Development and Validation of Stability Indicating RP-HPLC-PDA Method for Determination of Acebrophylline and It’s Application for Formulation Analysis and Dissolution Study

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ABSTRACT

A stability-indicating RP-HPLC-PDA method has been developed and subsequently validated for the determination of Acebrophylline in commercial capsules. The proposed HPLC method utilizes waters Cosmogel C18 column (150 mm x 4.6 mm, 5.0 μ particle size) and mobile phase consisting of methanol:acetate buffer (20:80 v/v) pH adjusted to 6 with glacial acetic acid at a flow rate of 0.85 mL/min and column was maintained at 50°C. Quantitation was achieved with UV detection at 274 nm based on peak area with linear calibration curves at concentration ranges 0.5-200 μg/mL for ACB (R² > 0.999). ACB and its drug products were exposed to acid, base and neutral hydrolysis, oxidation, heat and photolytic stress conditions. Under all these conditions, degraded products were well separated. The method was validated according to the ICH guidelines with respect to accuracy, precision, linearity, specificity, limits of detection, limits of quantitation and robustness. Method has been successively applied to assay of pharmaceutical formulation and dissolution study and no interference from the tablet excipients was observed. As the proposed method could effectively separate the drug from its degradation products, it can be employed as stability indicating method.

KEY WORDS: Column liquid chromatography, Stability-indicating method, Dissolution Study, Acebrophylline.

INTRODUCTION

Acebrophylline is an anti-inflammatory and airway mucus regulator. It contains ambroxol and theophylline-7-acetic acid, the former facilitates the biosynthesis of pulmonary surfactant while later raises blood levels of ambroxol, by stimulating surfactant production [1]. Chemically acebrophylline is 1, 2, 3, 6-tetrahydro-1, 3-dimethyl-2, 6-dioxo-7H-purine-7-acetic acid with trans-4-{[2-aminomethyl-3, 5 dibromophenyl] methyl}amino] cyclohexanol. Literature survey revealed that various analytical methods like spectrophotometric [2-6], HPLC[5-8], and HPTLC[9-10] , have been reported for the determination of Ambroxol HCl and Theophylline -7-acetic acid, individually and in combination with some other drugs. Also one TLC Densitometric[11] method has been reported for estimation of Acebrophylline in single dosage form. Further, no stability indicating method has been reported in literature for determination of ACB in presence of their degradants. Therefore, the aim of present study was to develop and validate stability indicating HPLC method for the analytes in combination by following ICH method validation guidelines. To establish the stability indicating nature of the method, forced degradation of each API and drug product was performed under stress conditions and stressed samples were analyzed by the proposed method. The proposed LC method was able to separate both drugs from degradants generated during forced degradation studies. The method was successfully applied for dissolution study of combined drug formulations [12,13,14].

EXPERIMENTAL

Materials and Reagents

HPLC grade methanol was purchased from Merck (Mumbai, India). Analytical reagent grade Glacial acetic acid was purchased from Ana Lab Fine Chem. Industries (Mumbai, India). Pure drug sample of ACB with % purity (99.75) was kindly supplied as a gift sample by Glenmark Pharmaceuticals Ltd. Baddi .These samples were used without further purification. Two capsule formulations AB Phylline (B.No. AD92037), manufactured by Sun Pharmaceutical Industries Dadra, containing ACB 100 mg per capsule and Ascovent (B.No.05100282) manufactured by Glenmark Pharmaceuticals Ltd. Baddi were purchased from local market.

HPLC Instrumentation and Conditions

The HPLC system consisted of a binary pump (model Waters 515 HPLC pump), auto sampler (model 717 plus Auto sampler), column heater and PDA detector (Waters 2998). Data collection and analysis was
performed using Empower- version 2 software. Separation was achieved on Cosmogel C18 column (150 mm x 4.6 mm, 5.0 µ particle size) columns maintained at 50 °C using column oven. Isocratic elution with methanol: acetate buffer (20:80 v/v) pH adjusted to 6 with glacial acetic acid at a flow rate of 0.85 mL/min was carried out. The detection was monitored at 274 nm and injection volume was 20 µL. The peak purity was checked with the photodiode array detector.

