Development and Validation of a Novel HPLC-UV Method for the Quantification of Hyaluronic Acid in Anti-Aging Creams

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This study introduces a novel, rapid, and cost-effective high-performance liquid chromatography (HPLC-UV) method for the direct quantification of HA in anti-aging creams without requiring derivatization. Chromatographic separation was performed on a reversed-phase C18 (5 μm × 4.6 mm × 150 mm) column, using an acetonitrile-acetate buffer (80:20, v/v) mobile phase at 0.9 mL/min flow rate. UV detection at 230 nm provided high selectivity. The method demonstrated excellent linearity (R^2 = 0.9997) across 0.1–30 μg/mL, with limits of detection (LOD) and quantification (LOQ) of 0.03 and 0.1 μg/mL, respectively. Validation was conducted per ICH guidelines, confirming high accuracy, precision (RSD < 1.88%), robustness, and repeatability, with minimal matrix interference. Compared to existing HA quantification methods, this optimized HPLC-UV technique offers superior sensitivity, speed, and simplicity, making it an ideal tool for routine analysis in cosmetic formulations.

Keywords: hyaluronic acid, anti-aging creams, HPLC-UV, method validation, cosmetic analysis

Introduction

Skin aging is a complex, natural process influenced by both intrinsic and extrinsic factors over time. In addition to genetic and physiological mechanisms, external factors such as stress, smoking, UV radiation, hormonal imbalances, poor nutrition, and inadequate skincare play a significant role in accelerating the aging process. Understanding the pathophysiology of skin aging and adopting preventive measures are crucial for maintaining healthy, youthful skin [1, 2].

Wrinkles primarily form due to the decreased density of dermal proteins, including collagen and elastin, as well as a weakened connection between the skin layers. Fibrils serve as a biomechanical scaffold, providing structural support for cell attachment and anchoring macromolecules. Among the various collagen types, Type I and Type III are key fibril-forming collagens. Fibroblasts, which are essential for maintaining healthy skin, contribute to the production of HA, Type I, III, and VII collagens, and elastin. Studies suggest that low molecular weight HA (Fig. S1) has a greater ability to penetrate the deeper layers of the epidermis, making it particularly effective in reducing mimic

The global anti-aging cosmetics industry was valued at USD 20.25 billion in 2018, with an estimated annual growth rate of 5.4%. Anti-wrinkle products continue to gain popularity due to their advanced formulations that slow down the aging process. Key active ingredients in these formulations include alpha hydroxy acids, retinoids, and HA, with serums and creams being the most widely used product types [5].

HA is highly valued in the pharmaceutical, cosmetic, and biomedical industries due to its unique properties, including hygroscopicity, biocompatibility, and viscoelasticity. Since the natural hyaluronan content in the dermis declines with age, HA and its derivatives are commonly incorporated into skincare formulations to help maintain skin hydration, reduce wrinkles, and improve texture and elasticity. With its ability to promote cellular renewal, moisture retention, and anti-aging effects, HA remains an essential component in modern skincare. Notably, the molecular weight of HA influences its biological activity and skin penetration, yielding different beneficial effects on the skin [6, 7].

Despite its widespread use, the accurate and efficient quantification of HA in cosmetic formulations remains a significant challenge. Various analytical techniques have been explored for HA determination, including gel-filtration chromatography [8], enzyme-linked immunosorbent assay (ELISA) [9], and high-performance liquid chromatography (HPLC) [10]. However, these methods have been predominantly applied to herbal extracts, synovial fluid, and human plasma, rather than cosmetic formulations [11-13]. A review of the literature reveals that only two analytical techniques have been reported for HA quantification in pharmaceutical formulations and moisturizing creams [14, 15]. These existing methods rely on fluorimetric detection with pre-column derivatization agents (e.g., dansylhydrazine), which introduces several drawbacks, including low sensitivity, complex sample preparation, and time-consuming procedures. Despite HA's extensive application in dermatology and skincare, no validated method currently exists for

its direct quantification in anti-aging creams. The lack of a standardized, efficient, and sensitive analytical technique highlights the need for a more robust, user-friendly, and reliable approach that ensures accurate HA quantification in complex cosmetic matrices.

The formulation complexity of anti-aging creams presents additional challenges in HA extraction and quantification. These products often contain a wide range of ingredients, including beeswax, liquid paraffin, bentonite, borax, methylparaben, alcohol, fragrance, purified water, and panthenol, which may interfere with analysis. To overcome these difficulties, different extraction techniques, including liquid-liquid extraction (LLE) and solid-phase extraction (SPE), were evaluated to determine the most effective approach for HA isolation from cosmetic matrices.

