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Development and validation of an HPLC method for the determination of hyaluronic acid active substance in pharmaceutical formulations

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ABSTRACT: It is an analytical requirement to identify and determine high molecular weight APIs in pharmaceutical forms. For this reason, it is aimed to develop and validate an analytical method for the determination of hyaluronic acid in pharmaceutical form in this study. 0.1 M Na_2SO_4 was prepared as mobile phase. The separation of compound was performed with a OHpak Shodex SB-806M HQ (13 µm, 8,0 x 300mm, Japan) analytical column. Guard column which is OHpak SB-G, (8 µm, 100 Å, 6.0 x 50 mm, Japan) was integrated to the system before the analytical column. UV detection at 198 nm was used to monitor the eluent and flow rate of the mobile phase was set to 1 mL/min. The method has been validated in terms of system suitability, linearity, limits of detection (LOD) and quantity (LOQ), precision, accuracy, specificity, selectivity, and stability. The obtained findings showed that the analytical method has linearity higher than 0.99, accuracy, precision, selectivity and stability. The method was found to be precise, accurate and specific during the study.

KEYWORDS: Hyaluronic acid; HPLC-UV; microemulsion; analytical method; validation.

1. INTRODUCTION

In 1934, Karl Meyer and John Palmer described a new glucosamine isolation procedure from the vitreous of the bovine eye [1]. This high molecular weight polysaccharide consisting of N-acetyl D-glucosamine and D-glucuronic acid has been named as hyaluronic acid (HA) (Figure 1) [1,2]. HA produced by synovial cells, fibroblasts and chondrocytes [2] is found in synovial fluid, skin, umbilical cord and vitreous part of the eye [3] and HA has been successfully isolated from biological sources such as umbilical cord, skin, synovial fluid, Streptococcus bacteria in later years [3]. In addition, HA is identical to all living organisms and is highly biocompatible. Due to its unique viscoelasticity and limited immunogenicity, HA and its derivatives have many clinical and preclinical applications such as drug delivery and drug targeting [1,3]. It is especially used as viscosupplement in the treatment of osteoarthritis and as an adjunct in eye surgery and also in wound regeneration. Today, many studies are conducted on nasal, oral, pulmonary, ophthalmic, topical, parenteral, and tissue engineering applications of HA [1,4–6].

HA is a disaccharide polymer that is composed of D-glucuronic acid and D-N-acetylglucosamine and linked by variable β-1,4 and β-1,3 glycosidic bonds and chains up to 8000 kDa, called high molecular weight HA (HMW-HA). HA can be as long as 25,000 disaccharide repeats. The size of sodium hyaluronate polymers can range from 5000 to 20,000,000 Da *in vivo*. HMW-HA has an immunosuppressive effect while its low molecular weight has the opposite effect leading to activation of the immune system [7,8]. Due to HA widely used in pharmaceutical and cosmetic products, the determination of HA in these products plays an essential role in terms of fruitful drug development. Until now, several different determination methods of HA [7,9] were developed and most of the reported methods are by derivatization but the effectiveness of the developed methods was not beneficial for pharmaceutical procedures in terms of being simple, accurate, precise, and sensitive.

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Compared to derivation, size exclusion chromatographic methods have the advantages of reducing analysis time, increasing sensitivity and flexibility, and lowering the cost of instruments and maintenance. One of the biggest disadvantages of derivatization is the lack of stability. The reaction products are unstable and probably have short half-lives due to spontaneous intermolecular rearrangement [9,10].

Microemulsions are homogeneous, transparent; thermodynamically stable dispersions, optically isotropic oil and water system stabilized with a suitable surfactant and co-surfactant [11]. Several pharmaceutical advantages, such as ease of preparation, transparency, and the potential to dissolve various drugs can be seen as advantages of microemulsions [12]. Surfactants are highly preferred to create these microheterogeneous systems. They may cause changes in many parameters of these microstructure systems, even if they are used in very small amounts. For all these reasons, there is a need to develop a useful analytical method for the separation and identification of drugs loaded on these systems [13]. Considering all these, this study aimed to develop a simple, specific, accurate, fast, precise HPLC method for the determination of HA in pharmaceutical and cosmetic products based on UV-detection.

