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Determination of the distribution of molecular masses of sodium hyaluronate by high-performance anion-exchange chromatography

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Abstract

Sodium hyaluronate, or hyaluronic acid (HA), is a glucosaminoglucan used in pharmaceuticals in ophthalmic surgery and for treatment of rheumatoid arthritis. The average molecular mass of the HA polymer used in these products is often in the range $1\cdot10^6$–$5\cdot10^6$. Size-exclusion chromatography has been used for analysis of molecular masses, including the distribution, up to about $3\cdot10^6$. In this work, an anionic exchange chromatography method is presented by which the peak molecular mass and an estimation of the distribution of the molecular masses of HA is possible in the range $0.1\cdot10^6$–$5\cdot10^6$.

Keywords: Pharmaceutical analysis; Sodium hyaluronate; Hyaluronic acid; Glucosaminoglucan; Polysaccharides

1. Introduction

Sodium hyaluronate, also called hyaluronan, is referred to as hyaluronic acid (HA) in this work. HA is an anionic linear polysaccharide and a naturally produced highly viscous glucosaminoglucan of the type $(–GlcNAc–GlcUA–)_n$, where GlcNAc is *N*-acetyl-D-glucosamine and GlcUA is D-glucuronic acid. HA is found in humans in tissues such as the vitreous body of the eye, cartilage, and synovial fluid of the joints [1]. The molecular mass of the HA polymer is in the range from 10^4 up to about 10^7 [2]. For the purification of HA from associated proteins and other components, various methods have been used, such as precipitation with cetylpyridinium chloride [3], cation-exchange [4], and anion-exchange chromatography [5]. Commercial pharmaceutical products of HA are successfully used in ophthalmic surgery and also for treatment of joint diseases such as rheumatoid arthritis. Several publications describe low-molecular-mass HA ($<0.5\cdot10^6$) as a strong inflammatory mediator in various tissues and producing a response that is evoked by chemokines and interleukines [6,7]. In the treatment of joint diseases, high-molecular-mass HA has been shown to be superior to low-molecular-mass HA in many respects [8,9]. Many pharmaceutical HA products therefore have been prepared to obtain an average molecular mass in the range $1\cdot10^6$–$5\cdot10^6$. Important steps in the process and analysis of pharmaceutical HA-based products are to determine both the molecular mass and the molecular mass distribution.

Methods that have been used for the determination of the molecular mass of HA include size-exclusion

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chromatography (SEC) [2], agarose gel electrophoresis [10], capillary electrophoresis (CE) [11], viscosimetry [12], and low-angle laser light scattering (LALLS) detection [13]. Unfortunately, mass spectrometry cannot be used for these high molecular masses ($>5\cdot10^5$). By light scattering, a weighted average molecular mass is obtained and the molecular mass distribution of HA up to $6\cdot10^6$ has been determined by this method [14]. Problems exist in the use of these methods for determining the molecular mass distribution; and even the widely used SEC technique can be used for molecular masses up to only about $3\cdot10^6$, and light scattering is extremely sensitive to particles present in samples to be analyzed. Thus, new techniques are needed to complement some of these methods.

In the present study, an ion-exchange chromatography (IEC) method is presented for the determination of the peak molecular mass and to estimate the distribution of the molecular masses of HA in the range $0.1\cdot10^6$–$5\cdot10^6$.

2. Materials and methods

Laboratory samples of sodium hyaluronate of different weighted average molecular masses ($0.1\cdot10^6$, $0.25\cdot10^6$, $0.5\cdot10^6$, $1\cdot10^6$, $3\cdot10^6$, $4\cdot10^6$ and $5\cdot10^6$), and of high purity (>97%), were obtained from Pharmacia, Uppsala, Sweden. These HA samples were used as molecular mass standards in this work. Concentration and weighted average molecular masses of HA standards were determined by the carbazole method, based on the analysis developed by Bitter and Muir [15] and by LALLS, respectively. Sodium sulfate and sodium phosphate (both analytical-reagent grade) were obtained from Merck (Darmstadt, Germany) and Tris(hydroxymethyl)-aminomethane (analytical-reagent grade) was from Sigma (Munich, Germany).

