ATATÜRK ÜNİVERSİTESİ YAYINLARI
ATATURK UNIVERSITY PUBLICATIONS

Research Article DOI: 10.5281/zenodo.12626292

Determination of Hyaluronic Acid in Ophthalmic Solution by HPLC–UV and Stability Analysis

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ABSTRACT

Objective: Hyaluronic acid (HA) is a naturally occurring, biocompatible polysaccharide with unique viscoelastic and hygroscopic properties. Its role as a natural lubricant and its excellent water-retaining properties make it well-suited for use in ophthalmic products. The objective of this study is determination of sodium hyaluronate in ophthalmic solution liquid chromatography (HPLC) method with UV detection and, stability of the product.

Methods: The analysis was carried out using a polymer column (PolySep-GFC- P5000) with a mobile phase consisting of (dH2O: 20 mM Phosphate Buffer pH:6.5), (95:5, v:v) at a flow rate of 1.2 mL/min. UV detection was set 205 nm.

Results: Calibration curve was linear over the concentration range of 80%-120% concentration of the ophthalmic product. The back calculated concentrations of the calibration standards were within ±15% of the nominal value (±20% for LOQ). The stability of ophtalmic solution was found to be 92.5% at the end of the days at room tempereture.

Conclusion: The developed method is fast and reliable for HA analysis in ophthalmic products. Accordingt to stability study, these products must be stored at +4 C during usage and storage.

Keywords: Hyaluronic acid, ophthalmic product, HPLC

INTRODUCTION

Hyaluronic acid, also called hyaluronan or hyaluronate (HA), is an important kind of glycosaminoglycans due to its various physiological functions. This polysaccharide consists of a repeating of glucuronic acid and disaccharide bound by glycosidic bonds (Figure 1). HA is abundant in all organs and especially in connective tissue such as umbilicalcord, synovial fluid, skin and the vitreous body. HA sources can obtain from animal sources such as rooster combs, bovine's eyes, and microbial production.

The molecular weight of HA is dependents on its source, for example human synovial HA averages about 7 million Da per molecule.[1] It has high hygroscopicity, viscoelastic nature and good biocompatibility. It does not produce toxic products when broken. HA is used in cosmetics, surgery, drug delivery system, rheumatology, otolaryngology and urology. Especially Its role as a natural lubricant and its excellent water-retaining properties make it well-suited for use in ophthalmic products.[2,3]

Size-exclusion chromatography (SEC) is a kind of chromatographic method which substances are separated by their size or molecular weight; The advantages of this method are good separation of large molecules like quaternary structure of purified proteins, peptids, polysaccharides, biotechnological drugs by preserving the biological activity of the particles. Breakdown of polymer is not necessary part of method development. While sample preparation steps decreases, sensitivity and simplicity of method increases.[4]

There are several methos reported on sodium hyaluronate determination in literature such as HPLC UV-Vis[1-7], electrophoresis[8] ,LC-MS[9-13], turbidimetric[14-16], chemiluminescence[20]. Most of studies are based on analysing disaccarides or monomers of HA after enzymatic digestion, acid-based degradation or derivatization of HA.

Received 10.05.2024
Accepted 13.06.2024
Publication Date 03.07.2024

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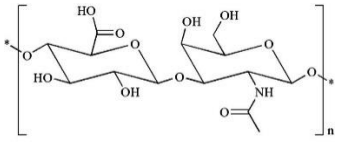
Cite this article: Arslan A, Özer Ünal D. Determination of Sodium Hyaluronate in Ophthalmic Solution By HPLC–UV and stability analysis *Pharmata*.2024;4(3):77-80



HA

Figure 1. Structures of hyaluronic acid

K. Ruckmani et al. developed a SEC chromatography method to analyse HA and applied in house pharmaceutical preparations.

High performance liquid chromatography (HPLC), is a widely used analysis technique to separate the components in mixtures and determine their quality and quantity. In this technique, analytes carried by the mobile phase pumped by pumps reach the chromatographic column. They interact with the column in different ways and reach the detector at different times. HPLC-UV absorption detection technique was used with a polymer column (PolySep-GFC- P5000) in this study. The developed method was used for determination of the HA, and evaluate stability of HA in a commercial ophthalmic formulation.

