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Research Article

HPLC Analysis of Lamivudine in Pharmaceutical Formulations: Method Development and Validation

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Abstract

A novel, rapid and efficient high performance liquid chromatography (HPLC) method was developed and validated to analyse the quantification of Lamivudine (LAM) in different pharmaceutical formulations, including pure form, commercial tablet, and nanostructured lipid carrier (NLC), a novel drug carrier system. Accurate analysis of the amount of active ingredient in pharmaceutical formulations is very important for assessment of the quality and therapeutic efficacy of formulations. In method, we used distilled water: methanol (MeOH) (60:40, v:v) as mobile phase and analysed on C18 column. To analyse the eluent, the method was performed at 270 nm, with a flow rate of 1 mL/min, in 10 min. The calibration curve obtained showed linearity in the concentration range 2-60 µg mL^{-1}. The average recovery of pharmaceutical preparations (Zeffix®,100 mg 28 tablets and NLC formulation) was 99.55%. The limit of detection (LOD) of the method was 1.49 µg mL^{-1} and, limit of quantification (LOQ) was 0.51 µg mL^{-1}. The method also allowed the determination of the amount of LAM contained in the existing commercial formulation and the newly developed NLC formulation and the verification of the homogeneity of the pharmaceutical formulations. The results showed that the developed HPLC method can be used reliably in both formulation development and stability studies in NLC drug carrier systems.

Keywords: Lamivudine, HPLC, Pharmaceutical Formulation, Nanostructured Lipid Carriers

Farmasötik Formülasyonlarda Lamivudinin HPLC Analizi: Yöntem Geliştirme ve Validasyonu

Öz

Yeni, hızlı ve etkili bir yüksek performanslı sıvı kromatografi (HPLC) yöntemi geliştirildi ve saf form, ticari tablet ve yeni bir ilaç olan nanoyapılı lipit taşıyıcı (NLC) dahil olmak üzere farklı farmasötik formülasyonlardaki Lamivudin (LAM) miktarının analiz edilmesi için doğrulandı. Farmasötik formülasyonlardaki aktif madde miktarının doğru analizi, formülasyonların kalitesinin ve terapötik etkinliğinin değerlendirilmesi açısından çok önemlidir. Yöntemde mobil faz olarak distile su: metanol (MeOH) (60:40, v:v) kullanıldı ve C18 kolonunda analiz edildi. Eluenti analiz etmek için yöntem 270 nm'de, 1 mL/dk akış hızıyla 10 dakikada gerçekleştirildi. Elde edilen kalibrasyon eğrisi 2-60 µg mL^{-1} konsantrasyon aralığında doğrusallık gösterdi. Farmasötik preparatların (Zeffix®, 100 mg 28 tabletleri ve NLC formülasyonu) ortalama geri kazanımı %99,552 idi. Yöntemin tespit sınırı (LOD) 1.49 µg mL^{-1}, miktar sınırı (LOQ) 0.51 µg mL^{-1} idi. Yöntem aynı zamanda mevcut ticari formülasyonda ve yeni geliştirilen NLC formülasyonunda bulunan LAM miktarının belirlenmesine ve farmasötik formülasyonların homojenliğinin doğrulanmasına da olanak sağladı. Elde edilen sonuçlar, geliştirilen HPLC yönteminin NLC ilaç taşıyıcı sistemlerde hem formülasyon geliştirme hem de stabilite çalışmalarında güvenilir bir şekilde kullanılabileceğini göstermektedir.

Anahtar Kelimeler: Lamivudin, HPLC, Farmasötik Formülasyon, Nanoyapılı Lipid Taşıyıcılar

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1. Introduction

Lamivudine (LAM) is a first generation nucleoside reverse transcriptase inhibitor (NRTI) approved for the treatment of human immunodeficiency virus-1 (HIV-1) infection in 1995 and hepatitis B virus (HBV) infection in 1998. Its chemical structure is shown in Figure 1 [1,2]. According to IUPAC nomenclature, 2‘,3’-Dihydroxy-3'-thiocytidine is called 4-Amino-1-((2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl)-1,2-dihydropyrimidinone [3]. LAM has the appearance of a white powder. It has a molecular weight of 229.26 g/mol and is a hydrophilic active substance with a solubility of 70 mg mL^{-1} in water at 20°C (LogP = -0.95). According to the current 2016 World Health Organisation (WHO) guidelines, LAM is recommended as first- and second-line antiretroviral (ARV) therapy from infancy to adolescence [4]. LAM, which is widely use in the treated of HIV, is a highly effective agent. It also has a long half-life [5]. This explains the administration of LAM once or twice a day. In addition, LAM, which is one of the best tolerated and safe drugs among all ARV agents, continues to be frequently preferred in single or combined preparations for the initial treatment of HIV patients [6].

