A Validated Stability-Indicating Liquid Chromatographic Method for the Determination of Retapamulin in Topical Dosage Form

Santaji Nalwade1,2* and Vangala Ranga Reddy2

1Department of Chemistry, Jawaharlal Nehru Technological University, Kukatpally, Hyderabad 500 072, A.P., India and 2Analytical Research and Development, Integrated Product Development, Dr. Reddy’s Laboratories, Ltd., Bachupally, Hyderabad-500 072, India

*Author to whom correspondence should be addressed. E-mail: santajin@rediffmail.com

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A sensitive, stability-indicating reversed-phase high-performance liquid chromatographic method is developed and validated for the quantitative determination of retapamulin in topical dosage form. The chromatographic separation is achieved on a C18 reversed phase (XTerra RP 18 250 × 4.6 mm, 5 µm) at 30°C. The mobile phase comprises a mixture of 0.05M potassium dihydrogen phosphate buffer (pH 6.1), acetonitrile and methanol in the ratio of 35:50:15 (v/v/v). The flow rate is set at 1.0 mL/min and chromatograms are extracted at 243 nm using a photodiode array detector. The method is validated with respect to linearity, accuracy, precision, robustness and forced degradation studies, which further prove the stability-indicating supremacy of the method. During forced degradation studies, retapamulin is observed to be liable to oxidative and base hydrolysis stress and stable in thermal, photolytic and acid hydrolysis stress. The degradation products are well separated from the retapamulin peak, thus proving the stability-indicating superiority of the method. The method is found to be sensitive for retapamulin, with a detection limit of 25 ng/mL and a quantification limit of 80 ng/mL. The proposed method is found to be very sensitive and accurate for the determination of retapamulin in topical dosage form. The method is also demonstrated to be robust, because it is resistant to small variations of chromatographic variables such as pH, mobile phase composition, flow rate and column temperature.

Introduction

Retapamulin (3a,4R,5S,6β,9R,9aR,10R)-6-ethenyl-5-hydroxy-4,6,9,10-tetramethyl-1-oxodecalhydro-3a,9-propano-3H-cyclopenta [8]annulen-8-yl[(1R,3S,5S)-8-methyl-8-azabicyclo [3.2.1]oct-3-yl] sulfonyl acetate) belongs to a class of topical pleuromutilin antibiotics and is available in the form of ointment for the treatment of bacterial skin infections. Retapamulin ointment is as effective as oral cephalexin for the treatment of secondary infected dermatitis (1). Retapamulin has a good safety profile and is effective against causative pathogens such as Staphylococcus aureus and Streptococcus pyogenes (2, 3). Retapamulin is not substantially effective against Gram-negative organisms (4). Retapamulin is a commercially available 1% (w/w) ointment with the brand names Retarel (Ajanta Pharmaceuticals, India) and Altargo (GSK Pharmaceuticals, London, UK) containing white soft paraffin and butylated hydroxytoluene as inactive ingredients.

Stability testing plays an important role in the process of drug product development. The purpose of stability testing is to provide evidence about how the quality of a drug substance or product varies with time under a variety of environmental conditions, such as temperature, humidity and light. Stability testing is useful for the determination of the shelf life of drug products and for monitoring degradation products generated during stability studies. According to the International Conference on Harmonization (ICH) guideline Q1A (R2) on the stability testing of new drug substances and products, the stability testing of a new drug substance or product should be conducted under different stress conditions to validate the stability-indicating superiority of the analytical methods used for the analysis of stability samples (5). In addition, the need for stability studies on a drug candidate arises because the chemical integrity of the drug substance should be maintained until the compound is delivered to the intended site of action. Furthermore, a stability-indicating assay provides assurance for the detection of changes in the identity and purity potency of the product.

Currently, retapamulin is not officially found in any pharmacopoeia and no chromatographic method has been reported for the determination of retapamulin in ointment or in any other formulation. The present work describes an analytical method that is stability-indicating, rapid, simple, robust and economical for the determination of retapamulin in ointment. The method is useful for routine analysis in quality control laboratories because of its short run time and low cost.

Experimental

Materials and reagents

Retapamulin, 1% (w/w) ointment, was purchased from an Indian market (Retarel ointment by Ajanta Pharma, No. S226; Altargo ointment by GSK Pharma, Lot No. C547701). Potassium dihydrogen orthophosphate, orthophosphoric acid, high-performance liquid chromatography (HPLC)-grade acetonitrile, methanol, sodium hydroxide, hydrochloric acid and hydrogen peroxide were obtained from Merck (Mumbai, India). Double distilled water was used throughout the experiment.

