DERIVATIZATION OF NEOMYCIN SULPHATE AND AREA UNDER CURVE METHOD FOR ESTIMATION OF NEOMYCIN SULPHATE AND CLOBETASOL PROPIONATE IN CREAM

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ABSTRACT
Neomycin sulphate is official in IP 2018, USP 2004 and BP 2003; which includes Microbial assay (Cylinder Plate assay) for the estimation therefore chemical derivatization of Neomycin Sulphate is done using 1-Fluoro-2,4-Dinitrobenzene (DNFB); also known as Sanger’s reagent, to make it detectable by UV with good accuracy and precision. A fixed dose combination of Neomycin Sulphate, Clobetasol Propionate and Chlorocresol (preservative) in cream formulation is used to treat different types of Skin infections. The literature review reveals that methods like UV, HPLC, HPTLC, GC-MS etc. have been reported for Neomycin Sulphate and Clobetasol Propionate individually and along with other drugs. But no reported method found in combination for Neomycin sulphate and Clobetasol propionate. A simple, accurate, precise and economical UV spectrophotometric i.e. Area Under Curve Method (AUC) was developed. All the dilutions of drugs were prepared in Methanol: Acetonitrile (50:50). Area under curve was integrated in the wavelength range of 235.0-245.0 nm, 252.0-262.0 nm and 223.0-233.0 nm for CSP, NMS and CCS respectively. The AUC method for Clobetasol Propionate (CSP), Neomycin Sulphate (NMS) and Chlorocresol (CCS) was found to be linear over the range of 0.8-1.2 µg/ml, 8.0-12.0 µg/ml and 1.6-2.4 µg/ml respectively. The result of analysis was analysed and validated statistically and recovery study was found within range of 98-102%. The % RSD was not more than 2.0 % which indicates good precision. All the validation parameters were carried out according to ICH Q2 (R1) guidelines.

Keywords: Neomycin Sulphate, Clobetasol Propionate, Area Under Curve Method, 1-Fluoro-2, 4-Dinitrobenzene (DNFB), Sanger’s reagent.

1. INTRODUCTION
Neomycin sulphate is (2R,3S,4R,5R,6R)-5-amino-2-(aminomethyl)-6-[(1R,2R,3S,4R,6S)-4,6-diamino-2-[(2S,3R,4S,5R)-4-[(2R,3R,4R,5S,6S)-3-amino-6-(aminomethyl)-4,5-dihydroxy oxan-2-yl]oxy-3-hydroxy-5-(hydroxymethyl)oxolan-2-yl]oxy-3-hydroxycyclohexyl]oxyxane-3,4-diol; sulfuric acid an aminoglycoside antibiotic found in many topical medications such as creams, ointments, and eyedrops. It is wide range antibiotic used in many bacterial infections [1, 2]. Neomycin sulphate is official in IP 2018, USP 2004 and BP 2003; which includes Microbial assay (Cylinder Plate assay) for the estimation. Clobetasol Propionate is official in IP 2018, USP 2017 and BP 2003; which includes HPLC method for estimation, but this combination is not available in any pharmacopoeia [3-5].
1.1. Derivatization

Neomycin sulphate is estimated by Microbial assay (Cylinder Plate assay) as per pharmacopoeia which was time consuming and lengthy procedure with less sensitivity of method for quantification. Neomycin Sulphate molecule is lacking chromophore which is needed for quantitative estimation by UV therefore derivatised to a Chemical form which is quantifiable by UV.

Derivatization of Neomycin Sulphate was done by using 1-Fluoro-2, 4-Dinitrobenzene. After derivatization procedure, yellow powder of derivatized Neomycin Sulphate i.e. Neomycin Dinitrobenzene (NDB) was formed. In this chemical reaction the halogen atom of 1-Fluoro-2, 4-Dinitrobenzene is reactive and yellow coloured liquid compound is replaced by primary and secondary amines [6].

