Apigenin in Apigenin Nanoliposomes: Development and Validation of an Analytical Technique Based on HPTLC

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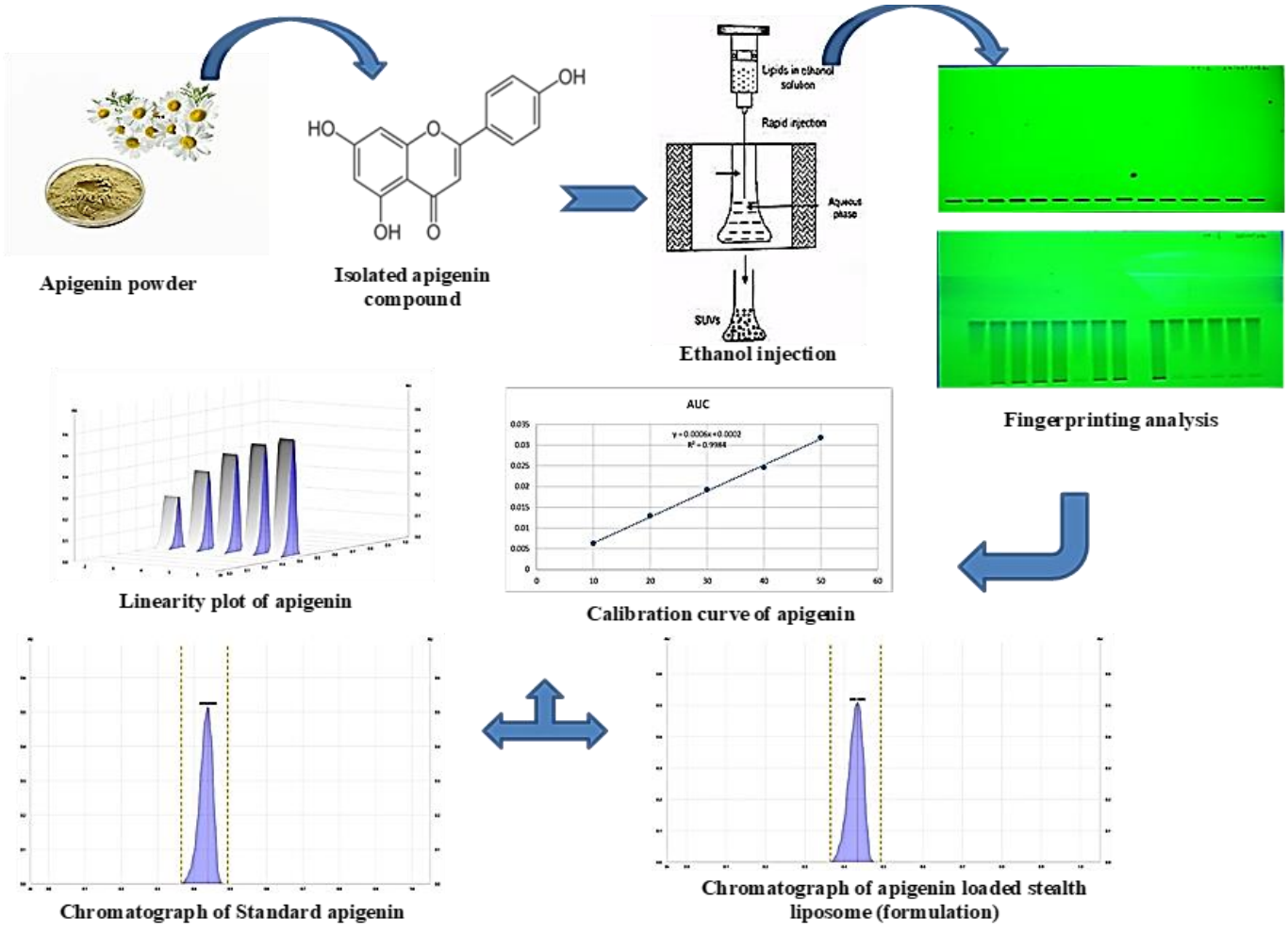
Abstract