**Preparation of Standard Solutions and Calibrations**

Standard stock solution of ACB (1000 µg/mL) was prepared in methanol. To study the linearity range, serial dilutions of ACB were prepared from 0.5-200 µg/mL, in mobile phase and injected on to column. For the construction of calibration curves, eleven standard solutions in concentration range mentioned above were prepared and injected on to column. Calibration curves were prepared as concentration of drugs versus peak area response. The system suitability test was performed from six replicate injections of standard solution containing 100 µg/mL of ACB. Typical chromatogram obtained from a standard solution is shown in Fig.1.

![Fig.No.1-Densitogram of Acebrophylline](image)

**System Suitability Test (SST) and Formulation Analysis**

The SST ensures the validity of the analytical procedure as well as confirms the resolution between different peaks of interest. All critical parameters tested met the acceptance criteria on all days. Adequate resolution of ACB peaks ensured the specificity of the method. The system suitability assessment for the analytical HPLC method established instrument performance parameters such as peak area, % R.S.D., column efficiency (N) and USP tailing factor (T)

For formulation analysis quantity of powder from 20 capsules equivalent to 100 mg of ACB was weighed and transferred to a 100 mL volumetric flask containing about 70 mL of methanol, ultrasonicated for 5 min and solution was filtered through Whatman paper No. 41 into a 100 mL volumetric flask. Filter paper was washed with the solvent, adding washings to the volumetric flask and volume was made up to mark. The solution was suitably diluted with mobile phase to get a concentration of 100 µg/mL of ACB. The sample solution was then filtered using 0.45 µ nylon syringe filter and 20 µL of the test solution was injected and chromatogram was recorded for the same and the amounts of the drugs were calculated.

**Method Validation**

The stability indicating RP-LC-PDA method was validated in terms of precision, accuracy, specificity, sensitivity, robustness and linearity according to ICH guidelines. Assay method precision (inter-day and intra-day) was determined using three concentrations and three replicates. Standard solutions containing 20, 40 and 80 µg/mL of ACB were used for precision study. Assay method was evaluated with the recovery of the standards from excipients. Three different quantities (50%, 100% and 150%) of the standards were added to preanalyzed formulation and were analyzed using the developed HPLC method. Values of Limit of Detection (LOD) and Limit of Quantitation (LOQ) were calculated by using σ (standard deviation of response) and b (slope of the calibration curve) and by using equations, LOD = (3.3 x σ)/ b and LOQ = (10 x σ)/ b. Calculated values were confirmed by repeated injection of samples containing amounts of analyte in the range of LOD and LOQ. To determine the robustness of the method, the final experimental conditions were intentionally altered and the results were examined by changing one parameter at the time. The parameters considered (± values) for the robustness study were, flow rate (± 5%), column temp. (± 2°C), measurement wavelength (±1nm), injection volume (± 2 µl), % organic (± 5%), buffer strength (± 5 mM), pH (± 0.5) and effect of column from different suppliers were studied. The stability of the drug solution was determined using the samples for short-term stability by keeping at room temperature for 12 h and then analyzing. The long-term stability was determined by storing at 4°C for 30 days. Auto-sampler stability was determined by storing the samples for 24 h in the autosampler. For method development and optimization, retention factor (k) was calculated using the equation: k = (tR - tM)/tM. Where, tR = retention time, tM = is the elution time of the solvent front.

**Dissolutions**

A calibrated dissolution apparatus (USP II) was used with paddles at 50 rpm and bath temperature maintained at 37±1°C. Freshly prepared and degassed 900 mL of 0.1 N HCl solution was used as the dissolution
medium. Six capsules (with sinkers) were evaluated for each drug product tested. Dissolution samples were collected at 5, 10, 15, 20, 25, 30, 35, 40 and 45 minutes with replacement. At each time point, a 5 mL sample was removed from each vessel sample, filtered through a nylon filter (0.45μm, 25 mm) and analyzed by proposed method. The amount of ACB in the test samples was calculated, as percentage dissolved, from the measured peak area for the test samples by using equation 1 and alternatively by using peak areas of sample (S₁) and Standard(S₂) using equation 2.

\[
\text{Dissolved} \% = \frac{\text{Conc. estimated by the method} \times 900 \times 100}{(1000 \times DL)} \ldots (1)
\]

\[
\text{Dissolved} \% = \frac{900 \times DL \times (\text{Peak Area} (S₁)/\text{Peak Area} (S₂)) \times \text{Conc. (std.)} \times 100}{... (2)}
\]

Where, DL= is drug load, which is 100 mg.