2.1. Method A—Determination of hyaluronic acid $0.1\cdot10^6$–$1\cdot10^6$

The HA samples were chromatographically analyzed using a Waters Alliance 2690 HPLC and a 996 UV photodiode array detection system (Waters, Milford, MA, USA), equipped with a strong anion-exchange-chromatography column, PL-SAX-4000 (4000 Å, 8 μm, 150×4.6 mm I.D.), and a PL-SAX pre-column, both purchased from Polymer Labs. (Church Stretton, UK). The functional group of this polymer column is a quaternary amine group. The column, in an oven at 45 °C, was equilibrated with 10 m*M* sodium phosphate, 20 m*M* sodium sulfate, at pH 7.0 (mobile phase A). Mobile phase B consisted of 10 m*M* sodium phosphate, 225 m*M* sodium sulfate, pH 7.0. Elution was carried out by a linear gradient (0–50 min 0–100% B, 50–60 min 100% B, 60–70 min 0% B). The flow-rate was 0.5 ml/min, except during the equilibration time, where an increased flow of 1 ml/min was used at 60–69 min. The injection volume of the respective HA standard was 15 μl, diluted to 1 mg/ml in 10 m*M* Tris–HCl, 20 m*M* sodium sulfate, pH 8.0. Detection was carried out by measuring the absorbance at 210 nm. Three separate analyses, including the standard curve ($0.1\cdot10^6$, $0.25\cdot10^6$, $0.5\cdot10^6$ and $1\cdot10^6$), followed by the $0.25\cdot10^6$ HA standard, analyzed as an unknown sample, were performed. All injections were made in duplicate.

2.2. Method B—Determination of hyaluronic acid $1\cdot10^6$–$5\cdot10^6$

In this method, mobile phase A contained 175 m*M* sodium sulfate, and higher-molecular-mass HA standards were used for the standard curve. Other analytical conditions were as described in Method A. Three separate analyses, including the standard curve ($1\cdot10^6$, $3\cdot10^6$, $4\cdot10^6$ and $5\cdot10^6$), followed by the $4\cdot10^6$ HA standard, analyzed as an unknown sample, were performed.

3. Results and discussion

Many techniques have been used for the analysis of the molecular mass distribution of high-molecular-mass HA. The most widely used methods are probably the SEC method and the light scattering techniques. Another chromatographic technique, which is similar to that of the presented IEC method, is high-performance anion-exchange chromatography coupled to pulsed amperometric detection (HPAEC–

PAD), which has been used for the molecular mass determination of neutral and anionic polysaccharides. By this method, sialic acid polymers have been determined up to a degree of polymerization (DP) of about 80, corresponding to a molecular mass of $2.4{\cdot}10^4$ [16]. Nevertheless, this is not sufficient for the analysis of high-molecular-mass HA.

In the present paper, we describe an IEC method for determining the peak molecular mass and the molecular mass distribution of HA. The basic principle of this method is that molecular species of HA with increased molecular mass elute at increased retention times when analyzed by anion-exchange chromatography. The elution order of the different molecular mass species is thus reversed compared to the SEC method. The obtained retention times were about 49–54 and 43–45 min for $0.1{\cdot}10^6$–$1{\cdot}10^6$ and $1{\cdot}10^6$–$5{\cdot}10^6$, respectively (Figs. 1 and 2). A gradient (20–225 m*M* sodium sulfate) was used for elution of the lower-molecular-mass species of HA ($0.1{\cdot}10^6$–$1{\cdot}10^6$). For the resolution of the higher-molecular-mass species of HA ($1{\cdot}10^6$–$5{\cdot}10^6$), a more gradual gradient (175–225 m*M* sodium sulfate) was used. Sodium sulfate was used as the eluting salt because of its low absorbance at 210 nm and its strong eluting properties. We used a simple point-to-point line for the peak molecular mass standard curve, which did not give an optimal fit. However, for determining the actual samples this model was considered to be satisfactory. Examples of the standard curves, $0.1{\cdot}10^6$–$1{\cdot}10^6$ and $1{\cdot}10^6$–$5{\cdot}10^6$ are shown in Fig. 3.

The peak molecular mass, based on the peak retention times of the analyzed HA samples, which were analyzed as unknown samples, showed values that were very close to the nominal molecular masses ($0.25{\cdot}10^6$ and $4{\cdot}10^6$), as calculated from the standard curve (Table 1). In the analysis of the molecular mass distribution of unknown samples, the integrated peaks were manually split at the retention times for the respective molecular mass standard, as depicted in Fig. 4. The peak fractions, for the integrated and split peaks, estimated the molecular mass distribution for the analyzed samples. This simple method was used to estimate the distribution of the molecular masses of HA. The $0.25{\cdot}10^6$ HA sample contained 64% of the HA $0.1{\cdot}10^6$–$0.5{\cdot}10^6$, while the $4{\cdot}10^6$ sample contained 22% of $3{\cdot}10^6$–$5{\cdot}10^6$. The dis-

