 50% powder (Alpha tocopheryl acetate) (Working standard)

Isopropyl alcohol (AR grade)

Purified Water (Milli-Q water (or) equivalent)

Methanol (HPLC grade)

Acetonitrile (HPLC grade)

Hydrochloric acid (AR grade)

Sodium Hydroxide (AR grade)

Hydrogen Peroxide 30% (AR grade)

7.0 DESCRIPTION OF ANALYTICAL METHOD

Chromatographic Conditions:

Column	:	Water Xterra, 150mmx 4.6mm, 5µ or equivalent
Flow rate	:	1.0ml/min
Wavelength	:	268 nm
Injection Volume	:	20 µL
Temperature	:	30°C
Retention time	:	About 15 minutes

Diluent preparation:

A mixture of 25 volume of methanol, 50 volume of Isopropyl alcohol and 25 volume of Acetonitrile.

Mobile phase preparation:

A mixture of 98 volume of Methanol and 2 volume of water and mix well. Filter through 0.45µ nylon membrane filter and degas.

Standard preparation:

Weigh accurately and transfer about 40.0mg of Vitamin E 50% powder working standard into a 100mL volumetric flask. Add 70mL of diluent, sonicate to dissolve and dilute to volume with diluent. Filter through 0.45µ nylon membrane filter and degas. (**Concentration:** 0.2IU/ml of Alpha tocopheryl acetate).

Test preparation:

Weigh 20 capsules and calculate the average net weight and crush to fine powder. Weigh accurately and transfer powdered of sample equivalent to about 20IU of Vitamin E into a 100mL volumetric flask. Add 60mL of diluent to disperse the sample, sonicate for 20 minutes with intermittent shaking (Maintain the bath temperature below 25°C) and dilute to volume with diluent. Filter through 0.45µ nylon membrane filter. (**Concentration:** 0.2IU/ml of Alpha tocopheryl acetate).

Procedure:

Inject the blank (Diluent) in single injection, standard preparation as five replicate injection in to the chromatogram and measure the response of the peak of Alpha tocopheryl acetate in term of standard area and test in duplicate. The test is not valid unless it meets the system suitability parameters. Calculate the system suitability with respect to the following parameters.

System suitability:

Theoretical plate	: NLT 2000 for Alpha tocopheryl acetate peak.
Tailing factor	: NMT 2.0 for Alpha tocopheryl acetate peak.
Relative standard deviation	: NMT 2.0% for five replicate injections of Alpha tocopheryl acetate peak.

Inject 20µl of the above solution as per following sequence.

Injection sequence:

S. No	Sample Name	No. of injections
1	Blank (Diluent)	1
2	Standard preparation	5
3	Test preparation	2
4	Standard Preparation (Bracketing standard)	1 Each after every 6 sample injection

Calculation:

Calculate the assay of Vitamin E acetate in IU/capsule as follows:

$$= \frac{AT}{AS} \times \frac{WS}{100} \times \frac{100}{WT} \times \frac{P}{100} \times ANW$$

Where,

AT = Average area of Alpha tocopheryl acetate peak in Test preparation

AS = Average area of Alpha tocopheryl acetate peak in standard preparation.

WS = Weight of Vitamin E 50% powder working standard taken in mg

WT = Weight of sample taken in mg.

P = Potency of Vitamin E 50% powder Working standard.

ANW = Average net weight of capsule in mg.

Calculate the assay of Vitamin E acetate in IU as follows:

$$= \frac{\text{IU/capsule}}{\text{LC}} \times 100$$

LC = Label claim of Vitamin E acetate in IU.

8.0 PARAMETERS TO BE VALIDATED:

Following parameters shall be selected for Validation	
S.No.	**VALIDATION PARAMETERS**
1	**System suitability**
2	**Specificity (Selectivity)** i) Interference from blank and placebo
3	**Linearity and Range**
4	**Interference from Degradants (Forced Degradation)**
	i) Acid degradation ii) Alkali Degradation iii) Oxidative Degradation
5	**Accuracy (Recovery)**
6	**Precision** i) System precision ii) Method precision iii) Intermediate Precision
7	**Stability of analytical solution**
8	**Filter paper study**
9	**Robustness** i) Wavelength change ii) Flow rate change iii) Oven temperature change

Note: More than one parameter may be performed at once with relevant sequence having common system suitability with bracketing preparation.