Apigenin is a flavonoid found in various fruits and vegetables, aimed at its extensive array of biological actions such as anti-oxidant, anti-viral, antibacterial, anti-inflammatory, including chemo preventive property. Therefore, this study aimed to develop, describe and evaluate apigenin nanoliposomes. In this study, ethanol injection method was employed to formulate apigenin nanoliposomes. The quantification of apigenin in the nanoliposomes was carried out using a HPTLC analysis technique. The defined standards were followed in development and validation of the approach based on ICH guidelines. An aluminium plate 60 F_{254} of silica gel was used as the stationary phase, toluene, ethyl acetate and acetic acid ratio of 6:3:1 (v/v/v) was the mobile phase. Validation was done on the linearity, range, detection limit, quantification limit, precision and robustness of the method. Reference standard for apigenin varied between 0.2-1 µg/band and a correlation value of 0.989 was observed. The results showed that the LOQ was 0.29 µg /band and LOD was 0.095 µg /band. % RSD readings for intraday and interday precision were determined to fall within the necessary range of 2 for the apigenin standard. The established analytical method for assessing apigenin in apigenin-loaded stealth liposomes has proven to be straightforward, reliable, accurate and robust method.

Keywords: Apigenin, HPTLC, ICH guidelines, Stealth liposome.



Graphical Abstract

Introduction

Polyphenols are secondary metabolic products that originate from plants and are in category of phyto-chemicals comprising of flavonoids[21]. Out of the over 6000 distinct varieties of flavonoids, quercetin, kaempferol, myricetin, apigenin and luteolin[10] are the five most prevalent forms found in plants. One of the main monomeric flavonoids that we consume on a daily basis, is apigenin[22]. It is found in a wide range of fruits, vegetables and herbs of the plant kingdom[23].

Apigenin, also known by the chemical name (5, 7 - dihydroxy - 2 - (4 - hydroxyphenyl) - 4H - 1 - benzoyl - ran - 4 - one) is yellowish molecule having molecular weight of 270.24 g/mol. It belongs to the subclass of flavones since it is found in nature as dimerbiapigenin, apigenin-7-O-glucoside and many acylated derivatives. Common fruits like grapefruits, plant-based drinks and vegetables comprising of parsley, oranges, onions, tea, chamomile and wheat sprouts are rich sources of this flavonoid.

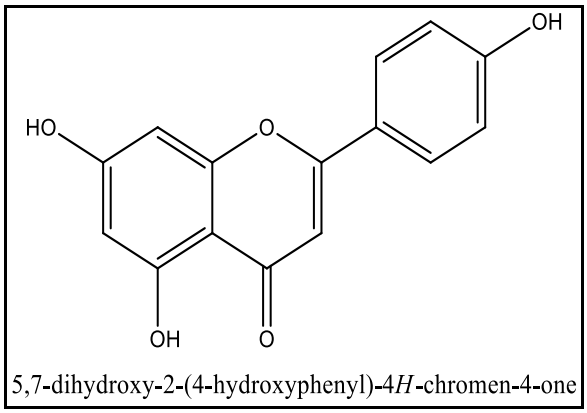


Fig. 1: Drug Apigenin

It should be noted that apigenin is an effective inhibitor of the cytochrome P450 (CYP) enzyme system which is in charge of metabolizing a wide range of pharmaceutical medications, even though it is frequently present in a variety of plants, some of which are commercially available as dietary and herbal supplements[12]. In addition to being widely distributed, apigenin has been isolated from a variety of plants including *Salvia officinalis* L[13] and *Chamomila recutita* L[18]. Reports of its many biological and pharmacological effects including anti-microbial, anti-oxidant, anti-tumor, anti-inflammatory, antiproliferative, antiviral and antidiabetic properties, have been made[16]. It is soluble in dimethyl sulfoxide, methanol, ethanol and dimethylformamide and demonstrates hydrophobic characteristics[17].

As research continues to grow, HPTLC is emerging as one of the best solutions for ensuring the quality of herbal products. The HPTLC method is simultaneously approach of samples and standards run together on same plate. In addition to being utilised for identification, the HPTLC can also be a tool for quality control[3]. After a review of the literature, it was discovered that apigenin has been measured using HPLC and HPTLC techniques in tablet dosage forms, dog and rat plasma and human urine, either by itself or in association with other flavonoids[8,11,15,19]. A novel, sensitive, specific, affordable, dependable, accurate and exact HPTLC approach was required for the quantitative determination of apigenin. Therefore, the purpose of this study is to develop, describe and evaluate apigenin nanoliposomes.