Equipment

An Agilent HPLC system (1100 series) was equipped with an online degasser, quaternary pump, auto-injector, column compartment and photodiode array (PDA) detector. The chromatographic separation was achieved on a C18 reversed phase column (250 × 4.6 mm, 5 µm; Waters XTerra RP 18), which was operated at 30°C. The detector was set at 243 nm. The data acquisition was performed on Empower software. A water bath...
equipped with an MV controller (Julabo, Seelbach, Germany) was used for hydrolysis studies. Photostability studies were conducted in a photostability chamber (Sanyo, Leicestershire, UK). Thermal stability studies were performed in a dry air oven (Mack Pharmatech, Hyderabad, India).

**Chromatographic conditions**

The method was developed by using a C18 reversed-phase column. The mobile phase was prepared by mixing 0.05M potassium dihydrogen phosphate buffer (pH 6.1), acetonitrile and methanol in the ratio of 35:50:15 (v/v/v). The mobile phase was pumped at a flow rate of 1.0 mL/min. The eluted compound, retapamulin, was monitored at 243 nm. The column temperature was maintained at 30°C. The injection volume for samples and standards was 20 µL. All samples were analyzed by using an HPLC method after filtration using 0.45 µm nylon membrane filters (Millipore, Bangalore, India).

**Preparation of standard solution**

A solution containing 200 µg/mL of retapamulin was prepared in the mobile phase. This standard solution was also used as the system suitability solution.

**Preparation of sample solution**

An accurately weighed ointment sample equivalent to 80 mg of retapamulin was transferred into a 100 mL volumetric flask, to which 75 mL of methanol was added. This was heated in a water bath at 60°C for 30 min and sonicated for approximately 20 min to dissolve. The solution was allowed to cool at room temperature and methanol was added to obtain a volume of 100 mL. From that solution, the working solution of 200 µg/mL was prepared in the mobile phase.

**Validation of the method**

The proposed method was validated as per ICH guidelines (6).

**System suitability test**

System suitability parameters must be checked to ensure that the system is working correctly during the analysis. Parameters such as tailing factor (must be < 1.5), theoretical plate counts (should be > 4,000) and retention times were used for the system suitability evaluation.

**Specificity**

Specificity is the ability of the method to measure the response of the analyte in the presence of excipient matrix and its potential degradants. Stress studies were performed for retapamulin ointment to provide an indication of the stability-indicating properties and specificity of the proposed method. Intentional degradation was attempted to determine the stress conditions (heat (105°C); photolytic sunlight for approximately 1.2 million lux hours and ultraviolet (UV) light at shorter and longer wavelengths for approximately 200 Wh/m²; acid (5N HCl), base (5N NaOH); relative humidity (RH) (40°C/75% RH) and oxidation (30% H2O2)) to evaluate the ability of the proposed method to separate the excipient peaks and major degradation products from retapamulin and its degradation products. For the acid, base and oxidation tests, the study period was 30 min, and for heat it was 12 h. The peak purity test was conducted for the retapamulin peak in the stressed samples by using the PDA in the wavelength range of 200–400 nm. The specificity of the method was also checked by injecting a solution containing the excipient without drug substances.

**Precision**

The repeatability studies were performed by analyzing six test samples solutions of retapamulin ointment during the same day and under the same experimental conditions. Intermediate precision was evaluated by analyzing six test solutions on different days. The percentage assays for retapamulin were determined and compared. The results were expressed as relative standard deviation (RSD) of assay values in percentage.

**Limits of quantitation and detection**

The limit of detection (LOD) was estimated as three times the signal to noise ratio and the limit of quantification (LOQ) was estimated as 10 times the signal to noise ratio. LOD and LOQ were achieved by injecting a series of dilute solutions of retapamulin. The precision of the method was performed by analyzing six test solutions prepared at LOQ (80 ng/mL) and LOD (25 ng/mL) levels and determined the percentage RSD of the peak area.

**Linearity**

The calibration curve of retapamulin was prepared to establish the linearity. The peak area of each concentration was plotted against the corresponding concentration to obtain the calibration graph; the plots were subjected to a linear regression analysis.

**Accuracy**

The accuracy of the method was assessed by analyzing spiked retapamulin samples using independent working standard solutions. The retapamulin was spiked into three different concentration levels, corresponding to 50, 100 and 150% of the target analyte concentration (200 µg/mL). The samples were prepared in triplicate at each level and then analyzed by using the proposed method. The percentage recovery of the added drug at each level was calculated against the corresponding working standard.