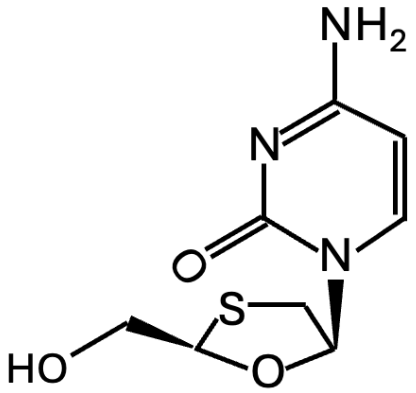

Figure 1. LAM's chemical structure.

There are various quantitative methods for the quantitative analysis of LAM. High performance liquid chromatography (HPLC) method is one of them [7,8]. HPLC quantification methods in published literature studies have been used for the determination of pharmaceutical preparations of LAM combined with other active ingredients [7-18]. In the literature studies, it was determined that there is no independent method for the quantification of LAM by HPLC to date. In the study, an independent method for the determination of LAM concentration in pharmaceutical formulations, including nanostructured lipid carrier (NLC) formulation, a novel drug carrier system, was developed and validated. This method is the first of its kind to allow accurate determination of LAM content in NLC formulations, marking a significant step forward in analytical techniques for advanced drug delivery systems.

The validation of the method was conducted in accordance with the International Conference on Harmonisation (ICH) Q2(R1) guideline, 'Validation of Analytical Procedures.' The evaluation included assessments of linearity, precision, accuracy, sensitivity, and stability. Utilizing a simple mobile phase, the method enables rapid analyses, completing each run within a short timeframe of 10 minutes. This efficiency is particularly crucial for the high-throughput analysis of multiple samples.

2. Materials and methods

a. Chemicals and reagents

Lamivudine (USP) (Batch: 22139215, EXP: 05.2026, Manufacturer: Mylan, Holland) was procured via donation from the company Nobel Pharmaceuticals Industry and Trade Inc. All solvents used in the HPLC device were HPLC grade and methanol was obtained from Sigma-Aldrich (USA). All chemicals used in the study were analytical grade and distilled water used in formulation and mobile phase preparation was obtained using Direct-Q®8 UV, Merck (Germany) water purification system.

Distilled water and MeOH at a ratio of 60:40, v:v was used as mobile phase for quantification analysis. Shimadzu (Japan), LC 20 AT, HPLC device was used. Separation of LAM was carried out at 270 nm using a C18 column (5 µm, 4.6×250 mm) EC 250/4.6 Nucleosil, Macherey-Nagel at a flow rate of 1.0 mL/min.

b. Preparation of mobile phase

The mobile phase was prepared by mixing 400 mL of methanol (MeOH) with 600 mL of water, followed by degassing in an ultrasonic water bath.

c. Preparation of standard and quality control samples

Standard solution of LAM was prepared using distilled water at a concentration of 100 µg mL^{-1} each. To generate a range of concentrations, eight distinct standard solutions ranging from 2 to 60 µg mL^{-1} (2, 5, 10, 20, 30, 40, 50, 60 µg mL^{-1}) were prepared through sequential dilution employing distilled water:MeOH (60:40, v:v). Additionally, a quality control (QC) samples were crafted at concentration of 50 µg mL^{-1}, derived from the stock standard solution.

d. Validation of HPLC Method of LAM

The HPLC method was validated for system suitability, linearity, accuracy, precision, sensitivity and limit of detection, specificity and stability.

A system suitability test was conducted for the chromatographic system prior to each analysis and validation procedure. This involved six consecutive injections of a calibration standard/system suitability solution and one injection of a control standard. Parameters such as the relative standard deviation (RSD) of the peak area, tailing factor, and column efficiency were evaluated based on the six suitability injections.

So as to examine linearity of method, calibration curves, regression equations and correlation coefficient (r) were calculated from the peak areas of samples diluted with LAM at 8 different concentrations ranging from 2-60 µg $mL^{-1}$for distilled water:MeOH (60:40, v:v).

