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Validation of a Simple HPLC/UV Method for Assay and In Vitro Release of Glycosaminoglycan from Pharmaceutical Formulations

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ABSTRACT: This study encompasses the validation of a simple, rapid, and sensitive HPLC/UV method developed in accordance with the guidelines set by ICH Q2(R2) for obtaining the active pharmaceutical ingredient from the glycosaminoglycan family in topical formulations. Previous methods reported for analyzing glycosaminoglycans in semisolid formulations are relatively complex and time-consuming, involving extraction, purification, and derivatization. This developed analytical method allows for straightforward extraction of the active pharmaceutical ingredient from the matrix, enabling the direct injection of samples. This method was performed and validated for the assay of the pharmaceutical gel and cream formulations to investigate the parameters of linearity (r = 0.9997 for the gel formulation and r = 0.9993 for the cream formulation), precision, accuracy, specificity, and robustness by HPLC/UV. Additionally, this method was used to determine the active ingredient in *in vitro* release studies. *In vitro* similarity correlation against commercial products was performed according to the Mann−Whitney U statistical test. The similarity results were 96.5−102.7% for the gel formulation and 98.0−106.0% for the cream formulation, which remained within the limits (75−133.33%) according to USP 1724. This proved that *in vitro* release profiles for both formulations were like those of the commercial product. In light of the research findings, we believe that the HPLC/UV analysis presented can be further enhanced in the future for determining the levels of active ingredients in various pharmaceutical formulations or for monitoring the levels of glycosaminoglycans in biological matrixes.

1. INTRODUCTION

Glycosaminoglycans (GAGs) are lengthy, unbranched chains of polysaccharides primarily consisting of repeated disaccharide units. These subunits are made up of hexuronic acid and amino sugar, connected by glycosidic bonds. The variations in disaccharide composition serve to classify the primary GAG classes: hyaluronic acid (HA), chondroitin sulfate, dermatan sulfate, keratan sulfate, and heparan sulfate. GAGs exhibit varying degrees of sulfation except for the unsulfated HA. The specific impact of GAGs is contingent upon their structure, particularly in terms of sulfation levels and polymer length. Alterations in GAG composition are commonly observed during physiological and pathological remodeling events, such as bone formation or scarring.[1]

Besides, GAGs find extensive use in diverse medical applications due to their multifaceted regulatory capabilities. For example, they can be found in various conventional drugs, including intraarticular injections, tablets, creams, gels, and drug carrier systems such as liposomes and nanocarriers. In the pharmaceutical industry, GAGs are commonly encountered in topical formulations, such as creams and gels, primarily leveraging their anti-inflammatory properties.[2,3]

Gel and cream formulations containing the active pharmaceutical ingredient (API) selected from the GAG family have been developed and manufactured by the Abdi İbrahim R&D Center for blunt trauma, phlebitis, and inflammation. This account focuses on the assay and *in vitro* release tests (IVRTs) of the API in these developed formulations.

The gel and cream formulations are topical formulations with different structures, leading to variations in assay and *in*

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vitro release testing when they contain the same API.[4,5] Creams are emulsions made from a mixture of water and oil and tend to have a thicker consistency. Due to their higher oil content, they are often used to moisturize the skin and alleviate dryness. Gels, on the other hand, possess a more slippery and intense texture, containing water along with gelatin or plant-based polymers to form a specialized gel structure. The abundant water content in gels provides a light and cooling sensation.[6] In our developed method, salt diluent was employed for extracting the API from the gel formulation, whereas a robust organic diluent was necessary to disrupt the matrix in the cream formulation. This approach allows for the effective extraction and analysis of the API from both formulations, accommodating their distinct structures and compositions.

The complex composition of gel and cream formulations poses challenges in planning and analyzing *in vitro* tests. Solubility, stability, and other physicochemical properties of the API may affect IVRTs.[7] All of these features require the selection of a suitable testing condition. Therefore, it is crucial to carefully select appropriate testing conditions that mimic physiological fluids to ensure more realistic results. The addition of certain organic solvents and surfactants to the release medium may be necessary to enhance the release rate. In this study, we preferred the dialysis bag method, a commonly employed technique in IVRTs of topical formulations, and pH 7.4 phosphate-buffered saline (PBS) was employed as the release medium. We conducted studies to prove the similarity between the *in vitro* release profiles of our developed gel and cream formulations and those of commercial products. Furthermore, we subjected the obtained results to statistical evaluation to validate our findings.[8]

There are various analytical methods for the assay of GAGs, but numerous challenges are encountered in the process. GAGs are complex and diverse biomolecules with intricate structures. Their heterogeneous structures and the distribution of different sulfate and acetyl groups can make the analysis complicated. The preparation of GAG samples is essential to ensure that they are in a suitable form for analysis. This process is critical for minimizing matrix effects and enhancing the analysis sensitivity. The analysis of large molecules like GAGs can lead to issues of specificity and selectivity, with the potential for cross-reactions with excipients and the risk of interference with other components like solvents in the analysis.[9−11]

Osago et al. (2014) systematically compared three different methods for the assay of GAGs.[11] Among these, there is an HPLC-based analysis, the carbazole test, and the Alcian Blue test. However, the Alcian Blue test can only detect sulfated GAGs, the carbazole test may produce false-positive results in the presence of salt, which means it cannot be adapted to IVRTs, and the HPLC-based analysis is based on the principle of breaking down GAGs with a series of enzymes and detecting their disaccharides.[12] Due to their lack of selectivity and their time-consuming and expensive nature, these methods are not suitable for calculating the amount of API in pharmaceutical formulations or integrating them into the laboratory. Upon conducting a thorough literature review, no validated HPLC/UV method was found for the selected API from the GAG family.