This study aims to develop and validate a novel HPLC-UV method for the rapid, accurate, and interference-free quantification of HA in anti-aging creams. Unlike previous approaches, this method allows for the direct quantification of HA without the need for derivatization, significantly improving efficiency, sensitivity, and reproducibility. By simplifying sample preparation and ensuring high analytical precision, this method provides a powerful tool for routine HA analysis in cosmetic formulations, regulatory quality control, and product standardization. If further validated, this technique could establish itself as a reference method for laboratories conducting HA analysis in anti-aging skincare products.

Experimental part

Instrumentation and reagents

Liquid chromatography (LC) analyses were conducted using an LC-20 system from Shimadzu (Japan). The system was equipped with an SPD-20A HT UV detector, calibrated at 230 nm, an LC-20 AT pump, a SIL AT-HT autosampler, and a CTO 10 AC column oven, all optimized for HPLC analysis. Chromatographic separation was performed isocratically at 30°C using a GL Sciences (Japan) C18 (ODS) column (4.6 mm I.D., 150 mm length, 5 µm particle size).

The mobile phase consisted of acetonitrile and water (100 mM acetate buffer, pH 5.6) with 1 mL/L triethylamine, delivered at a flow rate of 0.9 mL/min.

All chemicals used in the study were of high purity and suitable for analytical applications:

Hyaluronic acid (HA) was obtained from Sigma-Aldrich (St. Louis, MO, USA); Acetonitrile, triethylamine, and sodium acetate buffer solution (HPLC-grade) were sourced from Merck (Darmstadt, Germany); Ultrapure water was obtained using an ultrafiltration system from Water Human (Japan).

Method optimization

Chromatographic conditions were optimized to ensure optimal separation, resolution, and peak symmetry. Several parameters were systematically tested, including: Column selection: Various C18 columns with different lengths, internal diameters, and particle sizes were evaluated. The GL Sciences C18 (150 mm × 4.6 mm, 5 µm) column provided the best resolution. Mobile phase composition: Different ratios of acetonitrile, water, and buffer systems were tested to improve peak shape and retention time. Flow rate and temperature: The 0.9 mL/min flow rate at 30°C column temperature was determined to be optimal for achieving efficient separation.

Methods. A stock solution of HA (1 mg/mL) was prepared in an 80:20 acetonitrile:water mixture. Prior to experiments, the stock solution was diluted with acetonitrile to obtain working standard solutions at various concentrations. For HPLC analysis, 20 µL aliquots of the working standard solutions were injected, and chromatograms were analyzed based on peak areas corresponding to HA concentrations.

Preparation of the calibration curves. To establish the calibration curve, HA working standard solutions at various concentrations were analyzed. A linear least-squares regression analysis was performed to determine the linear concentration range for both substances. The calibration curve equations were derived using the formula y = ax + b, where x represents the concentration of the drug components in µg/mL, and y corresponds to the peak areas. Each concentration level was analyzed five times to ensure accuracy and reproducibility.

Uncertainty assessment. The main sources of uncertainty were evaluated, including contributions from the compound's purity ($u_{standard}$), weighing process ($u_{weighing}$), calibration curve parameters ($u_{calibration}$), recovery ($u_{recovery}$), and repeatability ($u_{repeatability}$). The combined uncertainty ($u_{combined}$) was calculated using the Formula S1. The overall expanded uncertainty ($u_{expanded}$) was determined at a 95% confidence level by multiplying the combined uncertainty by a coverage factor (k) of 2. The uncertainty calculations were performed in accordance with the EURACHEM Guide [15] and relevant literature [16].

Extraction process

A 1:1 mixture of ethanol and acetonitrile (1 mL) was added to an Eppendorf tube containing 0.1 g of the cream sample. The mixture was vortexed for 30 seconds, followed by centrifugation at 4000 rpm for 10 minutes. After centrifugation, 800 µL of the supernatant was filtered through a 0.45 µm polyethersulfone filter (Dainippon Seiki, Kyoto, Japan) and transferred to a 1.5 mL HPLC vial. For each duplicate, 20 µL of the sample was injected for HPLC analysis.

Results and discussion

Method development

Reverse-phase HPLC was selected as the preferred method for quantifying HA in anti-aging lotions. Preliminary tests were conducted using various column types and temperatures to determine the optimal chromatographic conditions. The best resolution, characterized by symmetrical and sharp

peaks, was achieved at 30 ºC using a C18 column (4.6 mm I.D., 150 mm length, 5 µm particle size).

Chromatographic separation was performed via isocratic elution at room temperature on a GL Sciences (Japan) C18 (ODS) column. The mobile phase consisted of acetonitrile and phosphate buffer (pH 5.6) in an 80:20 (v/v) ratio, with a flow rate of 0.9 mL/min. HA exhibited maximum absorption at 230 nm, as measured by a UV spectrophotometer. Under these conditions, the retention time of HA was 2.45 ± 0.02 min. The optimal conditions were established based on peak areas and resolution values.

