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A Validated Stability Indicating UHPLC Method for the Determination of Anti-inflammatory Corticosteroid Budesonide Epimers

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Abstract:

Background: Change in any one of the quality attributes of drug product is considered as a potential instability, and assessment of this change becomes mandatory as it is directly related to the safety and efficacy of the drug. Hence, stability testing is done.

Introduction: The aim of the proposed method was to develop simple, sensitive and economic stability indicating ultra high performance liquid chromatographic (UHPLC) method for the quantification of Budesonide in the presence of degradation products.

Method: Budesonide and its degradants were separated on Agilent Poroshell 120 EC-C18 (50mm x 4.6mm, 2.7µm) high efficiency column by using ACN: phosphate buffer (pH - 3.2): methanol (32: 66: 2, v/v/v) as the mobile phase. The injection volume was 20µL, flow rate was 1.5mL min^{-1} at 25°C and the detection wavelength was 240nm. The drug was subjected for stress studies as per ICH guidelines.

Results: The method was validated as per ICH Q2 (R1) guidelines in terms of accuracy, precision, system suitability, robustness and linearity. The linearity response was good with correlation coefficient 0.998 for both the epimers of budesonide when concentration plotted against area response.

Conclusion: The statistical analysis confirmed that accuracy, precision, selectivity and system suitability of the proposed method and this method can be effectively used for the analysis of Budesonide in the presence of degradants.

Keywords: Budesonide, Epimers, UHPLC, method validation, stress degradation, ICH guidelines.

1. INTRODUCTION

Budesonide chemically known as 11β, 21 dihydroxy-16α,17α-(butane- 1,1- diylbis(oxy)) pregna- 1,4- diene- 3, 20- one. Molecular formula is $C_{25}H_{34}O_6$. It is an anti-inflammatory corticosteroid which is used in the treatment of ulcerative colitis, Crohn's disease, chronic obstructive pulmonary disorder (COPD), asthma and in allergic rhinitis. An extensive literature survey was carried out for the determination of Budesonide epimers and all its related drugs. But no UHPLC stability indicating methods were reported for the estimation of Budesonide epimers. The present study was undertaken with an objective of developing a rapid, simple, cost effective, accurate, isocratic stability indicating UHPLC method for the estimation of budesonide.

1.1. Background:

The literature review reveals that various methods are employed for the Estimation of Budesonide alone or in combination with other drugs by LC-MS/MS, different Spectrophotometric techniques, HPLC, etc. in various pharmaceutical formulations. There is a need to develop and validate the proposed new UHPLC method. The method is validated for the parameters like accuracy, linearity, precision, specificity, robustness and system suitability.

1.2. Objectives:

- To optimize and develop the analytical method for the estimation of budesonide.
- To validate the proposed method in accordance with ICH guidelines.
- To validate the developed UHPLC method by using various analytical parameters such as accuracy, precision, specificity, robustness, linearity, stability and system suitability as per ICH guidelines.
- To perform forced degradation studies for the developed method and show that the method is specific in presence of the possible degradants.

2. MATERIALS AND METHODS

2.1. Instrumentation:

2.1.1. HPLC instrument specifications:

- HPLC : Agilent 1260 infinity UHPLC with DAD (Diode array detector)
- Pump : G1311B
- Auto Sampler : G1329B
- Detector : G1315D
- Software : Open LAB CDS (ChemStation Edition)
- Column oven : G1316A

2.1.2. Other instruments:

The other instruments used in the analysis are Shimadzu AUX220 weighing balance, S.V scientific digital hot air oven, Elico India L1 127 pH meter and Sub- Aqua 12 water bath.

2.2. Preparations:

The solvents and reagents used in this method were HPLC and AR grade. Mill Q HPLC water was used for all purposes in the analysis. Budesonide standard was obtained as a gift sample from Aurobindo Pharma Ltd., Hyderabad, India. List of reagents used were mentioned in table **1**.

