Journal of Chromatography, 472 (1989) 145–154
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 405

DETERMINATION OF STOKES RADII OF HYALURONATE OLIGOSACCHARIDES BY SEPHADEX GEL CHROMATOGRAPHY

EIJI SHIMADA* and GO MATSUMURA
School of Pharmaceutical Sciences, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142 (Japan)
(First received October 18th, 1988; revised manuscript received February 10th, 1989)

SUMMARY

Even-numbered hyaluronate oligosaccharides ranging from di- to hexadecasaccharides were chromatographed on Sephadex G-25, -50 and -75, and their available volumes (K_{av}) were examined. Log K_{av} ratios between any two samples of these oligomers were constant for the three Sephadex types used. The relationships and the use of the log K_{av} of a substance of known size simplified the processes for calculating Stokes radii of hyaluronate oligosaccharides.

INTRODUCTION

Ogston[1] proposed the following equation correlating the available volume (K_{av}) of a spherical molecule with its Stokes radius (R) in a gel:

$$K_{av} = \exp[-\pi L(R + r)^2] \quad (1)$$

where, in the case of Sephadex gel, L is the total concentration of the dextran chain expressed in length per unit volume and r is the radius of a straight dextran chain. Taking the natural logarithm of the above equation gives

$$\log K_{av} = -\pi L(R + r)^2 \quad (2)$$

Using Sephadex gel chromatography, Laurent and Killander[2] demonstrated the usefulness of Ogston's equation in the determination of the spherical equivalent radii (Stokes radius) of proteins and polysaccharides by use of the relationship

$$K_{av} = (V_e - V_0)/(V_t - V_0) \quad (3)$$

where V_e is elution volume, V_0 is void volume and V_t is total bed volume.

We are interested in the conformation of hyaluronate oligosaccharides in solution and have attempted to determine their molecular or Stokes radii in 0.2 M sodium chloride solution according to the procedure of Laurent and Killander. For this purpose, even-numbered hyaluronate oligosaccharides were chromatographed on

0021-9673/89/$03.50

some Sephadex gels. In the course of the studies, we noticed that the log K_{av} ratios for any two of these oligomers were independent of the Sephadex type used. These observations accord with eqn. 2. Therefore, by use of the ratios of log K_{av} of hyaluronate oligosaccharides to that of a substance with a known Stokes radius, *e.g.*, cellobiose or bovine serum albumin, the molecular sizes of the oligosaccharides could be determined.

In this paper, we discuss the relationships between log K_{av} values for hyaluronate oligosaccharides on various Sephadex types and a method for determining their Stokes radii.

EXPERIMENTAL

Materials

Even-numbered hyaluronate oligosaccharides, except N-acetylhyalobiuronic acid, were obtained from umbilical cord hyaluronic acid by limited digestion with testicular hyaluronidase (E.C. 3.2.1.35) and characterized as reported previously[3]. N-Acetylhyalobiuronic acid was prepared by treatment of hyaluronic acid with dimethyl sulphoxide containing 10% of 0.2 *M* hydrochloric acid for 16 h at 105°C according to the method of Inoue and Nagasawa[4].

A series of even-numbered hyaluronate oligomers from disaccharide (HA-2) to hexadecasaccharide (HA-16) having an N-acetylhyalobiuronosyl group as the repeating unit and commercial sugars (glucuronolactone, glucose, glucuronic acid, N-acetylglucosamine and cellobiose) were used. Bovine serum albumin (Wako Junyaku Kogyo), chymotrypsinogen and cytochrome *c* (Boehringer), ovalbumin (Sigma) and soybean trypsin inhibitor (Miles Labs.) were also employed.

Gel chromatography

Gel filtration was performed on Sephadex G-15, -25 (fine), -50 (superfine), -75, -100, -150 and -200 (Pharmacia) at room temperature, using 0.2 *M* sodium chloride solution as the eluent. Chromatographic columns (53–56 × 2 cm I.D. and 142–144 × 1.7 cm I.D.) were fitted with a sintered-glass filter disk at the bottom to facilitate the measurement of the total bed volume, V_t. Hyaluronate oligosaccharides and other sugars (about 0.5 mg of each) were applied in most instances as a mixture containing samples separable from each other on columns of various types of Sephadex. The samples were applied in 1 ml of 0.2 *M* sodium chloride solution. Blue Dextran 2000 (Pharmacia) of concentration 3 mg/ml was used to determine the void volume of the column, except for Sephadex G-15, for which hyaluronic acid (0.3 mg) was used. Column eluents were collected in 1.5- or 2-ml fractions, the exact volume of which were determined by measuring the combined volume of ten fractions.