For this study, samples were prepared at 80%, 100%, and 120% of the assay test concentration, and their recovery percentages were calculated. A total of nine samples were prepared, with three samples for each concentration level. The formula used to calculate recovery% is provided

in Equality 1 [19]. The accuracy was assessed by reporting the drug content of the samples at the specified concentrations as the arithmetic mean and standard deviation (SD).

Equality 1. $Recovery(\%) = \frac{Concentration\ of\ analysis\ results}{Theoretical\ concentration}\ x\ 100$

The precision of the method was analysed in terms of representing repeatability (intra-day) and intermediate precision (inter-day) by the evaluation of RSD%. For the representing repeatability (intra-day) parameter, the peak areas of the selected samples at low (40 µg mL^{-1}), medium (50 µg mL^{-1}) and high (60 µg mL^{-1}) concentrations were analysed six times. Intermediate precision (inter-day) analysis performed by peak areas measurements of six different samples at low concentration (40 µg mL^{-1}), medium (50 µg mL^{-1}) and six different samples at high concentration (60 µg mL^{-1}).

The limits of detection (LOD) and quantification (LOQ) of the HPLC method were determined based on the signal-to-noise (S/N) ratio, where LOD was calculated at an S/N ratio of 3:1 and LOQ at an S/N ratio of 10:1. The LOD represents the minimum detectable concentration, while the LOQ indicates the lowest concentration that can be reliably measured with accuracy and precision. These values are critical for the reliable detection and quantification of low analyte levels in samples.

For specificity assessment, distilled water:MeOH was used to confirm that it belongs to LAM only. The peaks of LAM-free solvents and LAM-containing solvents were compared.

Stability studies were performed by preparing LAM solutions in distilled water/MeOH (60:40, v:v) at the desired concentration. The samples were stored at room temperature (25°C) in tightly sealed containers, protected from light and contamination. Aliquots of the solution were taken at predetermined time intervals (6, 12, and 24 hours) and analyzed using the validated HPLC method. The recovery percentages were calculated based on the peak areas, and RSD% were determined from six replicate analyses at each time point. Stability was assessed by comparing the recovery values at each time interval with the initial values.

e. Process for pharmaceutical preparations

The average weight of Zeffix® tablets containing 100 mg LAM was determined and six tablets were finely crushed to obtain a homogeneous powder. A portion of this powder was accurately weighed to obtain a final concentration of 35 µg mL^{-1} when diluted to volume in a 100 mL volumetric flask with mobile phase (water/MeOH, 60:40 v/v) prior to analysis.

Lipid coated nanoparticles were prepared by hot homogenisation and ultrasonication [20-21]. Labrafil was used as liquid oil and stearic acid as solid oil in the formulation. Lauroglycol 90, a fat-soluble surfactant, added to lipid mixture, then hydrophobic active ingredient was dispersed in the molten lipid phase [20]. The water phase was prepared by mixing Tween 80 and water and brought to the same temperature as the oil phase. After the water phase was slowly added to the oil phase, the formulation was allowed to cool at room temperature and stored at 4°C for 24 hours.

Characterization studies were conducted to evaluate the physicochemical properties of the developed NLCs. Particle size, polydispersity index, and zeta potential were determined to assess the stability and uniformity of the formulations. Additionally, morphological analysis using transmission electron microscopy confirmed the spherical shape and uniform distribution of the nanoparticles. These characterization results confirm that the developed NLCs possess suitable properties for effective drug delivery, providing a promising platform for enhancing the therapeutic potential of the LAM.

The amount of active substance was calculated from the volume of prepared NLC formulations. The NLC formulation was then fractionated with MeOH and diluted with mobile phase according to scale. All formulations were sonicated for at least 10 minutes to help them dissolve. It was then filtered through a 0.45 μm membrane filter using a vacuum pump. An appropriated volume of the sample was diluted with mobile phase to ensure that the concentration of LAM in the final solution was within the linear regression operating range. It was then analyzed by HPLC.

3. Results and Discussion

3.1. Methodology Development and Optimization Process

In recent studies, there has been great interest in improving HPLC methods for the identification of drugs due to their important role in routine quality control analysis [21-23]. In this study, we recommend the use of an HPLC method as a suitable means for determining LAM in pharmaceutical dosage forms. Good separation was achieved with a C18 column (5 μm, 4.6×250 mm). Chromatographic conditions are adjusted to optimize the assay's performance. Method developed and then validated. The method employed a mobile phase comprised of distilled water: MeOH (60:40, v:v), analyse conducted at 270 nm. The retention time for the analysis with distilled water was 3.8 min at a flow rate of 1 mL/min. Volume of injection was 10 μL for analyse, total run time for assay was approximately 10 min. After numerous experiments with various solvent combinations, the choice of mobile phase was based on peak parameters (symmetry and tailing), running time, easy preparation and cost. The chromatogram of the mobile phase without LAM is displayed in Figure 2. The chromatogram obtained from standard LAM analysis using the developed method is shown in Figure 3. The chromatogram exhibits a distinct LAM peak with symmetrical characteristics, effectively separating from the solvent front. The observed retention time further facilitates rapid drug detection.