Table **1** List of chemicals/ reagents.

S. No.	Reagent	Grade
1	Sodium dihydrogen Orthophosphate dihydrate	AR
2	Orthophosphoric acid	AR
3	Acetonitrile	HPLC
4	Methanol	HPLC
5	Water	HPLC

2.2.1. Preparation of Buffer:
Transfer 3.17g of Sodium dihydrogen orthophosphate dihydrate into a 1000mL volumetric flask. Add 1000mL of HPLC grade water and is sonicated to dissolve it completely. Then adjust the pH of the solution to 3.2 by using Ortho-phosphoric acid by mixing well. Filter this solution through 0.22µm membrane filter.
2.2.2. Preparation of diluent:
Prepare a suitable quantity of degassed mixture of Water and Acetonitrile in the ratio of 50:50 (V/V).
2.2.3. Preparation of Stock A (1000µg mL^{-1}):
Transfer sample equivalent to 25mg into a 25mL volumetric flask and add 10mL of diluent and mix well. Make up the volume using diluent and this gives 1mg mL^{-1} solution
2.2.4. Preparation of Stock B (100µg mL^{-1}):
Pipette out 1ml of the Stock A solution into a 10mL volumetric flask and add about 5mL of the diluent and mix well. Make up the volume to 10mL by the use of diluent.
2.2.5. Preparation of working standard (8µg mL^{-1}):
Pipette out 800µL of stock B solution into a 10mL volumetric flask and add about 5mL of the diluent and mix well. Finally make up the volume to 10mL by using the diluent.

2.3. Chromatographic condition:

Column : Agilent Poroshell EC- C18 (50mm x 4.6mm, 2.7µ)
Column Temperature : 25° C
Flow Rate : 1.5mL min^{-1}
Injection Volume : 20µL
Run Time : 10min
Diluent : ACN: Buffer: Methanol (32:66:2)

2.4. Analytical method validation:
Analytical method validation is required for any new or amended method to ensure that it is capable of giving reproducible and reliable results, when used by different operators employing the same equipment in the same or different laboratories. The type of validation programme required depends entirely on the particular method and its proposed applications.
Parameters studied for method validation:
1. System suitability
2. Specificity
3. Precision
   - System precision
   - Method precision
   - Intermediate precision
4. Linearity
5. Accuracy
6. Range
7. Robustness
   - Effect of variation in organic phase
   - Effect of variation in wavelength of detection
   - Effect of variation in column temperature
   - Effect of variation in flow rate

2.4.1. System suitability:
System suitability is defined as the checking of a system, before or during the analysis of unknowns, to ensure the system performance. System suitability criteria may include factors such as plate count, tailing, retention, and/or resolution. System suitability criteria should also include a determination of reproducibility (% RSD).
2.4.2. Specificity:
Specificity is defined as the ability to assess unequivocally the analyte in the presence of components which might be expected to be present. Specificity describes about the method's ability responding to one single analyte only. It is done to measure the analyte in the presence of components such as impurity, degradation products and matrix components.
Specificity by forced degradation:
In order to confirm that during stability study or through its shelf life, any degradation product if found will not interfere with the main peaks of Budesonide Epimers, forced degradation on injection samples will be carried out.
Interference from the degradants:
A study was conducted to demonstrate the effective separation of the degradant peaks from budesonide epimer peaks in the injection samples. These injection samples were exposed to following stress conditions to induce degradation.
2.4.2.1. Acid stressed sample (1N HCl):
Transfer accurately weighed 10mg of sample into a 100mL volumetric flask, add about 10mL of diluent (ACN: Water, 1:1) and dissolve it completely. Then add about 5mL of 1N HCl and reflux it at 60°C for one hour with intermittent shaking. Cooled to room temperature and then this solution was neutralized with 1N NaOH solution. Made up the volume by using the diluent and is mixed well. And then the sample solution was prepared as per the test preparation.
2.4.2.2. Alkali stressed sample (0.05N NaOH):
Transfer accurately weighed 10mg of sample into a 100mL volumetric flask, add about 10mL of diluent (ACN: Water, 1:1) and dissolve it completely. Then add about 5mL of 0.05N NaOH and keep it for 30min with continuous shaking. Then this solution is neutralized with 0.1N HCl solution. Made up the volume by using the diluent and is mixed well. And then the sample solution was prepared as per the test preparation
2.4.2.3. Peroxide stressed sample (3.0% w/v H_2O_2):
Transfer accurately weighed 10mg of sample into a 100mL volumetric flask, add about 10mL of diluent (ACN: Water, 1:1) and dissolve it completely. Then add about 10mL of 3.0% w/v H_2O_2 solution and mix well. Keep this solution for one hour with intermittent shaking.

