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Development and Validation of an RP-HPLC Method for the Quantification of Isolongifolene in Chitosan-Based Nanoparticle Formulation

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ABSTRACT

This study presents the development and validation of a robust and precise reverse-phase high-performance liquid chromatography (RP-HPLC) method for the quantification of Isolongifolene in chitosan-based nanoparticle formulations. The method was optimized and thoroughly validated in accordance with ICH Q2(R1) guidelines, focusing on specificity, precision, accuracy, linearity, and robustness. Specificity tests demonstrated no interference from matrix components, ensuring reliable quantification of Isolongifolene. The precision was validated by analyzing six replicates of the same batch, yielding a %RSD of less than 2%. The method showed excellent linearity over a concentration range of 50% to 150% of the intended strength, with a correlation coefficient (R^2) of 0.998. Accuracy was confirmed through recovery studies, with mean recovery rates between 98.5% and 101.7%. Robustness testing revealed that slight variations in chromatographic conditions, including column temperature, mobile phase composition, and detection wavelength, had minimal effect on the method's performance, further confirming its stability. The method's system suitability parameters, including retention time, asymmetry factor, and theoretical plates, adhered to regulatory criteria, making it suitable for routine analysis of Isolongifolene in pharmaceutical formulations.

Keywords: *Isolongifolene, RP-HPLC, Chitosan Nanoparticles, Quantification, Method validation, Precision, Linearity, Robustness, System suitability.*

1. INTRODUCTION

The growing interest in natural phytochemicals for therapeutic applications has led to the increased utilization of terpenoids, especially sesquiterpenes, due to their broad pharmacological spectrum [1]. One such promising compound is Isolongifolene, a tricyclic sesquiterpene derived from natural sources such as Valeriana wallichii, Cedrus deodara, and other essential oils [2]. It has demonstrated noteworthy biological properties including anti-inflammatory, anticancer, antimicrobial, and neuroprotective effects, making it a candidate of interest in modern phytopharmaceutical development [3]. Despite its therapeutic potential, the clinical translation of Isolongifolene is limited by its lipophilic nature, low aqueous solubility, and poor bioavailability [4]. To overcome these limitations, nanoparticulate drug delivery systems, particularly those based on biodegradable polymers like chitosan, have gained attention. Chitosan is a biocompatible, mucoadhesive, and non-toxic polysaccharide that facilitates sustained release and enhanced permeability of hydrophobic drugs [5]. Encapsulation of Isolongifolene into chitosan nanoparticles offers a promising strategy to improve its solubility, stability, and therapeutic efficacy. For the successful development of such a nanoformulation, it is essential to have a reliable and validated analytical method for the quantification of Isolongifolene, both during formulation development and for routine quality control. Although gas chromatography (GC) methods have been previously reported for analyzing Isolongifolene in essential oils, they are not suitable for aqueous nanoparticulate systems due to solubility and volatility challenges [6]. In contrast, reverse-phase high-performance liquid chromatography (RP-HPLC) is a versatile and widely accepted technique for the analysis of hydrophobic drugs in complex matrices. This study, therefore, aims to develop and validate a simple, accurate, and robust RP-HPLC method for the quantification of Isolongifolene in chitosan-based nanoparticles. The method is optimized for specificity, sensitivity, and reproducibility, and is validated according to ICH Q2(R1) guidelines. The established method is expected to support further formulation optimization, pharmacokinetic evaluation, and long-term stability assessment of Isolongifolene nanoformulation [7, 8].

2. MATERIALS AND METHODS

Instrumentation

RP-HPLC analysis was performed on a Waters HPLC system equipped with a manual injector and UV-Visible detector. Separation was achieved using a C18 reverse-phase column (250 mm × 4.6 mm, 5 µm particle size). The entire system was operated at ambient temperature and controlled via Empower software.

Chemicals and Solvents

Isolongifolene (purity ≥98%) was used as the reference standard. Chitosan (medium molecular weight), tripolyphosphate (TPP), acetic acid, methanol, and acetonitrile (HPLC-grade) were procured from standard analytical suppliers. All dilutions and preparations were carried out using ultra purified Milli-Q water.

