Validation of Analytical Method for Vitamin A in Bioadhesive Ocular Cationic Nanoemulsion Loaded into Thermosensitive Gel Using RP-HPLC

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Abstract: *Various test methods have been previously documented for determining vitamin A levels in different dosage forms. This study specifically examines an isocratic reverse phase-high performance liquid chromatography (RP-HPLC) method designed for the direct extraction of vitamin A. The objective is to validate an analytical method for quantifying vitamin A in bioadhesive cationic nanoemulsions incorporated into thermosensitive gels. The method employs isocratic RP-HPLC with a YMC-Triart C18 column (L1), dimensions of 4.6 mm × 250 nm, particle size of S-5 µm, and a UV detector at λ = 265 nm. The mobile phase consists of HPLC-grade methanol, acetonitrile, and* n-*hexane in a ratio of 46.5:46.5:7. Validation parameters were assessed including selectivity, linearity, accuracy, precision, limit of quantification (LOQ), and limit of detection (LOD). Correlation coefficients were determined with an R^2 value of 0.9995 in the concentration range of 264–396 µg/mL (w/v). Recovery percentages ranged from 99.295% to 99.878%. Repeatability and intermediate precision relative standard deviations (RSD) were found to be 0.318% and 0.254%, respectively. The LOD was established at 2.018 µg/mL, and the LOQ was determined to be 6.114 µg/mL. The results affirm cost-effective and well-suited for the accurate measurement of vitamin A levels in bioadhesive thermosensitive gel formulations.*

Keywords: *analytical method; bioadhesive gel; HPLC; vitamin A*

■ INTRODUCTION

Within the realm of fat-soluble vitamins, vitamin A plays a crucial role in vision [1]. Functioning as an antioxidant molecule, vitamin A addresses oxidative stress by binding to peroxyl radicals and halting the oxidation chain on the eye's surface [2]. Moreover, it boosts anti-inflammatory responses, aids in treating dry eye disease, and diminishes the risk of age macular disease [3]. Nanosystems with bioadhesive properties, such as cationic nanoemulsions, have proven to be effective in transporting optimal concentrations of bioactive molecules to the eye [4]. Thermosensitive gels, a type of *in situ* gel containing a loaded nanoemulsion, offer improved permeation and a sustained-release drug profile and thus represent a viable alternative to commercial eye drops [5].

Knowing the retinol level in dietary supplements is crucial in determining the vitamin A dose, thereby mitigating the risk of overdosing and toxicity [6]. Various test methods for determining vitamin A levels in different dosage forms have been previously documented. A study has utilized ultraviolet (UV) spectroscopy [7], while others have employed high-performance liquid chromatography (HPLC) [8-10]. However, the determination of vitamin A levels in cationic nanoemulsions loaded onto thermosensitive gels as bioadhesive matrices has not been reported. This study employed isocratic RP-HPLC to assess the vitamin

A content in the matrix. Measuring the vitamin A concentration in cationic nanoemulsions loaded onto thermosensitive gels is challenging because vitamin A is insoluble in water and its stability is influenced by the medium, pH, antioxidants, and storage conditions. In particular, vitamin A is particularly susceptible to the effects of light, heat, and oxidation [11]. Vitamin A was encapsulated within nanoemulsion droplets and retained in the core of the poloxamer micelles, requiring additional time to ensure the complete release from the matrix. The cationic nanoemulsion consisted of an oil phase containing vitamin A and surfactants that dispersed the oil phase in water. Fortunately, the structural integrity of poloxamer-based gels is typically degraded rapidly in aqueous environments, leading to the rapid release of loaded drugs. Additionally, low-weight hydrophilic molecules readily diffuse through the interconnected porous network of the gel, resulting in the rapid early release of vitamin A [12].

Unlike the analysis of pure vitamin A active ingredients, which does not require a purification process during sample preparation, vitamin A in this bioadhesive gel needs to be purified first from residues that affect the determination of levels. The purification of vitamin A can be performed by saponification or direct extraction [13]. In accordance with the USP 46 <571> vitamin A assay chromatographic methods procedure 4, vitamin A was extracted with direct extraction method, using hexane in a liquid–liquid extraction process [14]. According to Ishimaru et al. [15], the retinoid content decreased by about 10% as a result of saponification treatment. Literature reports indicate that different extraction solvents, such as ethanol/hexane (50/50, v/v), have yielded good recoveries of retinyl palmitate [13]. To address this issue, following the USP method is suitable.