Fig. 2: UV Spectra of Neomycin Sulphate before derivatization

1.2. Area Curve Method (AUC)

The absorptivity values ($\varepsilon_1$ and $\varepsilon_2$) of each of the two drugs were determined at the selected wavelength range and AUC usually applicable when there is no sharp peak or when broad spectra are obtained. Total area under curve of a mixture at wavelength range is equal to the sum of area under the individual component at that wavelength range. This method is applicable when the $\lambda_{\text{max}}$ of the two components is reasonably dissimilar, the two components do not interact chemically and both the component must be soluble in same solvent. The methods deviated when overlapping of UV spectra of two drugs significantly and large difference in labelled strength [7-14].

Clobetasol Propionate is [(8S,9R,10S,11S,13S,14S, 16S, 17R)-17-(2-chloroacetyl)-9-fluoro-11-hydroxy-10,13, 16-trimethyl-3-oxo-6,7,8,11,12,14,15,16-octahydro- cyclopenta[a] phenanthren-17-yl] propanoate Gluco- corticoid used in treatment of to treat a variety of skin conditions like eczema, psoriasis, dermatitis, and allergies, rash [15,16].

Fig. 3: UV Spectra of NDB at 350.00 nm after derivatization

Fig. 4: Clobetasol Propionate

Fig. 5: Chlorocresol
Chlorocresol is 4-Chloro-3-methyl phenol is generally used as preservative in cream. According to literature review there are numbers of analytical methods available for estimation of both drugs either alone or with other drugs. UV spectrophotometric methods, HPLC methods, HPTLC method, LC-MS method are available for NMS, CSP and CCS in combination with other drugs but no methods were found on this combination. Individual drugs are available in Pharmacopoeia but this combination is not available in any pharmacopoeia. So the aim of the present research work is to develop simple, accurate and precise method for routine analysis [16-24].

2. MATERIAL AND METHODS

2.1. Instrument
A double beam UV/Visible spectrophotometer (Shimadzu-1800, Software-UV Probe, Version 2.42) having matched quartz cells of light path 1 cm. Spectra were automatically obtained by UV-Probe system software. An Electronic analytical balance REPTECH-0.1 mg Sensitivity, an Ultrasonicator (Athena Technology) was used in the study.

2.2. Material
Neomycin Sulphate was received as Gift sample from Daiwik Pharmashphere, Vapi, Clobetasol Propionate was received as Gift sample from Avik Pharmaceutical Ltd., Vapi, Chlorocresol was received as Gift sample from Oxford Laboratories Pvt. Ltd., Vapi, Methanol (UV Grade-Thomas Baker, Mumbai), Acetonitrile (UV Grade -Tomas Baker, Mumbai), 1-Fluoro 2, 4-Dinitrobenzene (SRL, Vapi) were used.

2.3. Methodology

2.3.1. Preparation of Standard Stock Solution
The standard stock solution of NDB was prepared by dissolving 10 mg of powder in 10 ml of Methanol: Acetonitrile (50:50) in 10 ml of volumetric flask (1000 µg/ml). For CSP, 5 mg of API was weighed in 25 ml of volumetric flask, dissolved with Methanol (100µg/ml). For CCS, 5 mg of API was weighed in 25 ml of volumetric flask, dissolved with Methanol (200µg/ml).

2.3.2. Preparation of Working Standard Solution
Accurately pipetted out from Standard stock solutions of NDB, CSP, CCS were transferred into 100 ml of volumetric flask separately and prepared 8, 9, 10, 11, 12 ppm, 0.8, 0.9, 1.0, 1.1, 1.2 ppm and 1.6, 1.8, 2.0, 2.2, 2.4 ppm with methanol. The resultant solution would be NDB (100 µg/ml), CSP (10 µg/ml) and CCS (20 µg/ml).