**Range**

The range of an analytical method is the interval between the upper and lower levels (including these levels) that have been determined with precision, accuracy and linearity using the proposed method. The range is normally expressed in the same units as the test results obtained by the analytical method.

The range of the proposed method was validated by verifying that the analytical method provides acceptable precision, accuracy and linearity when applied to samples containing the analyte at the extremes of the range and within the range, as per the ICH requirements (80 to 120% of target concentration).

**Robustness**

To prove the reliability of the analytical method during normal usage, some small but deliberate changes were made in the analytical method. The flow rate was changed by 0.2 units, from...
0.8 to 1.2 mL/min. The column temperature was changed by 5°C, from 25 to 35°C. The pH of the buffer solution was changed by 0.2 units, from 5.9 to 6.3. The organic composition of the mobile phase was changed from 90 to 110% of the composition for both methanol and acetonitrile. The system suitability parameters (i.e., theoretical plates and tailing factor) were evaluated for the studies.

Solution stability
Injections of the standard and sample solutions were performed after they were stored at room temperature, protected from light and under refrigerated conditions over time in tightly packed containers. The stability of the drug solution was evaluated by calculating the similarity factor between the freshly prepared drug solution and the stored drug solution. The stability of the sample solution was evaluated by calculating the percentage assay against the freshly prepared standard. The study was conducted for each 24 h interval up to 48 h.

Results and Discussion
Method development and optimization
The primary objective for the development of an HPLC method for the determination of retapamulin was that the method should be able to determine the drug with a run time under 10 min. The method should be sensitive, stability-indicating, free of interference from degradation products and straightforward enough for routine use in quality control testing. During optimization of chromatographic conditions, different mobile phase compositions, HPLC columns, flow rates and organic modifiers such as acetonitrile and methanol were tested to achieve acceptable system suitability parameters. The optimum selected wavelength was 243 nm because of the high sensitivity of retapamulin at this wavelength (Figure 1).

Effect of stationary phase
Reversed-phase columns are silica-based bonded phases, and the C8-type bonded phase is most frequently used. On an XTerra RP 8 150 × 4.6 mm, 5 μm column, retapamulin was eluted with a solvent front peak (Table I; Figure 2A). The XTerra RP 18 250 × 4.6 mm, 5 μm column and pH of the mobile phase at 6.1 provided good retention and symmetrical peak shape of the analyte. The flow rate of the mobile phase was changed to obtain the expected retention time of the analyte.

Figure 1. UV spectra of retapamulin.
Effect of the organic modifier

Potassium dihydrogen phosphate (0.05M, pH 6.1) was used as the buffer for the mobile phase preparation. Because of the less aqueous solubility of retapamulin and the presence of soft paraffin in the sample, the maximum of organic modifiers were used to wash out the nonpolar components and the column for better column life. Different combinations of buffer, acetonitrile and methanol were tried. It was observed that methanol in combination with a buffer leads to more retention of retapamulin peaks. An increase in the organic modifier volume in the mobile phase produces a reduced retention time of retapamulin peak (Table I; Figures 2B–2D). During fine tuning of the method, a mobile phase combination of buffer–acetonitrile–methanol in the proportion of 35:50:15, v/v/v, provided a symmetric peak shape, an acceptable tailing factor and a short run time up to 10 min.

Effect of mobile phase buffer pH

The chromatographic performance was found to be poor toward acidic pH of the buffer and it does not show satisfactory performance at pH 6.8. Different trials were run at pH 3.0, 4.0, 5.0 and 6.8 of the mobile phase buffer. The retention time was not altered, with marginal differences, but the peak performance (tailing factor and theoretical plates) varied with different pH values. Some of the selective trials are included in Table I and Figures 2E and 2F.

Table II

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (min)</th>
<th>Theoretical plates</th>
<th>Tailing factor</th>
<th>RSD* (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retapamulin</td>
<td>5.0</td>
<td>7,685</td>
<td>1.02</td>
<td>0.23</td>
</tr>
</tbody>
</table>

*Six determinations of standard solution.
Optimization of sample solution preparation