Made up the volume with the diluents and is mixed well. And then solution was prepared as per the test preparation.

2.4.2.4. Thermal stressed (dry heat) sample:

Transfer about 40mg of sample into a Petri plate and is placed in hot air oven which is maintained at 40°C for about 24hours. From this weigh sample equivalent to 10mg into a 100mL volumetric flask and dissolve it by using 10mL of diluent. Finally make up the volume by the use of diluent. And then the sample solution was prepared as per the test preparation.

2.4.3. Precision:

The precision of an analytical method is the degree of agreement among individual test results obtained when the method is applied to multiple sampling of a homogenous sample. It is usually expressed as the standard deviation or RSD of series of measurement.

2.4.3.1. System precision:

The system precision is checked by using the standard analytes to ensure that the analytical system is working properly. The retention time and area response of six determinations were measured and calculated the % RSD. Injected each of blank, six replicates of standard analyte preparations and recorded the chromatograms.

2.4.3.2. Intermediate precision:

It is the agreement of complete measurements when the same method is applied many times within the same laboratory. This includes full analysis on different days, instruments, analyst, but would involve multiple preparation of samples and standards. It is done to ensure that the analytical results will remain unaffected with the change in the instrument, analyst, column and day.

2.4.4. Linearity:

The linearity of an analytical method is ability to obtain test results which are directly proportional to the concentration of the analyte in samples within given range. The linearity study was performed with the working standard at 5 levels from the range of 25% to 200%. Preparation of linearity stock solutions was given in table 2. Linearity was established by plotting a graph between concentrations versus peak area and the slope, intercept, correlation coefficient and regression coefficient (R^2) were determined.

Table **2** Linearity stock solutions preparations.

Level %	Stock B(100µg mL^{-1}) in µL	Total volume with diluents in mL	Conc. in µg
25	200	10	2
50	400	10	4
100	800	10	8
125	1000	10	10
200	1600	10	16

2.4.5. Accuracy:

Accuracy is a measure of the closeness of test results obtained by a method to the true value. Accuracy indicates the deviation between the mean value found and the true value. It is determined by applying the method to samples to which known amounts of analyte have been added. These should be analyzed against standard and blank solutions to ensure that no interference exists. The accuracy is then calculated from the test results as a percentage of the analyte recovered by the assay.

The accuracy studies were performed with Budesonide at five levels at 25%, 50%, 100%, 150%, and 200% of working concentration and triplicate injections were made. From the results calculate the percent recovery, the mean value and the % RSD for each concentration level.

Table **3** Accuracy stock solutions preparations.

Level %	Stock B(100µg mL^{-1}) in µL	Total volume with diluents in mL	Conc. in µg
25	200	10	2
50	400	10	4
100	800	10	8
125	1000	10	10
200	1600	10	16

2.4.6. Range:

The range of the method is the interval between the upper and lower levels of an analyte that have been determined with acceptable precision, accuracy and linearity. It is determined on either a linear or nonlinear response curve (where more than one range is involved, as shown below) and is normally expressed in the same units as the test results.

