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HAL Authorization

Integrative validation method of Roflumilast by quantification and detection in lipid-based Nanoparticles via Reverse Phase HPLC

Iqra Chaudhery ^a, Marya Nawaz Malik ^a, Tofeeq Ur-Rehman ^a, Humaira Fatima ^a, Asim.ur.Rehman ^a, Naveed Ahmed ^{a*} and Abdelhamid Elaissari ^b

^a Department of Pharmacy, Quaid-i-Azam University, 45320, Islamabad, Pakistan

^b Univ Lyon, University Claude Bernard Lyon-1, CNRS, ISA-UMR 5280, 69622 Villeurbanne, France

*Corresponding author e-mail: natanoli@qau.edu.pk

Abstract

Purpose: The basic aim of this study is to create an accurate, ideal, and precise **Reverse-Phase HPLC** method for detecting and quantifying roflumilast (ROF) in lipid-based nanoparticles for the very first time. High-performance liquid chromatography (HPLC) is nowadays an extensively utilized quantitative analytical technology in the pharmaceutical business.

Method: RP HPLC (Shimadzu 20A) ROF detection method was validated with a mobile phase of distilled water and acetonitrile having 40:60 concentrations. Samples were analyzed via isocratic elution of 10 µl injection volumes, a PDA detector, using a C18 Backmann column silica column (4.6*250-mm-wide) as the stationary phase and the ambient temperature of the column was sustained at 45°C with 35 MPa max pressure. A flow rate of 1 ml/min was run over 15 minutes (run time) and ROF was detected at wavelength of 254 nm with linearity range of 70 to 130% (40 to 300 µg).

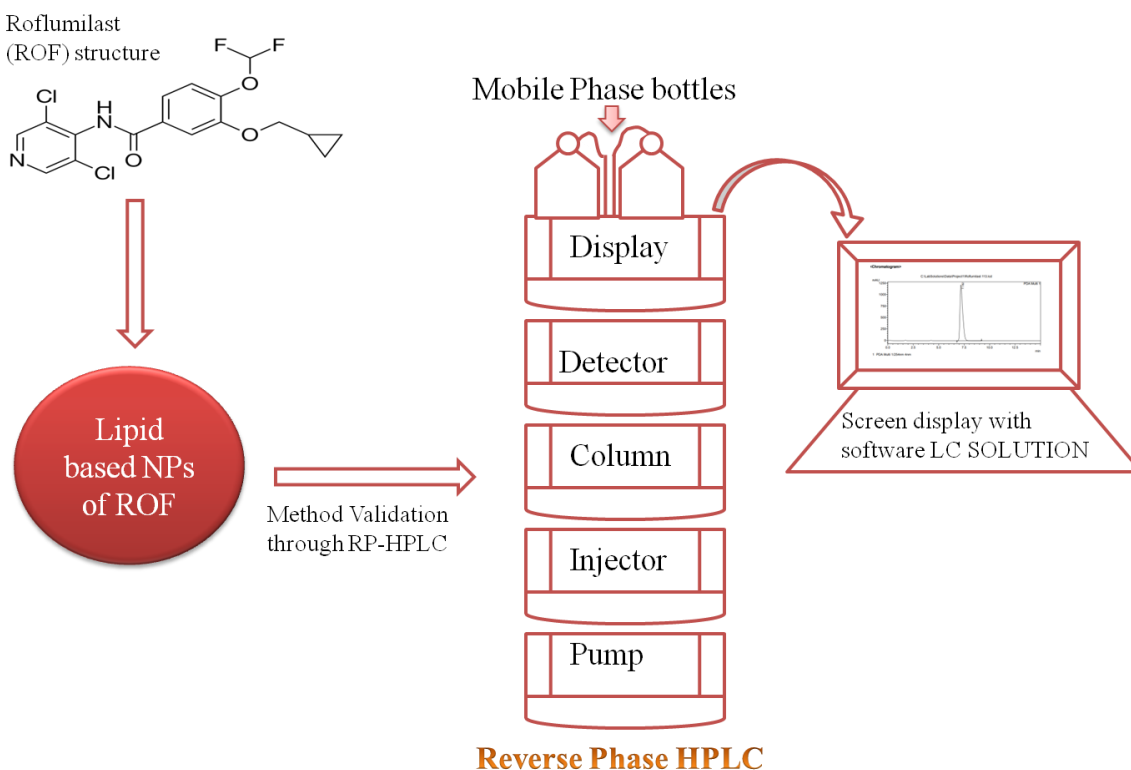
Results: All HPLC analytical parameters; accuracy, precision, linearity, and range were studied and optimized according to ICH guidelines. The retention time of Lipid-based nanoparticles of Rof was 7.1 ± 0.02 minutes and approximately 100 percent accuracy, specificity and precision were achieved with this method. The values of limit-of detection (LOD) and limit-of quantification-(LOQ)-were found to be -0.0041 and 0.031 µg/ml respectively.

