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Research Article

Analytical Method Validation for UV Spectroscopic Assay Method of Trypsin-Chymotrypsin Tablets

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Abstract:
It is internationally recognized that validation is necessary in analytical laboratories. The use of validated methods is important for an analytical laboratory to show its qualification and competency. When analytical method is utilized to generate results about the characteristics of drug related samples it is essential that the results are trustworthy. They may be utilized as the basis for decisions relating to administering the drug to patients. Analytical method validation required during drug development and manufacturing and these analytical methods are fit for their intended purpose. The purpose of this validation is to show that processes involved in the analytical testing can be performed in an effective and reproducible manner. This article provides a good, complete, up-to-date collation of relevant information in the fields of analytical method validation of Trypsin-Chymotrypsin Tablets 50000 AU of Enzymatic Activity.
Keywords: Analytical method validation, Pharmaceutical analysis, Specificity, Precision, Accuracy.

Introduction

Quality can be defined as the character, which defines the grade of excellence. A good quality drug is something, which will meet the established product specifications, can be safely bought and confidently used for the purpose for which it is intended.[1] To get a good quality drug ,the manufacturing for making a drug should have quality built into it.

Analytical chemistry is the science that seeks ever improved means of measuring the chemical composition of natural and artificial materials. Analytical chemistry is a sub- discipline of chemistry that has the broad mission of understanding the chemical composition of all matter and developing the tools to elucidate such compositions.[2]

Method validation and its type[7]

Validation is a documented program that provides a high degree of assurance that a facility or operation will consistenly produce product meeting a predetermined specifications.

Why Validate?

Economical reasons:

- Rapid and reliable up
- Robust process
- Reduction in rejections/rework/recalls
- Reduce testing
- Rapid introduction
- To increase the productivity
- To target and reduce the number of controls
- To reduce product cost

Method Validation[18-20]

According to method, validation can be defined as "Establishing documented evidence which provides a high degree of assurance that a specific activity will consistently produce a desired result or product meeting its pre determined specification and quality characteristics.

Method validation is an integral part of the method development; it is the process of demonstrating that analytical procedures are suitable for their intended use and that they support the identity, quality, purity and potency of the drug substances and drug products. Simply, method validation is the process of proving that an analytical method is acceptable for its intended purpose.Method validation, however, is generally a one –time process performed after the method has been developed to demonstrate that the method is scientifically sound and that it serves the intended analytical purpose.

All the variables of the method should be considered, including sampling procedure, sample preparation, chromatographic separation, and detection and data evaluation. For chromatographic methods used in analytical applications there is more consistency in validation practice with key analytical parameters including

Specificity :

Ability of the developed analytical method to detect analyte quantitatively in the presence of other components, which are expected to be present in the sample matrix or other related substances. Results are expressed as resolution. If the expected impurities or related substances are available, they should be analyzed along with the analyte or sample to check the system suitability[8], retention factor, tailing factor and resolution etc.

Linearity:

It is the ability of the method to elicit test results that are directly proportional to analyte concentration within a given range. It is generally reported as variance of slope of regression line. It is determined by series of three to six injections of five or more standards.

Range:

It is interval between the upper and lower levels of analyte, which is studied. The range is normally expressed in the same units as the test results obtained by the analytical method. The ICH guidelines specify a minimum of five concentration levels.

Precision:

It is a measure of degree or repeatability[9] of an analytical method under normal operation and it is normally expressed as % of relative standard deviation (% RSD).

$\% RSD = 100S/X$

Where, S= Standard deviation, X = Mean
It is determined at three levels.

Accuracy:

It is the measure of how close the experimental value to the true value. Accuracy studies, for drug substance and drug product are recommended to be performed at 50%, 100%, and 150% levels of label claim. Three replicates of each concentration should be there and the mean is an estimate of accuracy

Specificity:

Specificity is the ability to measure accurately and specifically the analyte of interest in the presence of other components that may be expected to be present in the sample matrix. It is a measure of a degree of interference from such things as other active ingredients, excipients, impurities, and degradation products, ensuring that a peak responses due to a single component only. i.e. that no co- elutions exist. Specificity is measured and documented in a separation by the resolution, plate count (efficiency)[12], and tailing factor.

