Method development and Validation of Reverse Phase HPLC Method for the Determination of Dithranol in Pharmaceutical Ointment Forms

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A simple and rapid reverse phase high performance liquid chromatography (RP-HPLC) method was developed and validated for quantitative determination of dithranol in Ointments. Dithranol was analyzed by using reverse phase Phenomenex C18 column (4.6mm x 25cm, 5microns) with mobile phase consisting of acetonitrile: water: TFA (60:40:0.05%) v/v. The flow rate was set 1.2ml/min and the analysis was performed at wavelength 254nm using Photo Diode Array (PDA) detector at 25°C temperature. The method was validated and stability studies were conducted under different conditions. The retention time for dithranol was around 10.2minutes. The developed method was successfully applied to estimate the amount of dithranol in ointment formulations and it is the stability indicating method.

Key words: RP-HPLC; PDA; Dithranol

INTRODUCTION

Dithranol (1, 8, 9-trihydroxyanthracene) accumulates in mitochondria where it interferes with the supply of energy to the cell, probably by the oxidation of dithranol releasing free radicals. This impedes DNA replication and so slows the excessive cell division that occurs in psoriatic plaques. Dithranol was used successfully by Ingram in 1953 (1) to treat chronic plaque psoriasis with the introduction of short contact therapy (SCT) by Schaefer et al. (2). In addition Dithranol may act by reducing the elevated levels of cGMP that occurs in psoriasis. Dithranol has a slower onset of action in controlling psoriasis, typically several weeks, compared to glucocorticoid steroids, but is without the potential for rebound reaction on withdrawal. It cannot be used on the face or genitalia. Very few instances of contact allergic reactions to dithranol have been reported. However, transient primary irritation of uninvolved skin surrounding the treated lesions is more frequently seen and may occasionally be severe. Some temporary discoloration of finger and hair nails may arise during the period of treatment but should be minimized by careful application. Dithranol may stain skin, hair or fabrics. Staining of fabrics may be permanent, so contact should be avoided.

MATERIALS AND METHODS

Chemicals and reagents:
Dithranol standard (Standardized known purity of Dithranol) Acetonitrile (HPLC Grade Rankem) Water (Milli-Q, Millipore), Tri Fluoro Acetic acid (Extra Pure Biochemical grade), Hydrochloric acid (GR grade, Merck), Sodium hydroxide (Pure, Merck), Hydrogen peroxide (Pure, Merck).

Instrumentation:
The HPLC system consisted of a Shimadzu equipped with solvent delivery module in a quaternary gradient mode and PDA detector. Data acquisition was performed by LC solution software. Analysis was carried out at 254nm with a reversed phase phenomenon C18 column (250x4.6mm, 5μm) at 25°C temperature with mobile phase consisting of acetonitril: water: TFA (60:40:0.05% v/v). The mobile phase was degassed and filtered through 0.45μm membrane filter before pumping into HPLC system. Injection volume 5ul, detection at 254nm, run time 40mins and 0.5% TFA in acetonitrile as the diluents.

Preparation of drug stock solution:
Weighed 5.09mg of Dithranol drug substance in to 10ml volumetric flask and dissolved in diluent. This solution was injected onto the chromatographic system.

Method Validation
System suitability:
The system suitability was assessed by replicate analysis of six injections of the drug at a concentration of 0.5 mg/ml. The acceptance criterion was ±2% for the %RSD for the peak area and 1% retention times for dithranol. The number of theoretical plates should not be less than 2500 and the tailing factor should not be more than 2.0. (3, 4, 13)

Linearity (Calibration curve):
The calibration curve was constructed with 5 concentrations ranging from 0.25 to 0.75 mg/ml. The peak area ratio of the drug was considered for plotting the linearity graph. The linearity was evaluated by linear regression analysis, which was calculated by the least square regression method (3, 4).

Accuracy and precision:
Accuracy of the method was carried out by recovery experiments. Quality control sample Solutions of 75, 100 and 125% containing the excipients used in the formulations were tested and the recovery was calculated in each of the case using the regression line equation developed under the linearity experiment. Demonstration of precision was done under two categories. The injection reproducibility was assessed by using 6 injections of the standard solution for dithranol and the relative standard deviation of the replicate injection was calculated. (3, 4)

Determination of limit of detection & limit of quantitation:
A series of dilute solutions were prepared in the range of 0.01%, 0.03%,0.06% and 0.09% of the assay concentration (0.5mg / ml) using the standard solutions. Equal volumes (5 μl) of each of the above solutions were injected in 6times and the areas were calculated due to dithranol peak.
The standard deviation for the 6 injections for each concentration was calculated. Based on the data obtained, the standard deviation at concentration 0 was calculated and this value was used for the calculation of the Limit of Detection and Limit of Quantitation. The limits of detection (LOD) and quantification (LOQ) were calculated using the following formulae:

\[
\text{LOD} = (3.3 \sigma / S) \quad \text{and} \quad \text{LOQ} = (10 \sigma / S)
\]

Where, \(\sigma\) is the standard deviation of the response and \(S\) is the slope of the regression line (3, 4).