A critical task in the development of the method was the sample solution preparation from the ointment formulation. Paraffin is the primary excipient of this formulation. The extraction of active ingredient from the paraffin is difficult. Paraffin is practically insoluble in water and alcohol and is freely soluble in ether and petroleum solvents. It melts on heating and reprecipitates on cooling. Therefore, the first trial was conducted using solvent extraction; diethyl ether and 2.0 pH water were used as solvents. Paraffin is soluble in diethyl ether and retapamulin was insoluble in 2.0 pH water, but reproducibility was the concern with this method (the recovery varied from 89.4 to 97.3%; \( n = 3 \)). The primary objective in the selection of solvent was to achieve 100% extraction of retapamulin from ointment by keeping the paraffin un-dissolved. By considering this, different solvents like ethanol, methanol, and acetonitrile were used for the extraction of the sample. The literature shows that the paraffin is slightly soluble in absolute ethanol. Therefore, methanol was finally used as the solvent for the sample stock solution preparation. Because paraffin starts melting from 40 to 60°C, the sample solutions were heated at different temperatures of 40, 50 and 60°C for a time interval of 30 min to 1 h. Heating the sample in 70% of methanol at 60°C for a time interval of 30 min, followed by 20 min of sonication and cooling to room temperature to resolidify the paraffin, produced reproducible results.

Validation of the Method

System suitability

System suitability tests were used to verify that the proposed method was able to produce acceptable results with high reproducibility. System suitability was analyzed in terms of tailing.

Table III

<table>
<thead>
<tr>
<th>Stress conditions</th>
<th>Net degradation (%)</th>
<th>Purity angle</th>
<th>Purity threshold</th>
<th>Purity flag</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid hydrolysis: 5N HCl/60°C/30 min</td>
<td>Stable 0.5%</td>
<td>0.231</td>
<td>0.354</td>
<td>No</td>
</tr>
<tr>
<td>Base hydrolysis: 5N NaOH/60°C/30 min</td>
<td>8.2</td>
<td>0.454</td>
<td>0.654</td>
<td>No</td>
</tr>
<tr>
<td>Peroxide oxidation: 30% H2O2/ambient/30 min</td>
<td>15.6</td>
<td>0.124</td>
<td>0.421</td>
<td>No</td>
</tr>
<tr>
<td>Photolytic: sunlight*</td>
<td>Stable</td>
<td>0.190</td>
<td>0.325</td>
<td>No</td>
</tr>
<tr>
<td>Heat stress: 105°C/12 h Heat Stress</td>
<td>2.0</td>
<td>0.435</td>
<td>0.524</td>
<td>No</td>
</tr>
<tr>
<td>Humidity stress: 40°C/75% RH</td>
<td>Stable</td>
<td>0.124</td>
<td>0.546</td>
<td>No</td>
</tr>
</tbody>
</table>

* 1.2 million lux hours/200 watt hours/square meter.
factor (must be <1.5), theoretical plate counts (should be >4,000) and retention time. The results for the proposed HPLC method are given in Table II. According to the results, the proposed HPLC method fulfills these requirements within the accepted limits. A standard chromatogram is depicted in Figure 3.

Specificity
All forced degradation samples were analyzed at an initial concentration of retapamulin with previously described HPLC conditions, using a PDA detector to ensure the homogeneity and purity of the retapamulin peak. During forced degradation studies, retapamulin was observed to be labile to oxidative and base hydrolysis stress.

Figure 4. Typical chromatograms of forced degradation studies of retapamulin under optimized method conditions: acid hydrolysis test (A); base hydrolysis test (B); peroxide oxidation test (C).
and stable in thermal, photolytic and acid hydrolysis stress. The degradation was performed on the drug product and placebo (without active ingredient). The chromatogram of the placebo (without active ingredient) showed no interference at the retention time of the analyte peak. The results from the forced degradation study are presented in Table III. The chromatograms of the forced degradation study are depicted in Figure 4.

**Precision**
The RSD for the assay values of retapamulin are presented in Table IV. The RSD for the precision and intermediate precision study was found to be less than 1% (it should be less than 2.0%), confirming the precision of the method. A sample chromatogram is depicted in Figure 3.

**LOD and LOQ**
The LOD and the LOQ for the retapamulin peak are reported in Table IV. The precision for the retapamulin peak at LOQ concentration was found to be less than 5% RSD (it should be less than 10.0%). The LOD and LOQ values are presented in Table IV.

**Linearity**
The result shows that an excellent correlation existed between the peak area and the analyte concentration. A linear calibration plot for the assay method was obtained over the calibration ranges tested; i.e., LOQ to 150% of the targeted level of the assay concentration (80 ng/mL to 300 μg/mL). The calibration graphs were obtained by plotting peak area ratios against the concentration of the drugs. The correlation coefficient was greater than 0.999 (Table IV). These results showed that an excellent correlation existed between the peak area and the concentration.

**Accuracy**
The percentage recovery of retapamulin in ointment varied from 99.8 to 100.8 (it should be 98.0 to 102.0%) and the RSDs of three samples at each level were found to be less than 0.4% (it should be less than 2.0%) at 50, 100 and 150% of target analyte concentrations. The recovery values for retapamulin are presented in Table V.

