DEVELOPMENT AND VALIDATION OF STABILITY INDICATING HPLC-UV METHOD FOR THE DETERMINATION OF AMPHOTERICIN B IN BULK AND DOSAGE FORM

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ABSTRACT

Objectives: To develop a simple, sensitive, precise and accurate reversed phase liquid chromatographic method for the estimation of amphotericin B from bulk dosage form.

Methods: Method was developed using a the Spheri-5, CYANO column (30 × 4.6 mm, 5 µm) column with a mobile phase consisting 10 mM sodium acetate buffer and acetonitrile (72:28, v/v with pH-4); at a flow rate of 1.2 mL min⁻¹. The UV detection was achieved at 408 nm, over a wide dynamic range of 0.039 to 40 µg mL⁻¹. The retention time of amphotericin B was 5.9 minutes.

Results: Method was successfully validated in accordance to ICH guidelines acceptance criteria for specificity, linearity, accuracy, precision, robustness, ruggedness and system suitability. Intra-day and inter-day assay accuracy and precision of the amphotericin B were less than 2%, and the average recovery were in the range of 98–102%. Amphotericin B was subjected to the stress conditions of oxidation, acid and base hydrolysis and thermolysis.

Conclusion: method was successfully applied for analysis of amphotericin B in the presence of excipients in commercially available ophthalmic and bulk dosage form.

Keywords: Amphotericin B, HPLC, Dosage form.

INTRODUCTION

Amphotericin B (AB) (Fig. 1), a macroyclic, polyene, antifungal antibiotic produced from a strain of Streptomyces nodosus and is considered as treatment of choice in fungal infection like keratitis due to Candida and Aspergillus species. AB is invariably fungistatic and occasionally fungicidal, depending on the concentration achieved in serum and the susceptibility of the pathogen's maximum activity is seen at a pH range from 6.0 to 7.5 [1]. AB has been administered by intravenous (i.v.), topical, intravitreal (i.t.v.), and intracameral (i.t.c.) routes for treatment of fungal keratitis due to Candida and Aspergillus species. AB is both heat labile and light sensitive; hence, the dry powder should be refrigerated and protected from light.

Therefore aim of the present work was to develop simple, precise and accurate RP-HPLC method for determination of AB in pharmaceutical and bulk dosage form. The established method was validated with respect to specificity, linearity, precision, accuracy and ruggedness. In addition, forced degradation studies were performed in order to prove the suitability of the method for the stability-indicating assay of AB.

MATERIALS AND METHODS

Chemicals and Materials

AB pure powder (Purity: 99.95%) of pharmaceutical grade was gifted by Cipla Ltd. (Mumbai, India). HPLC grade acetonitrile and methanol were from Sigma Aldrich Chemicals Pvt Ltd (Mumbai, India). Sodium acetate AR, glacial acetic acid AR, and ammonium solution (25%) were purchased from E. Merck Pvt Ltd (Mumbai, India). Ultrapure water was obtained from a MILLI-Q PLUS PF water purification system. AB suspension USP; 5% w/v (Amphotin®) Batch No RA1246103, manufactured by Health Biotech Limited, (Chandigarh, India) were purchased from local pharmacy within their shelf-life period.

Instrumentation and Chromatographic Conditions

The method was developed using a Waters HPLC system (Milford USA) consisted of a binary pump (model S15), auto sampler (model 717) and UV detector (model 2998). Separation was achieved on Spheri-5, CYANO column (30 × 4.6 mm, 5 µm) column with a mobile phase consisting of Acetonitrile: Sodium acetate buffer, pH 4 (28:72 v/v); at a flow rate of 1.2 mL min⁻¹. Detection was carried out with ultra-violet detector at 408 nm. Total run time was 8 min and volume of injection was 35 µL, prior to injection of analyte, the column was equilibrated for 30-40 min with mobile phase. Analysis was performed at ambient temperature. The data collection and analysis were performed using breeze · version 3.1 software.