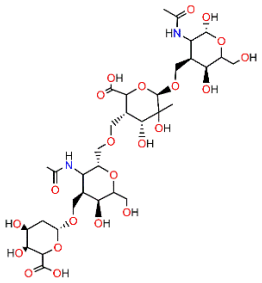

Figure 1. Chemical structure of hyaluronic acid.

2. RESULTS AND DISCUSSION

One of the most essential tools in terms of evaluating drug products is HPLC. Synthesis, manufacturing, and storage of the drug molecule and pharmaceutical formulation can cause drug-related impurities or degradants. This is the reason why an HPLC method should be able to separate, detect, and quantify the drugs and drug-related components as previously mentioned degradants and impurities. Knowing the performance characteristics and limitations and therefore the experimental and environmental effects that can change these characteristics are playing a key role in terms of method validation. So, the process that covers all the period of determination performance characteristics and limitations is being called as validation [14]. A method developed for an HPLC procedure must accurate, sensitive, and able to identify an analyte with high selectivity. For this purpose, validation of analytical methods should include assessments of linearity, selectivity, accuracy (recovery), precision, LOD, LOQ, and measurement uncertainty [15].

In our study, the UV spectrum of the HA in the mobile phase was scanned in the region between 190 and 800 nm. The λ_{max} value was determined as 198 nm (Figure 5).

The linearity of the method was observed in the concentration range of 5 to100 µg/ml, demonstrating its suitability. Each experiment at all concentrations was repeated three times on three separate samples to obtain the calibration data. The area value observed in HPLC was plotted against concentration and the calibration curve was drawn. The equation of the line and the correlation coefficient (R^2) value were calculated. R^2 indicating the functional linear relationship between the concentration of analyte and the area under the peak was above 0.999 across the concentration range used. The correct equation has been found as y = 6.8294X + 2.9334. The R^2 value was determined as 0.9996. The Calibration graph is shown in Figure 2.

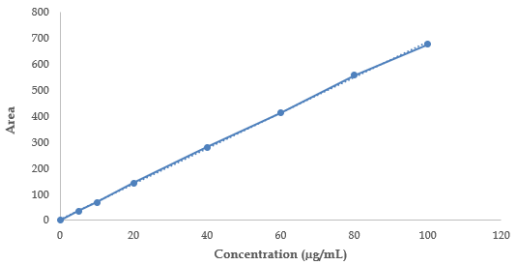

Figure 2. Calibration curve based on chromatographic data of hyaluronic acid.

Precision studies are performed for an analytical procedure to express the proximity between measurement sequences obtained from the same homogeneous samples under the same conditions. For analytical method validation following ICH guidelines, the solution at the lowest, middle, and top concentrations of the working range (5 µg/mL, 60 µg/mL and 100 µg/mL) was measured ten times consecutively, and mean, standard deviation (SD), coefficient of variation (RSD) (%), accuracy (%), precision values were calculated. The results obtained are shown in Table 1.

Table 1. Calculation of accuracy and precision values at the lowest and highest concentrations of the working range for the results of the precision study and analytical method validation as a result of HPLC data.

No	Theoretical Concentration (µg/mL)	Area	Practical Concentration (µg/mL)
1	5 µg/mL	37.88	5.117
2		38.02	5.138
3		37.20	5.018
4		37.61	5.078
5		37.55	5.069
6		37.91	5.121
7		37.25	5.025
8		37.23	5.022
9		37.71	5.092
10		37.01	4.990
		Mean	**5.067**
		SD	**0.051**
		RSD %	**1.007**
		Accuracy %	**101.34**
1	60 µg/mL	415.00	60.337
2		419.52	60.999
3		412.50	59.971
4		410.24	59.640
5		411.08	59.763
6		41824	60.812
7		405.86	58.999
8		417.67	60.728
9		417.62	60.721
10		408.43	59.375
		Mean	**60.611**
		SD	**0.907**
		RSD %	**1.497**
		Accuracy %	**101.018**
1	100 µg/mL	687.81	100.284
2		696.40	101.541
3		687.06	100.174
4		682.66	99.529
5		680.74	99.248
6		680.28	99.181
7		680.13	99.159
8		678.39	98.904
9		686.90	100.150
10		687.20	100.191
		Mean	**99.837**
		SD	**0.792**
		RSD %	**0.793**
		Accuracy %	**99.836**