In this paper, for the first time, a practical, simple, and rapid HPLC/UV method for simultaneously analyzing the assay and *in vitro* release testing of GAG derived from topical cream and gel is presented, following the ICH Q2(R2) guidelines.[13]

2. EXPERIMENTAL SECTION

2.1. Materials. Sodium dihydrogen phosphate, sodium perchlorate monohydrate, phosphoric acid, tetrahydrofuran, sodium formate, formic acid, potassium chloride, and hydrochloric acid were purchased from Merck, Schuchardt OHG, Darmstadt, Germany. The working standard was obtained from an API supplier. Highly pure water was prepared with a Millipore Milli Q plus purification system.

2.2. Chromatographic Conditions. The assay method for the API in pharmaceutical dosage forms was performed using a Waters HPLC system equipped with a UV detector set at 202 nm in accordance with the European Pharmacopoeia.[14] The system involved two mobile phases: Mobile Phase A, comprising sodium dihydrogen phosphate, and Mobile Phase B, comprising a mixture of sodium dihydrogen phosphate and sodium perchlorate monohydrate. An IonPac 2 mm × 250 mm column was utilized with a flow rate of 0.22 mL/min and a gradient system. The column temperature was set to 40 °C, and the sample temperature was maintained at 25 °C. The injection volume was 50 μL, and chromatographic data were processed using Empower 3 Build 3471 software.

These chromatographic parameters apply to both the assay and *in vitro* release testing of gel and cream formulations.

2.3. Assay Method Conditions. Considering the hydrophilic nature of the API and the current system conditions, pH 2.0 buffer and formate buffer were chosen as solvents.[15,16] While these solvents enabled the extraction of the API from the gel formulation effortlessly, tetrahydrofuran was added prior to the aforementioned solvents to degrade and dissolve the cream structure within the cream formulation. A pH 2.0 buffer was meticulously prepared in accordance with the European Pharmacopoeia monograph by dissolving 6.57 g of potassium chloride in pure water and subsequently adding 119.0 mL of 0.1 M hydrochloric acid, followed by dilution to 1000 mL with pure water.[15] An evaluation of the buffer capacity of the pH 2.0 buffer was undertaken using 1 M hydrochloric acid (HCl) and 1 M sodium hydroxide (NaOH). The results revealed a buffer capacity of β = 0.125 with 1 M HCl and β = 0.01 with 1 M NaOH. Formate buffer is a buffer consisting of sodium formate and formic acid with pH = 4.0. It is prepared by adding 2 mL of formic acid to 2 g of sodium formate and then diluting it with pure water to 1000 mL. Similar to the pH 2.0 buffer, a thorough assessment of the buffer capacity of the formate buffer was conducted using 1 M HCl and 1 M NaOH. The results demonstrated a buffer capacity of β = 0.036 with 1 M HCl and β = 0.054 with 1 M NaOH.[17]

2.3.1. Preparation of Standard Solution. The concentration of the API for the standard solution was determined as 0.178 mg/mL. It was prepared by dissolving it with a pH 2.0 buffer and formate buffer.

2.4. IVRT Conditions. Since the products were gel and cream formulations in the IVRT, pH 7.4 PBS was chosen as the release medium.[18,19] To enhance the release rate in the gel formulation and achieve a profile similar to that of the commercial product, 20% ethanol was added into the release medium. The procedure applied for gel and cream formulations was the same, which is described as follows.

As a result of the method development studies, a speed of 300 rpm was preferred with a 300 kDa dialysis bag. In order to ensure the sink condition, 100 mL of release medium was used, and 1 mL of sample was withdrawn from the medium at

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certain intervals (1, 2, 3, 4, 5, 6, 7, 8, and 24 h) and a heated medium was added as much as the drawn amount (1 mL) and analyzed.[20,21]

2.4.1. Preparation of Standard Solution for the IVRT. The concentration of the API in the standard solution was determined to be in accordance with the sample concentration providing 100% *in vitro* release and was prepared by dissolving it in the *in vitro* release medium.

3. ASSAY METHOD VALIDATION

The assay method validation of API from pharmaceutical dosage forms was performed, and the parameters (specificity, linearity, precision, accuracy, and robustness) were evaluated according to the ICH Q2 Validation of Analytical Procedures: Text and Methodology guideline.[13,22]

3.1. Specificity. Specificity testing was conducted to assess the method's capacity to selectively quantify the targeted substances within the analyzed sample. Blank, placebo, standard, and sample solutions were injected for specificity analysis. The spectrum of the injected solutions was taken.

3.2. Linearity. To establish the linearity of the method, the peak areas of solutions prepared at five different concentration levels (40, 60, 80, 100, and 120%) were measured. The calibration curves were constructed, and the linear regression analysis was calculated by the least-squares regression method.

3.3. Precision. The precision of the method was determined by system precision, repeatability, and intermediate precision and was expressed as the relative standard deviation.

3.3.1. System Precision. To assess the system precision, six consecutive injections were performed using the standard solution prepared at 100% working concentration, and the peak areas obtained from the injections and the relative standard deviation (RSD %) between them were measured. Standard deviation, confidence interval (CI 95%), and peak performance parameters (retention time, symmetry factor, theoretical plate number) were to show the sensitivity of the instrument.

3.3.2. Repeatability. Repeatability was examined by assaying six consecutive samples of formulation on the same day (intraday) and under equal experimental conditions against the standard solution.

3.3.3. Intermediate Precision. Intermediate precision was examined by performing the analysis of six consecutive samples of formulation against the standard on two different days (interday) and by another analyst performing the analysis with a different column, using a different HPLC/UV instrument.

3.4. Accuracy. To prove the accuracy of the method, the API was spiked into the placebo solution and shown at levels of 40, 100, and 120%. A total of nine samples, three for each level, were prepared and injected twice, and the RSD% and CI (95%) between the results were calculated.

3.5. Robustness. *3.5.1. Changes in Analysis Conditions.* Assay analyses were conducted by altering the analysis parameters, including flow rate, wavelength, and column temperature, and the method's robustness to these variations was assessed. Assay results and peak performance parameters obtained as a result of repeatability and changes in the analysis conditions were compared. Relevant parameters and changes for the robustness analysis are given in Table 1.