Preparation of Standard Stock Solution and Calibration Curves

The stock solutions of 1.0 mg mL⁻¹ of AB were prepared in methanol. Calibration samples and quality control samples (QC) were prepared by diluting working standard solution with methanol to give concentrations in the range of 0.039–40 µg mL⁻¹ for AB. QC samples at four different concentrations (0.039, 0.312, 5 and 40 µg mL⁻¹) as LLOQ, low, medium and high, respectively were prepared.
separately in five replicates, independent of the calibration standards. Test samples and quality control samples were then interpolated from the calibration curve to obtain the concentrations of the respective analyte. Calibration curves were plotted as concentration of drugs versus peak area response.

Analysis of Marketed Formulation
Three replicates of the required dilutions were prepared from suspension stock solution (Amphotin-Vit®) and sonicated for 10 min. For analysis of suspension dosage form of amphotericin B was filtered through whatman filter paper.

Method Validation
The method was validated in compliance with ICH guidelines[3]: in terms of specificity, Selectivity, linearity, precision, accuracy, limit of quantification, limit of detection, robustness and other aspects of analytical validation.

Specificity and Selectivity
The specificity of the method was checked by comparing chromatograms obtained from standard, sample and the corresponding placebo. The selectivity of the method was established from the resolution of the drug peak from the nearest and also among all other peaks. The analytes with a resolution factor greater than 4.2 exhibited the selectivity of the method.

Linearity
The linearity of the method was determined at seven concentration levels ranging from 0.039 to 40 µg mL⁻¹ for AB. The calibration curves were established by plotting the peak area versus concentration. The regression parameters of slope, intercept and correlation coefficient were calculated by fitted to the y = mx + c using weighing factor (1/x²).

Precision and Accuracy
The precision (% RSD) and accuracy (% bias) mean relative error (RME, %) of this analytical method were determined using QC samples (n = 5) in five replicates of LLOQ, LOQ, QC, and HQC 0.034, 0.312, 5.0 and 40 µg mL⁻¹, respectively. The accuracy of each sample preparation was determined by injection of calibration samples and four QC samples in five replicate for 5 days. The criteria for acceptability of the data included accuracy within ± 2% standard deviation (SD) from nominal values and precision of within ± 2% relative standard deviation (RSD).

Recovery
The recovery of the method was assessed by adding known amounts of AB to commercial suspension containing a known amount of the drug (standard addition method). The average recovery was obtained in the range of 98-102% for all with %RSD below 2%. The recovery of the added pure drug was calculated as:

% Recovery = [(C_0-C_3)/C_0] × 100

where C_0 is the total amount of drug measured after standard addition, C_3 the amount of drug in the formulation, and C_0 the amount of drug added to the formulation.

Limit of Detection and Limit of Quantification
The limit of detection (LOD) and limit of quantification (LOQ) were calculated from the standard deviation of responses and slopes using signal-to-noise ratio as per ICH guidelines. This study was carried out to determine the limit of detection (LOD, S/N = 3) and limit of quantitation (LOQ, S/N = 10) in order to apply this method for the quantification of AB.

Robustness
Robustness of the method was studied by changing the extraction time of AB from dosage form by ± 5 min, composition of mobile phase by ± 2% of organic solvent, flow rate by ± 0.1 mL min⁻¹ and buffer pH by ± 0.2. The changes in the response of the analyte were noted.

System-Suitability Test
System suitability testing was done by CDER (Centre for drug and evaluation research ) guideline [12]. It is used to verify that the resolution and repeatability of the system were adequate for the analysis intended. The parameters used in this test were asymmetry of the chromatographic peak, peak resolution, tailing factor, theoretical plates and repeatability, as %RSD of peak area for replicate injections.

