Journal of Chromatography, 328 (1985) 73–80
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17 654

COMPARISON OF RELATIONSHIPS BETWEEN THE CHEMICAL STRUCTURES AND MOBILITIES OF HYALURONATE OLIGOSACCHARIDES IN THIN-LAYER AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

EIJI SHIMADA* and GO MATSUMURA
School of Pharmaceutical Sciences, Showa University, 1-5-8, Hatanodai, Shinagawa-ku, Tokyo, 142 (Japan)
(Received February 13th, 1985)

SUMMARY

The R_M values of odd- and even-numbered hyaluronate oligosaccharides, comprised of N-acetylglucosamine and glucuronic acid residues, were determined by thin-layer chromatography (TLC). Previous retention time data of the acidic oligosaccharides obtained by high-performance liquid chromatography (HPLC) were converted into R_M values. By dividing the oligosaccharide structures into several fragments, the contributions of these fragments to chromatographic mobility (group constants) were estimated essentially from the difference between the R_M values of two oligomers having appropriate structures. The group constants of the bridging oxygen atoms at the β-1,4- and -1,3-glycosidic linkages of these oligomers were identical in HPLC but not in TLC. In the two types of chromatography, the mobility of a given hyaluronate oligosaccharide could be explained by a linear combination of group constants and the R_M value of N-acetylglucosamine or glucuronic acid, with the exception that the R_M value of the uronic acid in TLC was anomalous.

INTRODUCTION

In a previous paper[1] on the separation of hyaluronate oligosaccharides by thin-layer chromatography (TLC) we indicated that four oligosaccharide series [$(GlcUA–GlcNAc)_{1,2,3,4}$, $(GlcNAc–GlcUA)_{1,2,3}$, $GlcUA–(GlcNAc–GlcUA)_{1,2,3}$ and $GlcNAc–(GlcUA–GlcNAc)_{1,2}$] gave parallel plots of R_M values[2], $\log[(1/R_F)-1]$, against the number of hexose units. The results suggest that these oligomers migrate according to Martin's theoretical postulates[3,4] of the relationships between chemical structure and mobility in partition chromatography. There have been many reports on the effects of substituents on the paper or thin-layer chromatographic behaviour of aliphatic and aromatic compounds such as aliphatic acids[5], phenols[6], pyridine derivatives[7], etc., but few studies (*e.g.*, ref. 8) on the contribution of each constituent monosaccharide in homologous oligosaccharide series to chromatographic mobility. Thus we have investigated the contributions of N-acetylglucosamine (GlcNAc) and

0021-9673/85/$03.30

glucuronic acid (GlcUA) components to the mobilities of hyaluronate oligosaccharides on TLC by considering the difference in R_M value (ΔR_M) due to one monosaccharide increment and found a regular change in ΔR_M characteristic of the attachment of GlcNAc or GlcUA.

Nebinger *et al.*[9] also reported on the separation of acidic oligosaccharides by high-performance liquid chromatography (HPLC). This method, however, could not distinguish some oligomers, while TLC was ineffective to resolve other ones. Comparison of ΔR_M parameters obtained in the two types of chromatography has revealed that these observations were closely related to the behaviour of the bridging oxygen atoms at the β-1,4- and -1,3-glycoside bonds of the oligomers.

In this paper, we compare the relationships among the ΔR_M found for the hyaluronate oligosaccharides by TLC with those derived from HPLC data by Nebinger *et al.*

EXPERIMENTAL

TLC of hyaluronate oligosaccharides

TLC was done on a plate (10 × 5 cm) of Kieselgel 60 (plastic plate, thickness 0.2 mm, Merck). Hyaluronate oligosaccharides (about 5 μg of each) were separated with isopropanol–water (66:34) containing 0.05 *M* sodium chloride at room temperature. The R_F values were calculated by location of the highest density in the spots detected by the sulphuric acid charring technique[1].

TABLE I

STRUCTURES AND R_F VALUES OF HYALURONATE OLIGOSACCHARIDES

The data are based on a representative chromatogram at 20°C. N-Acetylglucosamine and glucuronic acid, $(GlcUA–GlcNAc)_{1,2,3,4}$, $GlcUA–(GlcNAc–GlcUA)_{1,2,3}$, $(GlcNAc–GlcUA)_{1,2,3}$ and $GlcNAc–(GlcUA–GlcNAc)_{1,2}$ were applied as a mixture.