Material and Methods

Materials: Apigenin was provided a complimentary sample by Aktin Chemicals, a Chinese company. Fisher Scientific in Bombay, India provided HPLC-grade methanol, toluene, ethyl acetate and acetic acid. The water implemented in the study was of analytical grade, provided by Merck in Mumbai, India. Utilizing a Millipore Milli 'Q' plus filtration system, high-purity water was produced. The solvents and other reagents that were utilized, were all of analytical grade.

Instrumentation: Throughout the study, a Hamilton microliter syringe (Linomat syringe, Hamilton-Bonaduz Schweiz, Camag, Switzerland) was utilised. A 20 x 10 cm, 1 mm thick pre-coated silica gel aluminium plate 60 F254 (E. Merck, Darmstadt, Germany) was used. The Linomat 5 (Camag, Switzerland) sample applicator and a 20 x 10 cm Twin trough chamber (Camag, Switzerland) were both employed. A UV chamber (Camag, Switzerland) and a TLC scanner 4 (Camag, Switzerland) with vision CATS version 3 software were also used in the investigation.

Preparation of apigenin stealth liposomes: The ethanol injection method was used to generate Apigenin stealth liposomes. 25 ml beaker was used to hold the required dosages of the medication, stearic acid, DSPE-PEG and cholesterol. The drug was dissolved properly by adding 2 ml of ethanol to the polymer and lipid mixture which was then heated on a hot plate at 45°C while ensuring thorough mixing with the aid of a magnetic bead. Subsequently, PBS was added drop by drop to the same beaker and the mixture was continuously stirred using the magnetic bead at a temperature of 60°C for 3 hours. To achieve a consistent smaller particle size distribution, the formulation was then sonicated with a probe sonicator for 10 minutes. The resulting liposomes were transferred to amber-colored bottles and stored at or below 4°C. The apigenin stealth liposomes were further analyzed using dynamic light scattering (DLS) to measure particle size, polydispersity index and Zeta potential (Zetasizer Nano ZS, UK).

HPTLC method development

Preparation of standard and sample solutions: Stealth liposomes coated with microencapsulated apigenin were used to conduct the test while 10 mg of apigenin served as the standard. They were carefully weighed before being divided into two distinct volumetric flasks and filled with 10 millilitres of methanol, the solvent. Methanol was used to alter the amounts in order to reach a 50 mg/ml concentration of apigenin solution. The working solution were made by

diluting the stock solutions using methanol which included 50 mg/ml apigenin and stealth liposomes loaded with apigenin.

Method Optimization: The mobile phase and the ratios were optimized after carrying out various trails. The chamber saturation of five minutes was given and the plates were allowed to develop to a distance of 70mm[1].

HPTLC method validation: The established analytical technique was verified by employing the subsequent specifications: linearity, range, precision, specificity, robustness, limit of detection (LOD) and limit of quantification (LOD), in compliance with the guidelines of ICH Q2 (R1).

Linearity and range: The linearity curve was used to calculate the limit and the range of the standard was established by applying different sample concentrations. It is also decided upon what wavelength the parameters would be applied at.

Limit of detection LOD: The following formula has been used to compute the limit of detection, which is based on the slope obtained from the linearity and the standard deviation:

$$LOD = 3.3*(\sigma / S)$$

where σ represents the standard deviation and S represents the slope of the calibration curve.

Limit of quantification LOQ: Based on slope and standard deviation, the limit of the quantification parameter is derived similarly to the limit of detection. The formula used is:

$$LOQ = 10*(\sigma / S)$$

where σ represents the standard deviation and S represents the slope of the calibration curve.

Precision: The intraday and interday precision parameters are determined independently. Interday precision is assessed by repeating the same protocol over three non-consecutive days. Intraday precision is evaluated by repeating the same procedure three times within the same day. The calibration curve with the highest concentration at the centre is selected for the precision study. According to the ICH criteria, the precision study calculations must have a relative standard deviation (RSD) of less than 2%.

Specificity: The standard apigenin, stealth liposome along with the solvent and mobile phase are applied on the sample plate and checked for the development of chromatograms.