**Specificity (Forced degradation) and stability:**

The specificity of the method was demonstrated through forced degradation studies conducted on the sample using Acid, Alkaline, Oxidative, and Photolytic degradations. The sample was exposed to these conditions and the main peak was studied for the peak purity (dithranol peak), thus indicating that the method effectively separated the degradation products from the dithranol active ingredient. (14, 15)

**Preparation of Drug stock solution:**

Accurately weighed 62.56mg of Dithranol was transferred into 100ml volumetric flask and volume was made up to the mark with diluents (conc. 0.5013mg/ml)

**Acid degradation:**

8ml of the drug stock solution was transferred into a 10ml volumetric flask and the volume was made up to the mark with 0.5N HCl. Initial sample was taken in to a HPLC vial and remaining solution was stored in a 40°C stability cabinet and periodically samples were withdrawn from stability chamber and transferred in to a HPLC vial and analyzed.

**Alkaline degradation:**

8ml of the drug stock solution was transferred into a 10ml volumetric flask and the volume was made up to the mark with 0.05N NaOH. Initial sample was taken in to a HPLC vial and remaining solution was stored in a 40°C stability cabinet and periodically samples were withdrawn from stability chamber and transferred in to a HPLC vial and analyzed.

**Oxidative degradation:**

8ml of the drug stock solution was transferred into a 10ml volumetric flask and the volume was made up to the mark with 3%H2O2. Initial sample was taken in to a HPLC vial and remaining solution was stored in a 40°C stability cabinet and periodically samples were withdrawn from stability chamber and transferred in to a HPLC vial and analyzed.

**Hydrolysis degradation:**

8ml of the drug stock solution was transferred into a 10ml volumetric flask and the volume was made up to the mark with HPLC water. Initial sample was taken in to a HPLC vial and remaining solution was stored in a 40°C stability cabinet and periodically samples were withdrawn from stability chamber and transferred in to a HPLC vial and analyzed.

**Valued Reverse Phase HPLC Method**

**Robustness and ruggedness:**

The robustness/ruggedness of the method was demonstrated through the study of the following variations.

1. Column to Column Variation
2. Day to Day Variation
3. Analyst to Analyst Variation

The above three parameters Viz., Column, Day and Analyst were studied through a matrix design involving the estimation on two different days using two different columns and 2 different analysts with a total of 4 determinations.

Under each of the conditions, samples were analyzed including a duplicate injection for each estimate (12, 13).

**RESULTS AND DISCUSSION**

**Method development and optimization:**

Dithranol hydrochloride is freely soluble Diluent: 0.05%TFA in Acetonitrile. The drug can be separated on a phenomenex column as phenomenex column is slightly polar and it has unique selectivity for polar compounds in both reversed and normal phase modes. The optimization of the method development was done by fixing one variable constant and changing the other variables among mobile phase composition, flow rate. The peak shape and symmetry were found to be good when a mobile phase composition at 25°C temperature with mobile phase consisting of acetonitril: water: TFA (60:40:0.05 v/v). The mobile phase was degassed and filtered through 0.45μm membrane filter before pumping into HPLC system. Flow rate observed good at1.2 ml/min and run time is 40 min. (5, 6, 7, 8) the concentration of the drug was calculated and is presented in the Table.8 With the optimized chromatographic conditions, a steady baseline was recorded. The retention times of Dithranol are around 10.2min respectively with a total run time of approx 40min. A typical chromatogram of sample solution is given in figure-1. The assay procedure was repeated for six times and mean peak area ratio and mean weight of standard drugs were calculated. The percentage of drug found in formulation, mean values were calculated. The results of analysis show that the amount of drugs was in good agreement with the label claim of the formulation. The method was validated as per ICH guidelines (6). The accuracy of the method was determined by recovery experiments. The recovery studies were carried out six times and the percentage recovery were calculated and presented in table 3. From the data obtained, added recoveries of standard drug were found to be accurate. The precision of the method was demonstrated by inter day and intraday variation studies. In the intraday studies, six repeated injections of standard and sample solutions were made and the response factor of drug peaks and percentage RSD were calculated. From the data obtained, the developed HPLC method was found to be precise.

**Method Validation**

**System suitability:**

The % RSD of peak area and retention time for the drug is within 2% indicating the Suitability of the system (Table 1). The efficiency of the column as expressed by number of theoretical plates for the 6 replicate injections was 7912 (mean %RSD) and the USP tailing factor was 1.38.