Forced Degradation Stability Studies
The forced degradation of the drug molecule can help to identify most likely degradation pathway and the intrinsic stability of the analyte. Specificity is the ability of method to measure the analyte response in presence of its potential impurities [13]. All forced degradation studies were performed at an initial drug concentration of 20 µg mL⁻¹. Acid hydrolysis was performed in 1.0 N HCl at 60 °C for 1 hr. The study in basic solution was carried out in 10 N NaOH at 60 °C for 1 hr. The oxidation studies were carried out at ambient temperature in 3% and 30% hydrogen peroxide (H₂O₂) for 1 hr. For study in neutral solution, the drug dissolved in water and kept at 60 °C temperature for 1 hr. Thermal degradation was performed at 60 °C for 7 days. Assessment of mass balance in the degraded samples was carried out to confirm the amount of impurities detected in stressed samples matches with the amount present before the stress was applied. Quantitative determination of AB was carried out in all the stressed samples against qualified working standard and the mass balance (% assay + % sum of all degradation products) was tabulated in Table 5.

Method Development
The chromatographic conditions were optimized with respect to specificity, resolution, and time of analysis. Hence we started the development activity with C₈ stationary phase of various manufacturers such as Zorbas, ODS (250 × 4.6 mm, 5 µm), Spherisorb ODS (250 × 4.6 mm, 5 µm) Symmetry shield C₈ (250 mm × 4.6 mm, 5 µm), and Phenomenex Luna C₈ (250 ×4.6 mm, 5 µm). The last two columns were found to be suitable. However, Symmetry shield C₈ (250 mm × 4.6 mm, 5.0 µ, column was used as a good resolution and minimum elution time were obtained. The stationary phase was not only the parameter which could give better resolution. Mobile phase, pH and organic modifiers also played very important role which leads to the best separation.

Different mobile phases containing acetonitrile, methanol, water and buffer were examined. Initially the methanol was used as an organic modifier which gives the poor baseline with baseline drift. Hence the response for the AB was reduced. To improve the resolution and response, acetonitrile was tried as an organic modifier. The base line was found good and response AB was improved. Effects of pH (3–7) and ionic strength (5–50 mM) were investigated using phosphate and acetate buffer and it was found that at higher and lower pH the tailing of the AB peak was more and resolution was also poor. The effect of buffer concentration on the retention of AB was also studied. The mobile phase containing acetonitrile: sodium acetate buffer (28: 72 v/v, pH 4), was selected as optimal for obtaining well-resolved peaks with acceptable system suitability parameters. Flow rates from 0.8 to 1.5 mL min⁻¹ were tested. Low flow rates led to an increase in retention time and the time of analysis. High flow rates led to a remarkable increase in column pressure and decrease in resolution. It was found that 1.2 mL min⁻¹ was optimal as it compromised between resolution and run time.

Effect of the wavelength on the response factor was observed over the wavelength range 303–420 nm. The detection wavelength, 408 nm was found optimal due to the high absorbivity at this wavelength for AB. Complete separation was achieved in < 8.0 min at ambient temperature (Fig 2). The average retention times ± RSD % for AB was found to be 5.9 ± 0.14 (n = 10).

Method Validation
Specificity
There was no cross interference from impurity, excipients or additives at the retention time of AB was found. Representative
chromatograms of blank and AB are shown in Fig. 2 & 3 respectively. The retention time of the analyte showed less variability with a relative standard deviation (R.S.D.) well within the acceptable limit of 5%.

![Fig. 2: Representative chromatogram of blank mobile phase](image1)

![Fig. 3: Representative chromatogram of Amphotericin B](image2)

**Linearity**

The calibration plot for the method was linear over the concentration in the range of 0.039 to 40 µg mL⁻¹ for AB. The correlation coefficient ($r^2$) of the regression was 0.9997. The best-fit linear equation obtained was $y = 100437.2x - 13335$. This result demonstrates linearity of this method over the specified range.