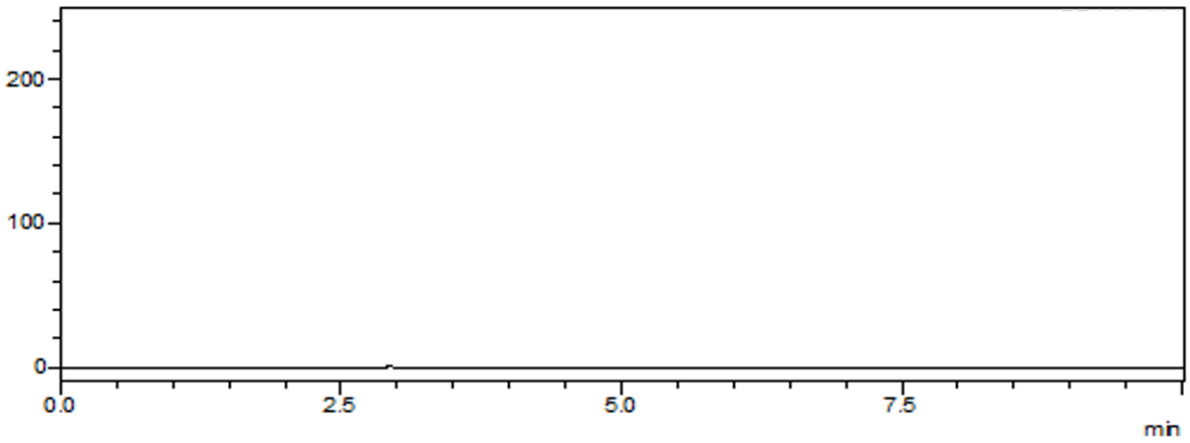

Figure 2. Chromatogram of the mobile phase

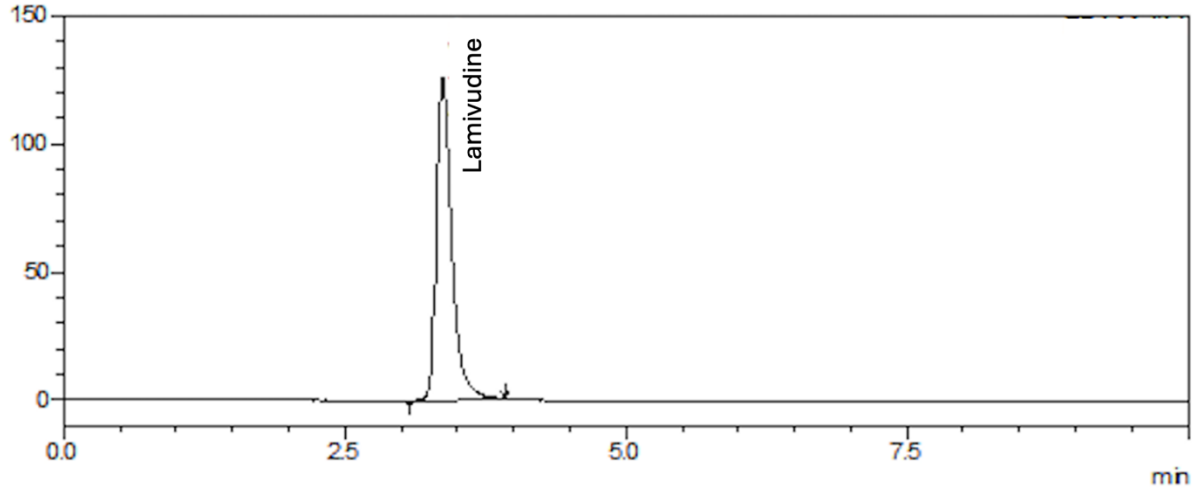

Figure 3. Chromatogram of the mobile phase containing LAM. (50 µg mL^{-1})

3.2.Method Validation

3.2.1.System Suitability

System suitability testing was performed prior to each analysis. It was measured based on the average of the result of six repeated injections of the control standard injection. Parameters such as area relative standard deviation, tailing factor and efficiency were determined for these six conformity injections. The control standard was measured relative to the average of the six injections of conformity. The tailing factor remained ≤ 1.6 and RSD% ≤ 1.26 during all sample analyses.

