Validated Reversed Phase High Performance Liquid Chromatographic Method for Determination of Three Novel Steroids in Bulk and Pressurized Metered - Dose Commercial Preparations Using a Common Mobile Phase

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Abstract: A simple and sensitive reversed phase high performance liquid chromatography (RP-HPLC) method has been proposed for determination of three widely used novel steroids in bulk as well as in their pressurized metered dose commercial preparations. The steroids are Budesonide, Fluticasone propionate and Ciclesonide. These steroids are widely used in treatment of different types of respiratory disorders mainly in the form of pressurized metered dose commercial formulations. The steroids were chromatographed using methanol: acetonitrile (60:40 v/v) as the mobile phase at a flow rate of 1.0 ml/min. The retention time (RT) of Budesonide, Fluticasone propionate and Ciclesonide was found to be 3.4161 ± 0.1305 min., 3.0512 ± 0.3694 min. and 4.841 ± 0.2421 min., respectively. The linearity of Budesonide, Fluticasone propionate and Ciclesonide were in the range of 1-10 µg/ml, 1-18 µg/ml and 2-20 µg/ml, respectively. The method was validated as per ICH guidelines and successfully applied for quantification of Budesonide, Fluticasone propionate and Ciclesonide in their pure and metered dose commercial formulations, contributing to improve the quality control and to assure the therapeutic efficacy.

Keywords: Budesonide; ciclesonide; fluticasone propionate; novel steroids; pressurized metered - dose preparations; reversed phase high performance liquid chromatography (RP-HPLC); analytical method validation.

1. Introduction

Budesonide, chemically known as \((11\beta, 16\alpha) - 16, 17\text{-}[\text{butytlidene bis (oxy)}\text{]-11, 21\text{-dihydroxypregna-1, 4-diene-3, 20- dione}}\). It is used orally for the treatment of Crohn’s disease. While its inhaled formulation are used for the treatment of asthma and various types of respiratory disorders. Ciclesonide, chemically known as \((11\beta, 16\alpha) - 16,\)
17-[(R)-cyclohexylmethylene] bi (oxy)-11-hydroxy-21-(2-methyl-1-oxopropoxy) pregna-1, 4-diene-3, 20-dione, is a new corticosteroid, for the prophylactic treatment of asthmatic attacks in adults. Fluticasone propionate, S-(fluoromethyl)-6α, 9-difluoro-11β, 17-dihydroxy-16α-methyl-3-oxoandrosta-1, 4-diene-17β-carbothioate, 17-propionate, is a synthetic steroid of the glucocorticoid family of drugs that is used to treat asthma and allergic rhinitis. The structures of Budesonide, Ciclesonide and Fluticasone propionate are represented in Figure 1, Figure 2 and Figure 3, respectively.
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As per literature, the methods available for determination of Budesonide include UV spectroscopy [1], Stability indicating HPLC [2], LC/MS method [3, 4] and TLC with UV detection [5]. One UV spectroscopy [6] and HPLC [7] methods were also reported for simultaneous determination of Budesonide and Formoterol in their combine dosage forms. One LC/MS/MS method was reported as well for determination of Budesonide and Fluticasone in human sputum sample [8]. Simultaneous HPLC analysis was also reported for determination of Budesonide along with triamcinolone acetonide in microdialysate and rat plasma [9]. Similarly for determination of Fluticasone propionate, the reported methods include LC-MS/MS [10], Stability indicating LC [11] and visible spectroscopy [12]. A RP - HPLC method is reported for determination of Fluticasone propionate along with Salmeterol xinafoate [13]. For determination of Ciclesonide and Fluticasone propionate individually, one HPTLC method was also reported [14]. For determination of Ciclesonide, the available methods include LC-MS/MS [15] and stability indicating HPLC method [16]. One liquid chromatography method is reported for simultaneous determination of Ciclesonide and Formoterol fumarate in dry powder inhalers [17]. RP - HPLC is found to be the most effective and widely used method where sensitivity as well as accuracy is concerned. According to literature, any RP - HPLC method could not be traced for the analysis of Ciclesonide and Fluticasone propionate separately in their bulk as well as their pressurized metered dose preparations. Considering the wide importance of the novel steroids in the treatment of respiratory disorders, we developed simple, sensitive, accurate as well as precise RP - HPLC method for quantification of pure steroids and application of the developed method for determination of their metered dose commercial formulations were carried out. After development of the method, it was successfully validated as per validation parameters of ICH guidelines [18].