3.5.2. Stability of Solution. In order to find the period during which the prepared solutions were stable, the standard and sample solutions were analyzed at certain time intervals, keeping the storage conditions constant (25 °C), the peak areas obtained were recorded, and the similarity % was calculated. Peak performance parameters were reported.

Table 1. Parameters and Changes for Robustness Analysis

Parameters	Changes
flow rate	0.20 mL/min and 0.24 mL/min
wavelength	200 and 204 nm
column temperature	38 and 42 °C

4. RESULTS AND DISCUSSION

Quantifying GAGs is a challenging process due to their complex structure. These polysaccharides consist of lengthy chains comprising various glucosamine and glucuronic acid units and are commonly embedded within a complex matrix alongside other components in the samples. When literature research is carried out, studies on determining the amounts of GAGs in biological materials are frequently included, while studies on the quantity determination of GAGs in pharmaceutical dosage forms are quite limited. The analytical methods available to quantify GAGs are spectrophotometry, chromatography, and mass spectroscopy, but each comes with its own challenges.[23−25] These challenges include identifying various subtypes of GAGs, obtaining pure and homogeneous samples, and separating them from other components. For example, spectrophotometric methods often exploit the abilities of GAGs to react with specific reagents, and the selectivity and accuracy of these methods can sometimes be limited.[26]

Many studies have been performed for detection of GAG in biological samples; the determination from pharmaceutical dosage forms remains a controversial issue. In a similar study, the method was validated by means of the limit of quantification, limit of detection, linearity, robustness, recovery, precision, and accuracy using the Bioanalytical Method Validation Guidance.[27]

As stated by Miriam Marques et al. (2023), using a UV detector for analysis also makes the HPLC method more cost-effective and sustainable for developing new products in the pharmaceutical industry.[28]

4.1. Assay Method Validation. *4.1.1. Specificity.* In standard and sample solution chromatograms, there should not be any peak from the blank and placebo at the retention time of the API peak and the spectrum of the API peak should indicate that it does not interfere with other peaks.

The chromatogram of the specificity analysis for the gel formulation is illustrated in Figure 1, while Figure 2 represents the specificity analysis of the cream formulation. As seen in the chromatograms, there is no interfering peak at the retention time of API in the blank and placebo solutions. The selectivity of the method has been proven for both formulations. In a separate study, no peaks from solvent and placebo were detected in the retention time of MPSP in standard, cream, and gel formulation solutions.[27]

4.1.2. Linearity. To assess the linearity of the calibration curve via the least-squares regression method, absorbance values were obtained from a standard solution prepared at five different concentrations: 40, 60, 80, 100, and 120%. The coefficient of determination (R^2) for the gel formulation is calculated at 0.9995, with a correlation coefficient (r) of 0.9997 between concentration and areas. For the cream formulation, the R^2 is calculated at 0.9987; the r between concentration and

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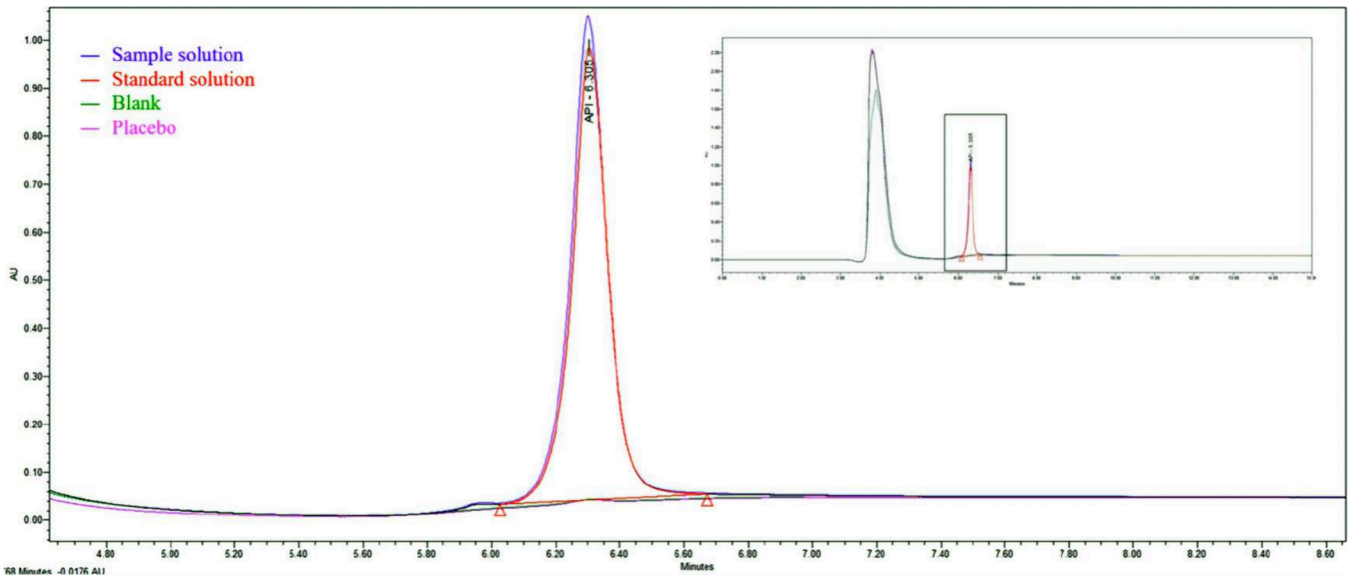

Figure 1. Specificity overlay chromatogram of API from the gel formulation obtained from the Empower 3 Build 3471 software system and using a Waters HPLC (UV:202 nm) with an IonPac 2 mm × 250 mm column at 40 °C. (One replicate for each sample.)