Robustness: The parameters of the procedure, such as slight variations in the mobile phase ratio, chamber saturation time and mobile phase volume, are intentionally modified for the robustness parameter. The resulting changes must meet the criteria of %RSD (relative standard deviation) being less than 2%.

Quantification: A stock solution with a concentration of 50 mcg/ml was prepared for the measurement of apigenin-loaded stealth liposomes and 3 mL of it was used. The technique for quantifying the Apigenin-loaded stealth liposomes was performed three times[8,7,20,6,2,14].

Results

Characterization of apigenin-loaded stealth liposome: The ethanol injection approach was used to prepare the apigenin-loaded stealth liposomes. The particle size, polydispersity index and zeta potential of the apigenin-loaded stealth liposomes were measured and found to be 356 nm, 0.29 and 1.33 mV respectively.

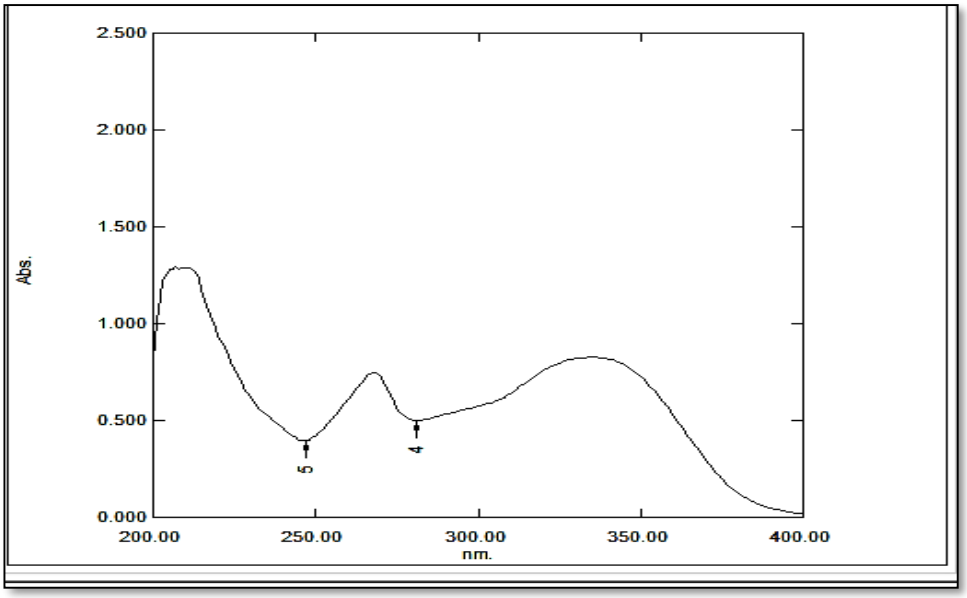


Fig. 2: Spectra of Apigenin

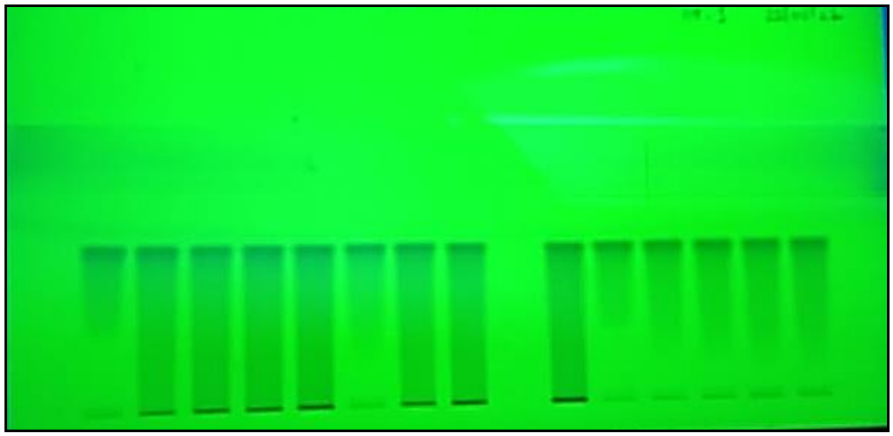


Fig. 3: Developed plate for standard Apigenin

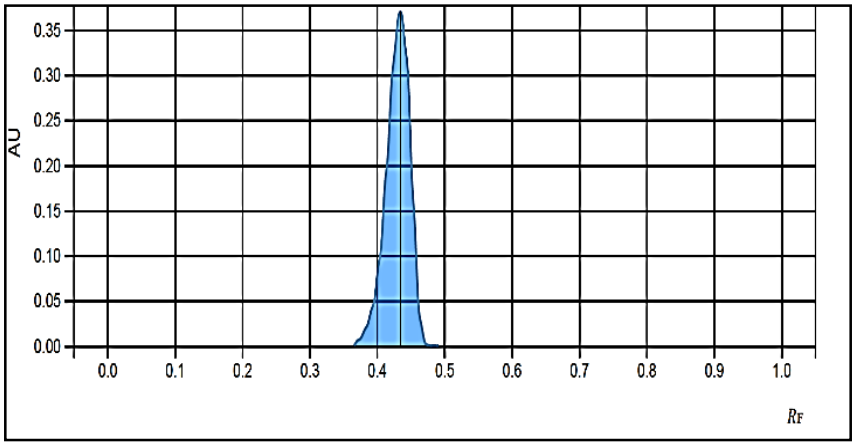


Fig. 4: Chromatogram of Apigenin

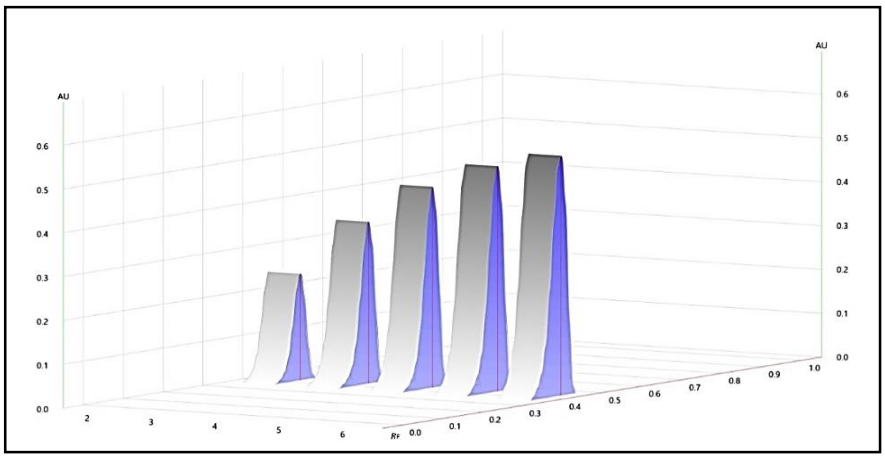


Fig. 5: Linearity of the standard Apigenin

Method development: After various trials, the mobile phase finalized and optimized was toluene: ethyl acetate: acetic acid (6:3:1 v/v/v) as it gave the better separation in all the trails. The spectra (Figure 2) of the standard were obtained and the peak maximum was obtained at 267nm. The Rf (Figure 3 and 4) value was found to be 0.435 for the standard apigenin.

Method validation

Linearity and range: The linearity (Figure 5 and 6) of the standard apigenin obtained was found to be in range of 0.2–1 µg/band range after various trails and the correlation coefficient was discovered to be 0.989.

Limit of detection and Limit of quantification: The LOD and LOQ were determined using a signal-to-noise ratio of 3:1 and 10:1 respectively. The LOD was found to be 0.095 µg/band and the LOQ was found to be 0.29 µg/band.

Precision*:* Precision measurements were performed both intraday and interday and the results showed that the %RSD values fell within the required range of ≤2 for the apigenin

standard. Table 1 displays the data that was acquired for both intraday and interday precision.

Specificity*:* The sample and standard had overlapping peaks whereas the mobile phase and solvent did not overlap the peaks, hence we can say that the developed method is specific.