Conclusion: This validated analytical method was so sensitive and site specific for verification of roflumilast in lipid-based formulations.

Keywords

Roflumilast, Reverse-phase High-performance liquid chromatography (HPLC), lipid-based nanoparticles, ICH-guidelines, limit of Detection (LOD) and limit of Quantification (LOQ)

Graphical Abstract



1. Introduction

FDA has authorized Roflumilast, selective phosphodiesterase-4 inhibitor, as anti-inflammatory medication for secondary inflammation and for severe chronic-obstructive-pulmonary-diseases [1]. PDE-4 is among the 11 enzymes that are responsible for the breakdown of cyclic AMP (adenosine-monophosphate) and/or cyclic GMP (Guanosine-monophosphate). PDE-4 inhibitors have a variety of anti-inflammatory effects, including inhibiting inflammatory mediator release and innate immunity. PDE-4 inhibitors might be valuable in the management of an assortment of provocative or autoimmune diseases [2]. PDE-4 is a course of enzymes that breakdown and inactivate c AMP [3], a post-messenger that is essential for OPC development. It was recently discovered that the atypical PDE-4 inhibitor roflumilast stimulates remyelination both *in vitro* and *in vivo* [4].

Once the right medicine has been identified, the next step is to create an accurate quantitative analytical procedure for obtaining dependable drug concentration data [5]. The purpose of any qualitative or quantitative analytically validated procedure is to acquire results that secure

sufficiently the unknown real values of the analyte in test samples [6]. Strategy validation is one method for demonstrating that the analytical method will produce dependable findings. If the validation does not provide the guaranteed results based on the findings, it will be incorrect and possibly will result in diagnostic mistakes such as designating a healthy patient to be unwell and vice versa [7, 8].

The scrupulous explored studies reveal that few analytical methods are available to detect ROF but this proposed method helps to detect and quantify drug along with its excipients in pharmaceutical dosage forms without any separation [9, 10]. Roflumilast is a medicine of class **IV** having minimal solubility and less permeability, to overcome this problem nanoparticles formation has been selected [11]. Nanoparticles delivered medicine to the site of action at low concentration, enhance bioavailability, and can help in disease diagnosis [12]. Herein, the current exploration was carried out to attain a new, simple, accurate, rapid, and cost-effective scheme for the real-time approximation of ROF in lipid-based curative medication. Consequently, the basic objective of the developed method, is to quantify ROF from lipid-based nanoparticles because quantification analysis of lipid-based formulations is slow and difficult to identify drugs due to undistinguishable chromospheres [13]. Hence, mostly drugs prior to detection and validation must be extracted from these lipids (excipients) presented in any dosage forms to avoid any interaction. However, the importance of lipid compositions in nanoparticles is a key feature to access their size, drug encapsulation, drug release profile and prolonged stability [14-16]. Efficient and sensitive detectors are required to identify drug from these lipid-based tiny creatures of different chain lengths, head groups and polarities [17].

1.1 Technical considerations and objectives of study

Previously for the quantitative validation of drugs there was a necessity to separate the drug by extraction from other components of lipid-based formulations due to their interference. But this method is so accurate, rapid, and sensitive that did not require any lipid extraction prior to analysis. High performance liquid chromatography possesses a preferable procedure for the separation and quantification of many drugs that were not detected and quantified by UV or other possible techniques [18]. The cyclopropane substituent, which is unstable at acidic pH, is one component of ROF's structure that suggests apparent unstability. Its amide group makes it therefore more susceptible to hydrolysis, making it difficult to stabilize the medication pharmaceutically [19].