Samples	*Abbreviations*	R_F
Oligosaccharides		
GlcUA–GlcNAc	HA-2	0.59
$(GlcUA–GlcNAc)_2$	HA-4	0.48
$(GlcUA–GlcNAc)_3$	HA-6	0.37
$(GlcUA–GlcNAc)_4$	HA-8	0.26
GlcUA–GlcNAc–GlcUA	HA-3	0.39
$GlcUA–(GlcNAc–GlcUA)_2$	HA-5	0.29
$GlcUA–(GlcNAc–GlcUA)_3$	HA-7	0.20
GlcNAc–GlcUA	HA-2′	0.52
$(GlcNAc–GlcUA)_2$	HA-4′	0.41
$(GlcNAc–GlcUA)_3$	HA-6′	0.31
GlcNAc–GlcUA–GlcNAc	HA-3′	0.59
$GlcNAc–(GlcUA–GlcNAc)_2$	HA-5′	0.49
Monosaccharides		
GlcNAc		0.70
GlcUA		0.45

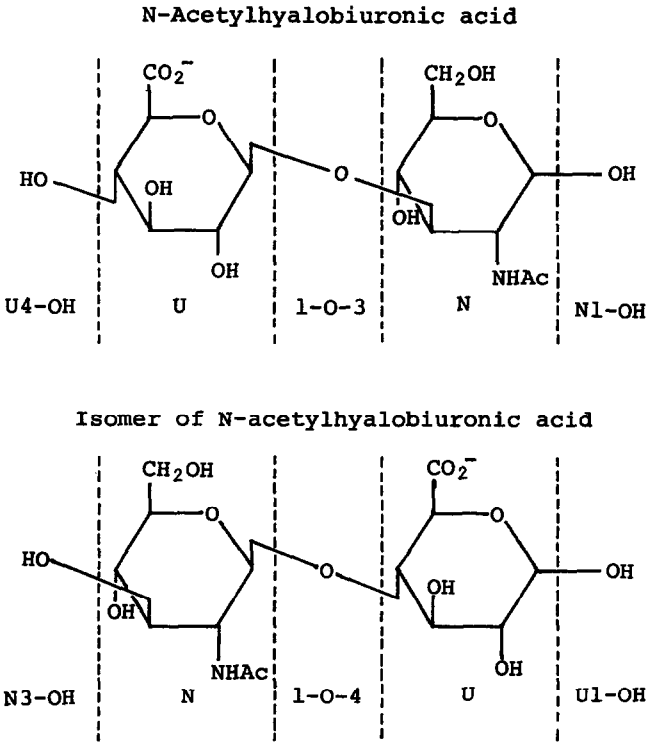

Fig. 1. Structural subdivision of hyaluronate disaccharides for the calculation of group constants.

The preparation and characterization of the twelve hyaluronate oligosaccharides used in this experiment have been described in previous papers[1,10]; their structures are presented in Table I together with the R_F values.

Contributions of oligosaccharide components to mobility in TLC

The partition theory of Martin and co-workers[3,4] and the R_M concept introduced by Bate-Smith and Estall[2] can be employed to derive an equation[5,8,11] for the chromatographic mobility of a substance having various numbers (m, n, etc.) of identical substituent groups (X, Y, etc.)

$$R_M = C + P + mX + nY + \ldots.$$

where C is a constant depending on the experimental conditions, P is a constant for the parent molecule and X, Y, etc., are constants characteristic of the structures of substituent groups.

When the structures of N-acetylhyalobiuronic acid (HA-2) and its isomer (HA-2') are conveniently divided into five fragments as illustrated in Fig. 1, the structures of the various hyaluronate oligosaccharides used can be constructed from the fragments U4-OH, N3-OH, 1-O-3, 1-O-4, U, N, U1-OH and N1-OH. On the basis of Martin's predictions, the R_M values for these oligomers can be expressed by linear combinations of the constant C and several other constants, *i.e., U4-OH, N3-OH,*

1-O-3, 1-O-4, U, N, U1-OH or *N1-OH*; the latter constants are herein called group constants. For instance, the R_M values for HA-2, -2', -3, -3' and -4 are given by:

$$R_M(\text{HA-2}) = C + (U4\text{-}OH) + (U) + (1\text{-}O\text{-}3) + (N) + (N1\text{-}OH) \quad (1)$$

$$R_M(\text{HA-2'}) = C + (N3\text{-}OH) + (N) + (1\text{-}O\text{-}4) + (U) + (U1\text{-}OH) \quad (2)$$

$$R_M(\text{HA-3}) = C + (U4\text{-}OH) + (1\text{-}O\text{-}3) + (1\text{-}O\text{-}4) + 2(U) + (N) + (U1\text{-}OH) \quad (3)$$

$$R_M(\text{HA-3'}) = C + (N3\text{-}OH) + (1\text{-}O\text{-}3) + (1\text{-}O\text{-}4) + (U) + 2(N) + (N1\text{-}OH) \quad (4)$$

$$R_M(\text{HA-4}) = C + (U4\text{-}OH) + 2(1\text{-}O\text{-}3) + (1\text{-}O\text{-}4) + 2(U) + 2(N) + (N1\text{-}OH) \quad (5)$$