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Table 1. System suitability study of dithranol Injection

<table>
<thead>
<tr>
<th>Sample name</th>
<th>Sample ID</th>
<th>Retention time (min)</th>
<th>Theoretical Plates</th>
<th>Area (WHSV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dithranol Injection_1</td>
<td>10.12</td>
<td>6995</td>
<td>5843515</td>
<td></td>
</tr>
<tr>
<td>Dithranol Injection_2</td>
<td>10.12</td>
<td>7046</td>
<td>5857901</td>
<td></td>
</tr>
<tr>
<td>Dithranol Injection_3</td>
<td>10.12</td>
<td>7184</td>
<td>5856099</td>
<td></td>
</tr>
<tr>
<td>Dithranol Injection_4</td>
<td>10.11</td>
<td>7234</td>
<td>5842305</td>
<td></td>
</tr>
<tr>
<td>Dithranol Injection_5</td>
<td>10.09</td>
<td>7314</td>
<td>5825982</td>
<td></td>
</tr>
<tr>
<td>Dithranol Injection_6</td>
<td>10.08</td>
<td>7375</td>
<td>5841613</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>10.11</td>
<td>7192</td>
<td>5844549</td>
<td></td>
</tr>
</tbody>
</table>

Linearity:
The calibration curve constructed was evaluated by its correlation coefficient. The peak area of the drug was linear in the range of 50% to 150%. The average areas for each of the Concentration obtained was plotted against the concentration of the analyte. The correlation Coefficient for the data was calculated as 0.9999 for Dithranol indicating a strong correlation between the concentration and the area under the curve. A linear regression graph was drawn between the Concentration of the analyte and the areas. The regression line was observed to be $y = 1E+08x - 277454$ for Dithranol. Regression analysis was done at confidence level of 0.05 (5%) using the t-statistic. The results revealed that there was a strong correlation between the amount added and amount found (Table 3).

Accuracy- Results of recovery experiments

Table 3. Accuracy- Results of recovery experiments

<table>
<thead>
<tr>
<th>Spike level</th>
<th>Amount added (conc. mg/ml)</th>
<th>Area response</th>
<th>Amount found (conc. mg/ml)</th>
<th>% of recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>75%</td>
<td>0.375</td>
<td>4438965</td>
<td>0.3786</td>
<td>100.420</td>
</tr>
<tr>
<td>75%</td>
<td>0.375</td>
<td>4456890</td>
<td>0.3801</td>
<td>100.826</td>
</tr>
<tr>
<td>75%</td>
<td>0.375</td>
<td>4430545</td>
<td>0.3779</td>
<td>100.230</td>
</tr>
<tr>
<td>100%</td>
<td>0.502</td>
<td>5756895</td>
<td>0.5047</td>
<td>100.537</td>
</tr>
<tr>
<td>100%</td>
<td>0.502</td>
<td>5769594</td>
<td>0.5058</td>
<td>100.748</td>
</tr>
<tr>
<td>100%</td>
<td>0.502</td>
<td>5796658</td>
<td>0.5081</td>
<td>101.214</td>
</tr>
<tr>
<td>125%</td>
<td>0.628</td>
<td>7265986</td>
<td>0.630</td>
<td>100.285</td>
</tr>
<tr>
<td>125%</td>
<td>0.628</td>
<td>7259847</td>
<td>0.629</td>
<td>100.201</td>
</tr>
<tr>
<td>125%</td>
<td>0.628</td>
<td>7242531</td>
<td>0.628</td>
<td>99.962</td>
</tr>
</tbody>
</table>

Mean: 100.495

% of RSD: 0.382

Calibration curve equation values:
Slope : 11469400
Intercept : 46263.5
R^2 value : 0.9997

Determination of limit of detection & limit of quantitation (Sensitivity):
The results indicated that the method was sensitive enough to detect a concentration of 0.000129 mg/ml and able to quantify at a concentration of above 0.0004257 mg/ml

Table 4 Raw Data for Dithranol

<table>
<thead>
<tr>
<th>Level (w.r.t working conc.)</th>
<th>Conc. mg/ml</th>
<th>Area SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.03%</td>
<td>0.0001510</td>
<td>1762</td>
</tr>
<tr>
<td>0.06%</td>
<td>0.0003021</td>
<td>3512</td>
</tr>
<tr>
<td>0.09%</td>
<td>0.0004532</td>
<td>5322</td>
</tr>
</tbody>
</table>

Accuracy:
Accuracy of the method was carried out by recovery experiments. Drug standard solutions Of 75, 100 and 125% containing the excipients used in the formulations were tested and the Recovery was calculated in each of the case using the regression line equation developed Under the Linearity experiment. A regression line Graph was drawn using the amount added on the x-axis and the amount found on the y-axis. The slope and intercept were calculated for the regression line (Method of Least Squares), and hypothesis was tested for the correlation between the amount added and amount found, at confidence level of 0.05 (5%), Using the t-statistic. The results revealed that there was a strong correlation between the amount added and amount found (Table 3).
Specificity (Forced degradation) and stability:
The specificity of the method was demonstrated through mobile phase and forced degradation studies conducted on the sample using acid, alkaline, oxidative, reductive and photolytic degradations (Table 4). Under each of the conditions, the chromatogram was studied using PDA detector. Using the chromatographic software, the Purity Angle and Purity Threshold were calculated.