This issue was solved by formulating lipid based nanoparticles of ROF to protect it from acidic hydrolysis. A thorough review of the current literature finds a lack of in-depth investigations describing the forced degradation of ROF. No ROF monograph has been published in any pharmacopoeia. To separate and identify ROF in lipid-based nanoparticles, a reversed-phase HPLC technique containing a diode PDA detector is used in the study. It uses a 1024 element photometric diode array, with measurement wavelength range from 190 to 900 nm and resolution of 1 nm. PDA transiently monitors the absorption of light by a liquid moving through and is a crucial as well as dependable technique for the depiction of non-polar molecules due to its precision and significance [20]. The goal of the work is to demonstrate the development and authentication of this approach to validate an RP HPLC Method for the identification of ROF in lipid based nanoparticles.

1.2 Analytical Comparison of proposed RP HPLC method

Although ROF quantification is not included in the pharmacopoeia, various methods such as LC-MS, UV and HPLC have been reported in the literature but not related to the detection of this compound along with its excipients specifically in nanoparticles [21, 22]. In 2012 A sensitive and reliable high-performance liquid chromatography–mass spectrometry (LC–MS/MS) was developed and validated for simultaneous quantification IC87114, roflumilast (RFM), and its active metabolite roflumilast N-oxide (RFN) using tolbutamide as an internal standard [23]. Separation of ROF was performed in another study on a C18 analytical column (250*4.6 mm, 5 µm), with a mobile phase-A of ACN and a phase-B of ammonium acetate buffer (5 mM, pH 4.2) containing triethylamine (0.5% v/v) having a complex mobile phase although used RP HPLC [24]. One more study determined only ROF and its metabolites in raw form not specifically in any dosage form. A new validated capillary electrophoresis method to determine Roflumilast (RFL) with an internal standard in tablet was published in 2019 but not for nanoparticles Another study was published in literature regarding detection of raw ROF in tablets through UV spectrophotometer [25, 26], but this method is not suitable for detection of ROF in every solvent, because it is not sensitive method as HPLC. LC-MS is a difficult and complicated method needs specifically a trained person to run and validate different chemicals. The phospholipids present in a formulation or in plasma can be responsible to generate considerable matrix ionization effects during LC/MS/MS analysis thus required drug extraction prior to analysis [27]. This study

simultaneously determines ROF in lipid based structure for the first time, which is unique, novel and can be utilized in any circumstances. Lipid, specifically phospholipids, interferes in detection of certain compounds resulting in alteration in drug properties and could be responsible for matrix effects [28, 29]. In this study simple mobile phases are used, that's why its handling is very simple and is easy to operate [30, 31]. This study was focusing on the development of a sensitive, specific, accurate, precise and stable reverse-phase HPLC method for the determination of ROF in lipid-based nanoparticles.

2. Methodology

2.1 Chemicals and Reagents

Roflumilast was purchased from Jiangsu Yongan pharmaceutical co. ltd., Huai'an, Jiangsu, China, Phospholipon 90 g (gifted from Lipoid France), cholesterol, Tween 80, ethanol, and Acetonitrile HPLC Grade (Sigma-Aldrich, Darmstadt Germany), Water for HPLC, micro filters 0.22 μm , white nylon HNWP 47 mm membrane filters, micro filters of 0.45 μm (AIJIREN China), syringes (SCHOTT USA).

2.2 Sample solution

Lipid-based nanoparticles of Roflumilast were prepared using a reported method with slight modification. An organic phase was prepared having the drug 0.2 mg/ml, lipid 0.22 mg and cholesterol 0.8 mg. Then it was poured into an aqueous phase comprising of Tween 80 (0.5%) and distilled water. After mixing both phases and complete hydration, homogenization was done for 15 minutes at 15000 rpm and the final volume was make up with distilled water [32]. Placebo was also prepared using the same procedure but without the drug.

2.3 Standard preparation

Accurately weighed quantity of working standard Roflumilast (0.02 g) was added into volumetric flask (100 ml). Then, 30 ml of the mobile phase (acetonitrile: water) was added to dissolve the drug by sonication. Finally, the volume was made upto 100 ml within similar solvent. The ultimate strength of the standard was 0.2 mg/ml.

2.4 Instrumentation

Shimadzu 20A Reverse phase high-pressure, liquid-chromatography equipment was linked to a PDA detector Mass-Spectrometer, (PDA Multi 1/254 nm 4 nm). ROF was detected using a C18 Backmann column silica column (1.8 m length, 4.6 mm wide) as the stationary Phase. The ROF

detection was accomplished by an isocratic system at ambient temperature with mobile phase comprised of Acetonitrile and dist. water in 60:40. The mobile phase was filtered and degassed prior to use by passing through 0.22 µm filters. The rate of flow level was adjusted at 1ml / min and injecting volume was 50 microliters.