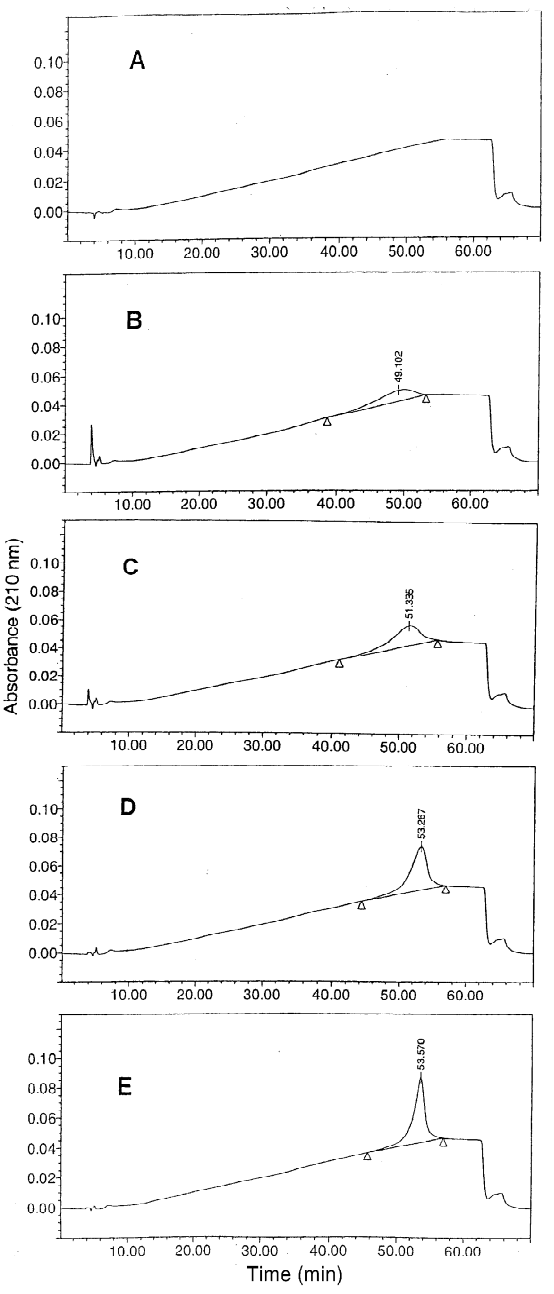

Fig. 1. Anion-exchange chromatography of hyaluronic acid (HA) using a PL-SAX column equilibrated with 10 m*M* sodium phosphate, 20 m*M* sodium sulfate, pH 7.0, in 45 °C, at a flow-rate of 0.5 ml/min. Elution by gradient, 20–225 m*M* sodium sulfate at 0–50 min (Method A). 15 μl HA, 1 mg/ml, was injected and detection was performed by measuring the absorbance at 210 nm. (A) Water blank, (B) HA $0.1{\cdot}10^6$, (C) HA $0.25{\cdot}10^6$, (D) HA $0.5{\cdot}10^6$, (E) HA $1{\cdot}10^6$. For further details see Materials and methods.