Figure S2 (a-e) presents chromatograms of the anti-aging cream sample, standard solution, and blank solution. Table S1 summarizes the chromatographic separation quality and system suitability parameters. Compared to a previous study where HA had a retention time of approximately 10 minutes [21], our method significantly reduces the retention time to 2.45 minutes, demonstrating improved efficiency and reliability. Moreover, all chromatographic system parameters remained within acceptable limits.

Validation of the method

The method was validated in accordance with International Conference on Harmonisation (ICH) guidelines [18,19], evaluating linearity, accuracy, precision, robustness, and uncertainty assessment to confirm its reliability for routine HA analysis [18,19].

Preparation of calibration curve. The calibration curve was established by analyzing HA standard solutions within a 0.1–30 µg/mL concentration range. The linearity was assessed using least-squares regression analysis, with each concentration measured in five replicates. The calibration equation obtained was: *y = 20571x + 4278.7*, where y represents the peak area, and x corresponds to the HA concentration (0.1–30 µg/mL). The method demonstrated excellent linearity (R^2 = 0.9997), significantly improving sensitivity compared to previous studies, where the lowest reported LOD was 0.3 mg/L [22].

Limit of detection (LOD) & Limit of quantification (LOQ). The following formula was used to calculate LOD and LOQ: LOD or LOQ=kSDa/b, where k=3 for LOD and 10 for LOQ, SD_a represents the standard deviation of the intercept, and b is the slope of the calibration curve. Table 1 provides a summary of the key analytical performance characteristics of the proposed method. In a previous study, the lowest reported LOD for biogenic amines was 0.3 mg/L [22]. However, in our current study, the LOD was determined to be 0.03 µg/mL, demonstrating the superior sensitivity and reliability of our method as a quality parameter.

Accuracy, Precision, and Recovery. The accuracy, precision, and recovery of the developed HPLC method were rigorously evaluated to ensure its suitability for the quantitative determination of hyaluronic acid (HA) in anti-aging cream formulations. These validation parameters were assessed using quality control (QC) samples prepared at three concentration levels: 0.1, 5.0, and 30.0 µg/mL.

Table 1. Analytical performance parameters of the method.

Parameters	Method
Concentration range [a] (µg mL^{-1})	0.1-30
Regression equation [b]	y = 20571x + 4278.7
Intercept± SD	4278.7 ± 14.3
Slope± SD	20571 ± 118
Correlation coefficient (r^2)	0.9997
LOD (µg mL^{-1})	0.03
LOQ (µg mL^{-1})	0.1

[a] Average of six determinations.

[b] Regression equation follows y = xC + b, where y represents the peak area and C is the concentration in µg/mL.

The method's accuracy was determined by analyzing HA-spiked anti-aging cream samples at different concentration levels. The recovery was assessed by comparing the peak areas of the extracted HA samples with those of the corresponding pure standard solutions prepared in the mobile phase. The relative recovery percentage was calculated using the formula:

$$\text{Recovery (\%)} = (C_M/C_A) \times 100,$$

where C_M - measured concentration and C_A - added concentration.

The mean relative recovery was found to be 99.50%, confirming excellent analytical accuracy. This indicates that the method effectively quantifies HA in complex formulations without significant matrix effects.

The precision of the method was assessed by evaluating both repeatability (intra-day precision) and reproducibility (inter-day precision). Intra-day precision: Determined by analyzing spiked QC samples within the same day at three different concentration levels. Inter-day precision: Evaluated by analyzing the same QC samples over five consecutive days to assess the method's long-term reproducibility. The relative standard deviation (RSD%) was calculated to quantify variability. An RSD% below 2% is generally considered acceptable for bioanalytical methods. In this study, the RSD% remained consistently below 1.88%, confirming the method's robustness.

The results of the accuracy, precision, and recovery studies are summarized in the Table 2.

These results demonstrate high accuracy, as indicated by recovery values close to 100%; excellent precision, with RSD% consistently below 2% and strong repeatability and reproducibility, making the method highly reliable for routine quality control applications in cosmetic and pharmaceutical formulations.

Table 2. Accuracy and precision of the method.

Existing Conc. (μg mL^{-1})	Added Conc. (μg mL^{-1})	Found Conc. (Mean ± SD) (μg mL^{-1})	Recovery (%)	RSD of Recovery	RSD of Intraday Variation	RSD of Interday Variation
5.00	0.10	4.96 ± 0.08	99.20	1.21	0.82	1.40
	5.00	9.93 ± 0.20	99.30	1.17	0.58	1.62
	30.00	35.01 ± 0.91	100.02	1.01	0.35	1.88
Mean relative recovery =			99.50			

Note: For each concentration, n = 3.