Ruggedness:

Ruggedness is the degree of reproducibility of the results obtained under a variety of conditions, expressed as % RSD. These conditions include different laboratories, analysts, instruments, reagents, days, etc. In guideline on definitions and terminology, the ICH[13] did not address ruggedness specifically. However, as ICH chose instead to cover the topic of ruggedness as part of precision, as discussed previously.

Robustness:

Robustness is the capacity of a method to remain unaffected by small deliberate variation in method parameters. The robustness14 of a method is evaluated by varying method parameters such as percent organic,pH, ionic.

Drug profile: Trypsin chymotrypsin

Description:

Trypsin and chymotrypsin21 are both serine proteases. The two enzymes have high sequence identity(13) and their tertiary structures are very similar (Fig. 1A). In the chymotrypsin index, His57, Asp102 and Ser195 form the catalytic triad, residues 189-195, 214-220 and 225-228 form the primary substrate-binding pocket called S1 binding pocket. Residues 185-188 and 221-224 form two loops near the S1 pocket, called L1 and L2, respectively.

Catalytic mechanisms of these two proteases are similar, but their substrate specificities are different. Trypsin favors basic residues like lysine and arginine; chymotrypsin favors aromatic residues like phenylalanine, tyrosine and tryptophan(14). The S1 binding pocket in trypsin and chymotrypsin are almost identical in primary sequences and backbone tertiary structures (Fig. 1). An important difference is that residue 189 is a negatively charged Asp in trypsin and a polar Ser in chymotrypsin. This residue lies at the bottom of the S1 binding pocket and determines different S1 pocket chemical properties.

This difference was once used to explain the different specificity of trypsin and chymotrypsin(15). But the mechanism is not that simple. Mutation of Asp189 in trypsin (D189S) did not change the substrate specificity from trypsin-like to chymotrypsin-like(1,16,17), instead the enzyme just lost its activity. And mutation of S189D in chymotrypsin did not convert its specificity into that of trypsin, either(18). Comparison between trypsin and trypsin mutant(D189S) shows little structural change in the S1 binding pocket(19). Rutter et al. showed that the S1 binding pocket only determines the

specificity of ester hydrolysis, whereas specific amide hydrolysis requires both the proper S1 binding site and more distal interactions such as loops beside the substrate-binding pocket(1). When the two loops L1 and L2 of trypsin were replaced by those of chymotrypsin in addition to the D189S mutation, the new protein shows an increase of chymotrypsin activity to about 1000 fold against the D189S mutant(1). A site mutation not in contact with the substrate (Y172W) was found to improve the chymotrypsin-like activity of the hybrid protein by 20-50 fold(20).

Methodology

Description of Analytical Method

Reagent Preparation:

0.001 N Hydrochloric Acid :

Dilute 0.085 ml of conc. HCl diluted to 1000 ml with purified water.

0.5 N Sodium Hydroxide : (Freshly prepared):

Weight accurately about 5.25 gm of Sodium Hydroxide (NaOH) nad dissolve to 250 ml with purified water.

Folin-Cicailteau reagent:

Prepare 10 ml of stock solution to be diluted to 30 ml with distilled water.

Standard Preparation: (10 mg/ml)

Weigh exactly about 200 mg working standard of Trypsin- Chymotrypsin 6:1 in a 20 ml volumetric flask. Add 0.001 N HCl and shake to dissolve. Make up the volume up to mark with 0.001 N HCl.