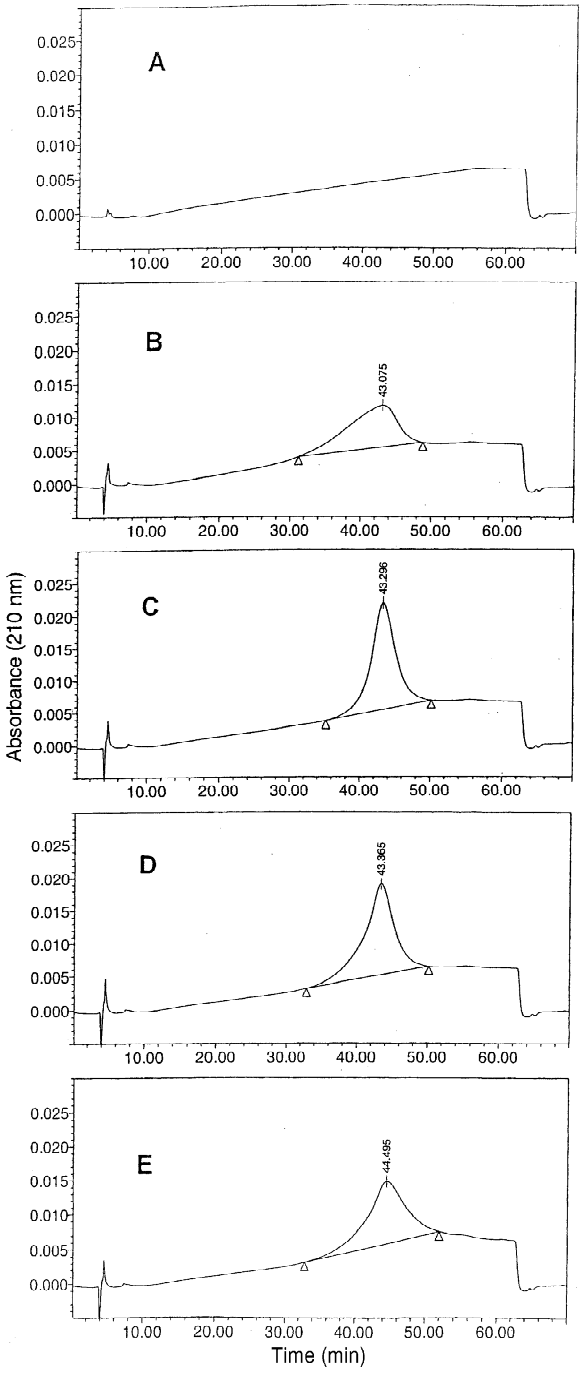

Fig. 2. Anion-exchange chromatography of hyaluronic acid (HA). Elution by gradient, 175–225 m*M* sodium sulfate (Method B). Other analytical conditions as in Method A. (A) Water blank, (B) HA $1{\cdot}10^6$, (C) HA $3{\cdot}10^6$, (D) HA $4{\cdot}10^6$ and (E) HA $5{\cdot}10^6$.

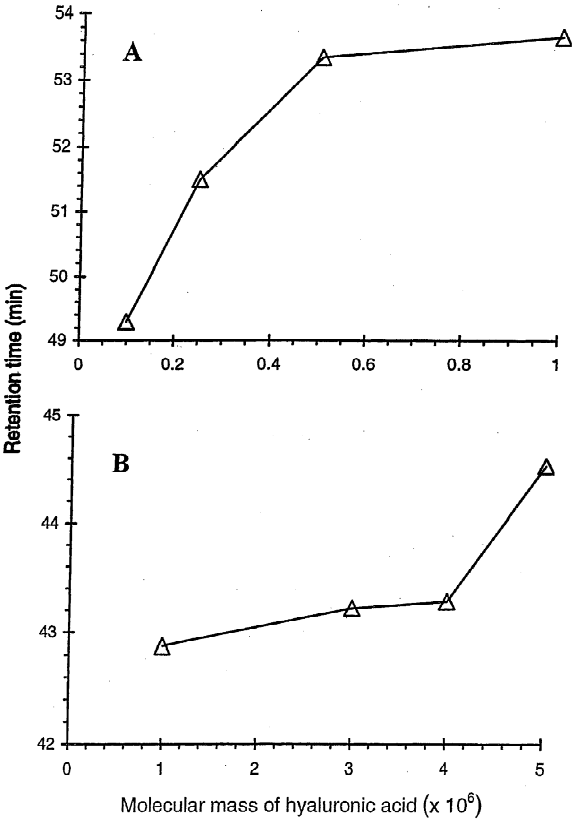

Fig. 3. Standard curves with retention times plotted against molecular mass of hyaluronic acid (HA). (A) HA $0.1{\cdot}10^6$–$1{\cdot}10^6$, eluted by a gradient 20–225 m*M* sodium sulfate (Method A), (B) HA $1{\cdot}10^6$–$5{\cdot}10^6$, eluted by gradient 175–225 m*M* sodium sulfate (Method B). Each point represents the average value obtained from duplicate injections. A simple point-to-point model was used.

tribution of the $4{\cdot}10^6$ HA sample showed an unexpected high percentage distribution of low-molecular-mass species (49% $<1{\cdot}10^6$). The same sample also contained a high degree of high molecular HA (23% $>5{\cdot}10^6$), which together may contribute to a weighted average molecular mass of about $4{\cdot}10^6$, as was obtained by LALLS. The precision of the determination of the peak molecular masses obtained from the retention times from the analyses ($n=3$) of the two samples was high (2 and 11% RSD for $0.25{\cdot}10^6$ and $4{\cdot}10^6$, respectively) and the precision in determining the molecular mass distribution was also considered to be acceptable (Table 1).