As Table 1 shows, the percentage of accuracy values for 5, 60 and 100 µg/mL solutions were found 101.34, 101.018 and 99.836, respectively, for bulk HA solution. Obtained results showed that the accuracy percentage was approximately 100 % and the standard deviation was less than 2% which is the acceptance criteria. According to these results, the analysis system for the determination is verified. In addition, the repeatability of the measurements was denoted as high due to the low values of standard deviation. Evaluation of these results highlights that the equipment used for the study was correct and hence the developed analytical method is highly repetitive. The accuracy of the method in this calculation has been confirmed by calculating the average recovery (%) values. SD and RSD% values were also calculated for this parameter. Area values of three parallel samples at three different concentrations (20, 40 and 60 µg/mL) were

measured. The concentration amount corresponding to this area value was found by placing it in the calibration equation. Accuracy and recoverability results are shown in Table 2.

Table 2. Accuracy and recoverability study results as a result of HPLC study (p> 0.05).

Theoretical Concentration (µg/mL)	Area-1	Area- 2	Area-Mean	Practical Concentration (µg/mL)	Recovery (%)
20	143.59	141.45	142.52	20.439	102.194
20	137.56	144.22	140.89	20.200	101.002
20	139.91	141.36	140.64	20.163	100.815
			Mean	**20.267**	**101.337**
			SD	**0.122**	**0.610**
			RSD %	**0.602**	**0.602**
40	272.82	277.92	275.37	39.892	99.729
40	270.24	266.63	268.44	38.876	97.191
40	272.71	271.73	272.22	39.430	98.576
			Mean	**39.399**	**98.499**
			SD	**0.415**	**1.038**
			RSD %	**1.054**	**1.054**
60	408.43	405.86	407.145	59.187	98.645
60	406.79	404.96	405.875	59.001	98.335
60	410.24	411.08	410.660	59.702	98.503
			Mean	**59.297**	**98.828**
			SD	**0.363**	**0.605**
			RSD %	**0.612**	**0.612**

The mean recovery data of HA in samples were 101.337, 98.499 and 98.828 %, respectively. The mean R.S.D. (%) was found 0.612, 1.054 and 0.612. R.S.D % results were found as acceptable for the study. Recovery (%) of HA is given in Table 2. The average of the concentrations, SD and RSD%, corresponding to the area value injected into the HPLC for ten consecutive times of the solutions at a concentration of 40 µg / mL and prepared on two different days were calculated. The results obtained are shown in Table 3. The fact that the method is repeatable shows that the RSD % is less than 2 %.

Table 3. Repeatability study results based on HPLC data.

No	Theoretical Concentration (µg/mL)	Area	Practical Concentration (µg/mL)	No	Theoritical Concentration (µg/mL)	Area	Practical Concentration (µg/mL)
1	40	279.83	40.545	TE-2-1	40	287.37	41.649
2	40	286.07	41.458	TE-2-2	40	296.53	42.990
3	40	286.52	41.524	TE-2-3	40	292.49	42.399
4	40	294.74	42.728	TE-2-4	40	294.37	42.674
5	40	288.86	41.867	TE-2-5	40	294.48	42.690
6	40	282.80	40.980	TE-2-6	40	298.28	43.246
7	40	293.01	42.475	TE-2-7	40	288.33	41.789
8	40	284.27	41.195	TE-2-8	40	294.50	42.693
9	40	293.87	42.601	TE-2-9	40	295.27	42.806
10	40	287.33	41.643	TE-2-10	40	307.70	44.626
		AVE	**41.702**			**AVE**	**42.756**
		SD	**0.685**			**SD**	**0.781**
		RSD %	**1.643**			**RSD %**	**1.826**

