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ORIGINAL RESEARCH

Molecular characteristics of some commercial high-molecular-weight hyaluronans

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ABSTRACT: Commercially available hyaluronan (HA) samples were investigated by the method of size exclusion chromatography (SEC). The fractions eluted from the SEC column were on-line molecularly characterized by using a multi-angle laser light scattering (MALLS) photometer. Along with the SEC-MALLS technique, the high-molecular-weight HA biopolymers were (off-line) analyzed by capillary viscometry. Copyright © 2002 John Wiley & Sons, Ltd.

INTRODUCTION

Hyaluronan (HA) is a nonbranched glycosaminoglycan whose polymeric chain is composed of regularly alternating units of D-glucuronic acid and *N*-acetyl-D-glucosamine linked by β-(1→4) and β-(1→3) linkages, respectively. This polysaccharide, present in all tissues and body fluids of vertebrates, is under physiological (pH) conditions a polyanion with corresponding counter-cations of H, Na, K, etc. (Fraser *et al.*, 1997).

The molecular weights of HA biopolymers cover the range from hundreds of thousands up to several millions of Daltons. The aqueous solutions of such high-molecular-weight hyaluronans exhibit shear-dependent viscosity and frequency-dependent elasticity. As a result of these properties, ultrapure viscous HA (usually Na^+ salt) solutions have been introduced among preparations serving for various (medicinal) purposes, such as a 'viscosurgery' aid during operations of the anterior chamber of the eye (Healon®), and a 'viscosupplementing' tool when administered intra-articularly into the (osteo-)arthritic knee-joint (Peyron, 1993).

The characteristic feature of a high-molecular-weight (bio-)polymer is sample polymolecularity/polydispersity. Thus the most exact molecular characterization of a 'native' hyaluronan is the determination of the distribution function of the sample molecular weights. For such purposes analytical (high-performance) size exclusion chromatographic (SEC) separation/fractionation technique coupled on-line to a multi-angle laser light scattering (MALLS) photometer has been gaining favor.

The aim of the present paper is: (1) to report the defined averages of the molecular weights of some commercially available high-molecular-weight hyaluronans as determined using the highly sophisticated SEC-MALLS method, and (2) to report the limiting viscosity values of these samples observed by the method of capillary viscometry.

EXPERIMENTAL

HA samples

The high-molecular-weight hyaluronans—sodium hyaluronates—investigated have been marketed by Lifecore Biomedical Inc., Chaska, MN, USA, CPN Ltd, Ústí nad Orlicí, Czech Republic, Sigma Chemical Co., St Louis, MO, USA, and by Genzyme Corp., Cambridge, MA, USA (Table 1).

SEC-MALLS analysis

The distribution functions of the molecular weights of the HA samples were determined using an Alliance 2690 separation module (Waters, Milford, MA, USA) equipped with two on-line detectors, namely with a UV–vis spectrophotometer (Model 996 PDA; Waters) and a MALLS photometer (DAWN DSP-F; Wyatt Technology, Santa Barbara, CA, USA). The operator variables/settings were as follows.

SEC. The columns were two stainless steel (both 7.8 mm × 30 cm)

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Abbreviations used: HA, hyaluronan; MALLS, multi-angle laser light scattering; SEC, size exclusion chromatography.

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Table 1. Molecular characteristics of some commercial HA samples

	Producer					
	Lifecore 1-9100-1	CPN	Sigma 35H0439	Lifecore P9710-2	Genzyme B22157	F1750762[a]
Producer specification				Molecular weight, viscosity average 1.2×10^6 Da Intrinsic viscosity 20 dl/g	Molecular weight 1.26×10^6 Da Intrinsic viscosity 21.65	
Parameter determined						
M_n; in kDa	232.7	350.7	589.7	678.2	894.1	855.9
M_w; in kDa	426.2	659.4	1017	1215	1340	1378
M_Z; in kDa	767.3	1050.4	1658	1690	1669	2205
$[\eta]$; in dl/g	7.33	8.72	11.27	17.5	17.21	16.75

