Research Article

Design of Experiment Centered Multivariate Optimization Strategy for RP-HPLC Method to Quantitate Retapamulin in Topical Cream and Microemulsion

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Abstract

Background: Retapamulin is the first pleuromutilin antibacterial approved for the treatment of impetigo. The objective of the current research was to utilize the design of experiments approach for development and optimization of robust RP-HPLC method for the quantitation of Retapamulin in marketed cream and in-house developed microemulsion based formulations with an oily matrix.

Methods: The impact of various chromatographic conditions (independent variables) was assessed using Plackett–Burman design on critical analytical attributes (response) to screen initial experimental conditions. The Box-Behnken design was employed to optimize the selected chromatographic factors on the responses. Further, validation of optimized RP-HPLC was carried out as per the ICHQ2(R1) guideline.

Results: Pareto ranking analysis showed that % organic phase, flow rate, and volume of injection were found statistically significant (p < 0.05) variables influencing the retention time, number of plates, and tailing of the Retapamulin peak. The optimized RP-HPLC method with the stationary phase, C18 (250 mm × 4.6 mm, 5 µm) column, and mobile phase as a mixture of methanol and potassium dihydrogen orthophosphate buffer (50 mM, pH 7.0, 90:10 % v/v, isocratic), the flow rate of 1.0 mL/min, 10 µL injection volume, 25°C column oven temperature, 247 nm as detection wavelength, was successfully validated based on ICHQ2(R1) guideline.

Conclusion: RP-HPLC method was successfully used to separate (retention time 4.34 ± 0.2 min) and assay Retapamulin in microemulsion and marketed cream. The outcomes of the investigation exhibited the effective application of a multivariant approach in the optimization of the RP-HPLC for routine analysis of Retapamulin.

Introduction

Retapamulin (RTPL) is the first pleuromutilin antibacterial (a potent inhibitor of bacterial protein synthesis) approved for the treatment and management of impetigo, secondarily infected traumatic lesions, and secondarily infected dermatitis. RTPL is marketed as topical cream and ointment for the treatment of impetigo. The literature review revealed that RTPL is not official in any pharmacopoeia. Additionally, literature unveils only a single research article describing the liquid chromatographic method for the quantitative estimation of RTPL in ointment. Recently, high performance thin-layer chromatographic (HPTLC) method for estimation of RTPL in cream and microemulsion formulations was reported from our laboratory. RTPL was well separated (Rf = 0.60 ± 0.02) on the HPTLC plate (ALUGRAM Xtra SIL G/UV 254) using optimized chromatographic conditions. Further, the literature survey disclosed no single Design of Experiments (DoE) supported RP-HPLC method for assay of RTPL in cream and microemulsion based formulation matrix. Therefore, an attempt was made to design, develop, and optimize new robust RP-HPLC method, considering the application of the DoE approach to overcome reproducibility issues related to the performance of the analytical method at various product development stages. Rational selection of optimal chromatographic conditions is the major challenge in the development of any RP-HPLC method. The multivariate approach is cost-effective, efficient, and requires a less number of experimental runs. Consequently, this can be a more realistic and meaningful approach in the optimization of the separation method instead of using the empirical approach of changing...
"one factor at a time." Frequently, a large number of experimental runs are involved in the development of RP-HPLC methods, and in most situations, further, development is required during the scale-up phase, which ultimately slowdowns the overall drug development process. Accordingly, the DoE approach assists towards screening of critical chromatographic variables and enables to understand the impact of individual variable and their inter-relationship on selected-response during optimization of robust RP-HPLC method. Therefore, designing an appropriate experimental strategy improves the control of any sources of variability. Additionally, it also enables to utilize necessary strategies to improve overall HPLC method performance.

The current study elaborates on the utilization of the DoE approach for the optimization of a robust RP-HPLC method for the quantitation of RTPL in topical formulations (cream and microemulsions). Plackett–Burman design (PBD) was utilized to evaluate and screen chromatographic factors, namely pH of the mobile phase, % organic phase, flow rate, injection volume, column oven temperature and detection wavelength considering their significant impact on the peak retention time ($T_R$), peak tailing ($T_w$), and the number of plates ($N$) of RTPL. Considering the flexibility, and for better understanding of the effect of factors (individually as well as inter-relationship), Box-Behnken experimental design (BBD) was chosen and executed for optimization of the selected RP-HPLC conditions. The proposed RP-HPLC method offers trouble-free sample preparation options and a cost-effective tool for routine analysis of RTPL in quality control laboratories.