2.3.3. Selection of wavelength range for estimation of NDB, CSP and CCS
Appropriate dilution were prepared for NDB (10 µg/ml), CSP (1 µg/ml) and CCS (2 µg/ml) from the working standard solution and the solution were scanned in the wavelength range of 200-400 nm. For NDB, the absorption spectra obtained were showing the absorption maxima ($\lambda_{\text{max}}$) at 257 nm and Area Under Curve (AUC) in absorption spectra were measured between the wavelength range 252.00 to 262.00 nm where good linear area was found in selected concentration range. For CSP, the absorption spectra obtained were showing the absorption maxima ($\lambda_{\text{max}}$) at 240 nm and Area Under Curve (AUC) in absorption spectra were measured between the wavelength range 235.00 to 245.00 nm where good linear area was found in selected concentration range. For CCS, The absorption spectra obtained were showing the absorption maxima ($\lambda_{\text{max}}$) at 228.00 nm and Area Under Curve (AUC) in absorption spectra were measured between the wavelength range 223.00 to 233.00 nm where good linear area was found in selected concentration range.

2.3.4. Preparation of Calibration curve
Calibration curve for NDB consisted of five different concentrations of standard solution ranging from 8.0-12.0 µg/ml. The solution was prepared by pipetting out 4.0, 4.5, 5.0, 5.5 and 6.0 ml of working stock solution of NDB (100 µg/ml) into series of 50 ml of volumetric flasks and the volume was made up to mark with methanol. Each solution was scanned against methanol as blank and corresponding spectra was recorded. Calibration curve for CSP consisted of five different concentrations of standard solution ranging from 0.8-1.2 µg/ml. The solution was prepared by pipetting out 4.0, 4.5, 5.0, 5.5 and 6.0 ml of working stock solution of CSP (10 µg/ml) into series of 50 ml of volumetric flasks and the volume was made up to mark with methanol. Each solution was scanned against methanol as blank and corresponding spectra was recorded. Calibration curve for CCS consisted of five different concentrations of standard solution ranging from 1.6-2.4 µg/ml. The solution was prepared by pipetting out 4.0, 4.5, 5.0, 5.5 and 6.0 ml of working stock solution of CSP (20 µg/ml) into series of 50 ml of volumetric flasks and the volume
was made up to mark with methanol. Each solution was scanned against methanol as blank and corresponding spectra was recorded. Absorbance at 252.00-262.00 nm, 235.00-245.00 nm and 223.00-233.00 nm was measured and the plot of absorbance vs. concentration was plotted and the straight - line equation was determined for NDB, CSP and CCS accordingly.

2.3.5. Analysis of marketed formulation
Accurately weighed 5gm Cream in 100 ml volumetric flask and 50 ml tris buffer was added for dispersion of Neomycin sulphate and stirred for 15 to 20 minutes then added 25 ml of Methanol for dispersion of CSP and CCS and again stirred for 10 minutes. The solution was centrifuged for 3 minutes, filtered with whatman paper no. 42. The resultant solution would be CSP 25 µg/ml, CCS 50 µg/ml and NDB 250 µg/ml. This solution undergoes derivatization procedure in water bath for 45 minutes at 100°C, and allowed to cool at room temperature. Finally the volume was made up with mixture of Methanol: ACN (50:50).

3. RESULTS
3.1. Validation of Proposed method
3.1.1. Linearity
The linearity response was determined by analysing 5 independent levels of calibration curve in the range of 0.8-1.2 µg/ml for CSP, 1.6-2.4 µg/ml for CCS and 8.0-12.0 µg/ml for NDB (n=5). The calibration curve of absorbance vs. concentration was plotted and correlation coefficient and regression line equations for CSP, CCS and NDB were shown in figs. 6-8.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Mean AUC ± S.D. (n=5)</th>
<th>% R.S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>2.91 ± 0.0071</td>
<td>0.2455</td>
</tr>
<tr>
<td>45</td>
<td>3.261 ± 0.0051</td>
<td>0.1563</td>
</tr>
<tr>
<td>50</td>
<td>3.679 ± 0.0088</td>
<td>0.2353</td>
</tr>
<tr>
<td>55</td>
<td>4.05 ± 0.0097</td>
<td>0.2406</td>
</tr>
<tr>
<td>60</td>
<td>4.419 ± 0.0049</td>
<td>0.1126</td>
</tr>
</tbody>
</table>