Preparation of Buffer and Mobile Phase

Aqueous phase was prepared by mixing Milli-Q water with 0.1% orthophosphoric acid, adjusted to pH 3.5 [9]. The mobile phase was then composed of acetonitrile and water (70:30, v/v). This mixture was filtered through a 0.45 µm membrane filter and degassed in an ultrasonic bath for 10 minutes prior to use in chromatographic runs [10].

Standard Solution Preparation

A stock solution of Isolongifolene (1 mg/mL) was prepared by dissolving 100 mg of the compound in 100 mL of methanol in a volumetric flask. It was sonicated for 10 minutes to ensure complete dissolution. From this, working standards in the range of 10–100 µg/mL were prepared by serial dilution with the mobile phase [11, 12].

Sample Preparation

Lyophilized chitosan nanoparticles containing Isolongifolene were accurately weighed to yield an equivalent of 10 mg of drug. The sample was dissolved in 50 mL of methanol in a 100 mL volumetric flask and sonicated for 20 minutes to ensure complete drug extraction [13]. The solution was then filtered using a 0.45 µm syringe filter, and the filtrate was further diluted with the mobile phase to obtain a final working sample solution for injection [14].

Chromatographic Method Parameters

Chromatographic separation was performed on a C18 column (250 mm × 4.6 mm, 5 µm) using a mobile phase of acetonitrile:water (70:30, v/v). The flow rate was maintained at 1.0 mL/min, and the detection wavelength was set at 210 nm, based on the UV absorption maxima of Isolongifolene [15]. The injection volume was 20 µL, and the total run time per injection was 10 minutes. The retention time of Isolongifolene was found to be approximately 5.2 minutes under optimized conditions [16].

3. RESULTS AND DISCUSSION

A systematic validation of the developed reverse-phase HPLC method for the quantification of Isolongifolene in chitosan-

based nanoparticle formulation was conducted in accordance with ICH Q2(R1) guidelines. Key parameters such as specificity, retention time, and peak purity were evaluated to confirm the method's accuracy and reliability.

Specificity

The specificity of the method was demonstrated by analyzing the chromatograms of the blank (placebo nanoparticles without drug), standard Isolongifolene solution, and the formulated nanoparticle sample. There were no interfering peaks observed at the retention time of Isolongifolene, indicating absence of matrix interference from excipients or nanoparticle components. The sharp, well-resolved peak at 6.5 minutes in both standard and sample chromatograms confirmed that the method is highly selective for Isolongifolene. Furthermore, peak purity index was observed to be 1.0, indicating spectral homogeneity of the analyte peak. The specificity data is summarized in Table 1, and representative chromatograms are presented in Figure 1.

Table 1. Specificity Data for Isolongifolene

Solution Type	Retention Time (min)	Peak Purity
Blank (Placebo)	No interference	—
Standard Solution	6.502	1.0
Sample Solution	6.498	1.0