Materials and Methods

Materials
The reference standard of RTPL (99.90 % w/w) was obtained from Ajanta Pharma Ltd., Mumbai, (India). Retarel (1% w/w RTPL) cream, Ajanta Pharma Ltd., India, Batch No GV001, SV386 was purchased from the local market. Topical microemulsion formulation containing RTPL (1% w/w) was prepared in-house (unpublished data). Potassium dihydrogen phosphate, methanol, acetonitrile, and chloroform were procured from E. Merck KGaA (Darmstadt, Germany).

Instruments
Instruments used for the development of HPLC methodology were as per the following details; digital analytical balance, (ML 204/A01, Mettler Toledo, USA) HPLC Systems, LC2010CHT and LC2010AT Prominence, (Shimadzu, USA), ultrasonic bath (FS-6, Frontline, India); water purification system, (Q-5UVS, Millipore, USA), digital pH meter, (FEP20, Mettler Toledo, USA); HPLC column, C18, 100A, 250 mm x 4.6 mm, 5 μm (Luna, Lot no- 608807-25, Phenomenex, India).

Table 1. Factors and their levels for Plackett–Burman screening design for optimization of proposed HPLC method of Retapamulin.

| Factor                        | Lower level | Upper level |
|-------------------------------|-------------|-------------|
| A. pH of Mobile phase         | 6.5         | 7.5         |
| B. % Organic phase            | 75          | 95          |
| C. Flow rate                  | 0.5 mL/min  | 1.5 mL/min  |
| D. Injection volume           | 10 µL       | 20 µL       |
| E. Column oven temperature    | 25 °C       | 35 °C       |
| F. Wavelength                 | 245 nm      | 249 nm      |

Standard and test samples (Cream and microemulsion)

(A) 10 mg of RTPL standard was dissolved in 5 mL of methanol and volume made up with methanol up to 10 mL to give a standard stock solution of strength 1000 µg/mL of RTPL. (B) 1g of cream (equivalent to 10 mg of RTPL) was dissolved in the 5 mL mixture of chloroform and methanol (1:1 v/v) and volume made up with methanol up to 100 mL to give the final sample solution with strength 100 µg/mL of RTPL. (C) 1g quantity of microemulsion (equivalent to 10 mg of RTPL) was dissolved in the 10 mL methanol and volume made up with methanol up to 100 mL to give the final sample solution with strength 100 µg/mL of RTPL.

Screening of chromatographic factors using Plackett–Burman design

PBD was applied to study and evaluate the influence of chromatographic factors, namely pH of mobile phase (A), the concentration of organic phase (B), flow rate (C), injection volume (D), column oven temperature (E) and detection wavelength (F) on the $T_R$, $T_w$ and $N$ of RTPL. A total of 15 trials was carried out including 3 center points to identify which factors have greater effects on dependent variables. The selected chromatographic (independent) factors and implementation of PBD for their screening purpose are as shown in Tables 1 and 2, respectively.

Optimization of RP-HPLC method using Box Behnken design

DoE concept was applied to study and understand the influence of a multivariate approach in the optimization of the RP-HPLC chromatographic factors. For the optimization of the RP-HPLC method based on the DoE approach, response surface design is the best choice. Considering, flexibility and simplicity, BBD was chosen and executed for optimization of selected experimental conditions based on outcomes of the initial screening of factors by PBD. Outcomes of screening design suggested
DOE Supported RP-HPLC for Quantitation of Retapamulin

**Table 2.** Matrix and execution of Plackett-Burman screening design for optimization of proposed HPLC method of Retapamulin.