Table 1: Linearity data of NDB at 252.00-260.00 nm

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Mean AUC ± S.D. (n=5)</th>
<th>% R.S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0</td>
<td>0.2523 ± 0.00037</td>
<td>0.1466</td>
</tr>
<tr>
<td>4.5</td>
<td>0.3344 ± 0.00089</td>
<td>0.2674</td>
</tr>
<tr>
<td>5.0</td>
<td>0.4160 ± 0.00141</td>
<td>0.3399</td>
</tr>
<tr>
<td>5.5</td>
<td>0.5054 ± 0.00055</td>
<td>0.1083</td>
</tr>
<tr>
<td>6.0</td>
<td>0.5932 ± 0.00045</td>
<td>0.1753</td>
</tr>
</tbody>
</table>

Table 2: Linearity data for CSP at 235.00 - 245.00 nm

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Mean AUC ± S.D. (n=5)</th>
<th>% R.S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.76x - 0.1347</td>
<td>0.9994</td>
<td></td>
</tr>
<tr>
<td>0.76x - 0.1347</td>
<td>0.9994</td>
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<tr>
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<td></td>
</tr>
<tr>
<td>0.76x - 0.1347</td>
<td>0.9994</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 6: Calibration curve NDB at 252.00-262.00 nm

Fig. 7: Calibration Curve for CSP at 235.00 - 245.00 nm

Fig. 8: Calibration curve CCS at 223.00-233.00 nm
Table 3: Linearity data for CCS at 223.00-233.00 nm

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Mean AUC ± S.D. (n=5)</th>
<th>% R.S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>0.9728 ± 0.0052</td>
<td>0.5361</td>
</tr>
<tr>
<td>9</td>
<td>1.3136 ± 0.0080</td>
<td>0.6128</td>
</tr>
<tr>
<td>10</td>
<td>1.6048 ± 0.0017</td>
<td>0.1114</td>
</tr>
<tr>
<td>11</td>
<td>1.9274 ± 0.00564</td>
<td>0.2925</td>
</tr>
<tr>
<td>12</td>
<td>2.2474 ± 0.0120</td>
<td>0.5372</td>
</tr>
</tbody>
</table>

3.1.2. Precision

3.1.2.1. Repeatability
Aliquots of 5 ml of working solution of CSP (10 µg/ml), CCS (20 µg/ml) and NDB (100 µg/ml) were taken into three separate series of 50 ml volumetric flask and volume was made up to mark with Methanol to give a solution containing 1.0 µg/ml of CSP, 2.0 µg/ml of CCS and 10.0 µg/ml of NDB. Solution was analysed six times (n=6) and % R.S.D. was calculated and shown in table 4.

3.1.2.2. Intraday Precision
Aliquots of 4.5, 5.0 and 5.5 ml of working solution of CSP (10 µg/ml), CCS (20 µg/ml) and NDB (100 µg/ml) were taken into series of 50 ml of volumetric flask. Using methanol, volume was made up to the mark to give solution containing 0.9, 1.0 and 1.1 µg/ml of CSP, 1.8, 2.0 and 2.2 of CCS and 9.0, 10.0 and 11.0 of NDB. Solution was analysed for three times (n=3) on the same day within short interval of time and % R.S.D. was calculated and shown in table 4.