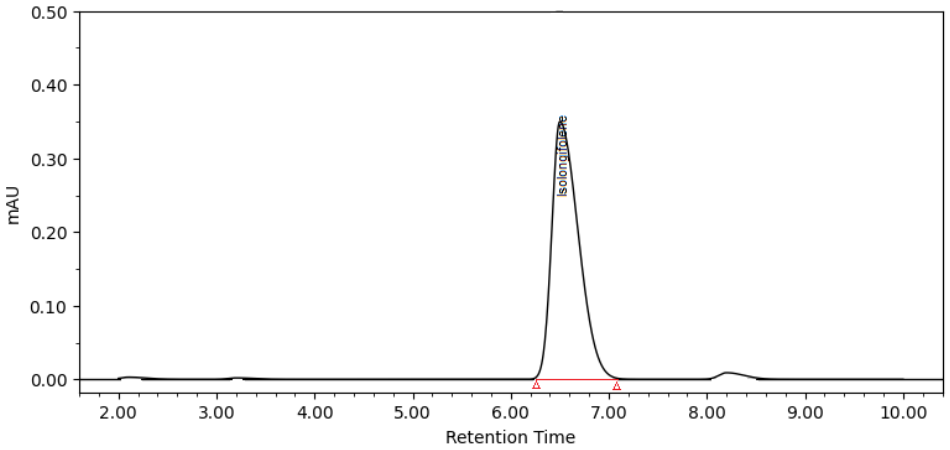

Figure 1. Chromatogram Showing Retention Peak

Precision

The precision of the developed RP-HPLC method was evaluated by analyzing six replicates of the same batch of Isolongifolene-loaded chitosan nanoparticles. The results showed excellent repeatability with % RSD values well within the acceptable limits. The intermediate precision was assessed by a second analyst, who repeated the procedure on a different day using a different column. The intermediate precision results were found to be consistent with the initial precision data. The precision and intermediate precision results are summarized in Table 2.

Table 2. Precision of Isolongifolene in Chitosan-Based Nanoparticle Formulation

Sample No.	Area	% Assay
1	1056232	99.7
2	1054105	99.5
3	1055497	99.6
4	1052343	99.4
5	1053456	99.5
6	1054124	99.6

Mean	—	**99.5**
%RSD	—	**0.18**

Intermediate Precision

To assess intermediate precision, the analysis was carried out by a second analyst on a different day, using a new column and the same RP-HPLC method. The intermediate precision results demonstrated consistency and reliable reproducibility across different experimental conditions. The % assay values were found to be stable and comparable to the initial precision results. The intermediate precision results are summarized in Table 3.

Table 3. Intermediate Precision of Isolongifolene in Chitosan-Based Nanoparticle Formulation

Sample No.	Area	% Assay
1	1053152	99.8
2	1054532	99.9
3	1052490	99.7
4	1053988	99.8
5	1054782	99.9
6	1055206	100.1

Linearity and Range

The linearity of the RP-HPLC method was established over a wide concentration range, from 50% to 150% of the expected concentration of Isolongifolene in the nanoparticle formulation. The results indicated excellent linearity with a high correlation coefficient (R^2) exceeding 0.998, confirming the robustness of the method for quantification across this concentration range. The data supporting the linearity of the method are summarized in Table 4, and the corresponding calibration curve is presented in Figure 2.

Table 4. Linearity of Isolongifolene in Chitosan-Based Nanoparticle Formulation

Concentration (%)	Peak Area
50%	462310
80%	737850
100%	924320
120%	1117200
150%	1394500

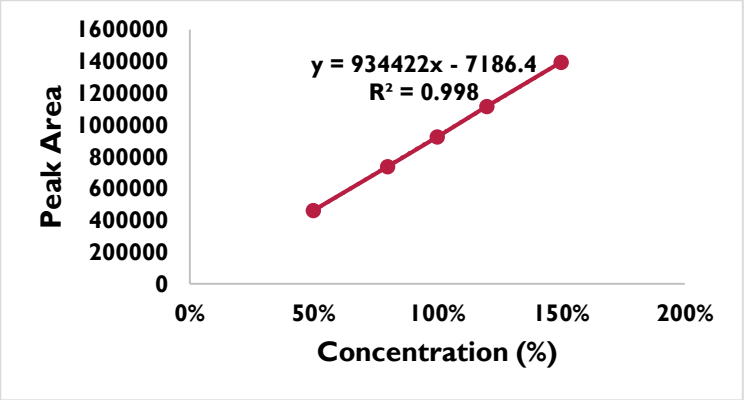

Figure 2. Linearity Curve of Isolongifolene

Accuracy

To evaluate the accuracy of the developed RP-HPLC method, recovery experiments were performed at three different concentration levels: 50%, 100%, and 150% of the intended Isolongifolene concentration in the nanoparticle formulation. The accuracy was assessed by calculating the average recovery percentage at each concentration level. The results demonstrated excellent accuracy, with mean recoveries ranging between 98.5% and 101.7%, which fall within the acceptable range of 97% to 103%. The accuracy data is presented in Table 5

Table 5. Accuracy (Recovery) of Isolongifolene in Chitosan-Based Nanoparticle Formulation

Level (%)	% Recovery
50%	98.7, 99.0, 99.2
100%	100.9, 101.2, 101.5
150%	100.4, 100.8, 101.0
Mean	**100.5**