METHODS

Apparatus and Reagents

The HPLC system of Agilent Infinity 1260 Series with UV detector were used for analysis HA. The HPLC system consisted of 1260 Quat pump, 1260 ALS, 1290 Thermostat, 1260 TCC, 1260 DAD. Chemstation was used as instrument software.

Hyaluronic acid was obtained from Bloomage Freda Biopharm Co.,Ltd. Potassium dihydrogen phosphate and potassium hydroxide were purchased from Merck. Brand of opthalmic product is Artelac Splash® (0.24% (w/v)Hyaluronic acid) from Bausch + Lomb Health and Optic Products TIC. Inc.

Chromatographic Conditions

PolySep-GFC- P5000 (300×7.8mm) was chosed as column and (dH2O: 20 mM Phosphate Buffer pH:6.5), (95:5, v:v) was used as the mobile phase with a flow rate of 1.2 mL/min. The detector was set at 205 nm and the injection volume was 100 µL.

Preparation of Solutions

3% HA stock standard solution was prepared by weighing 3 g sodium hyaluronate into 100 mL of ultra pure water. The calibration standard solutions were prepared by dilution of stock solution with dH_2O. Calibration curves were constructed at five concentration levels ($1.920x10^{-1}$-$2.880x10^{-1}$ %, w/v **(**weight/volume percentage concentration)). Eye drop wasn't diluted. 20 mM potassium dihydrogen phosphate was prepared by weighing 1.3608 g into 500 mL of ultra pure water and pH are adjusted to 6.5 by 1M KOH solution. Opthalmic solution was analyzed directly without dilution or extraction procedure.

RESULTS

Chromatographic separation of compound and interference came from sample was developed succesfully. Any interference at retention times of analyte wasn't seen (Figure 2). It means that method is selective for HA in ophthalmic product.

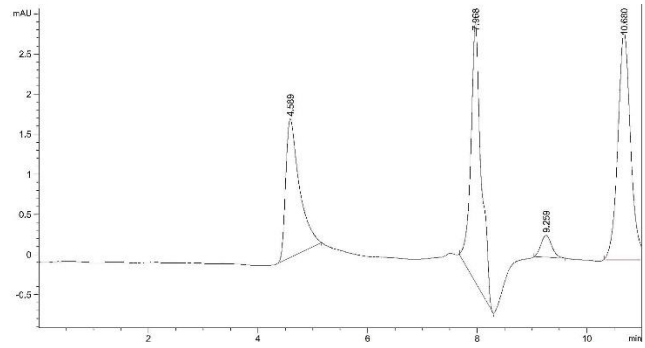


Figure 2. Chromatograms of ophthalmic solution, HA Rt: 4.589 min

System suitability parameters, tailing factor, theoritical plates, injection precision and capacity factor determined and summarized in Table 1. Values were found within acceptable limits .[23]

Table 1. System suitability parameters of hyaluronic acid

Parameter	Value (HA)	Limit (FDA guidline)
Tailing	0.735	$T \leq 2$
Theoretical Plates	5393	$N > 2000$
Injection Precision (RSD)	0.03	RSD% < 1% , $n \geq 5$
Resolution (R)	9.30	$R > 2$
Capacity Factor	4.62	$k' > 2$

HA: Hyaluronic acid, T: Tailing, N: Theoretical Plates, RSD: Relative Standart Deviation, R: Resolution, k': Capacity Factor

"The limit of detection (LOD) and lower limit of quantification (LOQ) were determined to evaluate the sensitivity of the analytical method. The calculated LOD and LOQ level based on signal to noise (S/N) ratio of 3:1 and 10:1, respectively. LOD and LOQ were found to be 0.012% and 0.039% (w/v), respectively. The method LOQ level is 0.192 % (w/v), the first point of calibration curve.

Calibration curve was plotted area of the peak versus HA concentration (1.920x10-1; 2.160x10-1; 2.400x10-1 ;2.640x10-1 ; 2.880x10-1 %, w/v). The linearity of the method was evaluated by correlation coefficient and equation of the standard curve.

They were found (r^2=0.9959) and (y =-127.29577.x-0.678689) respectively. Standart solutions were analyzed 2 times with an interwal 3 days. Accuracy of calibration curve was expressed as mean(%,w/v); precision was expressed as relative standard deviation (RSD %).The back calculated concentrations of the calibration standards were within ±15% of the nominal value (±20% for LOQ) (Table 2). The working range used to determine HA quantification was found acceptable accurate, precise, and linear (Figure 3).