9.0 DETAILS OF VALIDATION PARAMETERS:

9.1 SYSTEM SUITABILITY:

Purpose:

To establish system suitability as per methodology.

Study Design:

Sequence shall be in following provisional manner.

S.No.	Description of solution	No. of Injections
1	Blank (Diluent)	1
2	Standard preparation	5

Evaluate the following system suitability parameters:

1) % RSD of area of Alpha tocopheryl acetate peak in five replicate standard injections.

2) Theoretical plates for Alpha tocopheryl acetate peak in standard injection.

3) Tailing factor for Alpha tocopheryl acetate peak in standard injection.

Acceptance Criteria:

1) % RSD of area of Alpha tocopheryl acetate peak in five replicate standard injections should not more than 2.0%.

2) Theoretical plates for Alpha tocopheryl acetate peak in standard injection should not less than 2000.

3) Tailing factor for Alpha tocopheryl acetate peak in standard injection should not more than 2.0.

9.2 SPECIFICITY (SELECTIVITY)

9.2.1 Interference from blank and placebo

"The specificity is the ability of an analytical procedure to measure accurately an analyte in presence of componenets that may be expected present in sample matrix".

Purpose: To demonstrate that the placebo not interfering with the analyte peak.

Study Design:

Sequence shall be in following provisional manner.

S.No.	Description of solution	No. of injections
1	Blank (Diluent)	1
2	Standard preparation	5
3	Blank (Diluent)	1
4	Alpha tocopheryl acetate Working standard	1
5	Plain placebo for Alpha tocopheryl acetate	1
6	Working standard for Ascorbic acid,Sodium selenate, Cyanocobalamin, Folic acid, carbonyl iron, Zinc sulphate monohydrate	1
7	Placebo + Working standard for Ascorbic acid, Sodium selenate,Cyanocobalamin, Folic acid, carbonyl iron, Zinc sulphate monohydrate	1
8	Plain placebo + Alpha tocopheryl acetate Working standard	1
9	Sample preparation for Alpha tocopheryl acetate	1

Acceptance Criteria:

i) There should not be any interference due to blank, Placebo peak with analyte.

ii) Peak purity index not less than 0.995 according to Lab solution software.

9.3 LINEARITY AND RANGE:

"The linearity of the analytical method is it's ability to elecit test results data directly proportional to the concentration of the analyte in samples within given range".

Purpose:

To Establish the linearity of analyte within the specified range.

Study Design :

To demonstrate the linearity and range of analytical method over the range of 50% to 150% of targeted concentration.

Linearity stock solution, linearity level, expected concentration, linearity stock dilution and calculated concentration are tabulated below.

Linearity Stock solution	100.0	1	1	1	1	1000 ppm (con. ppm)
	100	1	1	1	1	

Lin level	Exp conc (ppm)	Lin Stock Vol (ml)	Dil to (ml)	Calc conc (ppm)
50%	100	2	10	100
75%	150	3	10	150
100%	200	4	10	200
125%	250	5	10	250
150%	300	6	10	300

Sequence shall be in following provisional manner.

S.No.	Description of solution	No. of Injections
1	Blank (Diluent)	1
2	Level – 1 (50%)	3
3	Level – 2 (75%)	3
4	Level – 3 (100%)	3
5	Level – 4 (125%)	3
6	Level – 5 (150%)	3

Acceptance criteria:

(i) To conclude the linearity, the squared correlation coefficient r^2 should not be less than 0.995.

(ii) To conclude the range, % RSD for peak area of linearity level of 50%, 75%, 100%, 125% and 150% should be not more than 2.0.

9.4 INTERFERENCE FROM DEGRADANT (forced degradation)

Study design:

To evaluate the interference from degradants, carry out a forced degradation study by stressing the test preparation under the following maximum stress conditions.