2. Materials and method

2.1. Chemicals and reagents

All chemicals used were of analytical grade and HPLC grade water was used throughout. Pure Budesonide (BUD), Fluticasone propionate (FP) and Ciclesonide (CIC) were of pharmacopeial grade. HPLC grade methanol and acetonitrile were used during the study (Ranchem, Rfcl Ltd., New Delhi, India). Water from Milli Q (Millipore Bedford, MA) was employed for total method development. Various pressurized metered dose preparations of Budesonide, Ciclesonide and Fluticasone propionate were obtained commercially.

2.2. Apparatus

The instrumentation comprises of a Shimadzu’s HPLC (LC-2010-HT, Shimadzu, Singapore) equipped with UV-Visible detector, ODS Phenomenex C18 column (250 mm X 4.6 mm i.d., 5 mm), Hamilton 20 μL glass syringe, a Citizen analytical balance (Sartorius Mechatronics India Pvt. Ltd., Bangalore, India.), an Equitron - ultra sonic sonicator (Labnet scientific services, Chennai, India) were used for method development.

2.3. Optimized chromatographic conditions

Chromatographic estimations were performed under the following conditions: ODS Phenomenex C18 column (250 mm X 4.6 mm i.d., 5 mm) was used as a stationary phase at
ambient temperature. The mobile phase comprised methanol: acetonitrile (60:40, v/v), was pumped at a flow rate of 1 mL min\(^{-1}\). The mobile phase was filtered through Nylon 0.45 mm, 47 mm membrane filter and was degassed before use. The source of radiation was D2 lamp emitting a continuous ultra violet radiation between 180 nm to 400 nm. The elution was monitored at 243 nm for Budesonide, 236 nm for Fluticasone propionate and 242 nm for Ciclesonide. The injection volume was 20 µL for each sample.

2.4. Preparation of the standard solutions

All reagents were tested for stability in solution and during the actual analysis; the behavior of the analytes remained unchanged up to 48 h from their preparation at ambient temperature using the selected solvent as well as up to 96 h from the preparation in refrigerator using the same. The steroids were found to be stable during each kind of experimental measurements. Each measurement was done at ambient temperature. Standard solutions of the steroids were prepared individually having final concentration as 1 mg/ ml for each steroid. One ml of aliquots from these solutions were taken and diluted up to 10 ml using methanol (100 µg/ml). From these stock solutions, various dilutions were made for Budesonide, Ciclesonide and Fluticasone propionate separately for calibration curves. Evaluation was done by measuring peak areas with linear regression for each plate.

2.5. Procedure for pressurized metered dose commercial preparation analysis

The preparation of the sample for metered dose formulation analysis was done exactly as was described in Indian Pharmacopoeia - 2007 [19]. The samples from the metered dose formulation were collected in methanol and the contents were shaken well. Solutions were filtered through Whatman filter paper No. 41 and same HPLC conditions were applied as mentioned above. All the brands of the pressurized metered dose preparations containing Budesonide, Ciclesonide and Fluticasone propionate were tested according to the procedure described above. The formulations were already available in the market and hence were procured from the registered medical store.

2.6. Validation parameters

2.6.1. Linearity and range

A calibration curve was plotted over a concentration range of 1 - 10 µg/ml for Budesonide, 1 - 18 µg/ml for Fluticasone propionate and 2 - 20 µg/ml for Ciclesonide. Stock solutions of Budesonide, Ciclesonide and Fluticasone propionate, having concentration 100 µg/ml were prepared separately and further diluted for calibration curves of each drug separately. Accurately measured standard stock solution of Budesonide, standard stock solution of Fluticasone propionate and standard stock solution of Ciclesonide were used for further preparations of standard solutions. 20 µL of each solution was injected under operating chromatographic conditions described above.