However, this preparation requires additional extraction time to ensure complete release of vitamin A from the matrix complex and ensure that impurities from the excipients do not interfere with the analysis. Extending the sample preparation time increases the probability of the oxidative degradation of vitamin A. The antioxidant butylated hydroxytoluene (BHT) is required to prevent the heat- and light-induced degradation of vitamin A [16]. Also, these methods are quite costly with a larger proportion of hexane (20/300, v/v), particularly when many samples must be analyzed. Additionally, hexane evaporates, posing a risk to vitamin A adhering to the surface of the round-bottom flask, which subsequently degrades due to the evaporation temperature of 65 °C, whereas vitamin A remains stable at temperatures below 15–20 °C [17]. Furthermore, owing to the low vitamin A content in the preparation, preventing the loss of vitamin A during analysis is imperative to obtain as high a recovery as possible, ideally 100%.

The novelty lies in enhancing the sample preparation process to ensure complete release from the matrix, minimize impurities, and prevent vitamin A degradation, resulting in the accurate and reliable determination of vitamin A levels. Therefore, validation of the analytical method is required to prove that the alternative step sample preparation method remains valid. Accordingly, the primary objectives of this study were to develop a method for the analysis of vitamin A and validate an analytical method for the quantification of the vitamin A content in bioadhesive cationic nanoemulsions loaded onto thermosensitive gel preparations using isocratic RP-HPLC. This validated method is expected to be widely applicable in routine quality-control analyses of complex matrices owing to its simplicity, cost-effectiveness, and efficiency.

■ EXPERIMENTAL SECTION

Materials

The reagents used for analysis were of HPLC grade, including acetonitrile (Merck), *n*-hexane (Merck), methanol (Merck), tetrahydrofuran (THF, Merck), *n*-hexane (Merck), and ethanol (Merck). The reference standard employed was retinyl palmitate certified reference material (certified purity 94.6%, U_{crm} = ±0.9%, k = 2.8, as is basis, Sigma Aldrich). Other materials included BHT (Sigma Aldrich) and a hydrophobic PTFE syringe filter (membrane solution). The vitamin A component in the bioadhesive ocular cationic nanoemulsion loaded into thermosensitive gel preparation consisted of capric triglyceride (The Nisshin Oillio Group), vitamin A palmitate (USP units/g 1810000,

Sigma Aldrich), Capryol® 90 (Gattefosse), and Transcutol® HP (Gattefosse) obtained from PT Menjangan Sakti, (Indonesia), Kolliphor® El (Sigma Aldrich), Poloxamer 188 (Sigma Aldrich), Poloxamer 407 (Sigma Aldrich), cethalconium chloride (Sigma Aldrich), sodium hydroxide (Merck), and aqua pi (PT Otsuka Indonesia).

Instrumentation

The instrument used for analytical method validation and vitamin A determination was HPLC (LC-2030C (-48) Shimadzu Corp. Liquid chromatography-mass spectrometer (LC-MS) (SCIEX Triple Quad™ 4500, Ref QTRAP 455, Singapore) was used to verify the results of the specificity test.

Procedure

Chromatography system, mobile phase, and solvent preparation

The chromatography system and mobile phase for determining vitamin A were obtained from a modified version of the USP 46 <571> vitamin A assay chromatographic methods procedure 4 [14]. The HPLC was employed for vitamin A content determination, utilizing a YMC-Triart C18 column (L1), 4.6 mm × 250 nm S-5 µm, and a UV detector at 265 nm. Key analysis parameters included a flow rate of 1.5 mL/min, injection volume of 20 µL, and a column temperature set at 40 °C. The mobile phase comprised HPLC-grade methanol, acetonitrile, and *n*-hexane in a ratio of 46.5:46.5:7. The diluent was a mixture of THF and acetonitrile dissolved in 0.1% w/v BHT at a 1:1 ratio.

Preparation of working standard solution

The vitamin A palmitate reference standard, certified with a purity of 94.6% was carefully weighed to an equivalent of 33 mg retinol in a 100 mL volumetric flask. It was then diluted with the diluent up to the limit mark, shaken homogeneously, resulting in a concentration of 330 µg/mL retinol (100% assay). The working standard was stored in a tightly closed dark bottle in a cold room at 2–8 °C.

System suitability

System stability was assessed using a standard solution, with a requirement for a relative standard deviation not exceeding 5.0%. Another aspect of the system's stability was based on the chromatographic <621> system suitability. The system was initially confirmed by injecting a standard solution (equivalent to 330 µg/mL retinol) for six times [14].

Pre-validation

Pre-validation was conducted during the stability testing of the standard and sample solutions. Both solutions underwent testing under normal laboratory conditions for 0, 4, or 8 h at room temperature. Stability was confirmed when the relative standard deviation (RSD) of the assay was < 2.0% [18].