For the reproducibility study, the area values of ten 20 µg/mL solutions prepared by 2 different analysts were tested and the mean, SD, and RSD% of the obtained values were calculated. The results obtained are shown in Table 4. It shows that the method is less than 2 % of the RSD that is available again. There was no significant difference between the area values calculated from ten injections for 20 µg/mL concentration ($p > 0.05$). The Limit of Detection (LOD) and Limit of Quantitation (LOQ) tests for the procedure are performed on samples containing very low concentrations of analyses. In the calibration equation, the SD values of the areas corresponding to the lowest value were calculated and the average of these values was divided by the slope of the selected calibration curve and multiplied by 3.3 and the detection limit was determined by multiplying the detection limit by 10. In LOD and LOQ studies, 3 samples were studied. The results obtained are shown in The solution of HA active substance, which was prepared at a concentration of 60 µg/mL using the mobile phase, was measured in HPLC at 15, 30, 60 and 120 minutes and 24 and 48 hours and it was determined whether there was any change in the values. The results obtained are shown in Table *6*.

The specificity of the HPLC method is demonstrated by the separation of the analytes from other potential components such as impurities, degradants, or excipients [16, 17]. In this regard, the optimum amount of contents in microemulsion formulation for IPM, Tween 80, ethanol and 0.9 % NaCl solution were found 7.02 %, 25.575 %, 25.575 %, 41.83 %, respectively.

Table 5Table 4. Reproductivity study results based on HPLC data.

	No	Theoretical Concentration (µg/mL)	Area-1	Area- 2	Area-Mean	Practical Concentration (µg/mL
ANALYST 1	1	20	148.63	145.99	147.31	21.140
	2	20	139.28	147.89	143.59	20.595
	3	20	145.25	152.55	148.90	21.373
	4	20	141.45	149.19	145.32	20.849
	5	20	146.66	147.21	146.94	21.086
	6	20	145.72	142.69	144.21	20.686
	7	20	149.67	143.98	146.83	21.069
	8	20	150.94	150.45	150.69	21.636
	9	20	155.30	148.13	151.71	21.785
	10	20	154.10	148.83	151.47	21.749
					Mean	**21.197**
					SD	**0.406**
					%RSD	**1.917**
	No	**Theoretical Concentration (µg/mL)**	**Area-1**	**Area- 2**	**Area-Mean**	**Practical Concentration (µg/mL**
ANALYST 2	1	20	146.90	151.06	148.98	21.385
	2	20	145.62	148.40	147.01	21.097
	3	20	141.95	152.50	147.23	21.128
	4	20	152.82	147.86	150.34	21.584
	5	20	155.70	148.36	152.03	21.832
	6	20	147.57	142.67	145.12	20.820
	7	20	157.06	151.23	154.15	22.141
	8	20	151.98	147.54	149.76	21.499
	9	20	144.85	152.23	148.54	21.321
	10	20	157.78	143.77	150.78	21.648
					ORT	**21.445**
					SS	**0.365**
					%VK	**1.700**

The solution of HA active substance, which was prepared at a concentration of 60 µg/mL using the mobile phase, was measured in HPLC at 15, 30, 60 and 120 minutes and 24 and 48 hours and it was determined whether there was any change in the values. The results obtained are shown in Table 6.

The specificity of the HPLC method is demonstrated by the separation of the analytes from other potential components such as impurities, degradants, or excipients [16, 17]. In this regard, the optimum amount of contents in microemulsion formulation for IPM, Tween 80, ethanol and 0.9 % NaCl solution were found 7.02 %, 25.575 %, 25.575 %, 41.83 %, respectively.

Table 5. Area and standard deviation (SD) values of the 5 ppm sample to calculate LOD and LOQ values.

Sample Concentration	5ppm
Area-1	34.67
Area-2	35.17
Area-3	34.07
Area-Mean	34.64
SD	0.550

$$LOQ = \frac{SD}{Slope\ of\ Calibration\ Curve} \times 10 = \frac{0.550}{6.8294} \times 10 = 0.805$$

$$LOD = \frac{SD}{Slope\ of\ Calibration\ Curve} \times 3.3 = \frac{0.550}{6.8294} \times 3.3 = 0.266$$

Table 6. Stability test results for 60 µg/mL concentration (p> 0.05).