Table 2. Accuracy and precision results of the calibration standard

Conc.(%,w/v)	Mean(%,w/v)	RSD%	CV%	n
$1.920x10^{-1}$	$1.852x10^{-1}$	3.550	1.079	6
$2.160x10^{-1}$	$2.051x10^{-1}$	5.051	1.219	6
$2.400x10^{-1}$	$2.388x10^{-1}$	0.483	2.070	6
$2.640x10^{-1}$	$2.738x10^{-1}$	3.698	0.724	6
$2.880x10^{-1}$	$3.014x10^{-1}$	4.638	1.396	6

Conc.: Concentration, w/v: weight/volume, RSD: Relative Standart Deviation, CV: Coefficient of Variation, n: number of experiment

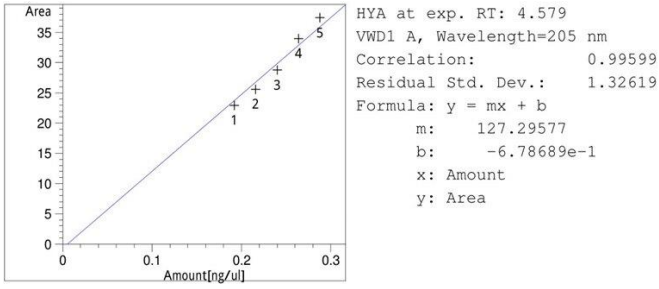


Figure 3. Calibration graph of HA

Stability of samples was performed. Samples containing HA at $2.400x10^{-1}$ % concentration in four replicate were kept at ambient temperature and, analyzed on days 1, 5 and 7. Recovery results of the samples are shown in Table 3.

Table 3. Recovery results for one week

Day	Conc.(%,w/v)	Mean(%,w/v)	RSD%	Accuracy	n
1.		0,239	0.417	99.58	4
5.	$2.400x10^{-1}$	0,227	5.417	94.58	4
7.		0,222	7.500	92.50	4

Conc.: Concentration, w/v: weight/volume, RSD: Relative Standart Deviation, n: number of experiment

DISCUSSION

Hyaluronic acid, due to its various physiological functions like high hygroscopicity, natural lubricant and good biocompatibility, is good candidate for ophthalmic products. Thus, routine analysis of HA in ophthalmic product is essential. HPLC- UV system was used for separation and detection. Polymeric column was chosen because of polymeric structure of HA .

This choice provides simplicity, repeatability and a fast analysis without sample preparation like derivative or digestion. In addition to this, while 100% aqua mobile phase system can't be used in C18 column because of phase collapse, it could be used in SEC coloumn to seperate and analyze HA which is stable in aqua phase at neutral pH. Literature survey shows us that pH effect the rate of the hydrolytic degradation of HA in aqueous solution extremely. HA is most stable at around neutral pH values than acidic or basic conditions.[21,22] Considering pH effect to HA stability, mobile phase composition was chosen at neutral pH.

Calibration range was suitable for quantitative of HA in commerical opthalmic product and, any sample preparation process also including dilution wasn't applied to product sample. Besides it wasn't used internal standard because of simple sample preparation procedure and without analyte loss. Weight/volume percentage concentration (%)" (w/v) was used as concentration unit because quantity of HA is defined on commercial product as % HA. The developed method was fast and reliable for HA analysis in ophthalmic products. The stability of ophtalmic solution was found to be %92.5 at the end of the days at room temperature. So, the ophtalmic products which is contain HA must be stored at +4 °C during usage and storage.

CONCLUSION

In literature, various degradation and derivatization process have been applied to determine hyaluronic acid content, but a few studies were about analysis of hyaluronic acid without these process. In this study, size exclusion chromatography technique were studied to quantify hyaluronic acid in ophthalmic product. The proposed method was simple, fast and reliable.

Ethics Committee Approval: Ethical approval and informed consent are not required in our study as no research was conducted on human or animal specimens.
Peer-review: Externally peer-reviewed.
Author Contributions: Concept -DÖÜ; Design- DÖÜ; Supervision-DÖÜ; Resources-DÖÜ; Data Collection and/or Processing-MA; Analysis and/or Interpretation-MA; Literature Search-MA; Writing Manuscript-MA; Critical Review-DÖÜ
Conflict of Interest: The authors have no conflicts of interest to declare.
Financial Disclosure: The authors declared that this study has received no financial support.

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