According to the guidelines, a RSD% value below 2% is required for the acceptability of the sensitivity of the method [24]. The RSD value of 1.26% was obtained in the developed method and is well below the value specified in the guideline. Therefore, the developed quantification method shows that it provides reliable and reproducible results throughout the study. This level of precision shows similar RSD% values for system suitability as other studies in the literature [20]. Therefore, the method fulfils both sensitivity and suitability criteria, strengthening its applicability for accurate quantification of LAM in pharmaceutical formulations.

3.2.2.Linearity

The construction of the calibration curve for the LAM standard involved plotting the compound concentration against the peak area. Solutions of standard containing 2 to 60 µg mL^{-1} (2, 5, 10, 20, 30, 40, 50, 60 µg mL^{-1}) of LAM were prepared, and 10 µL was injected into the HPLC column. Linear regression analysis, utilizing the least squares regression method, was utilized to evaluate the linearity. The regression equations, along with standard deviations of the slope (Sb) and intercept (Sa) of the regression line, were derived from the calibration graphs. The data obtained are shown in Table 1.

Table 1. Linearity of LAM Analysis: Linear Regression Parameters, Coefficient of Determination (R^2)

Solution	**Range**	**LR**	**Sa**	**Sb**	**R^2**
(60:40, v:v)	**(µg mL^{-1})**				
Distilled water/MeOH	2-60	y = 24456 x - 2986,5	32.501	342.65	0.9998

LR:Linear regression, x: LAM concentration, y: peak area

The calibration curve for LAM analysis was in the range of 2-60 µg mL^{-1}. The correlation coefficient (R^2 = 0.9998) showed a strong linear relationship between peak area and analyte concentration, indicating the reliability of the method for quantitative analysis. The regression equation was determined as y=24456x-2986.5.

3.2.3. Precision

QC samples were prepared and analysed for the precision. For repeatability (intra-day), samples were analysed on the same day. For intermediate precision (inter-day), samples were analysed on different days and evaluated. The results obtained are shown in Table 2.

Table 2. Precision

Solution (60:40, v:v)	**Added (µg mL^{-1})**	**Intra-day**		**Inter-day**	
		Found (µg mL^{-1})	**RSD%**	**Found (µg mL^{-1})**	**RSD%**
Distilled water/MeOH	40	41.06	0.07	40.26	0.13
	50	51.16	0.29	50.97	0.06
	60	61.04	0.12	60.06	0.12

SD represents the standard deviation obtained from six replicate determinations, while RSD denotes the relative standard deviation calculated based on the average of six replicate determinations.

The precision results of the developed quantification method show that it is reliable for the analysis of LAM. In the intra-day precision analysis, the RSD values of the samples were shown to be in the range of 0.07-0.29%. In the inter-day precision analysis, the RSD values were shown to be in the range of 0.06-0.13%. The data obtained in intra-day and inter-day precision analyses confirm that consistent results were obtained.

Overall, the low RSD values across intra-day and inter-day precision studies confirm the method's robustness and reliability, making it suitable for routine quality control analysis of LAM in pharmaceutical formulations.

3.2.4. Accuracy

The accuracy studies demonstrated the closeness of the measured values to the true values, confirming the absence of systematic errors in the method.

Table 3. Results of Accuracy

Concentration (μg mL^{-1})	**Concentration of analysis result (μg mL^{-1})**	**Recovery(%)**	**Average(%)**	**SD**
40	40.65	101.62	101.64	0.35
	40.65	101.62		
	40.67	101.68		
50	50.99	101.98	101.94	0.06
	50.98	101.97		
	50.93	101.87		
60	61.11	101.85	101.73	0.12
	61.04	101.73		
	60.97	101.61		
Average recovery% = 101.77		**SD = 0.18**		

When the accuracy results of the HPLC quantification method developed within the scope of validation studies were evaluated, it was shown that (Table 3) there were high recovery rates (average 101.77%) and low SD (0.18) values. These results show the reliability of the method by confirming the proximity of the value obtained in the method and the actual value. At different concentrations (40, 50 and 60 μg mL^{-1}), the average recovery percentages were 101.64%, 101.94% and 101.73%, respectively. These results show that the method provides accurate measurements within acceptable limits at all tested levels. In addition, it confirms that the method is reliable for LAM analysis.