Time	0 min	15 min	30 min	60 min	120 min	24 h	48 h
Area-1	417.67	421.94	412.50	418.24	420.81	406.79	400.49
Concentration-1	60.728	61.278	59.898	60.737	61.113	59.063	58.142
Area-2	417.62	415.00	410.24	429.89	420.17	404.96	405.42
Concentration-2	60.721	60.352	59.655	62.532	61.109	58.882	58.949
Area-3	408.42	419.52	411.08	424.04	405.86	394.60	407.11
Concentration-3	59.375	61.014	59.778	61.675	59.013	57.365	59.197
Area-Mean	414.57	418.82	411.27	424.056	415.61	402.116	404.34
Concentration- Mean	60.274	60.881	59.777	61.648	60.412	58.437	58.763
Concentration SD	0.779	0.390	0.099	0.733	0.989	0.761	0.450

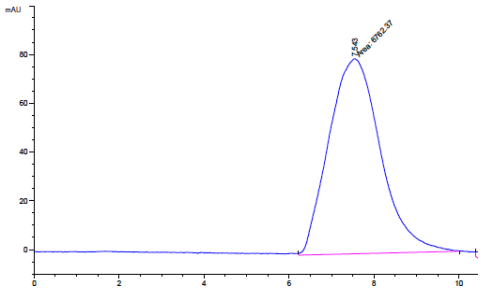

Figure 3. HPLC chromatogram of hyaluronic acid.

In order to prove that the field values in HPLC belong to the active substance, the field values of the HA solution (Figure 3), blank and the active substance loaded formulations were checked and the obtained HPLC chromatograms (Figure 4).

No interaction was found in between peaks of HA and the components of the microemulsion formulation and also the impurities. The retention time of the HA peak was at 7.543 min. The chromatogram blank microemulsion formulation (Figure 4a) showed no peak and retention time as well. The chromatogram of the formulation loaded with HA is Figure 4b. Drug content analysis of HA loaded microemulsion was found 9.9±0.2 mg/mL.

The HPLC method of HA has been developed and the method has been validated. Validation was evaluated in terms of linearity, accuracy, precision, specificity, and stability parameters according to the criteria recommended by the FDA. The determination coefficient (R^2), which indicates the linearity of the

calibration curve, was found to be 0.9996 while examining in terms of linearity. In the accuracy study at different concentrations, the accuracy values (%) calculated as a result of three parallel injections at the same concentration are above 90%. In evaluating the validation in terms of specificity, the HA peak came in almost 8 minutes as seen only in the mobile phase and the injection with the HA mobile phase, and no active ingredient peak was observed before and after this period in the empty injection.

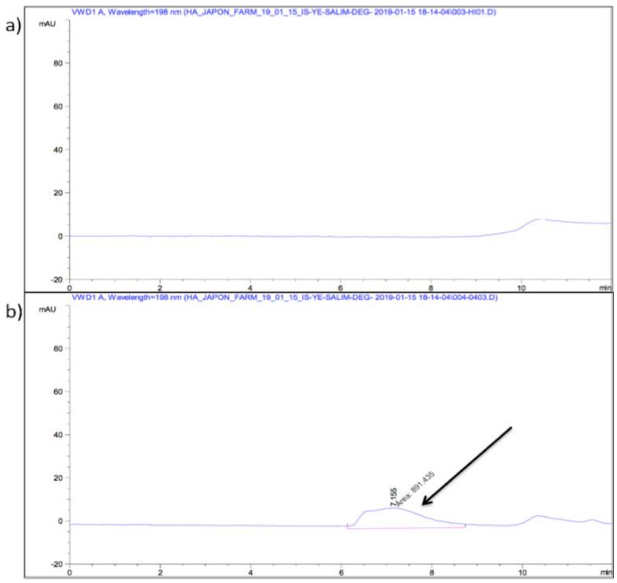

Figure 4. a) HPLC chromatogram of blank in microemulsion formulation, b) HPLC chromatogram of formulation loaded with hyaluronic acid.

3. CONCLUSION

In this study, a simple, accurate, precise HPLC method has been developed for simultaneous testing of hyaluronic acid. ICH guideline was used to validate the method. Results showed that the developed method is applicable for intended analysis because it has proven that the method is accurate and precise quantitative results under small variations of chromatographic conditions. Furthermore, the high recovery and low relative SD proved that this method is suitable for determining HA in dosage forms. Consequently, this method can routinely be used for HA determination.