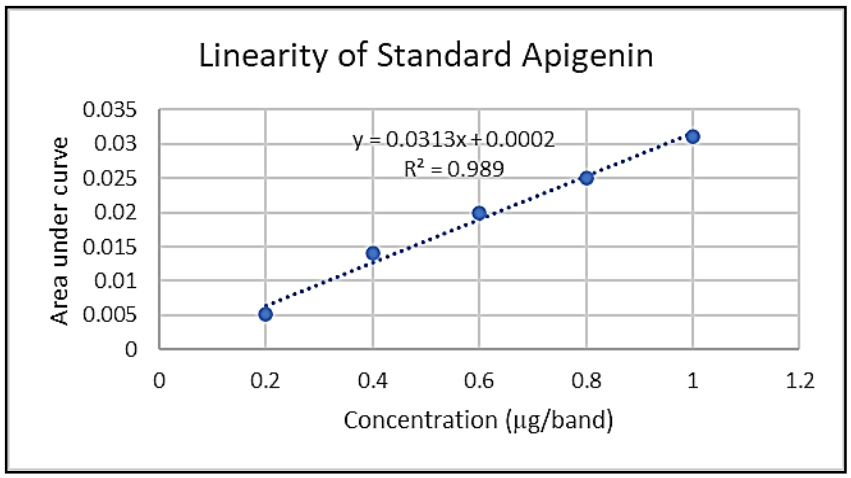


Fig. 6: Calibration curve of Apigenin

Table 1
Precision study data

Conc(µg/ml)	Intraday Precision	Interday Precision
	Peak area	Peak area
0.6	0.02015	0.02020
0.6	0.02063	0.02019
0.6	0.02055	0.02059
Mean	0.02044	0.02032
SD	0.00025	0.00022
%RSD	1.25793	1.12221

SD- standard deviation
%RSD- Percentage Relative standard deviation

Table 2
Results of Robustness parameter

Factors	Level	Rf value
Mobile Phase Composition (Toluene: ethyl acetate: acetic acid)		
5.9:3:1	-1	0.438
6:3:1	0	0.435
6.1:3:1	+1	0.439
%RSD		0.475%
Mobile Phase Volume (mL)		
19	-1	0.435
20	0	0.434
21	+1	0.439
%RSD		0.437%
Chamber saturation time (minutes)		
16	-1	0.434
20	0	0.438
24	+1	0.437
%RSD		0.436%

%RSD- Percentage relative standard deviation
Rf- Retention factor

Robustness: Following the performance of the robustness parameter (Table 2), it was noted that modifications regarding the mobile phase's composition produced a % RSD of less than 2%, indicating the robustness of the established approach.

Quantification: The apigenin stealth liposome was examined in triplicate and the findings are shown in table 3. The obtained results were analysed to determine their mean and standard deviation.

Table 3
Quantification data

Trail (n=3)	Apigenin loaded stealth liposome (µg/ml)
1	306.3
2	306.8
3	306.5
Mean ±SD	306.5 ±0.25

SD- Standard deviation

Discussion

The ethanol injection approach was utilized to create the stealth liposomes. After conducting multiple iterations of the solvent system trial procedure, the HP-TLC method was established. The mobile phase with a ratio of 6:3:1 v/v/v for toluene, ethyl acetate and acetic acid was determined to be the most suitable. The transition of the stationary phase was deemed satisfactory. The newly proposed technique underwent validation for various parameters including linearity, range, LOD, LOQ, accuracy, specificity and robustness in accordance with the ICH Q2 (R1) criteria.

Through multiple trials, it was determined that the linearity of the apigenin reference standard ranged from 0.2 to 1 µg/band, making it suitable for the range variable. The slope of the calibration curve was calculated using the formula derived from the linearity parameter and standard deviation, resulting in a LOD of 0.095 µg/band and a LOQ of 0.29 µg/band.

The precision data findings demonstrated the accuracy of the newly developed method. The % RSD for the peak area in the intra-day analysis was determined to be 1.12% and for the inter-day analysis, it was 1.25%. Both values met the criteria of being below 2%. To determine the concentration of the apigenin standard present in the stealth liposomes, triplicate measurements were performed. The concentration of the stealth liposomes was determined to be 306.5±0.25 mg/ml.

Conclusion

The guidelines provided in ICH Q2 (R1) were followed in the development and validation of the HPTLC method. It was discovered to be straight-forward, linear, precise, specific and robust. The technique offers a trustworthy and efficient way to examine the drug delivery mechanism for apigenin-loaded stealth liposomes. Overall, the developed method showed promising results and met the required criteria, indicating its suitability for the analysis of apigenin-loaded stealth liposomes.

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