Table 4: Summary of Validation Parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>NDB</th>
<th>CSP</th>
<th>CCS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wavelength (nm)</td>
<td>257 ± 5 nm</td>
<td>240 ± 5 nm</td>
<td>228 ± 5 nm</td>
</tr>
<tr>
<td>Linearity (n=5)</td>
<td>0.9994</td>
<td>0.9996</td>
<td>0.9996</td>
</tr>
<tr>
<td>Range</td>
<td>8.0 - 12.0 µg/ml</td>
<td>0.8 - 1.2 µg/ml</td>
<td>1.6 - 2.4 µg/ml</td>
</tr>
<tr>
<td>Accuracy (n=3)</td>
<td>99.533 - 100.530</td>
<td>99.733 - 100.655</td>
<td>99.866 - 100.515</td>
</tr>
<tr>
<td>Repeatability (n=3)</td>
<td>0.1486</td>
<td>0.2186</td>
<td>0.2283</td>
</tr>
<tr>
<td>Intraday Precision (n=3)</td>
<td>0.4314 - 0.4298</td>
<td>0.5982 - 0.5608</td>
<td>0.6637 - 0.6875</td>
</tr>
<tr>
<td>Interday Precision (n=3)</td>
<td>0.7436 - 0.7431</td>
<td>0.7905 - 0.7032</td>
<td>0.7888 - 0.7789</td>
</tr>
<tr>
<td>LOD (µg/ml)</td>
<td>0.1401</td>
<td>0.0096</td>
<td>0.0493</td>
</tr>
<tr>
<td>LOQ (µg/ml)</td>
<td>0.4247</td>
<td>0.0291</td>
<td>0.1495</td>
</tr>
<tr>
<td>Assay (%) ± S.D. (n=5)</td>
<td>99.8941 ± 0.4201</td>
<td>99.889 ± 0.3869</td>
<td>100.1092 ± 0.7124</td>
</tr>
</tbody>
</table>

3.1.2.3. Interday Precision
Aliquots of 4.5, 5.0 and 5.5 ml of working solution of CSP (10 µg/ml), CCS (20 µg/ml) and NDB (100 µg/ml) were taken into series of 50 ml of volumetric flask. Using methanol, volume was made up to the mark to give solution containing 0.9, 1.0 and 1.1 µg/ml of CSP, 1.8, 2.0 and 2.2 of CCS and 9.0, 10.0 and 11.0 of NDB. Solution was analysed for three times (n=3) on three different days and % R.S.D. was calculated and shown in table 4.

3.1.3. Accuracy
Accurately weighed 5 gm Cream was taken in in 100 ml volumetric flask and 50 ml Tris buffer was added for dispersion of Neomycin sulphate followed by stirring for 15 to 20 minutes and addition of 25x ml of Methanol for dispersion of CSP and CCS, stirred again for 10 minutes. The solution was centrifuged for 3 minutes, filtered the liquid with whatman paper no. 42. The resultant solution would be CSP 25 µg/ml, CCS 50 µg/ml and NDB 250 µg/ml. The solution undergoes derivatization procedure in water bath for 45 minutes at 100°C. Allowed to cool the solution at room temperature. Finally made up the volume with mixture of Methanol: ACN (50:50). Thus, this derivative solution would be Solution A for further accuracy study.

Each solution was scanned from 200-400 nm against methanol as a blank. Absorbance of solution was measured at selected wavelengths for CSP, CCS and NDB. The amount of CSP, CCS and NDB was calculated at each level (0%, 80%, 100%, 120%) and % recoveries were calculated and shown in table 4.

3.1.4. LOD and LOQ
The LOD (Limit of Detection) and LOQ (Limit of Quantitation) were estimated from the set of 5 calibration curves that were used to determine linearity of the method. The LOD and LOQ was calculated (shown in table 4) by using formula:
LOD = 3.3 x S.D. / Slope
LOQ = 10 × (S.D./Slope)
Where, S.D. = Standard deviation of the Y - intercepts of 5 calibration curves, Slope = Mean slope of 5 calibration curves

4. CONCLUSION
Derivatization of Neomycin sulphate was performed and achieved successfully and it was found to be easy and can be performed individually or in combination with other drugs, as this agent (DNFB) does not affect other drugs of the combination. The simple Area under curve method for simultaneous estimation of Neomycin Sulphate, Clobetasol Propionate and Chlorocresol in combined dosage form was estimated, developed and validated successfully. The method was found to satisfy the criteria of ICH Q2R1. Thus, this method can be utilized for routine analysis for simultaneous estimation of Neomycin Sulphate, Clobetasol Propionate and Chlorocresol in semisolid dosage formulation.

Conflict of interest
None declared

Source of funding
None declared

5. REFERENCES