Analytical performance characteristics

The assessment of linearity involved creating a series of 6 standard solutions with concentrations of 80, 90, 95, 100, 110, and 120% were analyzed by HPLC according to the level determination procedure. Subsequently, the slope and linear regression were calculated, with an acceptable value of R-squared (R^2) > 0.999 [14,18]. Precision is categorized as repeatability and intermediate precision (ruggedness). Repeatability was determined by testing the prepared sample 6 times, with an acceptable RSD value of less than 2.0%. To determine intermediate precision (ruggedness), 6 determinations containing 100% of the sample preparation were conducted by different analysts, with an acceptable RSD value of 2.0% [14].

Accuracy was evaluated through recovery experiments using the standard addition method with 3 concentrations (80, 100, and 120%) and 3 replicates for each concentration. Each mixture underwent testing [14]. The acceptance criteria mandated a recovery within the range of 95–105%, with a maximum RSD value of 2.0% for each concentration [19].

Specificity parameters were established using a placebo (blank sample), standard, and sample, adhering to the analytical method for determining vitamin A levels. Mass spectrometry analysis was employed to ensure the effective extraction of vitamin A from the dosage form matrix during the sample preparation process. The extracted samples and retinyl palmitate standard were dissolved in acetonitrile gradient grade

and subsequently injected into the LC-MS system. Identification of retinyl palmitate in both the samples and standards was based on parent ion masses coinciding with authentic standards. Detection of retinyl palmitate utilized a LC-MS equipped with an electrospray ionization (ESI) probe operating in positive ion mode. ESI (+)-MS/MS, *m/z* parent ion > *m/z* daughter ion 524.6/197.0, with ESI collision energy set at 40 V [20]. Qualitative analysis involved confirming HPLC chromatograms of standard and sample solutions with positive results at relatively similar retention times. In contrast, the placebo chromatogram did not exhibit a similar retention time. For quantitative analyses, specificity was ascertained by spiking the reference standard at an appropriate level, akin to the accuracy test. The test result was considered valid if it demonstrated no influence from the presence of foreign materials (reference standard) [14].

The limit of detection (LOD) and limit of quantification (LOQ) were determined using a successive series of linear standard solutions. LOD was defined as the lowest analyte concentration that produced a signal, as shown in Eq. (1);

$$\mathrm{LOD} = y_b + 3.3S_b \quad (1)$$

where y_b is the blank signal, and s_b is the standard deviation [21]. The LOQ represents the lowest amount of analyte that can be quantitatively determined and is defined in Eq. (2) [19].

$$\mathrm{LOQ} = \text{the average (blank)} + 10 \times S_0(\text{blank}) \quad (2)$$

Sample preparation

A sample of a vitamin A cationic nanoemulsion loaded onto a thermosensitive gel was formulated based on formula optimization in previous studies. Extraction was carried out using hexane containing 0.1% BHT. The sample, equivalent to 330 µg/mL retinol, was placed in a 500 mL separatory funnel containing 20 mL of absolute ethanol. Subsequently, 50 mL of *n*-hexane was added, and the mixture was shaken for 5 min. The solution was allowed to stand until the layers separated. The *n*-hexane extraction process was repeated twice, each time with a volume of 25 mL. Anhydrous sodium sulfate was employed to filter the *n*-hexane extract into a porcelain cup. The resulting *n*-hexane extract was evaporated to dryness in a fume hood at room temperature. Immediately, 10.0 mL of diluent was added, mixed to dissolve the residue, and the solution was filtered using a 0.2 µm hydrophobic PTFE syringe filter.

■ RESULTS AND DISCUSSION

Vitamin A levels were determined based on their correlation with retinol levels. The process for determining vitamin A levels involves an extraction method that has been widely employed by several researchers, yielding satisfactory results with various solvents [6]. In this study, the method referred to was USP 46 <571> vitamin A assay chromatographic methods procedure 4, which involved extracted a vitamin A sample using hexane in a liquid-liquid extraction process [14]. However, some alternative steps were made to the sample preparation process, included more efficient amounts of hexane and evaporated in a porcelain dish at room temperature under a fume hood under dark conditions. Antioxidants were required to prevent vitamin A degradation owing to repetitive the extraction process and increasing the extraction time. Synthetic antioxidants, such as BHT, were chosen for their lower cost, extended shelf life, and ready availability [22]. The addition of BHT antioxidants helps prevent the oxidative rancidity of fats and oils, preserves the activity of oil-soluble vitamins, and provides enhanced protection to vitamin A esters [23].

The analytical method was validated in accordance with the provisions of USP 46 <1225> validation of compendial procedures. In this case, determining the vitamin A levels in the preparation falls into category 1, involving the validation of analytical methods for quantifying the active ingredient components of drugs in pharmaceutical products. The parameters that must be determined for analytical method validation include accuracy, precision, specificity, linearity, and range [14,24]. Additional information was obtained to determine LOD, LOQ, and stability of the standard and sample solutions over 8 h.