2.4.7. Robustness:

The robustness of an analytical method is a measure of its capacity to remain unaffected by small but deliberate variations in the method parameters and also provides an indication of its reliability during the normal usage. Robustness of the developed analytical method was realized by deliberately changing the column temperature (± 2°C), change in the flow rate (± 0.1mL min^{-1}), change in the organic phase (± 1mL) and change in the wavelength of detection (± 2nm). The robustness parameters were mentioned in table 4.

Table **4** Robustness studies is conducted by altering the following parameters

S. No	Parameter	Actual method	Lower	Higher
1	Organic phase (ACN) ±1%	32%	31%	33%
2	Temperature ±2°C	25°C	23°C	27°C
3	Wavelength of detection ±2nm	240nm	238nm	242nm
4	Flow rate ±0.1mL min^{-1}	1.5 mL min^{-1}	1.4 mL min^{-1}	1.6 mL min^{-1}

3. RESULTS

3.1. Optimised chromatographic conditions:

The proposed new UHPLC method was optimised with a view to develop as a stability- indicating method for the estimation of budesonide. Chromatographic conditions were optimised by trying with different columns (C18, phenyl columns), different mobile phase compositions with phosphate buffer, Acetonitrile and methanol. A better chromatographic separation was achieved by the use of Agilent Poroshell C18 column (50 x 4.6mm, 2.7µm), mobile phase comprised of ACN, phosphate buffer and

methanol in the ratio 32: 66: 2(v/v/v) with flow rate of 1.5mL min^{-1} and a column temperature of 25°C at detection wavelength of 240nm and the elution time was 10minutes. Budesonide R and S Epimers had adequate retention, good peak shapes with less tailing and are eluted at 4.96 and 5.48 minutes respectively which were shown in the Fig.1.

3.2. Method validation:

The validation of the proposed method was performed in accordance with ICH guidelines. The validation parameters considered for the proposed analytical method were system suitability, specificity, precision, linearity, accuracy, range and robustness.

3.2.1. System suitability:

System suitability was performed by injecting one blank and budesonide standard of concentration 8µg ml^{-1} in five replicate injections and is found that the parameters are within the acceptable criteria.

3.2.2. Specificity:

Specificity was performed in the terms of the degradation studies. The budesonide sample was subjected to degrade in different stress conditions (acid, alkali, peroxide and thermal). The peaks obtained are homogeneous and has no co-eluting peaks, also the parameters are found within the limits indicating the specificity of the proposed analytical method. The chromatogram shows that there is no interference from the degradants which are shown in the Fig 2 to 5.

3.2.3. Precision:

The precision is observed from the data that the retention time and area response were consistent as evidenced by the values of relative standard deviation and were found to be within the acceptance criteria. Hence, it can be concluded that the system precision parameter meets the requirement of method validation.

Table **5** Results for forced degradation studies.

S.No.	Stress condition	Epimer R		Epimer S		Retention times of the degradants
		Retention time	% Assay	Retention time	% Assay	
1	As such	4.931	99.41	5.438	99.03	No degradants
2	Acid	4.785	75.99	5.279	84.34	1.997 and 3.034min
3	Alkali	4.853	88.17	5.354	85.33	0.339, 0.663. 0.939, 1.022 and 2.019
4	Peroxide	4.865	90.81	5.364	88.40	0.358
5	Thermal	4.808	82.76	5.302	79.89	No degradants

Table **6** Summary of method validation results of budesonide.