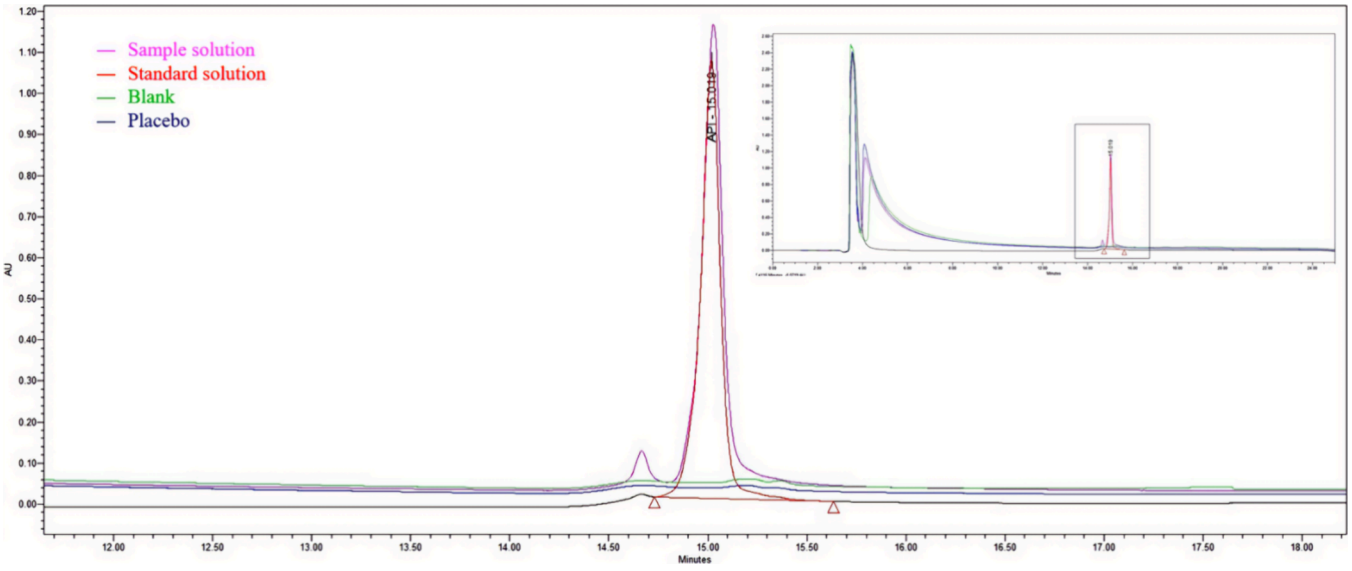

Figure 2. Specificity overlay chromatogram of API from the cream formulation obtained from the Empower 3 Build 3471 software system and using Waters HPLC (UV:202 nm) with an IonPac 2 mm × 250 mm column at 40 °C. (One replicate for each sample.)

areas is 0.9993. This indicates a value close to 1, indicating perfect linearity for the calibration curve of both formulations. When the results were converted to the mg/mL range, the results were determined as 0.07−0.214 mg/mL. Another study found this range to be 0.090−1.575 mg/mL, both showing remarkable linearity.[27] Dave and colleagues, in their study on GAG quantification from biological materials using the dimethyl methylene blue staining method, achieved a linearity with an r of 0.9960 (R^2 = 0.992) with a concentration range of 10−89 mg/L.[29] Although the margin of error may be higher at lower concentrations, we can obtain more meaningful results in terms of linearity compared to them.

ANOVA analysis of variance was performed with the data obtained for both formulations. Analysis results are listed in Table 2.

Two hypotheses were established: H_0 and H_a.

H_0 = coefficients equal to 0.

Table 2. ANOVA Analysis Results for Gel and Cream Formulations

	gel formulation	cream formulation
multiple r	0.9997	0.9993
R^2	0.9995	0.9987
significance F	4.69×10^{-6}	2.01×10^{-5}
P-value	4.69×10^{-6}	2.01×10^{-5}
X variable lower 95%	44486729	41195484
X variable upper 95%	48284463	47068046

H_a = coefficients are not equal to 0.

If $p > \alpha$, H_0 is accepted, there is a possibility that the coefficient will be 0 and, in this case, a linear graph cannot be obtained.

If $p < \alpha$, H_0 is rejected, H_a is accepted, and in this case, a linear graph can be obtained.

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In the 95% CI, the α value is 0.05. In the ANOVA analysis of variance, the p-value was found to be 4.69×10^{-6} for the gel formulation and 2.01×10^{-5} for the cream formulation. In this case, since $p < \alpha$, the H_a hypothesis is accepted. It has been proven that the graph is linear for both formulations.

The $y = mx + n$ graph was obtained. It was observed that the slope (m) of the graph obtained for the gel formulation would be between 44486729 and 48284463 and for the cream formulation it would be between 41195484 and 47068046 within the 95% CI. The fact that this range does not include the value of 0 indicates that the graph is linear for both formulations.

4.1.3. Precision. Instrumental precision, method precision, or intra-assay precision was performed by preparing six different samples from the same formulations.[30]

4.1.3.1. System Precision. The RSD% value between the areas obtained from six consecutive injections of the standard solution should not be more than 2.0%. In the validation assessments for gel and cream formulations, Table 3 presents

Table 3. Peak Areas Obtained from Six Consecutive Injections of the Standard Solution

injection	peak areas for gel formulation	peak areas for cream formulation
average of six injections	7904376	7301809
SD[a]	9931	85215
RSD[b] %	0.13	1.2
confidence level (95%)	7904376 ± 7946	7301809 ± 68185

[a]SD, standard deviation. [b]RSD, relative standard deviation.

the outcomes of the system precision parameter. The RSD% outcome for the six standards in the gel formulation validation is recorded at 0.13%, while the cream formulation validation yields an RSD% result of 1.2% for the six standards. Since both values are less than 2.0%, system precision is proven for both formulations.