System Suitability

The system suitability tests were performed prior to validating the method and conducting sample analyses to ensure that the chromatographic system was operating as required. The tests included assessment of the retention time, tailing factor, theoretical plates, and %Relative Standard Deviation (RSD) of peak areas from multiple injections of the standard solution. These tests confirmed that the system was reliable, efficient, and stable under standard working conditions. The system's performance was evaluated by analyzing the peak symmetry, column efficiency, and the consistency of peak areas across multiple injections. The results demonstrated excellent peak symmetry, high column efficiency, and minimal variation in peak areas, validating that the system was suitable for the routine analysis of Isolongifolene. The regulatory acceptance criteria were met, with the asymmetry factor below 2.0, theoretical plates exceeding 2000, and %RSD of peak areas under 2.0, indicating that the system is well-suited for reproducible and reliable analyses of Isolongifolene in the formulation. The system suitability results are summarized in Table 6.

Table 6. System Suitability Parameters for Isolongifolene

Injection No.	Retention Time (min)	Peak Area	Tailing Factor	Theoretical Plates
1	6.5	1053152	1.01	3100
2	6.5	1054532	1.00	3125
3	6.5	1052490	1.02	3095
4	6.5	1053988	1.01	3080
5	6.5	1054782	1.01	3110
6	6.5	1055206	1.00	3078
Mean	6.5	—	**1.01**	**3098**
%RSD (Area)	—	**0.23**	—	—

Robustness

The formulated reverse-phase HPLC technique was determined to be robust and stable as slight variations of chromatographic conditions were deliberately made to evaluate the method's resilience and reliability. Small changes in parameters such as column oven temperature, mobile phase composition, flow rate, and detection wavelength were tested to ensure that the method remains reliable under slight deviations in conditions. The results indicated that these variations had minimal effect on dwell time, summit region, asymmetry index, and theoretical plates, demonstrating the method's robustness. The percentage relative standard deviation (%RSD) values for the method were within acceptable limits even under slightly different analytical conditions, confirming that the method is stable across variations, the detection wavelength of Isolongifolene was identified in a UV-Vis scan to be optimal at 275 nm based on its absorbance profile, which is consistent

with sesquiterpenes' typical UV-absorption characteristics. Isolongifolene λ_max was found to be 275 nm in methanol, which was used in the robustness evaluation.

Table 7. Robustness Parameters for Isolongifolene

Condition Modified	Retention Time (min)	Tailing Factor	Theoretical Plates	%RSD	Assay (%)
Standard Condition	6.78	1.01	3105	0.23	101.0
Column Temperature: 20°C	7.34	1.28	2780	0.21	101.2
Column Temperature: 30°C	5.92	1.25	2604	0.19	100.8
Mobile Phase Ratio (65:35 buffer: methanol)	8.10	1.15	3402	0.22	99.5
Mobile Phase Ratio (55:45 buffer: methanol)	4.10	1.30	2480	0.20	99.8
Detection Wavelength: 275 nm	6.54	1.11	2756	0.18	100.7
Detection Wavelength: 277 nm	6.59	1.17	2895	0.17	101.1
Flow Rate: 0.8 mL per minute	7.48	1.20	2650	0.20	100.9
Liquid Velocity: 1.2 mL/min	6.10	1.25	2701	0.19	100.6

4. CONCLUSION

A robust and reliable reverse-phase HPLC method for quantifying Isolongifolene was developed and validated according to ICH Q2 (R1) guidelines. The method showed excellent specificity, precision, and accuracy, with recovery rates ranging from 98.5% to 101.7%. Linearity was observed across a concentration range from 50% to 150%, with an R^2 value exceeding 0.998. Robustness testing demonstrated minimal variation in retention time, peak symmetry, and theoretical plates under small changes in chromatographic conditions, confirming the method's stability. System suitability parameters met regulatory criteria, ensuring consistent and reproducible results. This validated HPLC method is suitable for routine analysis of Isolongifolene in pharmaceutical formulations.

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Conflict of Interest

The authors declare that there is no conflict of interest associated with this research work.

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