2.6.2. Precision

Intraday precision and inter day precision for the developed methods were measured in terms of % RSD. The experiments were repeated six times a day for intraday precision and on six
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different days for interday precision. The concentration values for both intraday precision and interday precision were calculated six times separately and percent relative standard deviation were calculated. Finally the mean of % RSD (% RSD = [S/X] 100, where S is standard deviation and X is mean of the sample analyzed) was calculated. The precision of the instrument was checked by repeated scanning of the same spot of both drugs six times without changing the condition of the instrument.

2.6.3. Recovery studies (Accuracy)

Accuracy of proposed method and interference from excipients was determined by recovery experiments. Recovery experiments were carried out by the standard addition method (spiking method). This study was performed by addition of known amounts of Budesonide, Ciclesonide and Fluticasone propionate (80%, 100% and 120% to that of labeled claim of commercial formulations) to a known concentration of the pressurized metered dose preanalyzed formulations and % of standard drugs recovered, were calculated.

2.6.4. Limit of detection (LOD)

Limit of detection was calculated using following equation as per ICH guidelines.
LOD = 3.3 × S/m where, S is the standard deviation of the peak areas of the drug and m is the slope of the corresponding calibration curve. It is expressed as signal to noise ratio of 3:1.

2.6.5. Limit of quantitation (LOQ)

Limit of quantification (LOQ) was calculated using following equation as per ICH guidelines.
LOQ = 10 × S/m where, S is the standard deviation of the peak areas of the drug and m is the slope of the corresponding calibration curve. It is expressed as signal to noise ratio of 10:1.

2.6.6. System suitability

Number of theoretical plates was determined by employing the formula, n =16(t/w)² where t=retention time and w = width of the peak. Tailing factor was derived from the formula t = w/2t where w = half of the width, t = retention time. The retention time was also observed as a system suitability factor.

3. Results and discussion

As per the literature, HPTLC method was reported for determination of two steroids, Ciclesonide and Fluticasone propionate individually [14]. As we know that HPTLC method development varies with various factors like size of the Plates, saturation of the Chamber, scanning position of the plates inside the scanner etc. RP-HPLC method provides more flexibility as compare to HPTLC in case of estimation of drugs and method development as well as validation. One of the most important purposes of the method development is application of the developed method for commercial formulation analysis. When we compare the results of formulation analysis by RP-HPLC and HPTLC for the same steroids, RP-HPLC proven to be more accurate and precise as compare to the previously developed HPTLC method. Excellent results of method validation as well as commercial formulation analysis were obtained in the
developed RP-HPLC method for all three Novel steroids.

### 3.1. RP - HPLC method optimization

Budesonide, Fluticasone propionate and Ciclesonide were soluble in methanol; therefore methanol was selected as common solvent for the steroids. Attempts were made to optimize a common mobile phase for the novel steroids which would give fast retention as well as detection at lower concentration. Various mobile phases were tried and retention time as well as peak area and peak symmetry was observed. Best results were obtained using Methanol: ACN in the ratio of 60: 40 v/v as mobile phase using C-18 column. The retention time (RT) of Budesonide, Fluticasone propionate and Ciclesonide was found to be 3.4161 ± 0.1305, 3.0512 ± 0.3694 and 4.841 ± 0.2421, respectively. The selected mobile phase gives best result in terms of faster retention, least tailing of the peaks as well as accurate results for each kind of chromatographic measurements throughout the development of the entire method. The chromatogram of Budesonide, Ciclesonide and Fluticasone propionate were represented in Figure 4, Figure 5 and Figure 6 respectively.