S.No.	Validation parameter	Acceptance Criteria	Results	
			Epimer R	Epimer S
1	System suitability	The column efficiency for the epimer peaks should NLT 2000 theoretical plates.	7253	7637
		The asymmetry factor for the epimer peaks should be NMT 2.0.	0.93	0.94
		The % RSD for the replicate injections of the standard preparation should be NMT 2.0%.	0.13	0.34
2	Specificity	Peak purity more than 997	998.14	999.53
3	Precision	RSD NMT 2.0%	0.2	0.4
4	Linearity	Regression coefficient NLT 0.998	0.998	0.998
		Correlation coefficient NLT 0.998	0.9983	0.9981
		Slope	17.78	10.83
		intercept	8.837	12.98
5	Accuracy	Mean recovery should be between 98% to 102%	99.33	98.63
6	Range	%RSD NMT 2.0%	0.34	1.02
7	Robustness	Change in organic phase ± 1% RSD NMT 2.0%	Unchanged 32%- 0.14	Unchanged 32%- 0.15
			33%- 0.06	33%- 0.12
			31%- 0.18	31%- 0.42
		Change in wavelength of detection ± 2nm RSD NMT 2.0%	Unchanged 240nm- 0.14	Unchanged 240nm- 0.15
			238nm- 0.02	238nm- 0.07
			242nm- 0.02	242nm- 0.03
		Change in column temperature ± 2°C RSD NMT 2.0%	Unchanged 25°C- 0.14	Unchanged 25°C- 0.15
			23°C- 0.25	23°C- 0.14
			27°C- 0.2	27°C- 0.25
		Change in flow rate ±0.1 mL min^{-1} RSD NMT 2.0%	Unchanged (1.5mL min^{-1})- 0.14	Unchanged (1.5mL min^{-1})- 0.15
			1.4mL min^{-1}- 0.9	1.4mL min^{-1}- 0.73
			1.6mL min^{-1}- 0.1	1.6mL min^{-1}- 0.23

3.2.4. Linearity:

Linearity studies have been performed with Budesonide standard at 5 levels from the range of 25% to 200% of working concentration of the standard solution. The linearity response of Budesonide was found in the range of 2-16 µg mL^{-1}. The calibration curve of the analytical method was assessed by plotting concentration versus peak area. The regression coefficient value was found to be 0.998 for both the epimers of budesonide.

3.2.5. Accuracy:

Accuracy was checked by performing the recovery studies at five different levels, with each level in triplicate (15 determinations). The prepared samples were then analyzed and the percentage recoveries were calculated. The recovery value of budesonide ranges from 98.80 to 99.65. The average recovery for 15determinations at 5levels for budesonide was found to be 99.33% and 98.63% for epimer R and S respectively.

3.2.6. Range:

The range is determined with the linearity and accuracy and %RSD was calculated and found to be within the acceptance criteria.

3.2.7. Robustness:

Robustness of the developed analytical method was realized by deliberately changing the column temperature (± 2°C), change in the flow rate (± 0.1mL min^{-1}), change in the organic phase (± 1mL) and change in the wavelength of detection (± 2nm). At these changes, area response and %RSD were found to be within the acceptance criteria and indicates that the proposed analytical method was robust.

4. DISCUSSION

Budesonide epimers R and S showed the retention times 4.96 and 5.48 mins respectively. The %RSD for system suitability studies was found NMT 2.0%. Two degradant peaks were found to have no interference with the epimer peaks in chromatogram of the acid stressed sample. Five degradant peaks are resolved from the base stressed sample and one peak of degradants is found in the chromatogram of alkali stressed sample. No degradant peaks were found for the thermal stressed sample.

The specificity of the method was confirmed by the purity of peak and is found to NLT 997.The linearity response of Budesonide was found in the range of 2-16 µg mL^{-1}. The regression coefficient value was found to be 0.998 for both the epimers of budesonide. The estimation at 5 levels states that the method is precise. The accuracy studies states that the mean recovery of budesonide was in between 98% to 102%. The robustness states that slight variations in the different analytical conditions of the proposed method are within the acceptance limits.

5. CONCLUSION

The developed analytical method gives a good resolution between Budesonide Epimers, unknown degradants and its related substances. There is no interference or co-elution of degradants along with budesonide epimer peaks. The method was validated and was found to be simple, sensitive, accurate, reproducible and precise. The proposed method can be employed for the estimation and quantification of Budesonide and the unknown degradants.

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