**Range**
From the results of the linearity, precision and accuracy studies presented in Tables IV and V, the working range of the proposed analytical method is from 50 to 150% of the target analyte concentration, which meets ICH requirements (80 to 120% of target analyte concentration).

**Robustness**
In all deliberately varied chromatographic conditions (flow rate, column temperature, pH of the buffer solution and organic composition), the chromatogram for the system suitability solution showed satisfactory results with no significant changes in system suitability parameters. The system suitability values are presented in Table VI.

**Stability in analytical solution**
The standard and sample solutions were found to be stable for approximately 48 h at room temperature. The similarity factor between freshly prepared standard and the 48 h standard solution was found to be 0.99 (it should be 0.98 to 1.02). The percentage difference between the 48 h sample and the initial sample assay was 0.9% (it should not be more than ± 2.0%).

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**Table IV**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Retapamulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOD (ng/mL)</td>
<td>25</td>
</tr>
<tr>
<td>LOQ (ng/mL)</td>
<td>80</td>
</tr>
<tr>
<td>Precision at LOQ (RSD; %)</td>
<td>4.3</td>
</tr>
<tr>
<td>Regression equation (y)</td>
<td>Slope (b) 321561.09</td>
</tr>
<tr>
<td></td>
<td>Intercept (a) 267.87</td>
</tr>
<tr>
<td>Correlation coefficient</td>
<td>0.9997</td>
</tr>
<tr>
<td>Precision (RSD and assay; %)</td>
<td>0.8 and 100.2</td>
</tr>
<tr>
<td>Intermediate precision (RSD and assay; %)</td>
<td>0.6 and 99.9</td>
</tr>
</tbody>
</table>

*Note: Linearity range is LOQ – 150% with respect to test concentration.
1Six determinations.
2Average of six determinations.

**Table V**

<table>
<thead>
<tr>
<th>Amount spiked (%)</th>
<th>Added (μg/mL)</th>
<th>Found (μg/mL)</th>
<th>Recovery (%)</th>
<th>RSD (%)</th>
<th>Mean recovery (%)</th>
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</thead>
<tbody>
<tr>
<td>50</td>
<td>100</td>
<td>99.9</td>
<td>99.9</td>
<td>0.2</td>
<td>100.0</td>
</tr>
<tr>
<td>50</td>
<td>100</td>
<td>100.2</td>
<td>100.2</td>
<td>0.2</td>
<td>100.5</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>99.8</td>
<td>99.8</td>
<td>0.2</td>
<td>100.1</td>
</tr>
<tr>
<td>100</td>
<td>200</td>
<td>201.1</td>
<td>100.6</td>
<td>0.2</td>
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<tr>
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<td>200</td>
<td>201.5</td>
<td>100.8</td>
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</tr>
<tr>
<td>150</td>
<td>200</td>
<td>200.3</td>
<td>100.2</td>
<td>0.1</td>
<td>100.2</td>
</tr>
<tr>
<td>150</td>
<td>300</td>
<td>300.2</td>
<td>100.1</td>
<td>0.2</td>
<td>100.2</td>
</tr>
<tr>
<td>150</td>
<td>300</td>
<td>300.6</td>
<td>100.2</td>
<td>0.1</td>
<td>100.2</td>
</tr>
</tbody>
</table>

*Note: Accuracy range is 50-150% with respect to test concentration.
1Amount of retapamulin spiked with respect to 100% test concentration of retapamulin.
2RSD (%) of the three determinations at each level.
3Mean recovery for three determinations.
Commercial Application

By using the optimized method, the commercial samples Retarel ointment (1%, w/w) and Altargo ointment (1%, w/w) were analyzed. The assay values were 99.5% ($n = 3$; 99.2, 99.5 and 99.9%) for Retarel ointment and 100.2% ($n = 3$; 100.1, 99.7 and 100.7%) for Altargo ointment. The results from the commercial samples proved the application of the proposed method for the analysis of commercial samples.

Conclusion

A reproducible isocratic reversed-phase HPLC method was developed for the quantitative analysis of retapamulin in pharmaceutical dosage forms. The proposed method is specific, accurate and precise for the determination of retapamulin from its pharmaceutical dosage form. The method was validated as per ICH guidelines and the results showed the stability-indicating superiority of the method. Hence, the method is suitable for the routine analysis and quality control of pharmaceutical preparations containing retapamulin as the active pharmaceutical ingredient.

References

6. ICH, Q2 (R1); Validation of analytical procedures: Text and methodology. International Conference on Harmonization, (2005).