| Experimental | pH | Organic phase (%) | Flow rate (mL/min) | Injection volume (µL) | Column oven temperature (°C) | Detection wavelength (nm) | Retention time (T<sub>R</sub>) (min) | Number of theoretical Plates (N) | Tailing factor (T<sub>f</sub>) |
|--------------|----|-------------------|--------------------|-----------------------|-----------------------------|---------------------------|-------------------------------|----------------------------------|------------------------|
| Run          | A  | B                 | C                  | D                     | E                           | F                          | R1                            | R2                              | R3                     |
| 1            | 7  | 85                | 1                  | 15                    | 30                          | 247                        | 5.993                         | 2550.22                         | 1.53                   |
| 2            | 8  | 95                | 1.5                | 10                    | 25                          | 245                        | 4.618                         | 2422.09                         | 1.804                  |
| 3            | 6  | 75                | 0.5                | 20                    | 25                          | 249                        | 8.547                         | 2168.37                         | 1.507                  |
| 4            | 6  | 95                | 1.5                | 10                    | 35                          | 249                        | 3.642                         | 2073.46                         | 1.565                  |
| 5            | 7  | 85                | 1                  | 15                    | 30                          | 247                        | 5.974                         | 2535.85                         | 1.54                   |
| 6            | 8  | 75                | 1.5                | 20                    | 35                          | 245                        | 5.202                         | 1178.19                         | 0.892                  |
| 7            | 8  | 95                | 0.5                | 10                    | 25                          | 249                        | 6.845                         | 2913.47                         | 1.821                  |
| 8            | 6  | 95                | 1.5                | 20                    | 25                          | 245                        | 3.642                         | 2088.71                         | 1.562                  |
| 9            | 8  | 75                | 0.5                | 10                    | 35                          | 245                        | 11.002                        | 1867.13                         | 1.501                  |
| 10           | 7  | 85                | 1                  | 15                    | 30                          | 247                        | 5.996                         | 2531.19                         | 1.553                  |
| 11           | 8  | 75                | 1.5                | 20                    | 25                          | 249                        | 5.202                         | 1167.51                         | 0.894                  |
| 12           | 8  | 95                | 0.5                | 20                    | 35                          | 249                        | 6.502                         | 2983.02                         | 1.805                  |
| 13           | 6  | 75                | 1.5                | 10                    | 35                          | 249                        | 6.017                         | 1715.19                         | 1.608                  |
| 14           | 6  | 95                | 0.5                | 20                    | 35                          | 245                        | 5.404                         | 1993                            | 1.686                  |
| 15           | 6  | 75                | 0.5                | 10                    | 25                          | 245                        | 8.885                         | 2340.53                         | 1.605                  |

that variation in chromatographic conditions, like % organic phase in mobile phase composition, flow rate, and injection volume (critical parameters), significantly (p < 0.05) affects the RTPL peak T<sub>R</sub>, T<sub>f</sub>, and N; whereas mobile phase pH (7.0), column oven temperature (25 °C) and detection wavelength (247 nm) were selected as constant chromatographic conditions. Thus, the factors selected for the optimization of the RP-HPLC method were % organic phase in mobile phase composition (X<sub>1</sub>), flow rate (X<sub>2</sub>), and injection volume (X<sub>3</sub>). The RTPL peak T<sub>R</sub>, T<sub>f</sub>, and N were selected as a response as depicted in Tables 3 and 4. In context to the optimization, minimum and maximum levels for % organic phase of mobile phase composition (X<sub>1</sub>), flow rate (X<sub>2</sub>), and injection volume (X<sub>3</sub>), were kept between 75 to 95 % v/v, 0.5 to 1.5 mL/min, and 10 to 20 µL, respectively. The optimum level of X<sub>1</sub>, X<sub>2</sub>, and X<sub>3</sub> for the desired response was derived using a polynomial equation developed using software (Design-Expert software 8.0). The selected model was analyzed for RTPL peak T<sub>R</sub>, T<sub>f</sub>, and N (response)

**Table 3.** Factors and their levels for Box-Behnken optimization design in HPLC method of Retapamulin.

| Factor                     | Lower level | Upper level |
|----------------------------|-------------|-------------|
| X<sub>1</sub>, % Organic phase (%) | 75          | 95          |
| X<sub>2</sub>, Flow rate (mL/min) | 0.5         | 1.5         |
| X<sub>3</sub>, Injection volume (µL) | 10          | 20          |

Results and Discussion

**Design, development, and optimization of the RP-HPLC method**

Simple and robust RP-HPLC method was developed and successfully applied for quantitation of RTPL (Figure 1) from pharmaceutical topical cream and microemulsion using the Luna C18 column and the mixture of methanol and phosphate buffer (pH 7) (90:10 % v/v) as a mobile phase. C18 column was selected as reverse phase stationary
material for the proposed RP-HPLC method based on preliminary trials performed, and literature survey. Additionally, the selected column was able to efficiently separate RTPL from the cream and microemulsion matrix. Further, initial investigations revealed that the change in chromatographic conditions like mobile phase pH, % organic phase, flow rate, the volume of injection, column oven temperature, and detection wavelength affect system suitability parameters of RTPL ($T_R$, $T_f$, and $N$). Therefore, PBD (screening design) was employed to evaluate and screen the effects of six chromatographic variables affecting the RTPL peak $T_R$, $T_f$, and $N$. Design Expert supported statistical analysis revealed that for the selected responses, the statistically significant ($p < 0.05$) chromatographic variables were the % organic phase in the mobile phase, flow rate, and volume of injection. Figure 2 (A-C) shows Pareto charts wherein the magnitude of the regression coefficient for each factor is represented with the length of the bar. The outcomes of PBD suggested that increasing the % of organic phase, flow rate, and injection volume resulted in a decrease in RTPL peak $T_R$. Thus, % of the organic phase, flow rate, and injection volume had an inverse effect on RTPL peak $T_R$. As the methanol proportion increased from 75 to 95 % v/v, the flow rate increased from 0.5 to 1.5 mL/min, and injection volume increased from 10 to 20 µL, RTPL peak $T_R$ decreased significantly ($p < 0.05$). On the other hand, mobile phase pH, column oven temperature, and detection wavelength had a statistically insignificant effect ($p > 0.05$) on RTPL peak $T_R$.