Suitability System

System suitability tests are integral to the analytical procedure, ensuring adequate performance of the

chromatographic system. In this study, longer chromatographic columns were chosen over shorter ones to achieve enhanced separation capabilities. This resulted in resolution values exceeding 2.0, tailing factor values below 2.0, and the observation of well-defined Gaussian-shaped peaks [25]. Parameters such as column plate number or theoretical plate (N), retention factor or capacity factor (k'), system repeatability (assay), signal-to-noise ratio (S/N), symmetry factor or tail factor (T), and resolution/peak-to-valley ratio (R_s) are used to assess chromatographic system performance, following <621> chromatography guidelines. Based on <571> vitamin A procedure 4, the required RSD for each system suitability parameter was less than 5%. As the RSD requirement exceeded 2.0%, data were obtained from six replicate injections [14].

Notably, the RSD linked to the repeatability of the retention time (t_R) should be less than or equal to 1%. Additionally, the T value should be two or less, the R_s value should be greater than two, N should be more than 2000, and the capacity factor should be greater than 2.0 [18]. According to Table 1, all system suitability parameters comply with the requirements. The results of the suitability testing were expected to ensure the validity of the analysis procedure throughout the analytical process.

Pre-validation: Standard Solution Stability Testing

Since chemical decomposition can occur during storage, it is important to assess the stability of a drug by analyzing samples and standard solutions. This allows the estimation of the maximum interval between sample preparation and analysis. The pre-validation method requirement focuses on ensuring stability, which must be sufficient for the timeframes of analysis. An acceptable criterion for stability is that the standard and sample solutions remain stable within 2% [18,26].

Pre-validation of the vitamin A palmitate standard and sample solution was conducted at 0, 4, and 8 h, revealing stability during storage and analysis. This evaluation, critical for reliable quantification, is indicated by the relative standard deviation of the examined area [9]. The pre-validation results are presented in Table 2, demonstrating that the standard and sample solutions remained stable for 8 h with standard RSDs of 0.385 and 0.629, respectively.

Table 1. System suitability result of vitamin A determination

Parameters	Specification	Average ± SD	RSD (%)
Peak area	RSD < 5.0 [14]	4,520,014.667	0.093
N	> 2000 [18]; RSD ≤ 5% [14]	28,303 ± 1167.202	4.124
k’	> 2.0 [18]; RSD ≤ 5% [14]	6.469 ± 0.021	0.323
Assay	RSD < 5.0 [14]	99.029 ± 0.092	0.093
S/N	RSD < 5% [14]	10,978.198 ± 383.043	3.489
T	≤ 2 [18]; RSD ≤ 5% [14]	1.249 ± 0.012	0.951
R_s	≥ 2 [18]; RSD ≤ 5%	4.023 ± 0.061	1.524
t_R	RSD ≤ 1% [18]	14.441 ± 0.026	0.179

Table 2. Stability profile sample and standard solutions

Time study (h)	Assay of standard		Assay of sample	
	µg/mL	%	µg/mL	%
0	331.651	100.630	331.318	100.503
4	330.943	100.360	329.366	99.758
8	329.657	99.869	328.045	99.254
Average ± SD	330.750 ± 1.011	100.286 ± 0.386	329.576 ± 1.646	99.838 ± 0.628
RSD (%)	0.306	0.385	0.500	0.629

Analytical Performance Characteristics Result

Linearity

Linearity is crucial throughout analytical procedures. As the Compendia did not specify the level of vitamin A in ophthalmic preparations, the linearity test was based on the Compendia's requirements for other vitamin A-containing preparations. Typically, vitamin A levels fall between 90–120%, and the linearity test range is set between 80–120% or 264–396 µg/mL. Furthermore, the ICH recommends a minimum of five concentrations, with the test concentration falling between 80 and 120% for drug substances or finished products [14]. Linearity is confirmed if the correlation coefficient (R) exceeds 0.999 [18]. Fig. 1 displays R and R^2 values of 0.9998 and 0.9995, respectively, indicating that the analytical method for determining vitamin A levels provides a linear response to concentration changes and assay measurements.

Precision (repeatability and intermediate precision/ruggedness)

The precision of an analytical procedure is vital for ensuring consistent and reproducible results. This was accomplished by applying the same procedure to multiple homogeneous samples and measuring the agreement among individual test results. Vitamin A level determination validation included a precision test performed using repeatability and intermediate precision parameters (ruggedness). Repeatability involves the use of analytical procedures within a short time in the laboratory, while intermediate precision considers variations within the laboratory, including those of different analysts [14].