4.1.3.2. Repeatability. The result of each of the samples studied must be within 85.0−115.0%. The RSD% between the assay results obtained from six samples should not be more than 2.0%. Table 4 presents the outcomes of the repeatability

Table 4. Repeatability Analysis Results for Gel and Cream Formulations

samples	assay results % for gel formulation	assay results % for cream formulation
average of six samples	101.4	97.6
SD[a]	0.26	0.8
RSD[b] %	0.3	0.8
confidence level (95%)	101.4 ± 0.2	97.6 ± 0.6

[a]SD, standard deviation. [b]RSD, relative standard deviation.

analysis for six samples each of gel and cream formulations. The average assay result for six samples in the gel formulation is 101.4%, and for the cream formulation, it is 97.6%. These findings fall within the range of 85.0−115.0%, which are the percentage values given in EMEA for biomolecules.[31] In addition, while the RSD% result for gel samples is 0.3%, this result is 0.8% for cream samples, with both RSD% values remaining below 2.0%. Repeatability of the analytical method was proven for both formulations. Conversely, in a study by Kızılçay and colleagues for the method they developed with size exclusion chromatography, they found the RSD % value to be 2.5%, exceeding the RSD % limit of 2.0%.[27]

4.1.3.3. Intermediate Precision. For this parameter, the assay result of each analyzed sample must be within 85.0−115.0%. The RSD% between the assay results obtained from the six prepared samples should not be more than 2.0%. Additionally, the RSD% between the assay results obtained from 12 samples of the analyses performed by two different analysts on different days with different HPLC/UV instruments and different columns should not be more than 2.0%.

In Table 5, the assay % results for the gel and cream formulations are presented, comparing the outcomes of the

Table 5. Intermediate Precision Analysis Results for Gel and Cream Formulations

	gel formulation		cream formulation	
samples	first day, first analyst assay % results	second day, second analyst assay % results	first day, first analyst assay % results	second day, second analyst assay % results
average of 6 samples	101.4	101.8	97.6	98.4
RSD[a] %	0.3	0.3	0.8	0.8
SD[b]	0.36		0.9	
RSD[a] %	0.4		0.9	
confidence level (95%)	101.6 ± 0.20		98.0 ± 0.5	

[a]RSD, relative standard deviation. [b]SD, standard deviation.

first analyst on the first day to those of the second analyst on the second day for six samples. The assay results for the gel formulation were 101.4 and 101.8%, respectively, and the RSD % result of both analyses was 0.3%. As a result of the analysis carried out by both analysts in 2 days, the RSD % result for 12 samples was found to be 0.4%. Since this value is less than 2.0%, the intermediate precision of the method is proven for the gel formulation.

The results of the cream formulation were 97.6 and 98.4%, respectively, and the RSD% result of both analyses was 0.8%. As a result of the analysis carried out by both analysts in 2 days, the RSD% result for 12 samples was found to be 0.9%. Since this value is less than 2.0%, the intermediate precision of the method is proven for the cream formulation.

4.1.4. Accuracy. The accuracy of an analytical method indicates how closely the determined value aligns with an accepted reference value or a conventionally acknowledged true value. Regarding this, placebo solutions were supplemented with active ingredients at levels of 40, 100, and 120%, each administered three times, and then the accuracies were calculated (Table 6). The accuracy % limit of each sample studied should be between 95.0 and 105.0%. The RSD% between the accuracy % values should not be greater than 5.0%. Table 6 presents the results of the accuracy % performed on formulations of both gel and cream. The gel formulation yielded an average accuracy result of 98.7%, while the cream formulation showed an accuracy result of 100.9%, both within the acceptable range. Furthermore, the % RSD value derived from nine samples in the gel formulation stands at 0.6%, whereas for the cream formulation, it is 2.1%. Both outcomes remain below the specified limit of 5.0%. When the accuracy results of the formulation in the study by Kızılçay and

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Table 6. Accuracy Analysis Results for Gel and Cream Formulations

levels	accuracy average % results for gel formulation	accuracy average % results for cream formulation
40%	98.9	101.2
100%	99.1	98.6
120%	98.0	102.9
Average	98.7	100.9
SD[a]	0.59	2.2
RSD[b] %	0.6	2.1
confidence level (95%)	98.7 ± 0.7	100.9 ± 2.5

[a]SD, standard deviation. [b]RSD, relative standard deviation.

colleagues were examined, it was found that the accuracy varied between 94.6 and 100.3%, and the RSD % values varied between 0.7 and 3.2%.[27] In the study conducted by Kavitha et al., although there was no recovery from a complex formulation, it was reported that the % accuracy results were between 82.2 and 93.6%.[32] With the HPLC/UV method we developed, 98.7% accuracy was achieved for the gel formulation and 100.9% accuracy was achieved for the cream formulation, and RSD % results were observed as 0.6 and 2.1%. This shows that the proposed method gives more accurate results.

Gels, creams, ointments, and other topical formulations constitute a minor yet noteworthy portion of the total pharmaceutical products available on the market. These products typically require tedious extractions and difficult sample preparation procedures. Therefore, it is difficult to accurately identify the active ingredients from semisolid formulations such as gels and creams. Schlegel and colleagues successfully achieved the accuracy of the active ingredient from a semisolid formulation as in our study.[33]

4.1.5. Robustness. 4.1.5.1. Changes in Analysis Conditions. The robustness of an analytical procedure is a measure of its capacity to remain unaffected by small changes in the method parameters and provides insights into its reliability during routine application. A comparison of the assay results and peak performance parameters obtained as a result of repeatability and changes in analysis conditions are given in Table 7. The repeatability and robustness analysis findings were evaluated, and it was determined that the assay results were similar and the peak performance parameters were appropriate. The analytical method for both formulations has proven robust to changes in the analysis conditions.

4.1.5.2. Stability of Solution. Standard and test solutions were prepared and injected at specific intervals over a duration of at least 28 h, with storage conditions maintained at a constant 25 °C. Absorbance values for both standard and test solutions were recorded, and the similarity % between initial and subsequent absorbances was calculated. It was confirmed that the standard solution remains stable for 28 h at room temperature (25 °C). The cream sample solution was found to remain stable for 43 h, while the gel sample solution exhibited stability for 103 h under the same conditions.