**Degradation Studies of API and Capsules**

ACB was subjected to various forced degradation conditions individually to effect partial degradation of the drug preferably in 10-20% range. The forced degradation study was performed for the drug product ACB bulk to determine whether any observed degradation occurred because of drug properties or was due to drug excipients interactions. Moreover, the studies provide information about the conditions in which the drug is unstable so that measures can be taken during formulation to avoid potential instabilities. Forced degradation of the drug product was carried out under thermolytic, photolytic, acid/base hydrolytic and oxidative stress conditions. For photolytic stress, drug product in the solid state was irradiated with UV radiation with peak intensities at 254 and 366 nm. The UV dose from the lamp of peak intensity at 366 nm was measured by use of a quinine monohydrochloride (2% solution in water) chemical actinometer as mentioned in the ICH guidelines. Minimum desired exposure (200 Wh/m²) was observed after irradiation for 28 h. A second photolytic stress test experiment with greater irradiation time, 52 h, was performed to establish the specificity of the method. Sample solution containing 1000 μg/mL of ACB was subjected to selected stressed conditions. Samples except for photo oxidation were protected from light. For Acid, base and water-induced degradation solutions containing 1000 mg/mL of the drug were prepared in 0.1N HCl, 0.1N NaOH and water and analysed after 5 h exposure. Oxidative degradation solution was prepared in water containing 30% v/v of H₂O₂ and analysed after 5 h. Sample for photolytic studies were exposed to short and long UV radiations for 28 h and were used. During dry heat study samples were exposed to 100 °C for 8 h in oven and analysed. After exposure to desired stress degradation condition, samples were diluted with mobile phase to achieve the nominal concentration of 100 μg/mL of ACB which was based on their label strength in standard solution. Formulation containing ACB was exposed to same stress conditions and stress degraded samples were analysed by following above procedure described for bulk analytes.

**RESULTS AND DISCUSSION**

**Optimization of the Chromatographic Conditions**

A well-defined symmetrical peak was obtained upon measuring the response of eluent under the optimized conditions after thorough experimental trials that can be summarized. Two columns were used for performance investigations, including Cosmogel C18 (5μ, 4.6×150 mm) and Qualisil BDS C8 (5 μ, 4.6×250 mm), the first column was the most suitable one since it produced symmetrical peak. Initially Methanol and water were tried in various ratios but at all the time peak shape and tailing was not acceptable. Acetonitrile and water, methanol and acetonitrile in mixture with water were tried in different ratios, with this there was always problem of peak shape, peak tailing and baseline disturbance. Water was replaced with acetate buffer and it was then tried in different ratios with acetonitrile alone and with methanol and acetonitrile combination, chromatographic conditions were improved but were not satisfactory. Acetonitrile was replaced with methanol and several trials were performed with different ratios. Methanol 20% with acetate buffer shown smooth base line, sharp peak with acceptable peak tailing. Therefore, Methanol 20 % was the organic modifier of choice giving symmetrical narrow peak. The effect of changing the concentration of acetate buffer on the selectivity and retention times of analytes was investigated using mobile phases with 10 - 50 mM acetate buffer and 25 mM of acetate buffer was found to be the most suitable giving highest number of theoretical plates. The effect of various flow rates on the formation and separation of peaks of the analytes was studied and a flow rate, 0.85 ml/min was optimum with reasonable time of analysis. Similarly effect of temperature was studied by varying the temperature from 35 – 55 °C; at lower temperatures the peaks were not symmetrical, whereas at 50°C the peak show good symmetry. The tailing factor was found to be always< 1.3 during analysis for all the peaks. The effect of pH was also studied at lower pH there was increased tailing and baseline disturbance when it was increased to 6 the peak shows good symmetry. UV detector response of ACB was studied and the best wavelength was found to be 274 nm showing highest sensitivity (Fig. 1). Development studies revealed that methanol: acetate buffer (20:80 v/v) pH adjusted to 6.0 with Glacial acetic acid at a flow rate of 0.85 ml/min, and column maintained at 50° C was suitable conditions for a stability indicating method. ACB was having retention time 4.1 min and degraded products of ACB were well separated.
Method Validation

The method was validated according to ICH guidelines. The following validation characteristics were addressed: linearity, range, accuracy, precision, specificity, sensitivity (LOQ and LOD) and robustness. Specificity of the method was determined by analyzing solutions containing drug product, excipients and stress degraded samples. All chromatograms were examined to determine if ACB and its stress degraded product co-eluted with each other or with any excipient peak. Peak purity of stressed samples of ACB was checked by using PDA detector. The purity angle within the purity threshold limit obtained in all stressed samples demonstrated the analyte peak homogeneity.