Repeatability is typically expressed as a measurement series SD or RSD (coefficient of variation) [19]. As shown in Table 3, the RSD value for repeatability, also known as the reproducibility test, was less than 2.0%, specifically 0.318, meeting the repeatability requirements.

Intermediate precision, also known as ruggedness, accounts for variation within a laboratory that may occur on different days or when different analysts or equipment are used. This evaluation helps assess the robustness of the method, ensuring reliable and consistent results [14]. Table 4 illustrates that the RSD value of ruggedness, performed by different analysts, was less than 2.0%, specifically 0.254%. These results indicate

Table 3. Repeatability data

No. sample	Sample concentration (µg/mL)	Assay (%)
1	326.438	98.921
2	327.197	99.151
3	327.789	99.330
4	326.693	98.998
5	328.361	99.503
6	329.170	99.748
Average	327.608	99.275
Standard deviation	1.041	0.315
RSD (%)	0.318	0.318

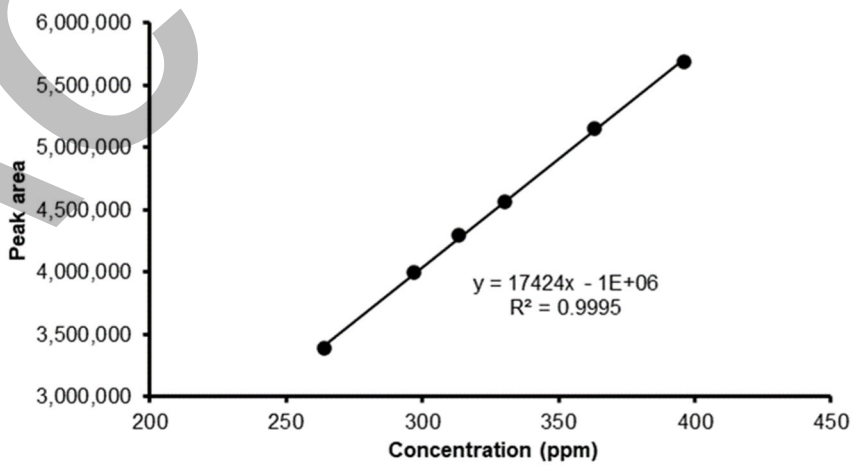


Fig 1. Linearity result

Table 4. Intermediate precision

No. Sample	Sample concentration (µg/mL)		Assay (%)	
	1st Analyst*	2nd Analyst	1st Analyst*	2nd Analyst
1	326.438	326.518	98.921	98.945
2	327.197	327.344	99.151	99.195
3	327.789	326.793	99.330	99.028
4	326.693	326.770	98.998	99.021
5	328.361	327.099	99.503	99.121
6	329.170	326.595	99.748	98.968
Average	327.231		99.161	
Standard deviation	0.832		0.252	
RSD (%)	0.254		0.254	

*) The data were taken from a repeatability test conducted by the first analyst and compared with a second analyst's

that the variation in vitamin A concentration during preparation did not impact the results.

Accuracy

The accuracy of an analytical method reflects how closely the test results align with actual values. It is crucial to assess the precision of sample preparation, as it directly influences the accuracy of the results. The addition method is commonly employed to determine percent recovery for accurate measurements. Furthermore, establishing the test concentration covering the specified range of sample concentrations analyzed at a minimum of three concentration levels within the analytical range is essential. The 80–120% concentration range is typically chosen, encompassing the sample concentration range and including concentrations close to the specified quantitation limit. The average percent recovery must be within 95–105% [19].

The accuracy of the analytical method, as indicated in Table 5, was satisfactory. The percentage reliability test conducted at three different concentration levels demonstrated results meeting the requirements with a RSD of less than 2.0%. These findings suggest that the analytical method is capable of producing accurate and reliable values across the 80–120% concentration range.