Robustness. The robustness of the method was evaluated by analyzing QC samples at three concentration levels (n=3), as described in the validation section. Several critical parameters were deliberately varied to assess the method's stability, including the mobile phase composition, flow rate, and column temperature. The mobile phase ratio (acetonitrile:buffer solution) was adjusted from 80:20 to 85:15 and 75:25, the flow rate was modified from 0.9 mL/min to 1.0 and 0.8 mL/min, and the column temperature varied from 30°C to 25°C and 35°C.

Despite these adjustments, no significant impact on peak area or resolution was observed, demonstrating the method's robustness. The recovery percentages remained within an acceptable range (93.17% to 101.27%), while the RSD values were consistently low (0.24% to 1.10%), indicating high precision and reproducibility under varied conditions. Table S2 presents the robustness data, further confirming the method's reliability for routine analytical applications.

Stability. The stability of HA in spiked anti-aging cream samples was evaluated at three concentration levels (1.0, 5.0, and 30 μg/mL) under different conditions. To assess the effects of freezing and thawing, the samples underwent four freeze-thaw cycles before analysis. Additionally, the stability of HA in spiked anti-aging creams was examined after 24 hours at room temperature and after 21 days at 20°C. The HA stock solutions stored at -20°C remained stable for at least 30 days, with no significant degradation observed. Similarly, after 30 days, there was no noticeable decrease in HA concentration in the anti-aging cream formulations, confirming the stability of HA under these storage conditions.

Uncertainty assessment. The uncertainty of the analytical method was evaluated and expressed as a percentage (%) at a 95% confidence level for all analytes in the calculated parameters. The results, summarized in Table S3, indicate that the uncertainty values are within acceptable limits. The contribution of uncertainty due to sample weighing was found to be negligible and, therefore, was not included in the final presentation.

Application of the method to real samples

The validated HPLC method was applied to analyze hyaluronic acid (HA) concentrations in anti-aging creams to assess its effectiveness in real-world cosmetic formulations. The results, summarized in Table 3, demonstrate the method's accuracy, precision, and reproducibility, making it a reliable tool for routine quality control analysis.

The recovery rates ranged from 86.38% to 94.44%, indicating high extraction efficiency, while the relative standard deviation (RSD%) remained below 2.10%, demonstrating excellent reproducibility. These results confirm the method's accuracy, precision, and suitability for routine quality control analysis of HA in cosmetic formulations.

Table 3. HA concentrations in anti-aging creams and method reproducibility.

Cosmetic product	Recovery, %	RSD, %	Hyaluronic acid (μg/mL)
Sample 1	94.32	0.66	25.10
Sample 2	89.76	1.25	23.74
Sample 3	91.14	1.08	24.15
Sample 4	94.44	0.48	25.60
Sample 5	92.71	0.94	24.87
Sample 6	87.10	2.10	22.61
Sample 7	90.22	1.18	24.02
Sample 8	86.38	1.48	22.77
Sample 9	88.90	1.33	23.48
Sample 10	93.20	0.72	24.90

Note: Each concentration value represents the mean of five replicates (n = 5).

The optimized extraction protocol, involving a simple ethanol-acetonitrile (1:1) mixture followed by centrifugation and filtration, ensures minimal sample preparation time while maintaining high recovery rates. The consistently low %RSD values across all samples confirm the method's robustness and reliability for routine HA quantification in cosmetic products.

Conclusions

Hyaluronic acid (HA) plays a crucial role in tissue hydration, lubrication, and cellular processes, making it widely used in ophthalmology, dermatology, orthopedics, and rheumatology. However, many existing analytical methods for HA quantification suffer from long retention times and labor-intensive sample preparation, limiting their suitability for high-throughput applications.

The developed HPLC method provides a rapid, user-friendly, and cost-effective solution with a short retention time of 4.2 minutes and an isocratic mobile phase (acetonitrile and 100 mM acetate buffer, pH 5.6, with 1 mL/L triethylamine), eliminating the need for complex derivatization. The method demonstrates strong linearity (0.1–30.0 μg/mL, $R^2 = 0.9992$), high sensitivity (LOD: 0.03 μg/mL, LOQ: 0.10 μg/mL), excellent precision (RSD < 1.88%), and a mean relative recovery of 99.50%, ensuring accuracy and reproducibility.

A key advantage is its fast and efficient sample preparation, involving a simple 1:1 ethanol-acetonitrile extraction, centrifugation, and filtration, which significantly reduces analysis time while maintaining robustness. This makes the method highly suitable for routine quality control applications in the cosmetic and pharmaceutical industries, providing a reliable and efficient tool for HA quantification.

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