**Table 4. Matrix of Box-Behnken design and responses for proposed HPLC method of Retapamulin.**

| RUN | Organic phase (%) | Flow rate mL/min | Injection volume µL | Retention time ($T_R$) | Number of theoretical Plates (N) | Tailing factor ($T_f$) |
|-----|-------------------|------------------|--------------------|-----------------------|----------------------------------|---------------------|
| 1   | 85                | 1                | 15                 | 6.01                  | 2031                             | 1.75                |
| 2   | 95                | 1.5              | 15                 | 3.14                  | 3789                             | 1.46                |
| 3   | 85                | 0.5              | 20                 | 6.73                  | 1938                             | 2.17                |
| 4   | 75                | 1                | 20                 | 8.55                  | 1168                             | 1.71                |
| 5   | 75                | 0.5              | 15                 | 8.55                  | 1368                             | 2.36                |
| 6   | 85                | 1.5              | 20                 | 4.33                  | 2088                             | 1.51                |
| 7   | 85                | 1                | 15                 | 6.5                   | 2150                             | 1.83                |
| 8   | 75                | 1                | 10                 | 8.89                  | 1240                             | 1.55                |
| 9   | 85                | 1                | 15                 | 5.97                  | 2235                             | 1.79                |
| 10  | 95                | 1                | 10                 | 5.25                  | 2913                             | 1.12                |
| 11  | 95                | 0.5              | 15                 | 6.51                  | 2983                             | 1.81                |
| 12  | 75                | 1.5              | 15                 | 5.21                  | 2168                             | 1.79                |
| 13  | 95                | 1                | 20                 | 6.12                  | 2379                             | 1.15                |
| 14  | 85                | 0.5              | 10                 | 6.87                  | 2068                             | 1.94                |
| 15  | 85                | 1.5              | 10                 | 3.52                  | 3022                             | 1.49                |

**Figure 1.** Chromatogram of (A) Retapamulin (100 µg/mL) working standard, (B) Retapamulin marketed cream, (C) Retapamulin loaded microemulsion formulation, (D) Purity curve for Retapamulin peak using proposed HPLC method.
Peak asymmetry resulting from either chromatographic fronting or tailing is not desirable for efficient chromatographic separation; $T_f$ dictates chromatographic peak shape. The Pareto chart discloses that the increase in the concentration of organic phase (methanol) increased $T_f$ of the RTPL peak. Thus, the % of organic phase had a direct effect on $T_f$, and this effect was statistically significant ($p < 0.05$). The mobile phase flow rate had an inverse effect on $T_f$ which indicates that as flow rate increases from 0.5 to 1.5 mL/min, the $T_f$ of RTPL peak decreases. On the other hand, the effect of injection volume, pH of the mobile phase, column oven temperature, and detection wavelength was found to have a statistically insignificant ($p > 0.05$) effect on $T_f$ of RTPL peak.

Columns with N higher than 2000 are considered as efficient since they favor narrow peak and better separation. There was a direct statistically significant ($p < 0.05$) relationship between % organic phase and N. An increase in N (N > 2000) for the RTPL peak was observed with an increase in % organic phase. The mobile phase flow rate and injection volume had an inverse effect on N (N < 2000) for the RTPL peak ($p < 0.05$). In contrast, the effect of pH of the mobile phase, column oven temperature, and detection wavelength was found to have statistically insignificant ($p > 0.05$) effect on N for the RTPL peak.

For the optimization of the RP-HPLC method, constraints in optimization design considered were: to obtain RTPL peak with $T_R$ between 3 to 7 mins, $T_f$ of RTPL peak between 1 to 1.5, and N > 2000 (specifications of desired response). Based on the results of PBD, % organic phase in mobile phase composition (methanol), flow rate, and volume of injection were found to be critical for the selected response. Experimental design for optimization study is reported in Table 3 and optimization results are shown in Table 4. All experiments were performed in a randomized manner to minimize the effects of uncontrolled variables.