Determination of samples in column eluents

Hyaluronate oligosaccharides, glucuronic acid and glucuronolactone were detected by the carbazole reaction[5]. Neutral sugars, except N-acetylglucosamine, which was measured according to the method of Reissig *et al.*[6], were analysed using the anthrone reaction[7]. Proteins and blue dextran were determined by absorbance measurements at 230 and 620 nm, respectively. The elution volume of sodium chloride on some Sephadex types was examined by adding the salt (150 mg) to the applied

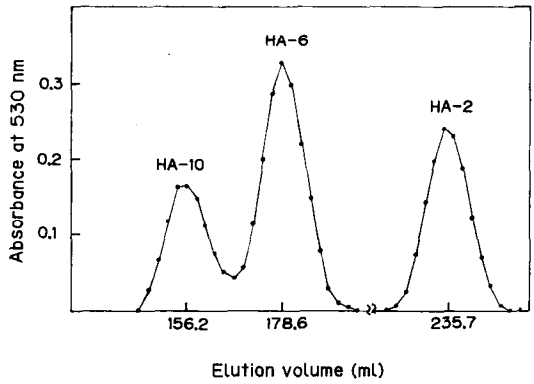

Fig. 1. Chromatography of hyaluronate oligosaccharides on Sephadex G-25. A mixture of HA-2, -6 and -10 was applied on a column (144 × 1.7 cm I.D.) of G-25 and eluted with 0.2 *M* sodium chloride solutes. Fractions of about 2 ml were analysed for uronic acid. The elution volume of Blue Dextran 2000 (V_0) was 141.5 ml.

volume and excess Cl^- concentration above the background of 0.2 *M* was titrated by the method of Fajans.

RESULTS AND DISCUSSION

Gel chromatography of hyaluronate oligosaccharides

The chromatogram of three hyaluronate oligosaccharides (HA-2, -6 and -10) on a long column (144 × 1.7 cm I.D.) of Sephadex G-25 is shown in Fig. 1, in which the elution volume required to elute half of the amount of a substance applied to the column was taken as V_e.

A mixture containing other oligosaccharide samples (HA-2, -4 and -8) was eluted through the same column in a separate experiment. Fig. 2 shows the chromatographic pattern of five hyaluronate oligosaccharides (HA-2, -4, -8, -12 and

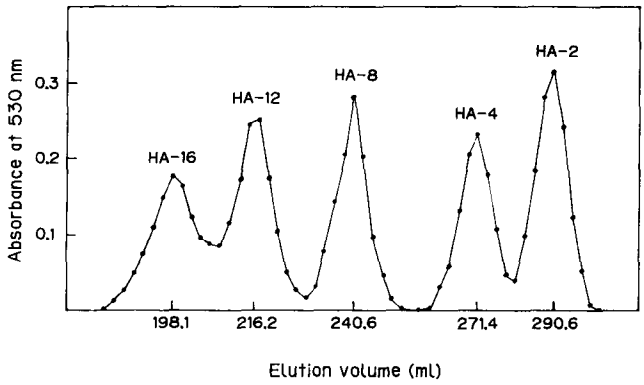

Fig. 2. Chromatography of hyaluronate oligosaccharides on Sephadex G-50. A mixture of HA-2, -4, -8, -12 and -16 was applied on a column (141.7 × 1.7 cm I.D.) of G-50. Assays were performed as in Fig. 1. V_0 was 125.6 ml.

TABLE I

K VALUES FOR HYALURONATE OLIGOSACCHARIDES ON THREE TYPES OF SEPHADEX

The figures in each row were obtained from separate experiments.