![Figure 4. HPLC chromatogram of budesonide](image1)

![Figure 5. HPLC chromatogram of ciclesonide](image2)
3.2. Method validation

3.2.1. Linearity and range

Linearity was found between the concentration range of 1 - 10 µg/ml, 1 - 18 µg/ml for Fluticasone propionate and 2 - 20 µg/ml for Ciclesonide. The average linear regression equation was represented as $Y = 105.75x + 9.065$ for Budesonide, $y = 43.30x + 69.51$ for Ciclesonide and same for Fluticasone propionate was found to be $y = 66.24x + 14.304$, where $X$ is the concentration of drug Peak areas and concentration were subjected to least square linear regression analysis to calculate the calibration equation and correlation coefficient. The linearity was found to be 0.999 for Budesonide, 0.999 for Fluticasone propionate and 0.999 for Ciclesonide in terms of correlation of coefficient ($R^2$). Relative standard deviations for determination of linearity of method were found to be 0.0174 for Budesonide, 0.0207 for Ciclesonide and 0.0276 for Fluticasone propionate determination (Table 1).

3.2.2. Precision

The intra-day and inter-day precision for Budesonide was found to be 0.1392 and 0.6935 respectively and the same for Ciclesonide was found to be 0.3459 and 0.7309 respectively. For Fluticasone propionate, the intraday and inter day precision was found to be 0.5058 and 1.5267 respectively in terms of %RSD. These values indicate that the method is precise. The %RSD for precision of the instrument by measuring the peak area was found to be 0.2047 for Budesonide, 0.2125 for Ciclesonide and 0.2091 for Fluticasone propionate. The %RSD for measuring the peak area (less than 2%), ensured proper functioning of HPLC system. The results depicted in Table 1.

3.2.3. Recovery studies (Accuracy)

The recovery study was carried out by spiking method. The formulations were preanalyzed and same types of sampling were made from the formulation for the recovery study. In the sample of formulations itself, 80%, 100% and 120% of the standard drugs were added for the respected steroids. Here at no addition, the estimated content of Budesonide, Ciclesonide and Fluticasone propionate were 47.26 µg/ml, 47.06 µg/ml and 47.22 µg/ml respectively. These are
the results of formulation analysis without spiking. After spiking 80%, 100% and 120% of the standards, the estimated contents were written in the table itself (column 2). Finally % of the standard that is recovered, were calculated, which is shown in the column 3 of Table 2. When the recovery study was carried out in preanalyzed sample of metered dose commercial formulations percentage recoveries were found to be 99.84 ± 0.8076, 99.46 ± 0.8787 and 99.71 ± 0.5262 for Budesonide, Ciclesonide and Fluticasone propionate respectively (Table 2).

3.2.4. Limit of detection (LOD) and limit of quantitation (LOQ)

The Values of LOD and LOQ for Budesonide were found to be 0.1220 µg/ml and 0.3699 µg/ml. The value of LOD was found to be 0.5304 µg/ml for Ciclesonide and 0.2041 µg/ml for Fluticasone propionate. Similarly the values of LOQ were found to be 1.6075 µg/ml and 0.6186 µg/ml for Ciclesonide and Fluticasone propionate respectively (Table 1).

Table 1. Validation parameters of developed RP - HPLC methods for budesonide (BUD), ciclesonide (CIC) and fluticasone propionate (FP)

<table>
<thead>
<tr>
<th>Validation parameters</th>
<th>BUD</th>
<th>CIC</th>
<th>FP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linearity range (µg/ml)</td>
<td>1-10</td>
<td>2-20</td>
<td>1-18</td>
</tr>
<tr>
<td>Scanning wavelength</td>
<td>243 nm</td>
<td>242 nm</td>
<td>236 nm</td>
</tr>
<tr>
<td>Regression equation</td>
<td>(Y = 105.75x + 9.065)</td>
<td>(y = 43.30x + 69.51)</td>
<td>(y = 66.24x + 14.304)</td>
</tr>
<tr>
<td>Correlation coefficient (R2)</td>
<td>0.999 ± 0.0174</td>
<td>0.999 ± 0.0207</td>
<td>0.999 ± 0.0276</td>
</tr>
<tr>
<td>Accuracy (Recovery study)</td>
<td>99.84 ± 0.8076</td>
<td>99.46 ± 0.8787</td>
<td>99.71 ± 0.5262</td>
</tr>
<tr>
<td>LOD (µg/ml)</td>
<td>0.1220</td>
<td>0.5304</td>
<td>0.2041</td>
</tr>
<tr>
<td>LOQ (µg/ml)</td>
<td>0.3699</td>
<td>1.6075</td>
<td>0.6186</td>
</tr>
<tr>
<td>Precision - intraday (%RSD)</td>
<td>0.1392</td>
<td>0.3459</td>
<td>0.5058</td>
</tr>
<tr>
<td>Interday (%RSD)</td>
<td>0.6935</td>
<td>0.7309</td>
<td>1.5267</td>
</tr>
<tr>
<td>Repeatability (%RSD)</td>
<td>0.2047</td>
<td>0.2125</td>
<td>0.2091</td>
</tr>
</tbody>
</table>