Polynomial equations for RTPL peak $T_R$, N, and $T_f$ (dependent variables) were obtained by multiple regression analysis as per the following:

$$T_R = 6.16 - 1.72 (X_1) - 1.56 (X_2) - 0.15 (X_3) - 0.01 (X_1X_2) + 0.03 (X_1^2) + 0.24 (X_2X_3) + 0.77 (X_3^2) + 1.07 (X_1^3) - 0.28 (X_2^3)$$  
(Eq. 1)

$$N = 2138.67 + 765 (X_1) + 338.75 (X_2) - 208.75 (X_3) + 1.5 (X_1X_2) - 115.50 (X_1X_3) - 201 (X_2X_3) + 42.17 (X_1^2) + 396.17 (X_2^2) - 255.83 (X_3^2)$$  
(Eq. 2)

$$T_f = 1.79 - 0.23 (X_1) - 0.25 (X_2) - 0.06 (X_3) - 0.06 (X_1X_2) - 0.03 (X_1X_3) - 0.05 (X_2X_3) - 0.17 (X_1^2) + 0.23 (X_2^2) + 0.24 (X_3^2)$$  
(Eq. 3)

The fitted full quadratic models were obtained for each average response ($p < 0.05$) by applying multivariate regression analysis. The correlation coefficients were found to be 0.9475, 0.9826, and 0.9631, for RTPL peak $T_R$, $N$, and $T_f$, respectively; indicating the fitting of the selected models. ANOVA results of the model for RTPL peak $T_R$, $N$, and $T_f$ are tabulated in Table 5. The precision value (response to deviation ratio) obtained was 17.64 for $T_R$, 35.40 for $N$, and 23.34 for $T_f$, which indicated satisfactory signal and significant model to navigate design space. In the present investigation, the coefficient of variation (CV) values obtained was 6.40, 6.14, and 2.59, for RTPL peak $T_R$, $N$, and $T_f$, respectively, therefore the model was accepted and considered good and reproducible. 13-15

In DoE based optimization, relationship between the selected factors and the response can be determined based on regression coefficient, its positive value indicates a favorable relationship, while negative value indicates an unfavorable relationship. It is clear from the equation 1 and 3 that mobile phase composition ($X_1$), flow rate ($X_2$), as well as injection volume ($X_3$), has an unfavorable effect on RTPL peak $T_R$ and $T_f$, respectively. However, as per equation 2, mobile phase composition ($X_1$), as well as flow rate ($X_2$) has a favorable effect while injection volume ($X_3$) has an unfavorable effect on RTPL N.

Lack of fit for the selected model was determined based on a pure error of the experiments\textsuperscript{14} and $p$-value was found to be $> 0.05$ for RTPL peak $T_R$, $N$, and $T_f$; indicating satisfactory and appropriate model ($p > 0.05$) without any lack of fit. The effects of % organic phase in mobile phase composition, flow rate and injection volume at a constant...
value of each factor (at medium level) on RTPL peak TR, N values, and Tf are shown in Figure 3-5, respectively. For the T<sub>R</sub> value of RTPL, response surface graph was found to be nonlinear in relationship to % organic phase (X<sub>1</sub>) and flow rate (X<sub>2</sub>), at a constant level of injection volume. Figure 3 (A-B) demonstrates that the optimum T<sub>R</sub> value of RTPL can be obtained with % organic phase in the range of 80 to 95 % and the flow rate between 0.9 to 1.2 mL/min. Figure 3 (C-D) reveals a nonlinear surface response plot, representing a nonlinear relationship between % organic phase (X<sub>1</sub>) and injection volume (X<sub>3</sub>). It was depicted from Figure 3 that the % organic phase in the range of 81 to 95 % of methanol and injection volume 10 to 18 µL favor lower T<sub>R</sub> value of RTPL. Figure 3 (E-F) displays the effect of flow rate (X<sub>2</sub>) and injection volume (X<sub>3</sub>) on the T<sub>R</sub> value of RTPL at a constant % organic. The surface response plot was found to be nonlinear and it was depicted from the plot that optimum T<sub>R</sub> value of RTPL could be achieved with a flow rate between 0.7 to 1.5 mL/min and injection volume 10 to 20 µL.