Oligosaccharide	*G-25*		*G-50*		*G-75*			
	1[a]	*2*[a]	*1*[a]	*2*[a]	*1*[a]	*2*[a]	*3*[a]	*4*[a]
HA-16			0.37		0.52			
HA-14				0.42		0.56		
HA-12			0.46				0.61	
HA-10	0.079			0.52	0.65			
HA-8		0.11	0.59					0.71
HA-6	0.22			0.66		0.75		
HA-4		0.31	0.74				0.83	
HA-2	0.51	0.49	0.84	0.84	0.89	0.89		0.90

[a] Experiment Nos.

-16) on a column (142 × 1.7 cm I.D.) of Sephadex G-50. The other samples (HA-6, -10 and -14) were co-chromatographed with HA-2 separately. Similar experiments were performed with a column (144 × 1.7 cm I.D., V_0 = 118.1 ml) of Sephadex G-75, in which HA-2 was usually co-chromatographed with other samples as a marker. The results are summarized in Table I in terms of K_{av}, which is hereafter denoted by K.

Relationships among log K for hyaluronate oligosaccharides and bovine serum albumin

Eqn. 2 suggests two important relationships between log K(A) and log K(B) as obtained from the gel filtration of substances A and B on the same gel. For a given Sephadex gel, L in eqn. 2 is constant and

$$\log K(\mathrm{B})/\log K(\mathrm{A}) = \{[R(\mathrm{B}) + r]/[R(\mathrm{A}) + r]\}^2 \quad (4)$$

where R(A) and R(B) are Stokes radii of A and B, respectively. If r is independent of the type of Sephadex gel, log K(B)/log K(A) will be constant for any Sephadex gel which does not exclude either of the two substances.

Next, we compare the K values for a substance A on two Sephadex gels (m and n). If R(A) and r in gel m are identical with those in gel n, the following relationship is obtained from eqn. 2:

$$\log K_n(\mathrm{A})/\log K_m(\mathrm{A}) = L_n/L_m \quad (5)$$

that is, the ratio log K_m(A)/log K_n(A) is determined by the dextran concentrations in gels m and n.

Various log K ratios of hyaluronate oligosaccharides were calculated from the data in Table I and are given in Table II. The log K/log K(HA-2) ratios for a given substance on the three Sephadex gels agree, within the limits of experimental error, but for a series of compounds run on a given gel the ratio increases with increasing chain length. Mean values ± S.D. for log K_{25}/log K_{50} and log K_{75}/log K_{50} were 3.9 ± 0.2 and 0.66 ± 0.02, respectively. The results indicate that eqn. 5 is reasonable.

TABLE II

RELATIONSHIPS AMONG LOG K VALUES FOR HYALURONATE OLIGOSACCHARIDES ON THREE TYPES OF SEPHADEX

Oligosaccharide	*log K/log K(HA-2)*			*log K_{25}/log K_{50}*	*log K_{75}/log K_{50}*
	G-25	*G-50*	*G-75*		
HA-16		5.70	5.61		0.66
HA-14		4.97	4.98		0.67
HA-12		4.45	4.37[a]		0.64
HA-10	3.77	3.75	3.70	3.9	0.66
HA-8	3.09	3.03	3.25	4.2	0.65
HA-6	2.25	2.38	2.47	3.6	0.69
HA-4	1.64	1.73	1.65[a]	3.9	0.62
HA-2	1.00	1.00	1.00	4.0[b]	0.65[b]

[a] The figures were based on the mean (0.893) of the three K(HA-2) values on Sephadex G-75 in Table I.

[b] The ratios (4.0 and 0.65) were calculated by use of the means (0.50 and 0.893, respectively) of K(HA-2) on Sephadex G-25 and -75 in Table I.

Andrews[8] reported on the relationships between elution volume and molecular weight for many proteins on Sephadex G-75 and -100. Each ratio of log K_{75} to log K_{100} on ten proteins was calculated from Andrews' data (Table 3 in his paper[8]), using estimated elution volumes of 74.1 and 96.7 ml for bovine serum albumin on Sephadex G-75 and -100 columns (50 × 2.4 cm I.D.), respectively. The average ratio (0.61 ± 0.03) also supports eqn. 5.