[a] HA sample was the gift of Dr E. Orviský, Research Institute of Rheumatic Diseases, Piešťany, Slovak Republic.
M_n, M_w and M_z represent the number-, weight- and z-average of the HA biopolymer molecular weights, respectively.

columns connected in series with a guard pre-column and thermostated at 35.0°C. Column packings were TSK gel PW (G6000 and G5000; 17-μm particles; Toso Haas, Montgomeryville, PA, USA). The mobile phase was 0.15 M aqueous NaCl solution; flow-rate, 0.4 mL/min; injected sample volume, 200 μL; and concentration of the injected sample, 0.1 mg/mL.

UV–vis spectrophotometer. Absorbance was at 206 nm.

MALLS photometer. The wavelength was 632.8 nm; flow-through cell type, K5.

The data acquisition and analysis softwares used were MILLENNIUM 2.15 (Waters) and ASTRA 4.50 (Wyatt Technology). The normalized (weight-) distribution functions of the molecular weights of the investigated HA biopolymers are represented in Fig. 1. The defined molecular-weight averages of the HA samples are listed in Table 1.

Capillary viscometry

The measurements were performed at 25.0 ± 0.05°C using an Ubbelohde dilution viscometer (Schott Glas, Mainz, Germany).

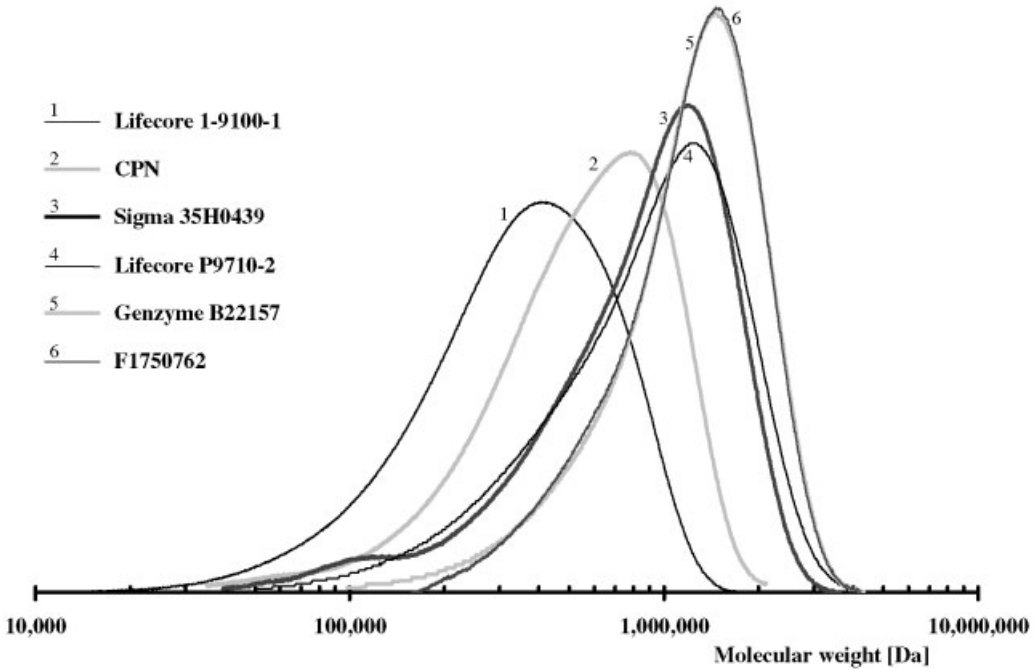

Figure 1. Normalized (weight-) distribution functions of the molecular weight of the investigated HA samples: Lifecore 1-9100-1 (1); CPN (2); Sigma 35H0439 (3); Lifecore P9710-2 (4); Genzyme B22157 (5); and F1750762 (6); see also Table 1.