2.5 Reverse phase HPLC analysis parameters

LC SOLUTION computer software was applied to handle the acquired information. ROF was detected at 254 nm wavelength and calibration gradient curve was constructed to quantify the ROF in analyzed samples. The calibration curve was created using MS-Excel. A calibration curve for each ROF was constructed using a proportion of the section area to the standard's point area. All calibration curves were built linearly, with the "x" variable weighted as 1/x. The calibration curve was created and then applied to all samples to determine the concentrations.

3. Validation methods

3.1. Accuracy and Precision

Initially, a placebo solution was used to assess procedure accuracy and precision with a defined but restricted matrix exposure. ROF standard was injected at six times with same concentration (0.2 mg/ml) and set a limit to equilibrate for half an hour at 25°C temperature before validation. For each concentration, three duplicates were validated and examined. This method was performed thrice a day to assess inter-day exactitude and truthfulness, yielding many validation samples examined. According to the ICH [33], accuracy must be evaluated utilizing a least amount of 9 fortitudes at 3 levels comprising the mandatory limit of concentrations [34]. To assess recovery, we used triplicates of three concentrations of ROF sample (75, 100, and 125 %) and compared the results with the standards. The ICH document recommends that repeatability should be assessed by employing a least amount of 6 resolves at 100% of the examination values [34, 35].

$$\% RSD = \frac{\text{Standard deviation}}{\text{Average Mean}} * 100 \quad \text{Equation (1)}$$

3.2. Specificity

Placebo, standards, and samples were evaluated to confirm the method's accuracy, precision, and linearity across a variety of common samples. In specificity, the analyte should be evaluated unambiguously along with the presence of components in the formulation such as contaminations, miscodifying, matrix etc. It is possible to investigate it experimentally by spiking the sample

product with suitable amounts of contaminants or excipients and demonstrating that the evaluated outcome is unaffected by the presence of these additional formulation fundamentals.

3.3. Linearity, detection, and quantification limits

Linearity and range are the aptitude of outcomes, which are clearly proportionate to the strength of segment in the experiment within a particular range. The response time test should be linear with at least 5 concentrations. For linearity and range, eight different concentrations were selected ranging from 0.04 to 0.3 mg/ml. **LOD** and **LOQ** were also defined using the following equations.

$$LOQ = 10 * \sigma/S \quad \text{Equation (2)}$$

$$LOD = 3.3 * \sigma/S \quad \text{Equation (3)}$$

$$\sigma = \text{Intercept} \quad S = \text{Slope}$$

3.4. Ruggedness and robustness

Ruggedness and robustness were measured by performing all the experiments with different analyst at some other places on some other days. It is the determination of the competence of a technique to remain untouched by tiny variations in systematic method parameters such as inter-laboratory factors, analyst to analyst variation, instrumental variation etc.

3.5 Matrix stability

Matrix stability was determined in different concentrations of working solvent. It was examined using standard solutions (SDS) of concentration 10 µg/ml in solvent's samples (formulation in mobile phase). Then these samples were stored at -18, 4, 25 and 45°C and analyzed three times within a month at 3rd, 15th and 28th day. The stability was evaluated by following equation.

$$\%SDS = \frac{St}{S^0} * 100 \quad \text{Equation (4)}$$

St was Concentration obtained after making a pause with t duration.

Where S⁰ was the initial peak area with delay in time without introducing extra pauses.

4. Results

4.1. Method Development

Till now, there has been no Pharmacopoeia procedure for the recognition and affirmation of ROF as already mentioned. The chromatographic settings were adjusted to produce distinct and sharp with high resolution peaks for the sample and standard. The retention periods for sample and standard were 7.1 and 7.2 minutes, respectively, in all matrices, with an overall run time of 15 minutes, allowing for rapid, accurate and efficient analysis of many samples. A sharp standard peak of sample and standard for ROF detection, was obtained having a retention time between 7 to 8 minutes at 254 nm shown in fig. 1A and 1B. The chromatogram of placebo (blank formulation) was also represented in figure 1C, as there was no sharp and high resolution peak was present to interfere with sample results. System suitability parameters were defined in table 1 for RP HPLC method validation of ROF.