**Accuracy and Precision**

Accuracy and precision (intra- and inter-day) were calculated at four different concentration levels of LLOQ, LOQ, MQC and HQC ($n = 5$), for analyte on five days are presented in Table 1. The results showed that the analytical method is accurate, as the % bias is within the acceptance limits of ± 2.0% of the theoretical value. The precision
around the mean value was never greater than ± 2.0% at any of the concentrations studied.

**Recovery**
The Value of recovery (%), standard deviation and % coefficient of variance (% COV), indicating method accuracy, is listed in Table 2.

**Limit of Detection and Limit of Quantification**
The LOD for AB was found to be 0.025 µg/ml at a signal to noise ratio of 3:1, while the limit of quantification was 0.039 ng/ml.

**Robustness & System Suitability Test**
The method was found to be robust and the results were not significantly affected by slight variation in composition of mobile phase, extraction time, flow rate and buffer pH (data not shown). The SST measured from six replicate injections of AB was capacity factor, theoretical plates column efficiency and tailing factor and the results are tabulated in Table 3. For all six injections the tailing factor was less than 1.5.

**Stability**
The stability of AB was investigated thoroughly under auto-sampler storage and bench-top storage (Table 4). The results obtained were well within the acceptable limits. At 4 °C, AB showed no degradation for a 24 hr period.

**Analysis of AB Dosage Form**
The proposed validated RP-HPLC method was successfully applied to determine AB in marketed opthalmic suspension (AB). Three replicates of the required dilutions were prepared from suspension stock solution and sonicated for 10 min. These solutions (35 µL) were injected for quantitative analysis. The amounts of AB were calculated by extrapolating the peak area from the calibration plot. The mean percentage of AB were found to be 100.46 ± 0.028, which are comparable to the corresponding labeled amounts.

**Results of Forced Degradation Studies**

**Degradation in acidic solution**
The AB was exposed to 0.1 N HCl at 60 °C temperature for 1 h. The AB rapidly underwent degradation with time in acidic condition.

**Degradation in basic solution**
The AB was exposed to 0.1 N NaOH at 60 °C temperature for 1 h, prominent degradation was observed.

**Oxidative conditions**
The AB was exposed to 30% and 3% fresh hydrogen peroxide preparation. AB has shown significant sensitivity towards the treatment of 30% hydrogen peroxide. The AB rapidly underwent degradation with time in 30% hydrogen peroxide. The major degradation product was eluted with solvent front.

**Thermal degradation**
The AB powder was exposed to dry heat at 60 °C for 7 days and the AB solution was kept at room temp for 7 days. No significant degradation (~4%) was observed.

**Degradation in neutral (water) solution**
No degradation was observed after 1 h at 60 °C temperature.

### Table 1: The Accuracy (% bias) and precision (% R.S.D.) of Amphotericin B

<table>
<thead>
<tr>
<th>Concentration (ng/ml)</th>
<th>Amphotericin B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LLOQ</td>
</tr>
<tr>
<td>Theoretical</td>
<td>0.039 µg/mL</td>
</tr>
<tr>
<td>%Bias_{intra-assay}</td>
<td>-1.82</td>
</tr>
<tr>
<td>%Bias_{inter-assay}</td>
<td>1.23</td>
</tr>
<tr>
<td>% RSD_{intra-assay}</td>
<td>2.11</td>
</tr>
<tr>
<td>% RSD_{inter-assay}</td>
<td>1.92</td>
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</table>

### Table 2: The recovery of Amphotericin B.

<table>
<thead>
<tr>
<th>Conc. (µg/mL)</th>
<th>%Recovery ± S.D</th>
<th>% COV</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.039</td>
<td>97.87 ± 0.034</td>
<td>0.87</td>
</tr>
<tr>
<td>0.312</td>
<td>101.87 ± 0.001</td>
<td>0.379</td>
</tr>
<tr>
<td>5.0</td>
<td>99.18 ± 0.026</td>
<td>1.02</td>
</tr>
<tr>
<td>40</td>
<td>99.75 ± 0.216</td>
<td>1.08</td>
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### Table 3: System suitability study (SST)