Further, for N, the response surface graph was found to be nonlinear in relationship to % organic phase in mobile

| Model (Significant) | p-value |
|---------------------|---------|
| X<sub>1</sub> | 0.0009  |
| X<sub>2</sub> | < 0.0001  |
| X<sub>3</sub> | 0.3296  |
| X<sub>1</sub>X<sub>2</sub> | 0.9710  |
| X<sub>1</sub>X<sub>3</sub> | 0.1843  |
| X<sub>2</sub>X<sub>3</sub> | 0.2808  |
| X<sub>1</sub><sup>2</sup> | 0.0133  |
| X<sub>2</sub><sup>2</sup> | 0.0033  |
| X<sub>3</sub><sup>2</sup> | 0.2346  |

| Lack of fit (Not significant) | p-value |
|------------------------------|---------|
| | 0.3186  |
| | 0.4525  |
| | 0.3150  |

It was depicted from Figure 3 that the % organic phase in the range of 81 to 95 % of methanol and injection volume 10 to 18 µL favor lower T<sub>R</sub> value of RTPL. Figure 3 (E-F) displays the effect of flow rate (X<sub>2</sub>) and injection volume (X<sub>3</sub>) on the T<sub>R</sub> value of RTPL at a constant % organic. The surface response plot was found to be nonlinear and it was depicted from the plot that optimum T<sub>R</sub> value of RTPL could be achieved with a flow rate between 0.7 to 1.5 mL/min and injection volume 10 to 20 µL.

Further, for N, the response surface graph was found to be nonlinear in relationship to % organic phase in mobile

Figure 3. Response surface and contour plots showing effect of % organic phase in mobile phase composition (X<sub>1</sub>), flow rate (X<sub>2</sub>) and injection volume (X<sub>3</sub>) at medium (0) level of each factor on retention time of Retapamulin peak (A-F).
phase composition ($X_1$) and flow rate ($X_2$), at a constant level of injection volume. Figure 4 (A-B) reveals that the optimum N value of RTPL can be obtained with % organic phase range of 90 to 95 % and the flow rate between 0.5 to 1.42 mL/min. Figure 4 (C-D) demonstrates a non-linear surface response plot, indicating a non-linear relationship between % organic phase ($X_1$) and injection volume ($X_3$). Mobile phase composition in the range of 82 to 95 % of methanol and injection volume 10 to 20 µL favor N value (N > 2000). Figure 4 (E-F) indicates the non-linear relationship of flow rate ($X_2$) and injection volume ($X_3$) at a constant % organic phase in mobile phase composition on the N value of RTPL. Higher N value could be achieved with a flow rate between 1 to 1.5 mL/min and injection volume 11 to 16 µL.

Furthermore, for $T_f$, nonlinear relationship was observed between % organic phase in mobile phase composition ($X_1$) and flow rate ($X_2$), at a constant level of injection volume. Figure 5 (A-B) reveals that optimum $T_f$ value of RTPL can be obtained with % organic phase in the range of 90 to 95 % and flow rate between 0.7 to 1.5 mL/min. Figure 5 (C-D) depicted nonlinear surface response, indicating a nonlinear relationship between % organic phase ($X_1$) and injection volume ($X_3$). % organic phase in the range of 88 to 95 % of methanol and injection volume 10 to 20 µL favor the $T_f$ value less than 1.5. Figure 5 (E-F) displays the effect of flow rate ($X_2$) and injection volume ($X_3$) at a constant % organic phase in mobile phase composition on the $T_f$ value of RTPL. The lower $T_f$ value could be achieved with a flow rate between 1 to 1.5 mL/min and injection volume 10 to 11 µL.

Based on the solutions provided by the software, experimental conditions with desirability 1 or close to 1 were selected. Optimal chromatographic conditions of % organic phase in mobile phase composition of 95 % v/v, flow rate 1.0 mL/min, and injection volume 10 µL were obtained considering desirability as well as optimum response values (RTPL peak $T_R$, $T_f$, and N). Further, replicate injections (n=6) of 100 µg/mL of the RTPL test sample were evaluated for confirmation of suggested chromatographic conditions. The results obtained exhibited less than a 5% difference between the observed and predicted RTPL peak $T_R$, $T_f$, and N values.

For quantitative estimation of RTPL from cream and microemulsion, a new RP-HPLC method was successfully developed and optimized with Phenomenex Luna C18 (250 mm × 4.6 mm, 5 µm) column, an isocratic mobile phase consisting of methanol: buffer (potassium dihydrogen orthophosphate, 50 mM, pH 7.0) (90:10 % v/v), flow rate 1.0 mL/min, injection volume 10µL, column oven temperature 25°C, detection wavelength of 247 nm and run time of 10 min. The developed RP-HPLC method was found to give RTPL peak at $T_R$ value 4.33 ± 0.02 min.