Degradation	Stress Condition
Acid degradation	Exposure to 5ml of 5N HCL and heat on water bath at 80°C for 30minutes
Alkali degradation	Exposure to 5ml of 5N NaOH and heat on water bath at 80°C for 30minutes
Oxidative degradation	Exposure to 5ml of 30% H2O2 and heat on water bath at 80°C for 30minutes

Sequence shall be in following provisional manner, For forced chemical degradation:

S.No.	Description of solution	No. of Injections
1	Blank (Mobile phase)	1
2	Standard preparation	5
3	Sample preparation (As such)	2
4	Sample preparation (Acid degradation)	2
5	Sample preparation (Alkali degradation)	2
6	Sample preparation (Oxidative degradation)	2
7	Standard preparation (Bracketing)	1

Chromatograph the samples of chemical and physical forced degradation into HPLC system equipped with diode array detector and evaluate the peak purity for the analytes in stressed samples and the degradation profiles under each stressed condition.

Acceptance Criteria:

1) There should not be any interference due to degradants with analyte in stressed samples.

2) The desired degradation should be 10-30% in acid, alkali and oxidative degradation, (if possible).

3) If about 10% to 30% degradation is not achieved by applying above stressed condition, same shall be documented and reported.

4) Peak purity should not be less than 0.950 according to Lab solution software.

9.5 ACCURACY (RECOVERY):

"The accuracy of an analytical method is the closeness of results obtained by that method to the true value. Accuracy may often be expressed as present recovery by the assay of known, add amount of analyte".

Purpose:

To establish the accuracy of the analytical method in the specified range.

Sequence shall be in following provisional manner

S.No.	Description of solution	No. of Injections
1	Blank (Diluent)	1
2	Standard preparation	5
3	Blank (Diluent)	1
4	Level – 1 Set – 1 (50%)	1
5	Level – 1 Set – 2 (50%)	1
6	Level – 1 Set – 3 (50%)	1
7	Blank (Diluent)	1
8	Level – 2 Set – 1 (100%)	1
9	Level – 2 Set – 2 (100%)	1
10	Level – 2 Set – 3 (100%)	1
11	Blank (Diluent)	1
12	Level – 3 Set – 1 (150%)	1
13	Level – 3 Set – 2 (150%)	1
14	Level – 3 Set – 3 (150%)	1
15	Standard preparation (BKT)	1

Study design:

To demonstrate the accuracy of the analytical method, prepare recovery samples by spiking known quantities of drug (at level 50%, 100% and 150% of targeted concentration) to placebo. Prepare the recovery samples in triplicate for each level and inject only one injection for each samples.

Acceptance criteria:

The mean % recovery at each level should be 98.0 to 102.0.

9.6 PRECISION

9.6.1 SYSTEM PRECISION

Purpose:

To establish the precision of the HPLC system being used for the analysis.

Study Design :

Sequence shall be in following provisional manner.

S.No.	Description of solution	No. of Injections
1	Blank (Diluent)	1
2	Standard preparation	5

Acceptance Criteria:

% RSD of area of analyte peak in five replicate standard injections should not be more than 2.0.

9.6.2 METHOD PRECISION

Purpose:

To establish the repeatability of test results obtained by the analytical method.

Study design:

To demonstrate the method precision, analyze six sample preparations as per the methodology representing a single batch and determine the assay for the same. Evaluate the method precision by computing the percentage and relative standard deviation of the assay results.

S.No.	Description of solution	No. of Injections
1	Blank (Diluent)	1
2	Standard preparation	5
3	Sample preparation-1	2
4	Sample preparation-2	2
5	Sample preparation-3	2
6	Standard preparation (BKT)	1
7	Sample preparation-4	2
8	Sample preparation-5	2
9	Sample preparation-6	2
10	Standard preparation (BKT)	1

Acceptance Criteria:

% RSD for assay of six sample preparations should not be more than 2.0.

9.6.3 INTERMEDIATE PRECISION

Purpose:

To demonstrate the reproducibility of test results obtained by the analytical method for the variability of instrument, column (different lot no) analyst and day. Analyse six sample preparations as per the methodology representing a single batch and determine the assay for the same. Evaluate the intermediate precision by computing the percentage and relative standard deviation of the assay results.

