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Supplementary Data File

Naringenin-Encapsulated Nano-cochleate Hydrogel for Topical Delivery: Cellular Anti-Inflammatory Activity and Dermatokinetic Profiling

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1. Analytical Method Validation of HPLC

1.1. System Suitability

System suitability experiments were conducted before the validation experiment to confirm chromatographic platform was working within the required analytical specifications. A standard solution (6 µg/mL) was injected six times under similar operating conditions. Performance indicators, including peak area reproducibility and retention time, were evaluated. The relative standard deviation (%RSD) of ≤2% for the peak area and retention times for NRG was set as the acceptance criterion.

1.2. Linearity

To obtain a linear response range of the method, five calibration levels (2–10 µg/mL) were prepared from the stock solution. The peak areas were plotted against their corresponding concentrations to get a calibration curve. The slope, intercept, and correlation coefficient were obtained from linear regression analysis.

1.3. Precision

Precision was examined at three concentration levels: 4, 6, and 8 µg/mL. Intra-day precision was assessed by performing three replicate injections of each level within a single analytical session, twice a day. Inter-day precision was evaluated by preparing fresh samples of the same levels and analysing them across three consecutive days, resulting in nine replicate readings per level. The degree of variability was expressed as the percentage relative standard deviation (%RSD) for each set of measurements. Lower %RSD values indicated consistent instrument performance and good method reproducibility across both short-term and intermediate-term intervals.

1.4. Accuracy

Accuracy was determined by conducting recovery experiments at the same three concentration levels (4, 6, and 8 µg/mL). Each level was prepared in triplicate and analysed to determine the proportion of the known amount of analyte successfully recovered by the method. The calculated percentage recovery and accompanying RSD values reflected the method's accuracy and precision. Recoveries of nearly 100%, with low variability, confirmed that the procedure accurately quantified the analyte without systematic deviation.

1.5. Sensitivity

The sensitivity of the analytical method was characterised by determining the limit of detection (LOD) and limit of quantification (LOQ), in accordance with ICH Q2(R1) guidance. The statistical method was applied to calculate these values. The standard error of the regression line, obtained through data-analysis tools, was combined with the slope of the calibration curve to compute LOD and LOQ.

$$LOD = \frac{3.3 \times \sigma}{S}$$

$$LOQ = \frac{10 \times \sigma}{S}$$

2. Results

The HPLC method developed was validated for Linearity, Inter-day and Intra-day studies, Accuracy, and Recovery according to the guidelines set by the International Conference of Harmonisation (ICH).

2.1. System Suitability

The results of the six replicate analyses at a 6 µg/ml concentration for the system suitability studies were within an acceptable range. The %RSD was found to be 0.77 and 1.21 for peak

area and retention time (RT), respectively, as shown in Table S1. NR was well eluted at 4 min, demonstrating excellent suitability and good repeatability of replicate sample analysis. Thus, it can be inferred that the developed method is suitable for NR analysis.

Table S1. Obtained system suitability parameters during the HPLC-UV method validation.

Conc. (µg/mL)	Peak area	RT (minutes)
6	159	4.012
6	159.8	4.065
6	160.1	4
6	161	4.123
6	159.67	4.101
6	162.5	4.089
Avg.	160.345	4.065
SD	1.24	0.04
%RSD	0.77	1.21

2.2. Linearity

The calibration curve showed a good linear relationship over the concentration range of 2–10 µg/mL for HPLC analysis. The linear equation was found to be y = 31.629x - 32.211 for the developed HPLC method. The correlation coefficient was found to be 0.999. The calibration curves are depicted in Figure S1.