Linearity, Range and Method Sensitivity

The data of peak area versus drug concentration was treated by linear least square regression analysis. Correlation coefficient $> 0.999$, revealed an excellent correlation between peak areas and analyte concentrations. Low values of LOD and LOQ indicates sensitivity of method. Linearity, range and method sensitivity data is presented in Table 1.

Table 1: Sensitivity, linearity, precision, recovery and SST data of analytes.

<table>
<thead>
<tr>
<th>Parameter / Analytes ($t_R$)</th>
<th>ACB (4.1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration Range (µg/mL)</td>
<td>0.5 – 200</td>
</tr>
<tr>
<td>Regression equation ($Y = b \times$ Concentration $\pm$ a) data</td>
<td></td>
</tr>
<tr>
<td>Intercept (a) $\pm$ SD</td>
<td>15500 $\pm$ 12503.6</td>
</tr>
<tr>
<td>Slope (b) $\pm$ SD</td>
<td>17000 $\pm$ 1059.73</td>
</tr>
<tr>
<td>Correlation coefficient (r)</td>
<td>0.999</td>
</tr>
<tr>
<td>Method sensitivity (µg/mL)</td>
<td></td>
</tr>
<tr>
<td>Limit of Detection</td>
<td>0.134</td>
</tr>
<tr>
<td>Limit of Quantitation</td>
<td>0.276</td>
</tr>
</tbody>
</table>

System suitability Test (SST) Parameter

- Peak Area, % RSD: 1888145, 0.42
- No of theoretical plates (±SD): 3783.98 ± 36
- USP Tailing Factor (±SD): 1.237 ± 0.013
- Capacity Factor: 2.7

* $t_{cap} = 2.015$ ($p = 0.05$; df = 5), $a = $ standard deviation (SD) of intercept, $b = $ SD of slope $c = $ resolution with respect to ACB, $t_R =$ Retention time

Formulation Analysis and System Suitability

Commercial two capsule formulations as described in materials and reagents section were analysed by the proposed method. Assay for ACB was always in the range of 100 ± 2.1 % and % RSD was always <1.5. Formulation analysis and SST results are presented in Table 1.
Accuracy and Precision

Accuracy and precision were established for ACB. Results for the accuracy of analytes tested in drug products by the technique of standard addition ranged from 98.3 to 101.5%. Results of precision and accuracy are summarized in Table 1.

Robustness

The standard solution of 100% concentration was prepared and injected in triplicate for every condition and % RSD of assay was calculated and variability of retention time, number of theoretical plates were studied. Results of robustness study for flow rate, % of organic phase and pH of mobile phase are shown in Table 2.

Results of robustness study for ACB were within 100 ± 1.6% indicating robustness of the method. The degree of reproducibility of the results has proven that the method is robust (Table 2).