Gel filtration of one or two hyaluronate oligosaccharides together with HA-2 on a short column (53–55 × 2 cm I.D.) of Sephadex G-25 or -50 resulted in similar K values to those on a long column (Table I). Such a short column was convenient for examining the chromatographic behaviour of mixed solutions composed of a few species. By means of this short column, hexadecasaccharide (HA-16) and bovine serum albumin (BSA) were co-chromatographed on Sephadex G-75, -100, -150 and -200. A representative elution profile of the two samples on Sephadex G-150 is shown in Fig. 3. The relationships among log K values for both samples (Table III) also follow eqn. 5.

Determination of molecular radii of hyaluronate oligosaccharides and other sugars

Eqn. 4 can be rearranged to

$$R(\mathrm{B}) = R(\mathrm{A})\,[\log K(\mathrm{B})/\log K(\mathrm{A})]^{1/2} + r\{[\log K(\mathrm{B})/\log K(\mathrm{A})]^{1/2} - 1\} \qquad (6)$$

This equation implies that if R(A), log K(B)/log K(A) and r are known, R(B) can be calculated.

Laurent and Killander[2] assumed that the radius of dextran chains (r) did not depend on the concentration of the polysaccharide; therefore, they used same r value of 0.7 nm for different Sephadex types ranging from G-25 to -200. The validity of their assumption is supported by the result that the log K ratio between two appropriate

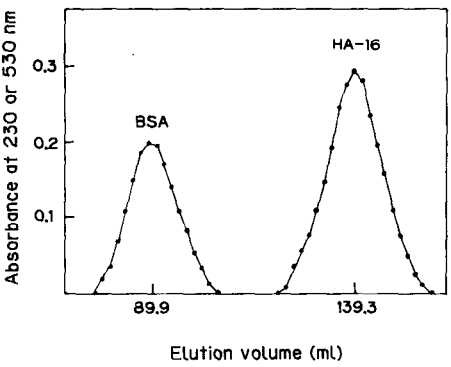

Fig. 3. Chromatography of hexadecasaccharide and bovine serum albumin on Sephadex G-150. A mixture of 1 mg of bovine serum albumin (BSA) and 0.5 mg of hyaluronate hexadecasaccharide (HA-16) was applied on a column (55 × 2 cm I.D.) of G-150. BSA was detected by measuring the absorbance at 230 nm. Assays to detect HA-16 were done as described before. V_0 was 54.9 ml.

samples (Tables II and III) is constant in spite of the use of different gels, as predicted in eqn. 4. Literature values of r, however, spread over a wide range of 0.2–0.7 nm, so the reinvestigation was required. For this purpose, the ratio (3.72) of log K(BSA) to log K(HA-16) shown in Table III and that (1.28) of log K(HA-2) to log K(cellobiose) in Table VI were utilized, because R(BSA) = 3.49 nm[2,9] and R(cellobiose) = 0.51 nm[2,10] are well documented. The following equations were used to calculate r:

$$(3.72)^{1/2} = \frac{R(\text{BSA}) + r}{R(\text{HA-16}) + r} = \frac{3.49 + r}{R(\text{HA-16}) + r} \tag{7}$$

$$(5.66)^{1/2} = \frac{R(\text{HA-16}) + r}{R(\text{HA-2}) + r} \tag{8}$$

$$(1.28)^{1/2} = \frac{R(\text{HA-2}) + r}{R(\text{cellobiose}) + r} = \frac{R(\text{HA-2}) + r}{0.51 + r} \tag{9}$$

TABLE III

LOG K RATIOS FOR HEXADECASACCHARIDE (HA-16) AND BOVINE SERUM ALBUMIN (BSA)

Sephadex	*K*		*log K(HA-16)/ log K_{75}(HA-16)*	*log K(BSA)/ log K_{75}(BSA)*	*log K(BSA)/ log K(HA-16)*
	HA-16	*BSA*			
G-75	0.52	0.087	1.00	1.00	3.73
G-100	0.61	0.17	0.76	0.73	3.58
G-150	0.72	0.30	0.50	0.49	3.67
G-200	0.79	0.40	0.36	0.38	3.89
				Mean ± S.D.	3.72 ± 0.11