<table>
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<tr>
<th>SST limits</th>
<th>CDER guidelines</th>
<th>Calculated</th>
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<tbody>
<tr>
<td>Repeatability of peak response ≤ 0% for 5 replicates</td>
<td>0.772</td>
<td></td>
</tr>
<tr>
<td>Resolution</td>
<td>&gt;2.0 general</td>
<td>5.44</td>
</tr>
<tr>
<td>Tailing factor</td>
<td>≤ 0.6</td>
<td>1.35</td>
</tr>
<tr>
<td>Column efficiency</td>
<td>&gt;2000 (plate count)</td>
<td>2594.74</td>
</tr>
<tr>
<td>Capacity factor</td>
<td>&gt;2</td>
<td>5.27</td>
</tr>
</tbody>
</table>

### Table 4: Bench top and auto sampler stability of Amphotericin

<table>
<thead>
<tr>
<th>Storage Conditions</th>
<th>Nominal Conc. (µg/ml)</th>
<th>Initial amount (µg/ml)</th>
<th>Amount after 20 hrs (µg/ml)</th>
<th>% deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Auto-sampler stability (4°C, 24 h)</td>
<td>0.312</td>
<td>0.318333</td>
<td>0.322667</td>
<td>-1.36126</td>
</tr>
<tr>
<td>2.5</td>
<td>0.312</td>
<td>2.462333</td>
<td>2.468</td>
<td>-0.23013</td>
</tr>
<tr>
<td>40</td>
<td>0.312</td>
<td>39.848</td>
<td>39.22267</td>
<td>3.150611</td>
</tr>
<tr>
<td>Bench-top stability for 20 hrs at ambient temp</td>
<td>0.312</td>
<td>39.848</td>
<td>39.22267</td>
<td>3.150611</td>
</tr>
<tr>
<td>5.0</td>
<td>0.312</td>
<td>5.10475</td>
<td>4.963</td>
<td>1.71</td>
</tr>
<tr>
<td>40</td>
<td>0.312</td>
<td>40.16433</td>
<td>40.45333</td>
<td>-1.33404</td>
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</tbody>
</table>

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Table 5: Forced degradation stability studies of Amphotericin B

<table>
<thead>
<tr>
<th>Stress condition</th>
<th>Time</th>
<th>% Assay</th>
<th>% degradation product</th>
<th>Mass balance (% assay + % degradation product)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid/0.1 N HCl/60°C</td>
<td>10 min</td>
<td>42.91</td>
<td>57.11</td>
<td>99.95</td>
</tr>
<tr>
<td>Base/0.1 N NaOH/60°C</td>
<td>10 min</td>
<td>5.0</td>
<td>95.61</td>
<td>99.46</td>
</tr>
<tr>
<td>Oxidation/3% H₂O₂/RT</td>
<td>60 min</td>
<td>96.074</td>
<td>3.72</td>
<td>99.23</td>
</tr>
<tr>
<td>Oxidation/30% H₂O₂/RT</td>
<td>60 min</td>
<td>8.89</td>
<td>91.11</td>
<td>99.95</td>
</tr>
<tr>
<td>Thermal degradation Powder</td>
<td>1 week</td>
<td>94.942</td>
<td>5.061</td>
<td>99.99</td>
</tr>
<tr>
<td>Water hydrolysis</td>
<td>60 min</td>
<td>94.691</td>
<td>5.27</td>
<td>99.98</td>
</tr>
</tbody>
</table>

CONCLUSION

The validated RP-HPLC method employed here proved to be simple, specific, accurate, precise, sensitive and robust. The validated method showed satisfactory data for all the validation parameters tested. The short retention time of 6.002 min allows the analysis of a large number of samples in a short period of time and is therefore more cost effective. The information presented here could be very useful for quality monitoring of bulk samples and as well as employed to check the quality of drug during stability studies in pharmaceutical formulations.

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REFERENCE

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