Validation of the optimized method

The optimized RP-HPLC method was assessed for specificity by injecting the reference and sample solution of RTPL (100 µg/mL). The method was found to be specific for quantitation of RTPL in cream and microemulsion.

Figure 4. Response surface and contour plots showing effect of % organic phase in mobile phase composition ($X_1$), flow rate ($X_2$) and injection volume ($X_3$) at medium (0) level of each factor on number of theoretical plates of Retapamulin peak (A-F).

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considering the higher peak purity value (≥ 0.9999) for the RTPL peak (Figure 1). The linearity of the optimized RP-HPLC method was established by constructing calibration curves (regression analysis) for data obtained for RTPL over a concentration range of 40-140 µg/mL (40, 60, 80, 100, 120 & 140 µg/mL; n=6). The correlation coefficient ($r^2$) value was found to be 0.995 and the regression analysis data are summarized in Table 6. The developed RP-HPLC method was found to be suitable for the quantitation of RTPL in cream and microemulsion formulations.

Limit of detection (LOD) and limit of quantitation (LOQ) were calculated according to ICH Q2(R1) based on the values of the standard deviation of the intercept and the slope of the calibration curve. LOD of RTPL was 9.97 µg and LOQ was 30.22 µg, respectively. The values of LOD and LOQ are indicative of the high sensitivity of the RP-HPLC method.

The repeatability (intraday precision) of the developed RP-HPLC method was checked by analyzing six independent sample solutions containing 60 µg/mL RTPL on the same day under the same set of RP-HPLC conditions. The intermediate precision of the method was performed on three different days in the same laboratory using different concentrations of RTPL (60, 80 & 100 µg/mL; n=6). The results of peak area measurement were expressed in the terms of percentage relative standard deviation (% RSD). The proposed RP-HPLC method displayed good precision with % RSD values less than 2.0 % (Interday precision 0.73-1.45; Intraday precision 0.48-1.17). The method's reproducibility was demonstrated by replicate analysis of the RTPL standard and test samples with 1.69 % RSD values which are less than 2.0 % for repeatability experiments. The accuracy was done by selecting the concentration of RTPL (80 to 120 µg/mL) covering the linearity range of the method and equivalent to 80 to 120 % of the target concentration (100 µg/mL). Based on the RTPL percentage recovery from the cream and microemulsion matrix, the accuracy of the optimized RP-HPLC was verified. For the proposed RP-HPLC method, accuracy results were found in the range from 99.74-101.11 % w/w of RTPL. Moreover, it exhibited high efficiency of the method without much demanding sample pretreatment for cream and microemulsion samples. The results of recovery and precision studies are revealed in Table 6.

The evaluation of the robustness of the method was done through small deliberate variations in the chromatographic parameters, keeping the others unchanged. The robustness of the proposed RP-HPLC method was evaluated by varying chromatographic conditions such as % organic phase (±10 % v/v), pH (±0.2), column oven temperature (±5 °C), flow rate (±0.2 mL/min), injection volume (±5 µL) and detection wavelength (±2 nm). The small deliberate changes in the optimized RP-HPLC method 

**Figure 5.** Response surface and contour plots showing effect of % organic phase in mobile phase composition ($X_1$), flow rate ($X_2$) and injection volume ($X_3$) at medium (0) level of each factor on tailing of Retapamulin peak (A-F).
DoE Supported RP-HPLC for Quantitation of Retapamulin

Table 6. Summary of regression analysis and method validation parameters of the proposed HPLC method for Retapamulin.

| Parameters                           | Values               |
|--------------------------------------|----------------------|
| Linear Regression Analysis           |                      |
| Best-fit values                      |                      |
| Slope                                | 384.1                |
| Y-intercept                          | -1439                |
| X-intercept                          | 3.745                |
| 1/slope                              | 0.002603             |
| Std. Error                           |                      |
| Slope                                | 4.921                |
| Y-intercept                          | 473.7                |
| 95% Confidence Intervals             |                      |
| Slope                                | 374.1 to 394.1       |
| Y-intercept                          | -2401 to -476.0      |
| X-intercept                          | 1.270 to 6.104       |
| Goodness of Fit                      |                      |
| R square                             | 0.995                |
| Sy.x                                 | 1009                 |
| Is slope significantly non-zero?     |                      |
| F                                    | 6093                 |
| DFn, DFd                             | 1, 34                |
| p-value                              | <0.0001              |
| Deviation from zero?                 | Significant          |
| Equation                             | Y = 384.1X - 1439    |