<table>
<thead>
<tr>
<th>sample name</th>
<th>Area %</th>
<th>Days</th>
<th>Peak purity index</th>
<th>Single point threshold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid degradation</td>
<td>96.74</td>
<td>2</td>
<td>1.000000</td>
<td>0.99997</td>
</tr>
<tr>
<td>Base degradation</td>
<td>77.01</td>
<td>Initial</td>
<td>1.000000</td>
<td>0.99997</td>
</tr>
<tr>
<td>Peroxide degradation</td>
<td>87.14</td>
<td>6</td>
<td>1.000000</td>
<td>0.99997</td>
</tr>
<tr>
<td>Hydrolysis</td>
<td>95.9</td>
<td>5</td>
<td>1.000000</td>
<td>0.99997</td>
</tr>
</tbody>
</table>

There was peak at the retention time of Dithranol in acidic, photolytic and oxidative degradations, while there was some degradation of the Dithranol under reductive and alkaline conditions. The Dithranol peak was tested for the peak purity using the chromatographic software. The purity threshold and the purity angle were estimated using the software and compared. In each of the cases, it was observed that the purity threshold was higher than the purity angle indicating that the peak observed was pure except in cases of reductive and alkaline degradations. However, the degradation products in these two conditions did not interfere with the Dithranol peak indicating that the method was specific for the estimation of Dithranol in the product under examination (Fig 3). Stability studies indicated that the samples were stable when kept at bench top for 12 hours (short-term), in auto-sampler for 24 hours. The results of these stability studies were given in Table 5, where the percent change was within the acceptance range of 99-101%.
Figure 3. Chromatogram of Dithranol Oxidation Degradation (0.5mg/ml)

Table 7. Stability Data for Dithranol, Description Area % Change

<table>
<thead>
<tr>
<th>Time period</th>
<th>Dithranol area</th>
<th>% of initial</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>5719197</td>
<td>100</td>
</tr>
<tr>
<td>40 C - 24hrs</td>
<td>5716035</td>
<td>99.94</td>
</tr>
<tr>
<td>40 C - 48hrs</td>
<td>5742149</td>
<td>100.4</td>
</tr>
</tbody>
</table>

Drug Product Recovery
Preparation of sample:
The formulation product ointment containing 1.5% Dithranol strength. From this sample 1.0 gram was weighed into 25 ml vol flask, dissolved and make up to the mark with diluent. This was taken for recovery analysis.

Table 8

<table>
<thead>
<tr>
<th>Drug</th>
<th>Amount mg/gm(1.5% strength)</th>
<th>%Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Labeled</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dithranol</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15.0</td>
<td>15.285</td>
</tr>
</tbody>
</table>

Robustness and ruggedness:
The three parameters Column, Day and Analyst were studied through a matrix design involving the estimation on two different days using two different columns with a total of 4 determinations. Under each of the conditions, samples were analyzed including a duplicate injection for each estimate and the assay content of the analyte was estimated. It can be observed from the results that the values are well within acceptance limits of 98-102%, with a RSD of less than 2.0%. Above experiments indicated that the method is rugged and provides consistent and reliable results.

CONCLUSION
A rapid, specific isocratic HPLC method has been developed for the determination of Dithranol using a HPLC with PDA detector. The method was validated for accuracy, linearity, specificity & stability, limit of detection & limit of quantization, robustness & ruggedness. The method uses a simple mobile phase composition, easy to prepare with little or no variation. The rapid run time of 40 min and the relatively low flow rate allows the analysis of large number of samples with less mobile phase that proves to be cost-effective and this method is useful for both assay and impurity methods. Efficient UV detection at 254 nm was found to be suitable without any interference from injectable solution excipient or solvents. The calibration curves were linear (r²=0.999) over a concentration range from 0.25 to 0.75 mg/ml. The relative standard deviation’s (R.S.D.) were <0.38% and average recovery was above 100.49%. Limit of detection (LOD) and Limit of quantification (LOQ) were 0.000129 mg/ml and 0.0004257mg/ml respectively. Stability experiments indicated that the solutions prepared were stable for a period of 5 days and the results obtained in this period were reliable and alkaline was degrading initially. The proposed HPLC method is fast, precise, accurate, sensitive, and efficient and can be used in routine analysis in quality control laboratories. The solution stability was found remain unchanged for 48 hrs.
REFERENCES


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