Table I: RP HPLC suitable parameters for validation of ROF

Sr. No	Parameters of system	Specification
1.	API concentration (sample and standard)	0.2 mg/ ml
2.	Mobile Phase	Acetonitrile: water (60:40)
3.	Wavelength	254 nm
4.	Detector	PDA Multi 1/254nm 4nm
5.	Column	Backmann C18 (4.6*250 mm: 5 micrometer particle size)
6.	Run time	15 minutes
7.	Retention time	7.1 ± 0.02 minutes
8.	Area	1885955
9.	Pump pressure	13.4 MPa
10.	Injection volume	50 µl
11.	Flow rate	1 ml/min

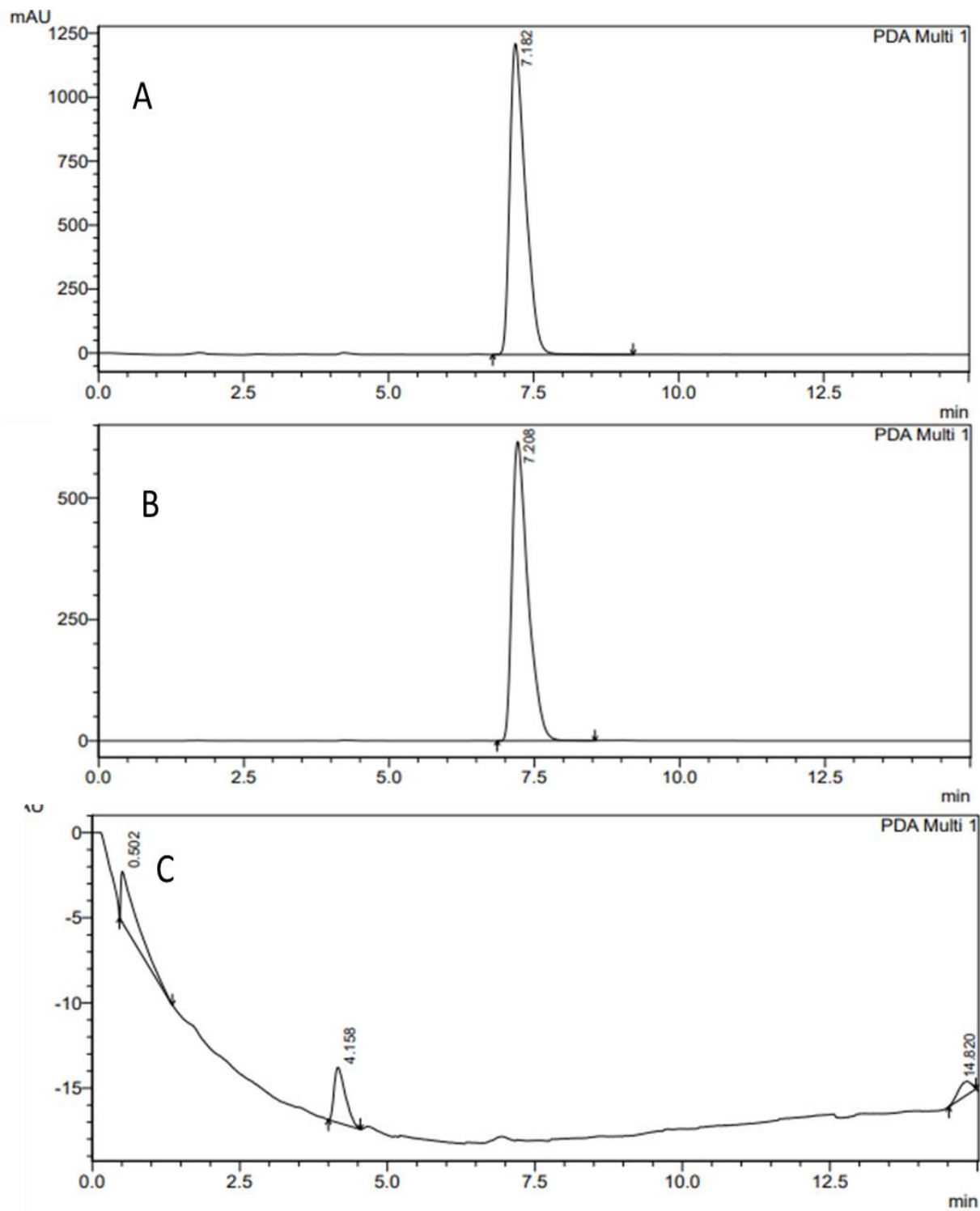


Fig. 1: Representing the Chromatograms of Sample and Standard were showing sharp with high resolution and characteristic peaks at 7.2 retention time. But no characteristic peaks were observed Placebo. A1: chromatogram of Standard, B1: chromatogram of Sample and C1: chromatogram of Placebo.