TABLE IV

STOKES RADII OF HYALURONATE OLIGOSACCHARIDES

Oligosaccharide	*Stokes radius (nm)*	*Oligosaccharide*	*Stokes radius (nm)*
HA-16	1.70	HA-8	1.21
HA-14	1.59	HA-6	1.03
HA-12	1.48	HA-4	0.83
HA-10	1.35	HA-2	0.60

A value of r to satisfy the above three equations was 0.20 nm, and agreed with that given by Siegel and Monty[11], who postulated dextran as a straight chain and employed this value for all of the Sephadex gels (G-75, -100 and -200) used. With a combination of r = 0.7 nm and R(BSA) = 3.49 nm, the Stokes radius of cellobiose is 0.11 nm from eqn. 7. This value is unacceptable and hence the r value of 0.7 nm could not fit our data. R(HA-2) from eqn. 9 was 0.60 nm on the basis of r = 0.20 nm. By use of these r and R(HA-2) values, and each average ratio [log K/log K(HA-2)] in Table II, molecular radii of the other hyaluronate oligosaccharides were calculated according to eqn. 6. The results are summarized in Table IV. As can be seen, the difference in radius per disaccharide unit tended to decrease slightly with increasing length of the sugar chain. This observation suggests that the segments of oligomer orient more randomly with increase in molecular weight. We noted from Table II a linear relationship between log K/log K(HA-2) and the number of hexose units. The chromatographic data for hyaluronate oligosaccharides reflect their conformations in solution.

Application of the method to some proteins and monosaccharides

Ovalbumin, chymotrypsinogen, trypsin inhibitor and cytochrome c (1 mg of each) were separately chromatographed together with BSA (1 mg) on a column (55 × 2 cm I.D.) of Sephadex G-100. Their Stokes radii, based on R(BSA) = 3.49 and r = 0.20 nm, listed in Table V were in good agreement with literature values.

Although some deviations were found with ovalbumin and cytochrome c, their co-chromatography gave 1.70 nm for the radius of cytochrome c based on R(ovalbumin) = 2.73 nm.

The elution volumes of some monosaccharides involving glucuronic acid (GlcUA) and N-acetylglucosamine (GlcNAc), which are component sugars of hyaluronic acid, were investigated on Sephadex G-15, -25 and -50. Their K values are summarized in Table VI. The value for HA-2 (0.80) on G-50 was significantly lower

TABLE V

STOKES RADII OF PROTEINS

Protein	*log K/log K(BSA)*	*Stokes radius (nm)*[a]
Ovalbumin	0.705	2.90 (2.73)
Trypsin inhibitor	0.451	2.28 (2.26)
Chymotrypsinogen	0.443	2.26 (2.24)
Cytochrome c	0.304	1.83 (1.64)

[a] The Stokes radii given by Laurent and Killander[2] (Table I in their paper) are shown in parentheses.

TABLE VI

STOKES RADII OF MONOSACCHARIDES

Chromatographic data for HA-2, cellobiose (C) and sodium chloride are also shown for comparison. The subscripts a and b indicate different lots of Sephadex G-15. The column sizes for lot a, lot b and G-25 was 55–57 × 2 cm I.D. and that for G-50 was 100 × 2 cm I.D.

Sample	*G-15*				*G-25*			*G-50*		
	K_a	K_b	$log\ K_a/log\ K_a(C)$	$log\ K_b/log\ K_b(C)$	K	$log\ K/log\ K(C)$	$R\ (nm)$	K	$log\ K/log\ K(C)$	$R\ (nm)$
HA-2	0.14	0.22	1.34	1.40	0.51	1.28	0.60	0.80	1.28	0.60
Cellobiose	0.23	0.34	1.00	1.00	0.59	1.00	0.51	0.84	1.00	0.51
GlcUA	0.20	0.28	1.10	1.18	0.60	0.97	0.50	0.85	0.93	0.48
GlcNAc	0.28	0.40	0.87	0.85	0.61	0.94	0.49	0.84	1.00	0.51
Glucose	0.32	0.43	0.78	0.78	0.66	0.79	0.43	0.87	0.80	0.44
Glucuronolactone	0.55	0.65	0.41	0.40	0.77	0.50	0.30	0.91	0.54	0.32
Sodium chloride	0.45	0.56	0.54	0.54	0.76	0.52	0.31	0.91	0.54	0.32

than that (0.84) in Table I. This may be due to the fact that the gel employed in the former experiment was a mixture of two lots. Although the available volumes of sample on lots a and b of G-15 differed from each other in spite of the similar column size, the log K ratio based on cellobiose agreed well; the mean $\pm$ S.D. for log K_b/log K_a of same sugar was 0.74 $\pm$ 0.02. Hence eqn. 5 seems to be satisfied even among different lots of the same Sephadex type.