Table 2: Result of robustness Study

<table>
<thead>
<tr>
<th>Parameter (Limit)</th>
<th>Level</th>
<th>Analyte Name</th>
<th>SST Parameters (SD) n=3</th>
<th>% Assay, % RSD, n=3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate, (± 0.02mL/min)</td>
<td>(-) 0.83</td>
<td>ACB</td>
<td>4.22 ± 0.016, 3780± 29</td>
<td>98.89, 0.76</td>
</tr>
<tr>
<td></td>
<td>(+) 0.87</td>
<td>ACB</td>
<td>4.09 ± 0.032, 3765 ± 23</td>
<td>100.87, 0.94</td>
</tr>
<tr>
<td>% of Organic (± 5%)</td>
<td>(-) 14</td>
<td>ACB</td>
<td>4.17 ± 0.018, 3772 ± 37</td>
<td>100.2, 1.4</td>
</tr>
<tr>
<td></td>
<td>(+) 26</td>
<td>ACB</td>
<td>4.31± 0.028, 3769 ± 25</td>
<td>101.79, 1.36</td>
</tr>
<tr>
<td>pH of Mobile Phase (± 0.1)</td>
<td>(-) 5.90</td>
<td>ACB</td>
<td>3.72 ± 0.014, 3790 ± 42</td>
<td>100.2, 1.4</td>
</tr>
<tr>
<td></td>
<td>(+) 6.10</td>
<td>ACB</td>
<td>4.27 ± 0.022, 3787 ± 35</td>
<td>101.79, 1.36</td>
</tr>
<tr>
<td>Wavelength (± 1 nm)</td>
<td>(-) 272</td>
<td>ACB</td>
<td>4.25 ± 0.024, 3772 ±45</td>
<td>100.85, 1.23</td>
</tr>
<tr>
<td></td>
<td>(+) 276</td>
<td>ACB</td>
<td>3.85± 0.030, 3782 ± 38</td>
<td>101.23, 1.75</td>
</tr>
<tr>
<td>Buffer strength (± 5 mM)</td>
<td>(-) 20</td>
<td>ACB</td>
<td>4.30± 0.026, 3754 ± 25</td>
<td>100.65, 1.45</td>
</tr>
<tr>
<td></td>
<td>(+) 30</td>
<td>ACB</td>
<td>3.97± 0.016, 3777 ± 40</td>
<td>99.89, 1.24</td>
</tr>
<tr>
<td>Column temp. (± 2 °C)</td>
<td>(-) 48</td>
<td>ACB</td>
<td>4.17 ± 0.024, 3768 ± 32</td>
<td>101.15, 1.62</td>
</tr>
<tr>
<td></td>
<td>(+) 52</td>
<td>ACB</td>
<td>4.01 ± 0.015, 3765 ± 36</td>
<td>100.85, 1.57</td>
</tr>
</tbody>
</table>

$t_R$ = retention time, N= No. of theorotical plates

Solution Stability Studies

Solution stability as described in method development under experimental section was studied. Result of short-term, long-term and the auto sampler stability of the ACB solutions was calculated from nominal concentrations and found concentration. Results of the stability studies were in the range of 98–102%.

Table 3: Result of stress degradation study

<table>
<thead>
<tr>
<th>Analyte → Stress condition</th>
<th>$t_R$ of Degraded Product</th>
<th>% Recovery</th>
<th>Peak angle, Threshold*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 N HCl at 80 °C, 1.5 h</td>
<td>2.67</td>
<td>91.3</td>
<td>0.221, 0.271</td>
</tr>
<tr>
<td>0.1 N NaOH at 80 °C, 1 h</td>
<td>2.67</td>
<td>87.56</td>
<td>0.218, 0.256</td>
</tr>
<tr>
<td>30 % H2O2 at 80°C, 2 h</td>
<td>2.67</td>
<td>89.92</td>
<td>0.210, 0.232</td>
</tr>
<tr>
<td>Short and Long UV, 28 h</td>
<td>Not detected</td>
<td>94.51</td>
<td>0.205, 0.242</td>
</tr>
<tr>
<td>100°C in Oven, 8 h</td>
<td>Not detected</td>
<td>95.65</td>
<td>0.223, 0.265</td>
</tr>
</tbody>
</table>

Stability Indicating Study

Analytes and its stress degradation product were well separated. Although the conditions used for forced degradation were attenuated to achieve degradation in the range 10–30%, this could not be achieved for thermal, short UV, long UV and photolytic degradation even after prolonged exposure. The drug was extensively degraded by acid hydrolysis, alkaline hydrolysis and oxidative condition. Chromatograms of acid, alkali and oxidative degradation of ACB and combined formulation are shown in Fig.3, 4, and 5, respectively. Stress conditions used, $t_R$ of degraded product, extent of degradation and peak purity data for the analytes is presented.
in Table 3. Chromatographic peak purity data was obtained from the spectral analysis report. Purity angle values which were always less than purity threshold values is indicative of a homogeneous peak thus established the specificity of the assay.

![Fig. No.6-Densitogram of Acid treated Acebrophylline formulation](image1)

![Fig. No.7-Densitogram of Base treated Acebrophylline formulation](image2)

![Fig. No.8-Densitogram of H$_2$O$_2$ treated Acebrophylline formulation](image3)

**Dissolution**

Dissolution studies carried out as described previously and about 90% of analytes dissolved within 15 min.

**Conclusions**

The developed method was found to be simple, sensitive, accurate, precise and robust and can be used for the routine quality control analysis of ACB in bulk drug and marketed formulation. As the method could effectively separate the drugs from their degradation products, it can be used to perform stability indicating studies of ACB in commercial drug formulation.

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