4.2 Method Validation

Roflumilast was tested for system compatibility utilizing a Backmann C18 column in conjunction with an acetonitrile: water (60:40) phase. International guidelines suggest quantifying injection accuracy, peak asymmetry, and retention. Each chromatogram's pictorial depiction is discussed in the supplemental data file.

Table 2: Table representing the linearity/range analysis for method validation of ROF.

Concentration ($\mu\text{g/ml}$)	Area
40	5784265.4
80	11757471.4
120	13072959.7
160	17139247.4
200	21939553.8
240	26005531.4
280	30460135.2
300	31487468.6
Slope	98547.90
Intercept	2213576.68
Linearity Equation	$y = 98547.90x + 2213576.68$
R^2	0.99
Range	300-40 $\mu\text{g/ml}$)

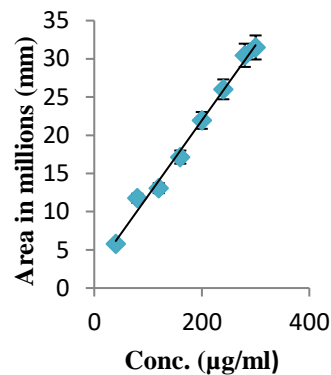


Fig 2: Calibration curve of data representing linearity and range of the RP-HPLC method from 0.04 mg/ml to 3 mg/ml.

4.3 Linearity, detection, and quantification limits

The linearity of the optimized HPLC technique for measuring ROF was tested by creating eight analytical curves with concentrations ranging from 70 to 130% (40 to 300 μg). Table 2 and Fig.2 summarized the regression analysis data of linearity and range. The correlation coefficient (R^2) value for ROF was 0.99 after running these selected concentrations. ROF has an **LOD of 0.0041 $\mu\text{g ml}^{-1}$ and a LOQ is 0.031 $\mu\text{g ml}^{-1}$** . The LOD and LOQ values showed that the suggested HPLC technique is sensitive for analyzing ROF in lipid-based nanoparticles. Over specified values of roughly five orders of magnitude, each target metabolite demonstrated excellent linearity, with R^2 values of approximately 1.

4.4 Accuracy and precision

The suggested RP-HPLC technique yielded accurate findings ranging from 100 to 101% w/w of ROF and data was represented in Table no. 3. ANOVA was used to calculate the significant analysis summary output of linearity and ranges. The technique's precision was measured using intraday and interday accuracy. The intraday precision was validated by analyzing six different sample preparations on similar day under the one and the same investigational circumstances.

Chromatograms were presented in supplementary data. The percent RSD for all experiments were determined to be between 0.04–1.11% respectively.

4.5 Robustness

According to ICH guidelines, all HPLC analytical parameters (precision, accuracy, repeatability, robustness') were optimized, studied, and summarized in table 3. The chromatographic parameter affects chromatographic output, and it was found to be compatible with the recognition criteria. Therefore, the suggested HPLC technique was determined to be suitable for quantification of ROF in lipid-based formulations.

Table 3: Data of validation method for ROF by reverse phase HPLC

Analysis	%Mean			Average mean	%RSD
Precision and Accuracy	Run 1	Run 2	Run 3		
75% (150 µg/ ml)	75.1	75	75.6	75.4 ± 0.59	0.77
100% (200 µg/ ml)	99.8	100.5	101.9	100.27 ± 0.86	0.94
125% (250 µ / ml)	126	125.5	126	125.9 ± 0.05	0.04
Inter day					
Analyst 1	99.5	100	99.8	99.9 ± 0.42	0.42
Analyst 2	101.1	100	100	100 ± 0.85	0.848
Intraday (another lab)	99.8	99.6	100	99.6 ± 0.45	0.447
Specificity					
Standard	100	101.3	100.3	100.6 ± 0.15	0.52
Placebo	No peak observed				
Sample	96.7	95	97	96.1 ± 0.2	0.43
Repeatability	100.1	101	99.7	101.1 ± 1.2	1.11
Robustness	101.2	101.8	102.2	102 ± 0.24 %	0.23
Percent recovery					
75%	75.1	76.3	75.8	76.1 ± 0.02	0.024
100%	99.8	101.5	100.9	101.2 ± 0.42	0.44
125%	126	126	125.6	125.7 ± 0.34	0.351