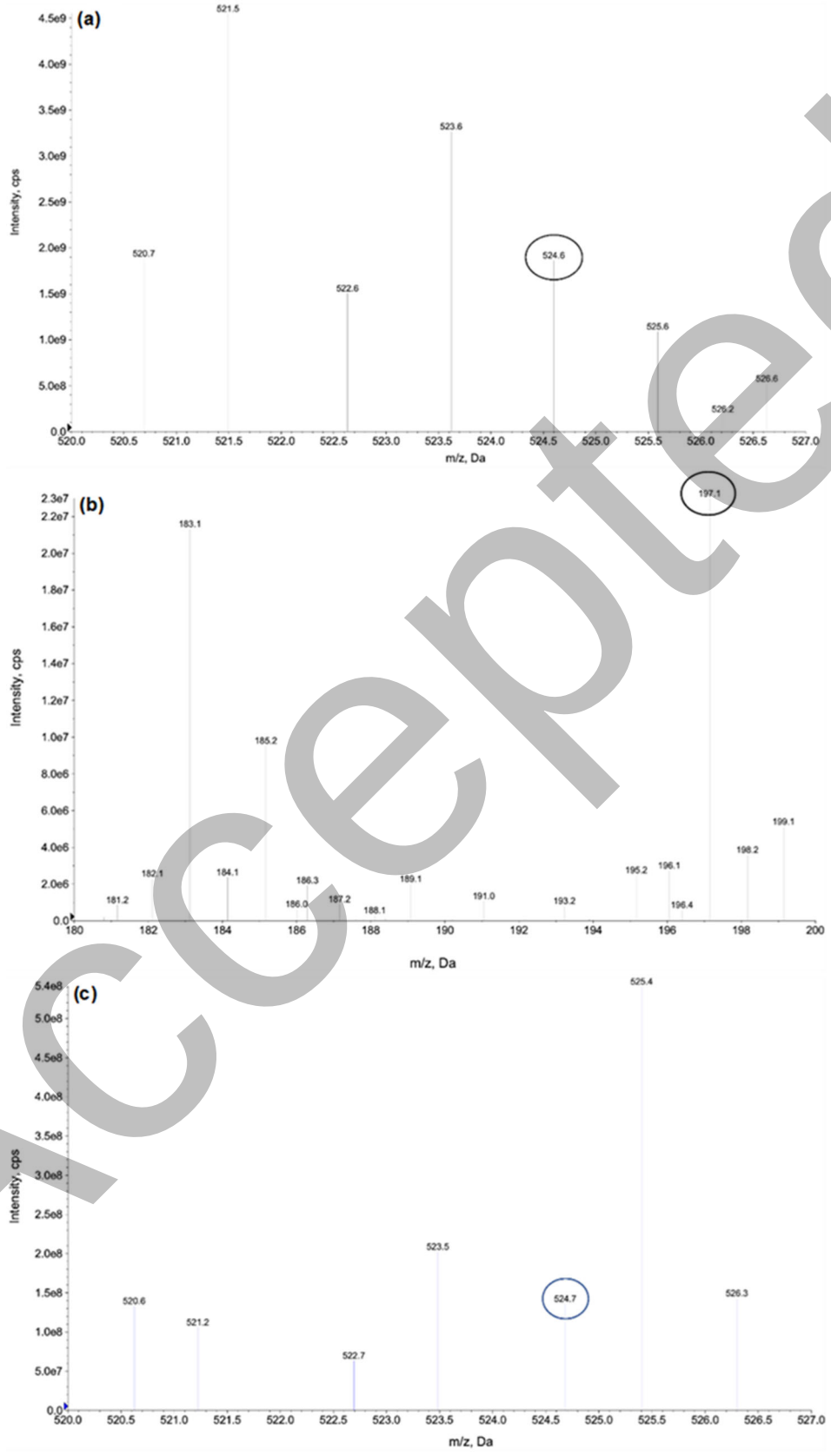
Specificity

According to ICH documents, "specificity" refers to the ability of an analytical procedure to determine the analyte without ambiguity, even in the presence of potential interferents such as impurities, degradation products, and matrix components. The lack of specificity in an analytical procedure can be compensated using complementary analytical procedures. Assay specificity is essential to ensure that impurities or excipients do not interfere with the procedure [14].

Analysis of the standard retinyl palmitate by MS (Fig. 2(a) and 2(b)) indicated the presence of a parent ion at and *m/z* of 524.6 and a daughter ion at an *m/z* of 197.1, confirming the identity of retinyl palmitate. Furthermore, the molecular fragment with parent and daughter ions at 524.7 and 197.3 *m/z*, respectively, appeared in the spectrum of the prepared gel sample (Fig. 2(c) and 2(d)). These findings are consistent with the MS/MS identification of retinyl esters by Goetz et al. [20]. Despite the non-dominance of the molecular fragment

Table 5. Percent recovery of vitamin A in cationic nanoemulsion thermosensitive gel

Recovery level	Standard cons. ± SD (µg/mL)	Mean (%) recovery ± SD	RSD recovery (%)
80%	264	99.295 ± 0.621	0.626
100%	330	99.878 ± 0.803	0.804
120%	396	99.570 ± 0.689	0.692