S.No.	Description of solution	No. of Injections
1	Blank (Mobile phase)	1
2	Standard preparation	5
3	Sample preparation-1	2
4	Sample preparation-2	2
5	Sample preparation-3	2
6	Standard preparation (BKT)	1
7	Sample preparation-4	2
8	Sample preparation-5	2
9	Sample preparation-6	2
10	Standard preparation (BKT)	1

Acceptance criteria:

1) % RSD for assay of six sample preparations should not be more than 2.0.

2) Cumulative % RSD for assay of twelve preparations (i.e. method precision and intermediate precision) should not be more than 2.0.

9.7 STABILITY OF ANALYTICAL SOLUTION:

Study design:

Prepare Standard and sample solution as per the methodology and store at room temperature. Chromatograph this solution at regular intervals by using same diluent. Calculate the % difference of analyte peak area for standard and sample preparations with that of initial. The study may be stopped if 2 consecutive failure of sample solution.

Sequence shall be in following provisional

S.No.	Description of solution	No. of Injections
1	Blank (Diluent)	1
2	Standard preparation	5
3	Standard preparation (Initial)	1
4	Sample preparation (Initial)	1
5	Standard preparation (Time interval)	1
6	Sample preparation (Time interval)	1

Acceptance criteria:

The sample and standard solution shall be considered stable for the final period till which the area difference between initial and next periodic interval should not be more than ±2%.

9.8 FILTER PAPER STUDY:

Study design:

The filter paper study of the analytical method shall perform by filtering sample solution through 0.45µ Nylon and PVDF membrane filter against that of unfiltered (centrifuged) sample.

Sequence shall be in following provisional manner.

S.No.	Description of solution	No. of Injections
1	Blank (Diluent)	1
2	Standard preparation	5
3	Sample preparation –Unfiltered sample (Centrifuged)	1
4	Sample preparation –Filter Set 1 (0.45µ Nylon membrane filter)	1
5	Sample preparation –Filter Set 1 (0.45µ PVDF membrane filter)	1
6	Standard preparation (BKT)	1

Acceptance criteria:

The % area difference of filter solution should not differ ±2.0 against that of unfiltered (Centrifuged) sample.

9.9 ROBUSTNESS:

Purpose: To establish the robustness of the analytical method.

Study Design:

The robustness of the analytical method can be established by demonstrating its reliability against deliberate changes in chromatographic conditions.

Sequence shall be in following provisional manner.

As such		
S.No.	**Description of solution**	**No. of Injections**
1	Blank (Diluent)	1
2	Standard preparation	5
3	Sample preparation	2
4	Standard Preparation (Bracketing standard)	1
According to each variable		
S.No.	**Description of solution**	**No. of Injections**
1	Blank (Diluent)	1
2	Standard preparation	5
3	Sample preparation	2
4	Standard Preparation (Bracketing standard)	1

Following variable shall be done according to deliberate changes in chromatographic parameters.

a) Flow rate change by ±10% mean (i.e 0.9 ml/min and 1.1 ml/minute)

b) Wave length change by ± 3nm (i.e. 265nm and 271nm)

c) Column oven Temperature change by ± 5.0°C (i.e. 25°C and 35°C)

Acceptance criteria:

System suitability should comply for each variable and % of drug not differ ±2% from mean assay value of method precision.

10.0 ABBREVIATION:

mg	:	Milligram
S.No	:	Serial Number
ml	:	Milliliter
%	:	Percentage
ID	:	Identification
API	:	Active pharmaceutical ingredient
HPLC	:	High performance liquid chromatography
B.NO	:	Batch number
mm	:	Millimeter
µm	:	Micrometer
min	:	Minutes
°C	:	Degree centigrade
nm	:	Nanometer
RSD	:	Relative standard deviation
µl	:	Micro litre
HCL	:	Hydrochloric acid
NaOH	:	Sodium Hydroxide
H_2O_2	:	Hydrogen Peroxide
WS	:	Working standard

11.0 REVISION HISTORY:

Protocol No.	Effective date	Reason for Review
ST/AMVAEP/23/033	13/01/2024	New Protocol prepared.