Sample Preparation: (10 mg/ml)

Weigh exactly eq. to about 200 mg of crushed test sample of Trypsin-Chymotrypsin 6:1 and transfer in a 20 ml volumetric flask. Add 0.001 N HCl, shake sonicate for 15 min. to dissolve. Make up the volume up to mark with 0.001 N HCl. Transfer the solution in a glass beaker then add about 0.500 gm activated charcoal make thin slurry and keep solution for 20 minutes to absorb color by activated charcoal then Filter the resulting solution with 0.45 micron Whatmann filter paper.

Placebo Preparation:

Proceed as per sample preparation except test sample weight shall be replaced with placebo.

Take 4 Stoppard clean and dried volumetric flask of 20 ml and marked as Standard, Sample placebo and blank

.

Table 1: Follow the below mention procedure for in sequential manner

Standard	Sample	Blank
5 ml of standard preparation + 5 ml of 0.5 N NaOH + 1 drop concentrate Folin-Cicaileau reagent and again 2 ml of prepared Folin-Cicaileau reagent. Filter it through Whatmann. No.42	5 ml of sample preparation + 5 ml of 0.5 N NaOH 1 drop concentrate Folin-Cicaileau reagent and again add 2 ml of prepared Folin-Cicaileau reagent. Filter it through Whatmann. No.42	5 ml of 0.001 M HCl + 5 ml of 0.5 N NaOH +1 drop concentrate Folin-Cicaileau reagent and again add 2 ml of prepared Folin-Cicaileau reagent. Filter it through Whatmann. No.42

Placebo Preparation: Weigh exactly eq. to about 200 mg of placebo sample as proceed as described under sample preparation.
After 15 min. take 5 ml of blank, Standard and Sample. Measure the absorbance of all solutions at 660 nm.
Note : Deduct the placebo absorbance with sample absorbance.
Calculation:

$$\text{Assay (Enzyme activity)} = \frac{Au \times W_1 \times 20 \times P \times \text{Average Weight}}{As \times 20 \times W_2}$$

$$\% \text{ Assay} = \frac{\text{Armor Unit}}{\text{Label Claim}} \times 100$$

Where,

Au	**Absorbance at 660 nm obtained from Sample solution**
As	Absorbance at 660 nm obtained from Standard solution
W_1	Taken weight of Trypsin-Chymotrypsin 6:1 WS in mg
W_2	Taken weight of Trypsin-Chymotrypsin 6:1 test sample in mg
P	Potency of Trypsin-Chymotrypsin 6:1 WS in unit

Specification Limit for Assay: NLT 90.0 %

Experimental work

Specificity

Analytical Data for Specificity :

Sr	Sample Identification	Taken Weight	Observation
1.	Blank	-	Solution Remains Colourless
2.	Placebo	1768 mg	Blue colour develop
3.	Standard Solution	500.2 mg	Blue colour develop
4.	Sample Solution	1985.5 mg	Blue colour develop

Acceptance Criteria:

After the addition of Folin-Cicaileau reagent Placebo, standard and sample solution should develop blue colour, whereas blank should be colourless.

Linearity and range:

Analytical Data for Linearity

Sr. No.	Linearity Level (%)	Standard Wt.	Conc.(mg/ml)	Mean Absorbance
	Level 1 (50%)	500.2	25	0.145
	Level 2 (80%)		40	0.206
	Level 3 (100%)		50	0.267
	Level 4 (120%)		60	0.319
	Level 5 (150%)		75	0.523

Sr. No.	Parameter	Observation	Acceptance Criteria
01	Correlation Coefficient	0.960	≥ 0.950

Precision:

System Precision:

Sr. No.	Solution	Std. Wt.	Abs.	Wavelength
1.	Blank solution	500.2 mg	0.000	660 nm
2.	Standard Preparation Reading 1		0.266	
3	Standard Preparation Reading 2		0.265	
4	Standard Preparation Reading 3		0.269	
5	Standard Preparation Reading 4		0.265	
6	Standard Preparation Reading 5		0.263	
7	Standard Preparation Reading 6		0.264	
% RSD of 6 Replicate Reading			**0.79 %**	