Table 2. K' and a parameters reported for HA–solvent, 25°C systems

K' (dl/g)	a	Solvent	HA molecular weight (Da)	Reference
0.00016	0.841	0.1 M aqueous NaCl	$>2.4 \times 10^6$	from Fouissac *et al.* (1993)
0.00029	0.80	0.15 M aqueous NaCl		from Li *et al.* (1997)
0.000346	0.779	0.15 M aqueous NaCl	$<1 \times 10^6$	Bothner *et al.* (1988)
0.00057	0.75	0.15 M aqueous NaCl		from Terbojevich *et al.* (1986)
0.000199[a]	0.829[a]	0.2 M aqueous NaCl	$\geq 40 \times 10^4$	Yanaki and Yamaguchi (1994)
0.000228	0.816	0.2 M aqueous NaCl	$>10^5$	Cleland and Wang (1970)
0.00036	0.78	0.2 M aqueous NaCl	7.7×10^4–1.7×10^6	Laurent *et al.* (1960)
0.000318	0.777	0.5 M aqueous NaCl	$>10^5$	Cleland and Wang (1970)
0.000278	0.78	0.1 M aqueous $NaNO_3$	4.2×10^5–1.4×10^6	This work

[a] Valid for measurements at shear-rate $\rightarrow 0\ s^{-1}$.

The diameter of the viscometer capillary was 0.53 mm and the flow-through time of the stock solvent used (0.1 M aqueous $NaNO_3$ solution) was 84.5 s. [The flow-through times of the stock solvent (η_0) as well as of the investigated HA solutions (η_i) were measured with a precision of 0.1 s for the run.]

The HA working solution ($\eta_i/\eta_0 \leq 2$) was diluted directly in the viscometer reservoir so as to fulfill the condition $\eta_i/\eta_0 \geq 1.1$. The viscometry data were evaluated according to the equations introduced by Kraemer and by Huggins, respectively (*Polymer Handbook*, 2002). The determined intrinsic viscosity values ([η]) of the investigated HA samples are listed in Table 1.

RESULTS

Table 1 lists the molecular characteristics of the six hyaluronan samples investigated. As is evident, the weight average of the HA biopolymer molecular weights (M_w) ranged between the values of 426.2 and 1378 kDa. The calculated values of M_w/M_n ranging from 1.50 up to 1.88 and of M_z/M_w falling within the interval of 1.25–1.80 indicated that all six HA samples were represented by relatively narrow distribution functions of the molecular weights (see Fig. 1).

The regression analysis of the dependence of the limiting viscosity number [η] on the HA sample molecular weight (M_w-average taken) written in the (conventional) form as

$$[\eta] = K' M_w^a$$

(system: HA in 0.1 M aqueous $NaNO_3$ solution at 25°C)

could be fitted with the K' and a numerical values of 2.78×10^{-4} and 0.78, where K' and thus [η] are in dl/g units. The values of the calculated pair of K' and a parameters were comparable with the values determined for similar HA–solvent–temperature systems (see Table 2 and Fig. 2).

DISCUSSION

Viscosity belongs among the main characteristics of a liquid, and the method of capillary viscometry is ranked to be the simplest and most precise technique of choice for determining the viscosity characteristics of a liquid/solution with Newtonian flow behavior. The relationship