3.2.5. Sensitivity

The LOD and LOQ values were determined on a signal-to-noise basis. LOD is the lowest concentration level that gives a peak height 3.3 times higher than the baseline noise. In the HPLC quantification method, LOD was 0.51 μg mL^{-1} and LOQ was 1.49 μg mL^{-1}. The calculated LOD and LOQ values are within acceptable ranges and indicate that high sensitivity

and reliable results can be obtained in the analysis of LAM. Low LOD and LOQ values indicate that the analyte at low concentration provides accurate and sensitive determination. The results show that the method fulfils the requirements of international validation guidelines such as ICH Q2(R1) [24]. The method is also suitable for routine LAM analysis, stability studies and quality control of pharmaceutical formulations containing LAM. The method shows high sensitivity and is a reliable method for the quantification of LAM.

3.2.6. Stability

Stability investigations showed that the samples remained stable when stored at room temperature for 6, 12 and 24 hours. The stability analysis results indicate that LAM in the distilled water/MeOH (60:40, v:v) solution remains highly stable under room temperature conditions for up to 24 hours.

The stability analysis results indicate that LAM in the distilled water/MeOH (60:40, v:v) solution remains highly stable under room temperature conditions for up to 24 hours. The recovery percentages at 6, 12, and 24 hours were 99.83%, 99.81%, and 99.57%, respectively, with very low RSD values ranging between 0.15% and 0.18%. These values demonstrate that there was no significant degradation of LAM over the studied time intervals.

The consistent recovery rates and minimal variation in RSD values confirm that the analyte's chemical stability is maintained in the given solvent system. These findings comply with stability study requirements outlined by international guidelines, such as ICH Q1A(R2). Thus, the method can be reliably used for analytical procedures where storage at room temperature for extended periods is required. This ensures that sample handling during routine laboratory work will not compromise the accuracy of the results.

3.2.6. Analysis of pharmaceutical formulations

Recovery studies involved the addition of a pure drug concentration to pre-analyzed tablet samples within the analytical concentration range of the developed method. The anticipated amounts of individual drugs were predicted using the aforementioned method. Satisfactory results were obtained from the recovery studies, with a recovery value of 99.74% ± 0.96 for Zeffix® tablets (100 mg) and 99.36% ± 1.25 for the NLC formulation (20 mg), demonstrating the accuracy of the developed HPLC method.

NLCs offer an important platform for the delivery of lipophilic and hydrophobic drugs because they have the advantages of increasing the bioavailability of drugs, providing controlled release and enabling specific targeting [22,25]. However, ensuring homogeneous distribution of the active substance in NLC formulations and verifying this distribution is critical in the evaluation of formulation quality and treatment efficacy.

Although NLC systems can improve the stability of the drug, there are some limiting factors that affect the homogeneous distribution of the active substance in the formulation. These factors include the distribution of the active substance in the lipid matrix, the influence of the formulation on processes such as ultrasonication and cooling, and changes in particle size as a result of aging over time [20,26]. Therefore, quantification of drug content in NLCs should be

considered as a critical quality control parameter both during the formulation process and during product stability.

The HPLC method developed in this study provided a high accuracy and sensitivity for the quantification of LAM in NLC formulations. The recovery results confirm that this method can be successfully applied to both commercial tablet formulations of LAM and newly developed NLC formulations. Ensuring the accurate quantification of LAM in NLCs is a critical step for verifying formulation homogeneity and clinical efficacy. [20].

This method can be used as an effective tool for both stability and formulation optimisation by verifying the homogeneous distribution of LAM content in NLC formulations.

4. Conclusions

A quantification method was developed and validated using HPLC for the determination of LAM. The method was validated according to ICH guidelines, with all validation parameters falling within the desired range. The results obtained prove that LAM quantification is a fast, simple, industry applicable method. Both Zeffix® tablets and the newly developed LAM-loaded NLC formulation were successfully analyzed using this method. Notably, the ability to quantify LAM in the novel lipid-based drug carrier system, NLC, highlights the method's effectiveness, as analyzing lipid-structured drug carriers like NLC, solid lipid nanoparticles, and lipid nanoparticles can be challenging due to their complex lipid structures. In addition, the method time of 10 min allows the analysis of LAM in many samples in a short time. Overall, this method is reliable, sensitive, and well-suited for industrial use in LAM analysis.

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