4.2. IVRT. IVRT is a crucial technique for monitoring the release and diffusion of drug products, playing a pivotal role in formulation development. Therefore, it is an essential tool, in addition to similar qualitative and quantitative compositions (Q1 Q2), to assess the similarity of microstructural arrangement (Q3) as proposed in the Topical drug Classification

Table 7. Robustness Analysis Results for Gel and Cream Formulations

conditions	assay % results	retention time (minute)	symmetry factor	theoretical number of plates
gel formulation				
repeatability analysis	101.4	6.858	0.9	26727
flow rate 0.20 mL/min	101.0	7.431	0.9	26985
flow rate 0.24 mL/min	101.2	6.361	0.9	27006
wavelength 200 nm	102.7	6.844	0.9	26974
wavelength 204 nm	103.2	6.843	0.9	27493
column temperature 38 °C	103.3	6.837	0.9	27368
column temperature 42 °C	102.7	6.846	0.9	27108
cream formulation				
repeatability analysis	97.6	15.722	1.6	69729
flow rate 0.20 mL/min	100.5	16.340	1.2	43210
flow rate 0.24 mL/min	99.5	15.288	1.2	52636
wavelength 200 nm	98.3	15.774	1.1	46895
wavelength 204 nm	102.6	15.786	1.2	46073
column temperature 38 °C	100.3	15.772	1.2	47968

System approach of classes 1 and 3.[34] Beyond its primary function in evaluating release kinetics, IVRT proves invaluable for detecting and evaluating formulation alterations. Its importance is particularly highlighted when seeking exemptions from the stringent demands of bioequivalence studies.

The IVRT was performed on the gel and cream formulations, and validation of the analysis methods was carried out. *In vitro* release result % (average) of six samples of the test gel formulation at the 24th hour was 92.4%, and *in vitro* release result % of six samples at the 24th hour for the commercial gel formulation was 87.7%, indicating a very similar *in vitro* release result between both formulations.

In vitro release result % (average) of six samples of the tested cream formulation at the 24th hour was 67.1%, and *in vitro* release results % of six samples of the commercial cream formulation at the 24th hour was 62.4%, indicating a very similar *in vitro* release result between both formulations.

Figure 3 illustrates the comparison of the *in vitro* release graphs for the gel and cream formulations with the commercial products.

A comparison of the *in vitro* release profiles for both formulations with commercial products revealed a similarity range of 96.5−102.7% for the gel and 98.0−106.0% for the cream. In accordance with the USP <1724> Mann−Whitney *U* statistical test, the limit for similarity was set at 75.0−133.33% for *in vitro* release analysis, and both formulations yielded results within the acceptable range.[35] The Mann−Whitney *U* statistical test is applied to establish the 90% CI for the ratio of slopes between the test and commercial reference products. For each comparison of test-to-reference slopes, denoted as T

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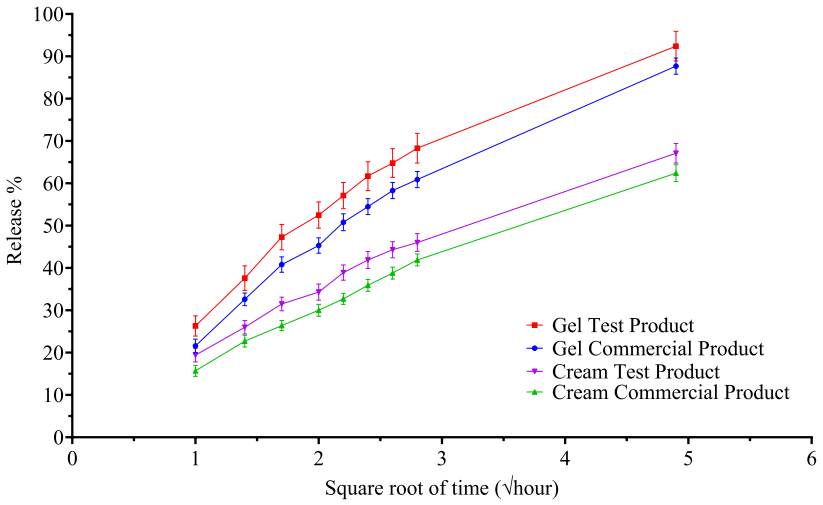

Figure 3. *In vitro* release graph of the gel and cream formulations compared to commercial gel and cream products. Experiments were conducted with $n = 6$ replicates. Values are expressed as means ± SD. The medium was pH 7.4 PBS:ethanol (80:20) at 300 rpm. The membrane was a 300 kDa dialysis bag.

and R, respectively, T/R slope ratios are computed by multiplying 100. After being calculated, these ratios are arranged in an ascending order. It is required that the eighth and 29th T/R ratios fall within the range of 75.0−133.33%.[36,37]

The formulation of gels and creams is difficult due to the multifaceted complexity of topical products. For ideal therapy, every drug concentration requires a distinct carrier, whether it is a structural matrix or other components. However, topical formulations are complex, and a thorough evaluation is required during formulation development in order to avoid any unwanted interactions and to meet patient acceptance. The structural behavior of formulations has a synergistic effect on drug release and diffusion. The *in vitro* performance of a topical product is a crucial aspect of quality for both innovative and generic products today. Also, the Mann−Whitney *U* statistical test is generally used in the pharmaceutical industry in order to calculate the similarity between test and commercial products for IVRT studies.[38]

5. CONCLUSIONS

Most methods for quantifying GAGs often include bioassays, and they are quite expensive, require precise work, and have low repeatability.[24,25,39] Accurately quantifying GAGs can be a challenge not only due to the complexity of analytical methods but also due to various factors that can occur during the preparation and analysis of samples. Therefore, GAG quantification often requires carefully planned experiments and meticulous laboratory techniques.

In this study, our objective was to develop a straightforward and cost-effective method that addresses the challenges presented in existing approaches. Thus, we elaborated and validated a simple, rapid, and sensitive HPLC/UV method for determining GAG in complex chemical matrices used in pharmaceutical dosage development, such as gels and creams. The linearity, precision, accuracy, specificity, and robustness parameter results showed the validation of the method. Our method was employed to extract GAG from formulations and to collect the *in vitro* release profile, aiming to demonstrate its similarity with that of the commercial product. The results confirmed the similarity of the gel and cream formulations with commercial products.