Table 2. Results of recovery studies of budesonide (BUD), ciclesonide (CIC) and fluticasone propionate (FP) by the developed RP - HPLC method

<table>
<thead>
<tr>
<th>Std drug added (%)</th>
<th>Estimated content (mcg/ml)</th>
<th>% Recoverya</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BUD</td>
<td>CIC</td>
</tr>
<tr>
<td>0</td>
<td>47.26</td>
<td>47.06</td>
</tr>
<tr>
<td>80</td>
<td>87.26</td>
<td>87.06</td>
</tr>
<tr>
<td>100</td>
<td>97.26</td>
<td>97.06</td>
</tr>
<tr>
<td>120</td>
<td>107.26</td>
<td>107.06</td>
</tr>
<tr>
<td>Mean recovery ± standard deviation</td>
<td>99.84 ± 0.8076</td>
<td>99.46 ± 0.8787</td>
</tr>
</tbody>
</table>

3.2.5. System suitability

The number of theoretical plates, tailing factors and retention times were represented in Table 3.
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Table 3. Results of system suitability of budesonide, ciclesonide and fluticasone propionate by the developed RP – HPLC method.

<table>
<thead>
<tr>
<th>System suitability parameters</th>
<th>Budesonide a</th>
<th>Ciclesonide a</th>
<th>Fluticasone propionate a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retention time (max% RSD)</td>
<td>3.4161 ± 0.1305</td>
<td>4.841 ± 0.2421</td>
<td>3.051 ± 0.3694</td>
</tr>
<tr>
<td>Tailing factor (%RSD)</td>
<td>0.6313 ± 1.1724</td>
<td>1.046 ± 0.3103</td>
<td>1.043 ± 0.2288</td>
</tr>
<tr>
<td>Theoretical plates (max%)</td>
<td>9848.5 ± 0.7446</td>
<td>11428 ± 0.7837</td>
<td>9531 ± 0.0913</td>
</tr>
</tbody>
</table>

a - mean value of six determinations

3.3. Application of the developed method for analysis of pressurized meter dose commercial formulations

Various brands of pressurized metered dose formulations containing Budesonide, Fluticasone propionate and Ciclesonide were tried and the results were shown in the table 4.

Table 4. Application of the developed RP - HPLC methods for pressurized metered dose commercial formulations of budesonide (BUD), ciclesonide (CIC) and fluticasone propionate (FP)

<table>
<thead>
<tr>
<th>Formulation</th>
<th>% Amount found a ± S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>BUD</td>
<td>CIC</td>
</tr>
<tr>
<td>Brand A</td>
<td>Brand C</td>
</tr>
<tr>
<td>Brand B</td>
<td>Brand D</td>
</tr>
</tbody>
</table>

a - mean value of three determinations

4. Conclusion

The newly developed HPLC method for estimation of Budesonide, Fluticasone propionate and Ciclesonide in bulk and pressurized metered dose formulations are found to be simple, rapid, sensitive, accurate, precise and reliable which indicates its adequacy for the routine pharmaceutical analysis. The statistical analysis proves that the developed method is suitable for determination of Budesonide, Ciclesonide and Fluticasone propionate as bulk drugs individually and in the commercial pressurized metered dose preparations without any interference from the excipients and with the use of common mobile phase.

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