4. MATERIALS AND METHODS

4.1. Materials

HA was a kind gift from Kuzey Kimya, Turkey. Sodium sulfate, sodium azide and sodium chloride were purchased from Sigma-Aldrich (Germany). IPM was obtained from Sigma, USA. Tween 80 and ethanol were purchased from Merck, Germany. ELGAFlex water system (UK) was used to obtain ultrapure water. Rest of the chemicals used were analytical grade.

4.2. Methods

4.2.1. Instrumentation

An HP Agilent 1100 series HPLC system (USA) equipped with a solvent pump, thermostable column, injection valve and a UV detector were used. The separation of compound was achieved with a OHpak Shodex SB-806M HQ (13 µm, 8,0 x 300mm, Japan) analytical column and also a guard column which is OHpak SB-G, (8 µm, 100 Å, 6.0 x 50 mm, Japan) was integrated to the system before the analytical column.

4.2.2. Chromatographic conditions

UV spectrometer (UV-1800-Shimadzu, Japan) was used to analyze the HA samples. The HA sample was analyzed in the range of 190–800 nm. The optimum wavelength was found as 198 nm (Figure 5). This wavelength is the wavelength of maximum response of HA in the mobile phase and it lets simultaneous determination of related impurities of HA.

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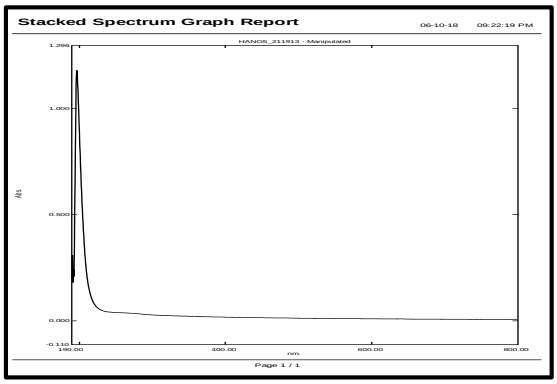

Figure 5. UV spectrum of hyaluronic acid.

The mobile phase was 0.1 M Na_2SO_4 and delivered at a flow rate of 1 mL/min which gave the best resolution within acceptable analysis time and column back pressure. The injection volume was 20 µL. The UV detector was operated at 198 nm. In addition, column was heated 40 ^oC throughout the analysis. The column was equilibrated for at least 1 hour with the mobile phase flowing through the system before the injection of the drug standards. The run time was set at 10 min with the system operating. The mobile phase was filtered and degassed through membrane filter of 0.22 µm porosity under vacuum.

4.2.3. Preparation of stock solutions and standard working solution

10 mg HA was dissolved in 100 mL 0.9% NaCl solution in order to prepare stock solution of HA, so 100 µg/mL stock solution was prepared. The prepared stock solutions were stored in the refrigerator at 4±1 ^oC in a clear glass volumetric flask and covered with aluminum foil in terms of light protection. 5, 10, 20, 40, 60, 80, 100 µg/mL HA concentrations in working solutions were chosen for the aim of drawing calibration curve. Dilution of stock solution in mobile phase was used to prepare further daily fresh working solutions. An aqueous 0.2 µm pore size membrane filter was used for all solutions to be filtered before injection.

4.2.4. Preparation and content determination of microemulsion formulation

The existence range of microemulsions was understood by using pseudo-ternary phase diagram. Microemulsion formulation was prepared by titration method [18]. In order to prepare microemulsion, IPM as oil phase, Tween 80 as surfactant, Ethanol as cosurfactant and 0.9 % NaCl solution as aqueous phase were used. HA was loaded microemulsion to a final concentration of 10 mg/mL. In order to determine the amount of HA in microemulsion, HA loaded formulation was dissolved in mobile phase and the amount of HA was analyzed by using HPLC.

4.2.5. Validation of HPLC method

The validation parameters consisted at system suitability, linearity, limit of detection (LOD) and quantitation (LOQ), precision, accuracy and specificity, selectivity, and stability.