4.6 Matrix stability

The stability of samples was determined and represented in figure 3. The obtained result explained that samples were most stable at room temperature and 4°C. Because at this temperature every excipient remained in diluted form as compared to other -18 and 45°C. Because these temperatures are considered extreme temperatures and may deteriorate samples, efficacy may compromise and detection peaks might be disturbed.

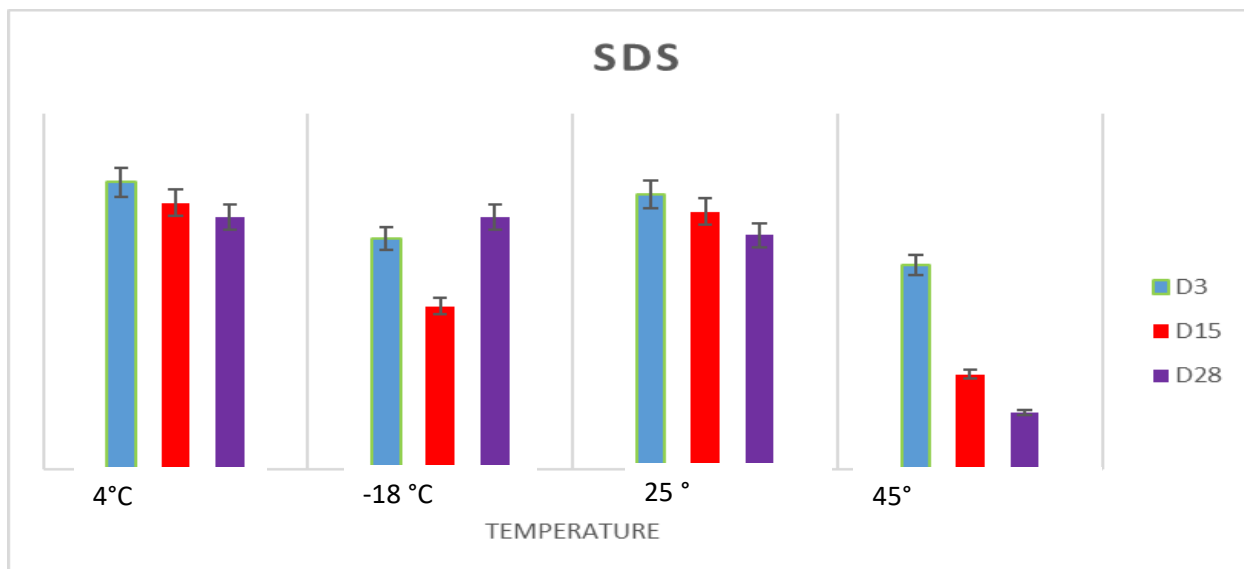


Figure 3: SDS of formulation in operating solvent in different temperature having $SD \pm 0.32$

Discussion

A simple RP-HPLC technique for ROF measurement was analytically verified under suitable conditions. This is the first technique to determine ROF in lipid matrix and has been verified using such a rigorous and detailed procedure. After in-depth research didn't find any relevant data related to the detect ROF in any lipid based formulations [36]. Furthermore, ROF determination in lipid-based formulations has received less attention since phospholipids are prevalent in natural matrices and constitute the principal class of body building materials that induce ion inhibition or enrichment in many studies [37]. Thus, these materials (*e.g.*, phospholipids) can cause a variety of analytical issues, including retention time changes, increased baselines, and diverging curves. When there is a strong matrix suppression in formulation, any tiny changes in retention periods between the analyte and internal standard might be resulted in various matrix effects [27]. When compared to the usage of standard and sample for quantification, the suggested reverse phase HPLC technique provides several advantages [16]. Matrix stability analysis showed that there was no big damage detected in lipid based nanoparticles of ROF.