(a)
Intensity, cps
m/z, Da
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523.6
520.7
524.6
522.6
525.6
526.6
526.2
(b)
Intensity, cps
m/z, Da
197.1
183.1
185.2
199.1
198.2
182.1
184.1
186.3
189.1
195.2
196.1
181.2
186.0
187.2
191.0
193.2
188.1
196.4
(c)
Intensity, cps
m/z, Da
525.4
523.5
520.6
524.7
526.3
521.2
522.7

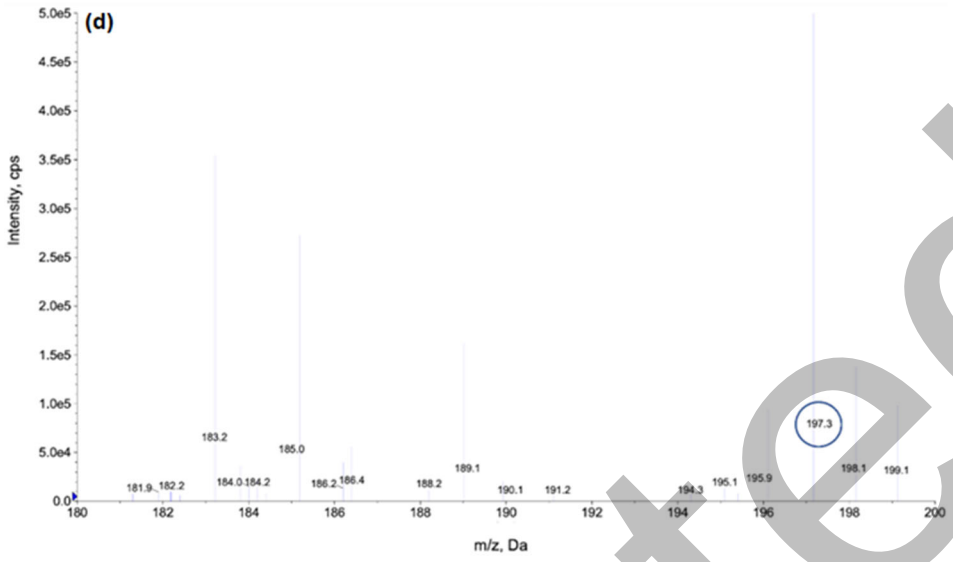

Fig 2. Mass spectrometry spectra of retinyl palmitate standard of a molecular fragment peak with a (a) parent ion at 524.6 *m/z*, (b) daughter ion at 197.1 *m/z*, (c) retinyl palmitate from gel matrix with a parent ion at 524.7 *m/z*, and (d) daughter ion at 197.3 *m/z*

with the parent ion, quantitative accuracy testing using spiked reference standards demonstrated that the foreign materials in the reference standard had no influence on the results.

Qualitative HPLC analysis demonstrated the positive response of the sample and vitamin A standard, with a peak at a relatively similar retention time of 15 min (Fig. 3(b) and 3(c)) and exhibited a negative response to the placebo (only excipient of the sample) (Fig. 3(a)). This indicates that this technique specifically detects vitamin A. The retention was notably shorter than that of the methodology used to determine vitamin A levels in fortified cereal matrices using reversed-phase HPLC with UV detection, as developed by Van Wayenberg et al. [10] with a retention time of 17.6 min and Yokota et al. [9] which show retention times of 17.6 and 30.967 min, respectively. The chromatogram was similar to that reported by Kwiecien et al. [27] with a retention time of 19 min. Additionally, specificity was determined by adding various concentrations of impurities to the standard solution. The observed specificity (Table 5) satisfies the required specifications at all concentrations;