The HPLC/UV method described in this article holds potential for further applications, particularly in the determination of the GAG amount in various pharmaceutical formulations. Moreover, it could be adapted for the future monitoring of GAG levels within biological matrices, offering versatility and applicability across different contexts.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c02161.

Linearity graphs (Figure S1 and Figure S2) for gel and cream formulations (PDF)

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■ REFERENCES

(1) Sodhi, H.; Panitch, A. Glycosaminoglycans in Tissue Engineering: A Review. *Biomolecules* **2021**, *11*, 29.
(2) Köwitsch, A.; Zhou, G.; Groth, T. Medical Application of Glycosaminoglycans: A Review. *J. Tissue Eng. Regen. Med.* **2018**, *12*, e23−e41.
(3) Goyanes, S. N.; D'Accorso, N. B. *Industrial Applications of Renewable Biomass Products: Past, Present and Future*; Springer International Publishing, 2017.
(4) Gupta, A.; Stratton, S. P.; Myrdal, P. B. An HPLC Method for Quantitation of Perillyl Alcohol in a Topical Pharmaceutical Cream Formulation. *J. Pharm. Biomed Anal* **2005**, *37* (3), 447−452.
(5) Chimalakonda, K. C.; Pang, E.; Weaver, J. L.; Howard, K. E.; Patel, V.; Boyne, M. T. Development and Validation of a Liquid-Chromatography Tandem Mass Spectrometry Method to Determine in Vitro and in Vivo Histamine Release. *J. Pharm. Biomed Anal* **2015**, *102*, 494−499.
(6) Allen, L. V.; Ansel, H. C. *Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems*, 10th ed.; Wolters Kluwer Health, Inc., 2013.
(7) Júnior, U. P. S.; Cabrera, S. P.; da Silva, T. M. G.; da Silva, E. M. S.; Camara, C. A.; Silva, T. M. S. Geopropolis Gel for The Adjuvant Treatment of Candidiasis−Formulation and In Vitro Release Assay. *Rev. Bras. Farmacogn.* **2019**, *29* (3), 278−286.
(8) U.S. Food and Drug Administration. *In Vitro Release Test Studies for Topical Drug Products Submitted in ANDAs*. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/in-vitro-release-test-studies-topical-drug-products-submitted-andas (accessed Dec 28, 2023).
(9) Herbst, Z. M.; Hong, X.; Sadilek, M.; Fuller, M.; Gelb, M. H. Newborn Screening for the Full Set of Mucopolysaccharidoses in Dried Blood Spots Based on First-Tier Enzymatic Assay Followed by Second-Tier Analysis of Glycosaminoglycans. *Mol. Genet. Metab.* **2023**, *140*, No. 107698.
(10) Kamilari, E.; Kontoyannis, C.; Lamari, F.; Orkoula, M. In Discrimination and Quantification of Glycosaminoglycan in Pharmaceutical Formulations Using Micro-Raman Spectroscopy, Proceedings of the 10th PanHellenic Scientific Conference in Chemical Engineering, 2015.
(11) Osago, H.; Shibata, T.; Hara, N.; Kuwata, S.; Kono, M.; Uchio, Y.; Tsuchiya, M. Quantitative Analysis of Glycosaminoglycans, Chondroitin/dermatan Sulfate, Hyaluronic Acid, Heparan Sulfate, and Keratan Sulfate by Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry. *Anal. Biochem.* **2014**, *467*, 62−74.
(12) Frazier, S. B.; Roodhouse, K. A.; Hourcade, D. E.; Zhang, L. The Quantification of Glycosaminoglycans: A Comparison of HPLC, Carbazole, and Alcian Blue Methods. *Open Glycosci* **2008**, *1* (1), 31−39.
(13) European Medicines Agency. *ICH Q2(R2) Validation of analytical procedures - Scientific guideline*. https://www.ema.europa.eu/en/ich-q2r2-validation-analytical-procedures-scientific-guideline (accessed Dec 28, 2023).
(14) *European Pharmacopoeia 11.3*. https://pheur.edqm.eu/subhome/11-3 (accessed Apr 09, 2024).
(15) *European Pharmacopoeia 11.3. 4.1.3.Buffersolutions*. https://www.drugfuture.com/Pharmacopoeia/EP7/DATA/40103E.PDF.
(16) Schieber, A.; Lopes-Lutz, D. Analytical Methods - Functional Foods and Dietary Supplements. In *Comprehensive Biotechnology*, 2nd ed.; Elsevier Inc., 2011; Vol. *4*, pp 487−499.
(17) Dubrovskii, A. V.; Kim, A. L.; Musin, E. V.; Ramazanov, B. R.; Tikhonenko, S. A. The Discovery of the Buffer Capacity of Various Types of Polyelectrolyte Microcapsules. *Polymers (Basel)* **2021**, *13* (22), 4026.
(18) Shazly, G.; Nawroth, T.; Langguth, P. Comparison of Dialysis and Dispersion Methods for in Vitro Release Determination of Drugs from Multilamellar Liposomes. *Dissolut Technol.* **2008**, *15* (2), 7−10.
(19) Anwar, S.; Jan, S. U.; Gul, R. Formulation And Evaluation Of Celecoxib Cream And Its Released Study. Int. J. Curr. *Pharm. Res.* **2020**, 15−19.
(20) U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research (CDER). *In Vitro Release Test Studies for Topical Drug Products Submitted in ANDAs Guidance for Industry DRAFT GUIDANCE*, 2022. https://www.fda.gov/drugs/guidance-compliance-regulatory-information/guidances-drugs.
(21) Eroğlu, I.; Gökçe, E. H.; Tsapis, N.; Tanriverdi, S. T.; Gökçe, G.; Fattal, E.; Özer, O. Evaluation of Characteristics and in Vitro Antioxidant Properties of RSV Loaded Hyaluronic Acid-DPPC Microparticles as a Wound Healing System. *Colloids Surf. B Biointerfaces* **2015**, *126*, 50−57.
(22) Lusina Kregar, M.; Dürrigl, M.; Rožman, A.; Jelčić, Ž.; Cetina-Čižmek, B.; Filipović-Grčić, J. Development and Validation of an in Vitro Release Method for Topical Particulate Delivery Systems. *Int. J. Pharm.* **2015**, *485* (1−2), 202−214.
(23) Pan, P.; Chen, M.; Zhang, Z.; Corte, A. D.; Souza, C.; Giugliani, R.; Pan, L.; Qiu, Y.; Amaravadi, L.; Wu, J. A Novel LC-MS/MS Assay to Quantify dermatan Sulfate in Cerebrospinal Fluid as a Biomarker for Mucopolysaccharidosis II. *Bioanalysis* **2018**, *10* (11), 825−838.
(24) Pepeliaev, S.; Hrudíková, R.; Jílková, J.; Pavlík, J.; Smirnou, D.; Černý, Z.; Franke, L. Colorimetric Enzyme-Coupled Assay for Hyaluronic Acid Determination in Complex Samples. *Eur. Polym. J.* **2017**, *94*, 460−470.
(25) Vogel, K.; Kuhn, J.; Kleesiek, K.; Götting, C. A Novel Ultra-Sensitive Method for the Quantification of Glycosaminoglycan Disaccharides Using an Automated DNA Sequencer. *Electrophoresis* **2006**, *27* (7), 1363−1367.
(26) Kubaski, F.; Osago, H.; Mason, R. W.; Yamaguchi, S.; Kobayashi, H.; Tsuchiya, M.; Orii, T.; Tomatsu, S. Glycosaminoglycans Detection Methods: Applications of Mass Spectrometry. *Mol. Genet. Metab.* **2017**, *120*, 67−77.
(27) Ergin Kizilçay, G.; Ertürk Toker, S.; Matur, D. Analytical Method Development and Validation of Mucopolysaccharide Polysulfate in Topical Formulations by Size Exclusion Chromatography. *Analytical Methods* **2021**, *13* (36), 4114−4119.