Data

| Parameters                           | Values               |
|--------------------------------------|----------------------|
| Number of X values                   | 36                   |
| Maximum number of Y replicates       | 1                    |
| Total number of values               | 36                   |
| Number of missing values             | 0                    |
| Linearity and range                  | 40-140 µg            |
| LOD                                  | 9.97 µg              |
| LOQ                                  | 30.22 µg             |
| Accuracy, %                          | 99.74-101.11         |
| Repeatability (RSD, %, n=6)          | 1.69                 |
| Precision (RSD, %)                   |                      |
| Interday (n=3)                       | 0.73-1.45            |
| Intraday (n=3)                       | 0.48-1.17            |
| Specificity                          | Specific             |

parameters resulted in lower values of % RSD, confirmed the robustness of the method for quantitative estimation of RTPL in cream and microemulsion (Table 7).

To perform the system suitability test, 100 µg/mL standard samples of RTPL were used (n=6). The quality attributes examined for the RTPL peak were T<sub>r</sub>, f<sub>r</sub>, and N, injection precision, and peak purity index. Table 8 shows the system suitability study results. The system was found suitable for the quantitative estimation of RTPL in cream and microemulsion samples (Table 8).<sup>13-15</sup>

Application of optimized and validated RP-HPLC method

RTPL content in marketed cream [Retarel (1% w/w RTPL)] and in-house developed microemulsion formulations was within the required range which is depicted in Table 9. The findings suggest the suitability of developed RP-HPLC method for routine analysis of RTPL in pharmaceutical formulations.

Conclusion

DoE based systematic and practical approach was implemented to develop an efficient and robust RP-HPLC method for the separation and quantitation of RTPL. The application of the PBD and BBD in screening and optimization resulted in the RP-HPLC method which was simple in implementation and chromatographically efficient in the estimation of RTPL in cream and microemulsion formulations. The optimized RP-HPLC method (with the chromatographic conditions: Phenomenex Luna C18 (250 mm × 4.6 mm, 5 µm) column, an isocratic mobile phase consisting of methanol

Table 7. Results of robustness study for the proposed RP-HPLC method for quantitative estimation of Retapamulin.

| Parameters                           | Change in parameters | Peak Area ± SD | %RSD |
|--------------------------------------|----------------------|----------------|------|
| Mobile phase composition (% Methanol) (±5%) | 80 29466± 339 | 1.15 |
| pH (±0.2)                            | 6.8 29220 ± 20   | 0.85 |
| Flow rate (±0.2 mL/min)              | 0.8 30299 ± 171  | 0.56 |
| Wavelength (±2 nm)                   | 245 29572 ± 185  | 0.62 |
| Injection Volume (±5 µL)             | 15 31543 ± 312   | 0.36 |
| Column oven temperature (±5 °C)      | 20 29338 ± 105   | 0.36 |
|                                     | 30 28997 ± 139   | 0.48 |
Table 8. System suitability test parameters of Retapamulin for proposed HPLC method.

| Parameters                      | Values                  |
|---------------------------------|-------------------------|
| Retention time (min)            | 4.3 ± 0.1               |
| Area (average)                  | 38504.17 ± 282.67       |
| Number of plates                | 2524.44                 |
| USP tailing factor              | 1.49                    |
| Repeatability                   | 1.69                    |
| Peak purity                     | 0.999                   |

Table 9. Assay results for pharmaceutical dosage form containing Retapamulin using the proposed HPLC method.

| Dosage forms                  | Values (Mean ± SD (n = 3), %) |
|-------------------------------|-------------------------------|
| Cream formulation            | 101.45 ± 0.52                 |
| Microemulsion formulation    | 101.04 ± 0.29                 |

and phosphate buffer (90:10 % v/v), flow rate 1 mL/min, injection volume 10µL, column oven temperature 25°C, detection wavelength of 247 nm and run time of 10 min) was successfully validated as per ICH Q2 (R1) guideline and found specific (peak purity ≥ 0.9999), sensitive (LOD – 9.97 µg and LOQ – 30.22 µg), accurate (99.74-101.11 % w/w) and precise (% RSD < 2.0 for RTPL). Moreover, the optimized and validated RP-HPLC method was successfully applied to separate and quantify RTPL in marketed topical cream and in-house formulated microemulsion.

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Author Contributions
RP: Conceptualization, design of work, writing – original draft, supervision, interpretation of data, software, NP: Investigation, formal analysis MP: Conceptualization, methodology, editing and revising the manuscript content. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest
The authors confirm no conflicts of interest.

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