4.2.6. Linearity

The calibration curve was used to analyze the linearity between peak area and concentration. For the drawing of the calibration curve, samples diluted in mobile phase from the stock solution to concentrations of 5, 10, 20, 40, 60, 80, 100 µg/mL were examined in parallel in three. After each injection, the area value observed in HPLC was plotted against concentration and the calibration curve was drawn. The equation of the line and the R^2 value are calculated.

The peak area of each concentration at which retention time and maximum absorption was obtained as a result of injection of standard solutions into HPLC was calculated. HA concentration versus peak areas were plotted and the calibration equation was determined. Each measurement was repeated 3 times.

4.2.7. Accuracy and recovery

The accuracy of an analytical method is defined as the closeness of test results obtained by the method to the true value. Field values of three parallel samples at three different concentrations (20, 40, and 60 µg/mL) were measured. The concentration amount corresponding to this area value was found by placing it in the calibration equation. Percentage recovery from the found value was calculated with the help of Equation 1 [9].

$$\% \text{ Recovery} = (C_{practical}/C_{theoretical}) \times 100 \text{ (Eq.1)}$$

$C_{practical}$: Concentration value obtained from the calibration equation of active substances

$C_{theoretical}$: Concentration value of the active substance

4.2.8. Precision

Precision is a measure of the degree of repeatability and reproducibility of the analytical method under normal operating conditions. It is performed by measuring the samples in the same concentration, in the number of statistically sufficient evaluations, and calculating the arithmetic mean, standard deviation (SD) and coefficient of variation (RSD%). RSD% less than 2% shows that the method provides precision. Accordingly, the standard solution at the same concentration (60 µg/mL) was measured fifteen times consecutively, and mean, SD and RSD% were calculated [19]. Accuracy and precision values were calculated at the lowest and highest concentrations of the working range (5 µg/mL and 100 µg/mL) for analytical method validation in line with the guidelines of the International Harmonization Conference (ICH) [20].

4.2.9. Stability of the solution

The solution prepared at a concentration of 60 µg/mL using the mobile phase of the HA active ingredient was measured at 15, 30, 60 and 120. minutes and 24 and 48 hours in HPLC and it was determined whether there was any change in the values. Each measurement was repeated 3 times.

4.2.10. Repeatability

A concentration (40 µg/mL) was selected from the stock solution prepared to create the calibration equation, and the solutions at this concentration prepared on two different days were injected into the HPLC ten times consecutively. The mean, SD and RSD % of the concentrations corresponding to the area value were calculated. The fact that the method is repeatable shows that the RSD % is less than 2 %.

4.2.11. Reproducibility

For the reproducibility study, the area values of ten solutions of the same concentration (20 µg / mL) prepared by dilution by 2 different analysts, starting from the same stock, were tested and the mean, SD and RSD % of the obtained values were calculated. The recoverability of the method indicates that the RSD % is less than 2 %.

4.2.12. Specificity/Selectivity

In order to prove that the field values found as a result of working with the formulations in HPLC belong to the active substance, the formulations without hyaluronic acid loaded and the active substance loaded formulations were read in HPLC and it was checked whether the HPLC field value of the HA active substance was separate from the field value of the empty formulation [21].

4.2.13. Limit of quantification and detection

Quantitation of HA should be able to analyze the expected concentrations easily and accurately. The limit of detection (LOD) is the lowest concentration at which the analyzed substance can be determined with acceptable accuracy and repeatability. The limit of quantitation (LOQ) is the lowest concentration at which the analyzed substance can be detected. These limits were determined by analyzing a series of low concentration samples. In the calibration equation, the SS values of the areas corresponding to the lowest value were calculated and the average of these values was divided by the slope of the selected calibration curve and multiplied by 3.3, the detection limit (Equation 2) was multiplied by 10 and the limit of determination (Equation 3) was determined. In LOD and LOQ studies, 3 samples were studied. σ is the standard deviation of the given sample; S refers to the slope of the calibration curve [22]

$$LOQ = \frac{10 \times \sigma}{S} \text{ (Eq. 3)} \qquad LOD = \frac{3.3 \times \sigma}{S} \text{ (Eq. 2)}$$

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