The photo diode array (PDA), utilized in this method also acknowledged as diode array detector [38] that can measure the entire wavelength range in real time other than UV visible regions. This is a sensitive tool utilized by RP- HPLC to detect the drug ROF from lipid based formulation

without any interference. Primarily, it eliminates the risks associated with handling a potent medication which is easily hydrolyzed in acidic conditions and this issue is addressed by formulating nanoparticles of ROF [19]. Secondly, it significantly decreases the cost and time restrictions associated with detecting ROF encountered due to complex mobile phase utilized in previous studies. Finally, rather than identifying the single drug present in the standard, it directly measured ROF in lipid-based formulations without extraction. The suggested RP-HPLC approach needed a minimal amount of detection that is simple, quick, and affordable.

However, when blank formulation was run, no new peaks were observed, and no interaction occurred between components and medication characteristics. As a result, these data represented in figure 1C supported the specificity and accuracy of the approach. After analysing standard, sample and blank from the identical matrix, the lack of interfering components of the formulation during the retention period of ROF was established. The inclusion of acetonitrile in the mobile phase of RP-HPLC helps to prevent peak tailing produced by the strong interaction between basic analyte and the acidic surface column [39].

Furthermore, the risk associated with the prospective use of the verified technique was assessed and proved to be less than 5% across the whole verified detection limit for all samples [40]. Finally, the values of uncertainties were computed, permitting accurate assessment, verification, and correlation of the data. From such an analytical standpoint, demonstrated that ROF detection in lipid-based formulations by RP- HPLC is precise enough to achieve clinical aims in the detection of medication levels in human bodies as well in future studies [41]. Cross inconsistency of other components along with drug most probable issue during LC/MS/MS analysis due to varying matrix effects. But this problem was not observed in the respective RP HPLC method for detection and quantification of ROF. And its deterioration was not monitored in sample (nanoparticles) peak like standards indicating no interactions and instability.

The validation findings showed that the technique will produce accurate and trustworthy results for standard and sample concentrations ranging from 150 to 280 $\mu\text{g/ml}$, with accuracy values ranging from 100 to 101 percent. In ordinary practice, ROF concentrations obtained in standard and sample after 6 repeated injections of 0.2 (200 $\mu\text{g/ml}$) indicating the repeatability and reproducibility of our validated method (data presented as supplementary material) with percent RSD 1.11. The linearity was proved using regression tolerance, regardless of the concentrations

investigated. Beyond these ranges, the results were not linear for calibration curve in figure 2. This analyzed data was utilised to pick the most excellent regression representation for calibration, and to identify the quantification limit (LOQ) and the range over which the technique may be regarded applicable. Limits of detection (LOD) and limits of quantification (LOQ) are needed for drug tests, to demonstrate the sensitivity of the proposed RP-HPLC technique. The detection limits (the least amount of analyte that can be identified in the sample but not quantified) was $0.0041 \mu\text{g ml}^{-1}$ while the quantification limits were estimated by multiplying the smallest and maximum contents, which was $0.031 \mu\text{g ml}^{-1}$. These discovered values were close to the benchmark of ROF and previously established data [10]. Matrix stability of ROF in lipid based nanoparticles was determined at different temperatures and proved no interaction by employing this respective method. This also indicated no deviation of active compounds peaks (standards and samples) in working solvents (mobile phase). So, this novel and innovative technique counteracts the degradation at different temperatures and provides a stable method to detect ROF in lipid-based nanoparticles. However, by employing this RP HPLC technique for ROF due to its precision, repeatability and avoidance of any related problems during verification and avoids interference of all the other entities present in formulations. The quantitative estimation of ROF in a lipid-based formulation was performed in triplicate ($n = 3$) using an optimal, validated HPLC technique and the devised approach proved successful. Matrix stability was determined to confirm the stability of our active compound in respective solvent system (mobile Phase). The suggested approach was successfully used to estimate ROF from lipid-based nanoparticle formulation.

Conclusions

In this research work, a high-throughput extraction and RP-HPLC technique is described that can prepare and analyze the ROF quality standard specimens and sample in a 24-hour timeframe. The suggested approach has been demonstrated to be highly repeatable over a wide variety of concentrations and nanoparticle types that have been extensively studied. Furthermore, the LOQ including all targets has been proven to be significantly below normal values, making this a suitable technique for investigations with restricted sample quantities. In comparison to prior approaches, the method developed here provides a quick, sensitive, and trustworthy technique for identifying three intensively investigated ROF in nano-based formulation. Furthermore, this approach may also be utilized in human *in vivo* studies for the detection of ROF nanoparticles.

Conflict of Interest

All Authors declare no conflict of interest.

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