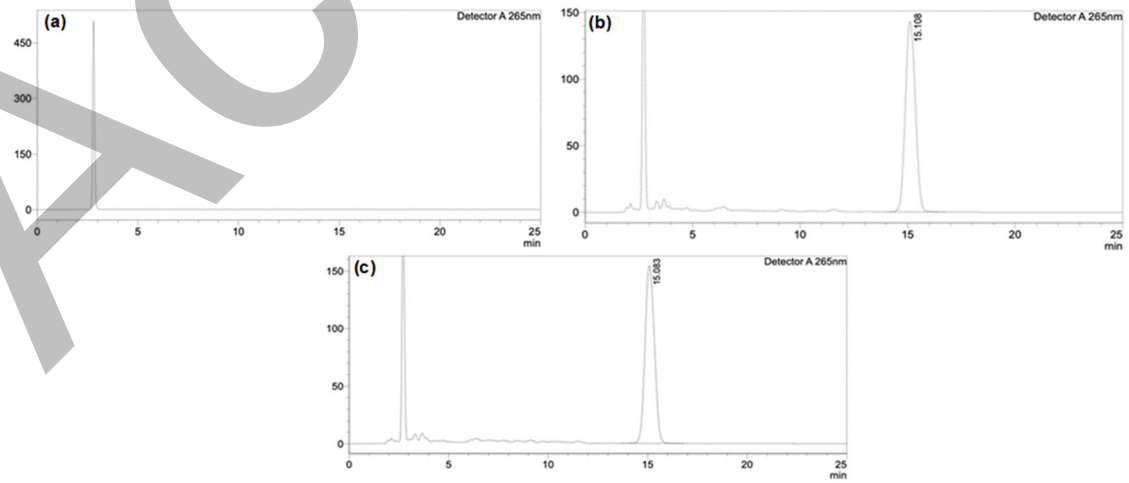

Fig 3. Chromatogram profiles of (a) placebo, (b) standard, and (c) sample

Table 6. Vitamin A assay

Sample	Peak area	Concentration (μg/mL)	Mean (μg/mL) ± SD	RSD (%)
1	4,502,255	326.438		
2	4,515,487	327.197	327.141 ± 0.677	0.207
3	4,525,793	327.789		

thus, the analytical procedure was considered specific.

LOD and LOQ

Based on the test results, the analytical method employed to detect vitamin A in thermosensitive gel preparations was found to be reliable and accurate. The LOD value obtained was 2.018 μg/mL, indicating the minimum amount of analyte detectable in the sample. Additionally, the LOQ value obtained was 6.114 μg/mL, representing the minimum amount of analyte that can be accurately and precisely determined under the stated experimental conditions. With these values, the validated analytical method proved effective in detecting and determining vitamin A levels in the form of retinol in vitamin A thermosensitive gel preparations.

Determination of Vitamin A

The vitamin A levels in cationic nanoemulsions loaded with thermosensitive gel preparations are presented in Table 6. The average vitamin A level is 327.141 μg/mL, closely approximating 100% of the targeted level of 330 μg/mL. The alternative step in the vitamin A preparation did not affect the accuracy, precision, specificity, and stability of vitamin A. This method has proven to have advantages, such as being able to maintain vitamin A stability for 8 h, providing a linear response to concentration changes and assay measurements, consistent and reproducible results for multiple samples and conditions, and the preparation process has no risk on vitamin A levels. In addition, the validated method is cost-effective and affordable because the extraction process uses a more efficient solvent, and the retention time is faster than in some previous studies.

■ CONCLUSION

The developed method using reverse-phase HPLC to determine the vitamin A concentration in ocular bioadhesive cationic nanoemulsions loaded onto thermosensitive gel preparations was validated. Enhanced the sample preparation by evaporated in a porcelain cup at room temperature, less hexane amount, repeated the extraction process, increased the extraction time, and added antioxidant BHT, ensured complete release of vitamin A from the matrix, reduced impurities, and prevented degradation. System suitability tests confirmed the effectiveness of chromatographic analysis, whereas stability tests demonstrated the stability of the standard and sample solutions over 8 h. The validated method is suitably accurate, precise, and consistent for the routine quality control of vitamin A levels in complex matrices. This study offers a simple, cost-effective, and efficient solution for the analysis of vitamin A in bioadhesive cationic nanoemulsions, thereby addressing a key challenge in pharmaceutical preparations.

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■ CONFLICT OF INTEREST

The authors declare no conflict of interest.

■ AUTHOR CONTRIBUTIONS

Conceptualization: Siti Fatmawati Fatimah, Akhmad Kharis Nugroho, Endang Lukitaningsih, and Ronny Martien. Methodology: Siti Fatmawati Fatimah, Akhmad Kharis Nugroho, and Endang Lukitaningsih. Software: Siti Fatmawati Fatimah, and Akhmad Kharis Nugroho. Validation: Akhmad Kharis Nugroho, Endang Lukitaningsih, and Ronny Martien. Formal analysis: Akhmad Kharis Nugroho, Endang

Lukitaningsih, and Ronny Martien. Investigation: Siti Fatmawati Fatimah, Akhmad Kharis Nugroho, Endang Lukitaningsih, and Ronny Martien. Resources: Siti Fatmawati Fatimah. Data curation: Siti Fatmawati Fatimah and Endang Lukitaningsih. Writing—original draft preparation: Siti Fatmawati Fatimah. Writing—review and editing: Siti Fatmawati Fatimah, Akhmad Kharis Nugroho, Endang Lukitaningsih, and Ronny Martien. Visualization: Siti Fatmawati Fatimah. Supervision: Akhmad Kharis Nugroho, and Endang Lukitaningsih. Project administration: Siti Fatmawati Fatimah, Akhmad Kharis Nugroho, Endang Lukitaningsih, and Ronny Martien. Funding acquisition: Akhmad Kharis Nugroho. All authors have read and agreed to the published version of the manuscript.

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