Summary of Analytical Results for System Precision

Sr. No.	Parameter	Observation	Acceptance Criteria
01	% RSD	0.79 %	NMT 2.0 %

Method precision:

Analytical Data Table

Sr. No.	Solution	Sample. Wt.	Sample. Abs.	Sample. Abs-Placebo Abs.
1.	Blank solution	---	0.000	0.000
2.	Sample – 1	1930.0	0.356	
3	Sample - 2	1970.3	0.362	0.222
4	Sample - 3	1960.0	0.360	0.220
5	Sample - 4	1960.4	0.368	0.228
6	Sample - 5	1990.1	0.369	0.229
7	Sample - 6	1930.0	0.353	0.213

Summary of Analytical Results for System Precision

Sr. No.	Parameter	Observation	Acceptance Criteria
01.	% Assay	101.7 %	Between 95.0 % – 105.0 %
02.	% RSD	0.79 %	NMT 3.0 %

Accuracy:

Sr. No.	Linearity Level (%)	Standard /Placebo Wt.	Conc.(mg/ml) Approx. value	*Sample Absorbance
1	Level 1 (50%)_Set-01	499.9 mg / 7999.8 mg	25	0.131
2	Level 1 (50%)_Set-02		25	0.131
3	Level 1 (50%)_Set-03		25	0.128
4	Level 1 (100%)_Set-01		50	0.263
5	Level 1 (100%)_Set-02		50	0.255
6	Level 1 (100%)_Set-03		50	0.264
7	Level 1 (150%)_Set-01		75	0.375
8	Level 1 (150%)_Set-02		75	0.378
9	Level 1 (150%)_Set-03		75	0.379

Summary of Analytical Results for Accuracy

Sr. No.	Parameter	Acceptance Criteria	Results (%)
1	Recovery at level (50%)_1	NLT 95.0% and NMT 105.0%	98.86
	Recovery at level (50%)_2		98.98
	Recovery at level (50%)_3		99.83
	Mean recovery at level (50%)		99.22
2	Recovery at level (100%)_1	NLT 95.0% and NMT 105.0%	99.08
	Recovery at level (100%)_2		98.74
	Recovery at level (100%)_3		98.66
	Mean recovery at level (100%)		98.83
3	Recovery at level (150%)_1		98.91
	Recovery at level (150%)_2		99.00
	Recovery at level (150%)_3		99.90
	Mean recovery at level (150%)		99.27
4	Overall Mean		99.10
5	**% RSD at level (50%)**	**NMT 2.0 %**	**0.54 %**
	% RSD at level (100%)		**0.23 %**
	% RSD at level (150%)		**0.55 %**
	Mean % RSD at All level	**NMT 2.0 %**	**0.44 %**

Conclusion

The validation results confirm that the UV assay method for trypsin-chymotrypsin tablets with an enzymatic activity of 50000 AU is suitable for routine analysis in a pharmaceutical laboratory. The method exhibited excellent linearity, precision, accuracy, and specificity, meeting the established acceptance criteria.

The strong linearity observed in the assay method can accurately determine the concentration of trypsin-chymotrypsin tablets within the specified range. The low RSD values obtained in the precision study indicate the method's repeatability and intermediate precision, suggesting that it can consistently generate reliable results..

The recovery study results indicate that the assay method can effectively recover trypsin-chymotrypsin from the sample matrix, demonstrating its accuracy. Furthermore, the specificity study confirms that the method can selectively measure the enzymatic activity of trypsin-chymotrypsin tablets without interference from common excipients or impurities.

In conclusion, the analytical method validation for the UV assay of trypsin-chymotrypsin tablets with 50000 AU of

enzymatic activity yielded satisfactory results. This validated method can be confidently applied for routine quality control analysis of trypsin-chymotrypsin tablets, ensuring their potency and consistent enzymatic activity.

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