(28) Miriam Marques, S.; Shirodkar, R. K.; Kumar, L. Analytical 'Quality-by-Design' Paradigm in Development of a RP-HPLC Method for the Estimation of Cilnidipine in Nanoformulations: Forced Degradation Studies and Mathematical Modelling of in-Vitro Release Studies. *Microchemical Journal* **2023**, *193*, No. 109124.
(29) Dave, M. B.; Chawla, P. K.; Dherai, A. J.; Ashavaid, T. F. Urinary Glycosaminoglycan Estimation as a Routine Clinical Service. *Indian Journal of Clinical Biochemistry* **2015**, *30* (3), 293−297.
(30) Ruckmani, K.; Shaikh, S. Z.; Khalil, P.; Muneera, M. S.; Thusleem, O. A. Determination of Sodium Hyaluronate in Pharmaceutical Formulations by HPLC-UV. *J. Pharm. Anal* **2013**, *3* (5), 324−329.
(31) European Medicines Agency. *EMA Guideline for Bioanalytical Method Validation*. https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-Bioanalytical-method-validation_en.pdf (accessed Dec 28, 2023).
(32) Kavitha, G.; Nadamuni, G.; Venkatesan, C. S.; Mukkanti, K.; Raju, V. N. S. M. V. G. A PH-Gradient SAX-HPLC Method for Determination of Trace Levels of Over Sulfated Chondroitin Sulfate And dermatan Sulfate In Heparin Sodium. *Int. J. Pharm. Sci. Res.* **2014**, *5* (11), 4916.
(33) Schlegel, L. B.; Schubert-Zsilavecz, M.; Abdel-Tawab, M. Quantification of Active Ingredients in Semi-Solid Pharmaceutical Formulations by near Infrared Spectroscopy. *J. Pharm. Biomed Anal* **2017**, *142*, 178−189.
(34) Shah, V. P.; Simona Miron, D.; Ștefan Rădulescu, F.; Cardot, J. M.; Maibach, H. I. In Vitro Release Test (IVRT): Principles and Applications. *Int. J. Pharm.* **2022**, *656*, No. 122159.
(35) *United States Pharmacopeial Convention All rights reserved, T. USP <1724> Semısolıd Drug Products-Performance Tests*, 2014. http://www.fda.gov/downloads/.
(36) Csorba, A.; Katona, G.; Budai-Szűcs, M.; Balogh-Weiser, D.; Fadda, A. M.; Caddeo, C.; Takács, Á. I.; Mátyus, P.; Balogh, G. T.; Nagy, Z. Z. Effect of Liposomal Formulation of Ascorbic Acid on Corneal Permeability. *Sci. Rep* **2023**, *13* (1), 3448.
(37) Akyol, S.; Çankaya, G.; Okuyucu Genç, C.; Özkırım Arslan, B.; Uz Gökalp, M. A Novel and an Alternative in Vitro Release Test for Hydrophobic Diflorasone Diacetate Ointment with Dialysis Method by Using Reciprocating Cylinder, USP Apparatus 3. Global. *Journal of Pharmacy & Pharmaceutical Sciences* **2021**, *8* (3), No. 555737.
(38) Manian, M.; Jain, P.; Vora, D.; Banga, A. K. Formulation and Evaluation of the In Vitro Performance of Topical Dermatological Products Containing Diclofenac Sodium. *Pharmaceutics* **2022**, *14* (9), 1892.
(39) Ozgen, G.; Turk Gezer, M.; Armagan, G.; Ballar Kirmizibayrak, P.; Yalcin, A.; Ozer, O.; Ozkirim Arslan, B.; Kayar, G.; Dude, U. K.; Kaymak Ozdemir, A. Establishment and Validation of an Automated System for the Antifactor IIa Assay: A Case Study of Potency Assessment of a Pharmaceutical Gel Formulation. *ACS Omega* **2024**, *9* (16), 18366−18374.

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