Faster elution of GlcUA on G-15 compared with cellobiose could be due to repulsion between the negatively charged monosaccharide and the gel matrix. A similar effect was observed in HA-2 having a GlcUA residue. By comparing the available volumes of glucuronolactone and sodium chloride, it was found that the sugar was reversibly adsorbed on the gel surface of G-15. Such a phenomenon was also noted for GlcNAc. The repulsion and adsorption effects were also found in the chromatograms on G-25, in which GlcUA was eluted slightly faster than GlcNAc. Molecular radii of monosaccharides (Table VI) were calculated from eqn. 6 by using log K(cellobiose), R(cellobiose) = 0.51 and r = 0.20 nm. GlcNAc was larger than glucose owing to its bulky substituent and their co-chromatography on G-50 demonstrated a faster elution of GlcNAc. This monosaccharide and cellobiose were found to have similar sizes under the present conditions. Glucuronolactone, with a compact structure, and sodium chloride showed similar mobilities on G-25 and -50. The calculated radius of the salt (0.3 nm) appears to correspond to the sum of their ionic radii (Na^+ and Cl^-). Squire[12] proposed an equation correlating elution volume with molecular weight and calculated the molecular weight of sodium chloride from its chromatographic data on Sephadex G-75. Although the calculated molecular weight showed a negative deviation of 17%, his and our data suggest that gel chromatography on Sephadex can also give information on the molecular weight and molecular size of a small molecule such as sodium chloride.

Sephadex gel chromatography has provided a powerful tool for the determination of the Stokes radius of a substance that does not interact with the gel grains and many investigators[2,12–14] have derived various equations to relate chromatographic data to molecular properties. We have chosen here an equation (eqn. 2 in this paper) given by Ogston[1], because it was useful for explaining our data. In the present method for determining the molecular size of a substance, its co-chromatography with a standard of known size, such as cellobiose or bovine serum albumin, is recommended. The use of the log K ratio was important in eliminating a complicated factor, L, included in eqn. 2 and therefore obtaining a reliable estimate of the radius. This method has the advantage that it needs only, one appropriate standard, but it should be taken into consideration that our calculation is valid when log K ratios of two specified samples in gels of different pore size and of the same material are constant.

REFERENCES

1 A. G. Ogston, *Trans. Faraday Soc.*, 54 (1958) 1754.
2 T. C. Laurent and J. Killander, *J. Chromatogr.*, 14 (1964) 317.
3 E. Shimada and G. Matsumura, *J. Biochem. (Tokyo)*, 88 (1980) 1015.
4 Y. Inoue and K. Nagasawa, *Carbohydr. Res.*, 141 (1985) 99.
5 J. Bitter and H. Muir, *Anal. Biochem.*, 4 (1962) 330.
6 J. L. Reissig, J. L. Strominger and L. F. Leloir, *J. Biol. Chem.*, 217 (1955) 959.
7 W. E. Trevelyan and J. S. Harrison, *Biochem. J.*, 50 (1952) 298.

8 P. Andrews, *Biochem. J.*, 91 (1964) 222.
9 J. M. Creeth, *Biochemistry*, 51 (1952) 10.
10 D. A. Rees and R. J. Skerrett, *Carbohydr. Res.*, 7 (1968) 334.
11 L. M. Siegel and K. J. Monty, *Biochim. Biophys. Acta*, 112 (1966) 346.
12 P. G. Squire, *Arch. Biochem. Biophys.*, 107 (1964) 471.
13 J. Porath, *Pure Appl. Chem.*, 6 (1963) 233.
14 G. K. Ackers, *Biochemistry*, 3 (1964) 723.