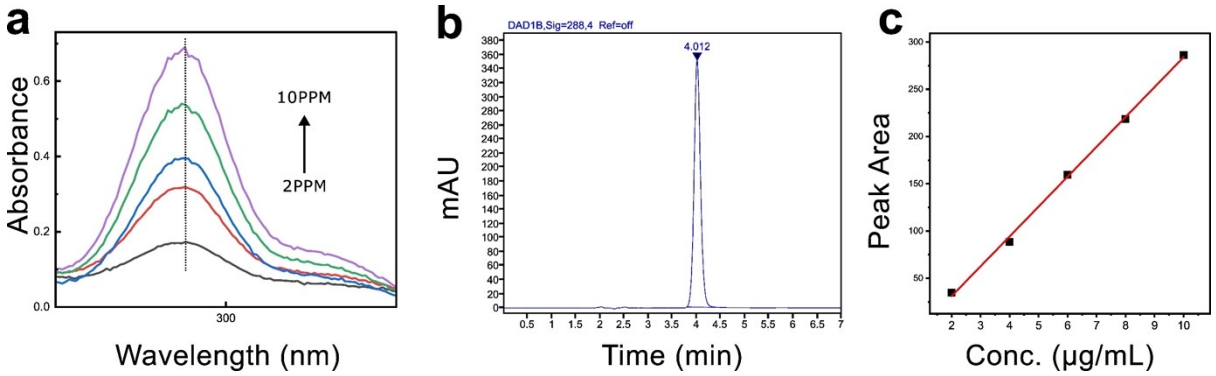

Figure S1. (a) UV/Vis absorption spectrum of NR; (b) HPLC chromatogram of NR; (c) HPLC Calibration curve of NR.

2.3. Precision

The results of both intra-day and inter-day studies confirm a high precision and repeatability of the developed methods. All data is expressed in RSD% (≤ 2%). The results for the Intra-day and Inter-day studies, using HPLC methods, are presented in Tables S2 and S3, respectively. It was found that the %RSD for intra-day and inter-day precision studies using the HPLC method was found in the limit.

Table S2. Intra-day Precision study of the HPLC-UV method for the determination of NR.

	Peak Area		
Concentration	**4 (µg/mL)**	**6 (µg/mL)**	**8 (µg/mL)**
Morning	88.58	159	218
	87.25	160.2	219.56
	89	159.65	220.56
Evening	88.65	159.8	217.56
	88.97	158.3	218.56
	86	161.25	218
Avg	88.07	159.70	218.70
SD	1.20	1.01	1.14
RSD	1.36	0.63	0.52

Table S3. Inter-day Precision study of the HPLC-UV method for the determination of NR.

	Peak Area		
Concentration	**4 (µg/mL)**	**6 µg/mL)**	**8 (µg/mL)**
Day 1	88.5	160	219.26
	87.59	161.89	221.5
	89.58	162	223
Day 2	88.95	167	224
	88.65	161	225.6
	89	162	229
Day 3	89	161.2	220.15
	88.6	163	219
	86	164	218
Average	88.43	162.45	222.16
SD	1.05	2.05	3.58
RSD	1.19	1.26	1.61

2.4. Accuracy

The average recovery of NR for each spiked sample in the skin was calculated from the peak area of NR using the calibration equation **(y = 31.629x - 32.211)** obtained from standard

solutions. An accuracy with a recovery rate of between 99% and 101% was obtained. The result of the study is reported in Table S4.

Table S4. Accuracy of the HPLC-UV method for the determination of NR.

Given Concentration **(µg/mL)**	**Peak Area**	**Calculated Concentration (µg/mL)**	**Recovery (%)**
4	93.25	3.96	99.16
4	93.31	3.96	99.21
4	93.89	3.98	99.67
6	160.58	6.09	101.58
6	159.50	6.06	101.02
6	159.60	6.06	101.07
8	223.50	8.08	101.05
8	221.50	8.02	100.26
8	219.80	7.96	99.59

2.5. Sensitivity

Sensitivity is typically assessed by calculating the LOD and LOQ. The sensitivity of the developed method was estimated by determining the LOD and LOQ. The LOD and LOQ were calculated by determining the standard error of each drug's obtained area and the slope of the linearity equation. The LOD and LOQ values of respective drugs were calculated accordingly. The LOD and LOQ of NR were found to be 0.47 µg/mL and 1.43 µg/mL, respectively.