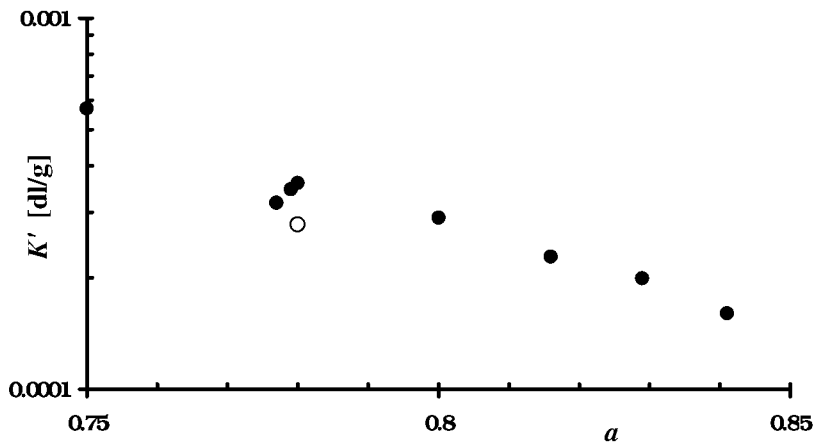

Figure 2. Graphic representation of the K' and a parameters for the systems of HA–aqueous NaCl (●) or $NaNO_3$ (○) solution, 25°C.

between the limiting viscosity number $[\eta]$ of a polymeric sample solution and the viscosity average of the polymer molecular weights (M_v) is described by a well-known Kuhn–Mark–Houwink–Sakurada equation $[\eta] = KM_v^a$. By using the proper tabulated *K, a* pairs (*Polymer Handbook*, 2002), the sample M_v-average can be calculated easily from the $[\eta]$ value determined using, for example, a conventional Ubbelohde capillary viscometer. Although such an approach to characterize the investigated polymeric sample by its M_v-average is frequently used with numerous types of polymers/biopolymers, in the case of the high-molecular-weight hyaluronans, the method of capillary viscometry for the determination of the sample molecular weight should be applied with great caution. Since high-molecular-weight HA biopolymer solutions behave like non-Newtonian liquids, the use of the viscometer/rheometer with 'nil' shear rate is recommended.

On summarizing the above facts, the way of calculating the *M* value of the HA sample from the $[\eta]$ determined by the Ubbelohde type viscometer, characterized by a mean shear rate value ranging from hundreds up to several thousands of seconds^{-1}, must be classified as invalid. On the other hand, the changes of the flow-through times of the HA solution due to, for example, the sample depolymerization/degradation (caused by a physical and/or chemical stressing condition) can be conveniently followed even by the conventional Ubbelohde capillary viscometer (Deeble *et al.*, 1989; Šoltés *et al.*, 2001).

Aqueous NaCl as well as aqueous $NaNO_3$ solutions are the most frequently applied solvents for dissolution of an HA sample prior its analysis performable in aqueous solution stage. The value of the power law exponent *a* ranging from 0.75 to *ca* 0.85 (Fig. 2) suggests that, under the conditions specified (Table 2), the HA biopolymeric chains (at adequately diluted state) have random, loosely oriented/stiffened conformations. In less diluted solutions, however, the HA macromolecules intertwine. The non-homogenous density of the entangled HA biopolymeric chains, forming a micro-heterogenic polymer network, is the main phenomenon resulting in the non-Newtonian flow behavior of the HA solutions. At a higher flow-rate of the eluent, injection of such a nonideal solution (of a gel-like consistency) onto the SEC column may lead even to polymer degradation, induced by high thinning of the viscous sample solution passing through the capillaries of the inlet column filter as well as flowing through interstitial channels between the SEC packing material(s). Thus all the above facts should be carefully evaluated at SEC analysis of high-molecular-weight HA samples with a minimal risk of sample degradation, and simultaneously by keeping the SEC column separation/fractionation performance for the high-molecular-weight HA species at maximum (Mendichi *et al.*, 1998; Mendichi and Šoltés, 2002; Mendichi and Giacometti Schieroni, 2002).

The operator variables/settings, specified in the section 'SEC-MALLS analysis', are an appropriate compromise warranting proper SEC separation/fractionation along with precise MALLS molecular-weight analysis of the hyaluronan samples investigated. Thus, the molecular characteristics determined for the six commercial high-molecular-weight HA samples can be classified as valid.

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