Table 1
Molecular mass distribution of hyaluronic acid (HA)

Analysis No	t_R (min)	M_p ($\times 10^6$)	Percentage molecular mass distribution of hyaluronic acid							
			$<0.1\cdot10^6$	$0.1\cdot10^6$–$0.5\cdot10^6$	$0.5\cdot10^6$–$1\cdot10^6$	$>1\cdot10^6$	$<1\cdot10^6$	$1\cdot10^6$–$3\cdot10^6$	$3\cdot10^6$–$5\cdot10^6$	$>5\cdot10^6$
1	51.38	0.24	29	64	2	5				
2	51.40	0.25	27	64	2	6				
3	51.41	0.25	29	63	3	6				
	Mean	0.25	28	64	2	6				
	RSD%	2	4	1	25	10				
4	43.35	4.1					48	7	24	22
5	43.41	4.0					51	3	22	24
6	43.38	3.3					49	6	21	23
	Mean	3.8					49	5.3	22	23
	RSD%	11					3	39	7	4

Gradients of 20–225 and 175–225 m*M* sodium sulfate were used for elution for analysis 1–3 and 4–6, respectively. Standard curves of HA with molecular masses of $0.1\cdot10^6$–$1\cdot10^6$ and $1\cdot10^6$–$5\cdot10^6$ were used in analyses 1–3 and 4–6, respectively. After plotting the standard curve in each analysis, one HA standard sample ($0.25\cdot10^6$ and $4\cdot10^6$ for analyses 1–3 and 4–6, respectively) was analyzed as an unknown and the obtained retention time (t_R) was used to calculate the peak molecular mass (M_p) from its respective standard curve. By manually splitting the integrated peak of this sample at the retention times for the respective molecular mass standard the percentage distribution at selected molecular mass ranges were obtained. All values are average values obtained from duplicate injections. Experimental conditions are as described in the Materials and methods section.

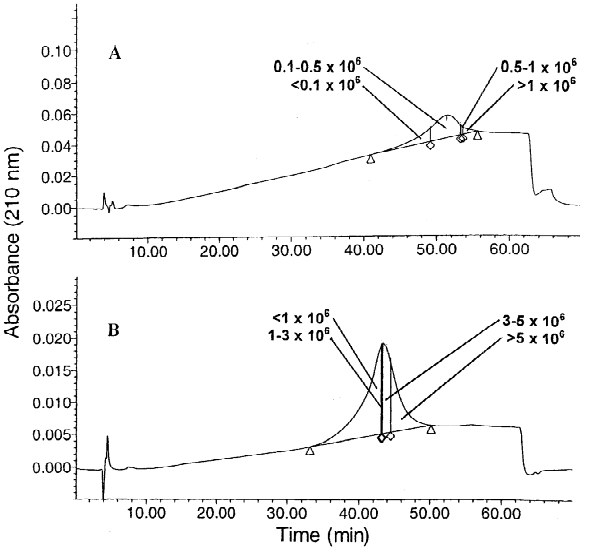

Fig. 4. Hyaluronic acid standards analyzed as unknowns. By manual splitting of the integrated peaks at the retention times for the respective molecular mass standards, the molecular mass distribution for the selected ranges was obtained, as indicated in the chromatograms. (A) HA $0.25\cdot10^6$, eluted by a gradient 20–225 m*M* sodium sulfate (Method A), (B) HA $4\cdot10^6$, eluted by gradient 175–225 m*M* sodium sulfate (Method B).

To assure the accuracy and evaluate the method, the estimation of molecular mass distribution by this chromatographic method should be compared with that of another, e.g. light scattering. This work is planned to be performed in the near future. By slight modifications to a suitable software program, such as that used for molecular mass determination by the SEC method, it may be possible to calculate a distribution curve for the molecular masses, and include the determination of polydispersity, etc. in the IEC analysis of unknown HA samples. Because the separation principles used in IEC and SEC are completely different, however, another approach may have to be used to achieve a successful evaluation. For our method, the HA samples to be analyzed must have a very high purity and a negligible content of proteins and other anionic carbohydrates. The IEC method is useful for determining the peak molecular mass and molecular mass distribution of HA that exceeds the upper limit of the SEC method, which is about $3\cdot10^6$. This method has another advantage in that small amounts of dust or molecular aggregates will not disturb the analysis, which can be a problem with the light scattering methods such as the LALLS.

In conclusion, this anion-exchange chromatog-

raphy method permits the determination of the peak molecular mass and the molecular mass distribution estimated for HA in the range $0.1 \cdot 10^6$–$5 \cdot 10^6$.

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