Eqns. 1 and 5 can be used to estimate the sum of the four group constants:

$$R_M(\text{HA-4}) - R_M(\text{HA-2}) = (1\text{-}O\text{-}4) + (U) + (1\text{-}O\text{-}3) + (N) = D \quad (6)$$

If the contributions of the free anomeric hydroxyl groups, *(U1-OH)* and *(N1-OH)*, can be assumed equal (assumption I), the following relationships can be derived from the R_M values for HA-2, -3 and -4, which have glucuronic acid at their non-reducing ends:

$$R_M(\text{HA-3}) - R_M(\text{HA-2}) = (1\text{-}O\text{-}4) + (U) = E \quad (7)$$

$$R_M(\text{HA-4}) - R_M(\text{HA-3}) = (1\text{-}O\text{-}3) + (N) = F \quad (8)$$

If the contributions of the other two hydroxyl groups, *(U4-OH)* and *(N3-OH)*, can be assumed equal (assumption II), the sum of *(1-O-3)* and *(U)*, and that of *(1-O-4)* and *(N)* can be obtained from eqns. 2 and 3, and from eqns. 1 and 4, respectively:

$$R_M(\text{HA-3}) - R_M(\text{HA-2'}) = (1\text{-}O\text{-}3) + (U) = G \quad (9)$$

$$R_M(\text{HA-3'}) - R_M(\text{HA-2}) = (1\text{-}O\text{-}4) + (N) = H \quad (10)$$

The quantities *D–H* can also be obtained from the following subtractions:

$$R_M(\text{HA-5,-6,-7,-8,-4',-5',-6'}) - R_M(\text{HA-3,-4,-5,-6,-2',-3',-4'}) = D \quad (6')$$

$$R_M(\text{HA-5,-7,-4',-6'}) - R_M(\text{HA-4,-6,-3',-5'}) = E \quad (7')$$

$$R_M(\text{HA-6,-8,-3',-5'}) - R_M(\text{HA-5,-7,-2',-4'}) = F \quad (8')$$

$$R_M(\text{HA-4,-5,-6,-7}) - R_M(\text{HA-3',-4',-5',-6'}) = G \quad (9')$$

$$R_M(\text{HA-4',-5',-6'}) - R_M(\text{HA-3,-4,-5}) = H \quad (10')$$

Contributions of oligosaccharide components to retention time in HPLC

Nebinger *et al.*[9] separated odd- and even-numbered hyaluronate oligosaccharides by HPLC, using an amino-modified silica gel column and a mobile phase of 0.1 *M* potassium dihydrogen phosphate, pH 4.75. Since the ratio of retention

times, t_0/t_R, in HPLC corresponds to R_F in TLC, their data can be compared with ours. Thus we calculated log $[(t_R/t_0)-1]$ by use of an estimated t_0 (3.5 min) and the t_R data (Table I in their paper), and obtained several group constants for the acidic oligomers in HPLC as described before. They employed thirteen oligosaccharides lacking HA-2′ but containing HA-7′ [GlcNAc–(GlcUA–GlcNAc)$_3$] and -8′ [(GlcNAc–GlcUA)$_4$] compared with our samples. Thus no subtractions involving R_M(HA-2′) were done, but instead those involving R_M(HA-7′) or R_M(HA-8′), *i.e.*, R_M(HA-8)$-R_M$(HA-7′) and R_M(HA-7′,-8′)$-R_M$(HA-6,-7) were carried out so as to calculate G and H.

Calculation of R_M values for N-acetylglucosamine and glucuronic acid

In order to correlate the chromatographic behaviour of hyaluronate oligosaccharides with that of GlcUA or GlcNAc, R_M values of these two monosaccharides were deduced from the R_M values of the oligomers and then compared with observed values. The R_M values for the two monosaccharides may be expressed as:

$$R_M(\text{GlcUA}) = C + (U4\text{-}OH) + (U) + (U1\text{-}OH) \quad (11)$$

$$R_M(\text{GlcNAc}) = C + (N3\text{-}OH) + (N) + (N1\text{-}OH) \quad (12)$$

Because of the validity of assumptions I and II (see Results and discussion), we can calculate R_M(GlcUA) and R_M(GlcNAc) from each R_M value for the oligosaccharides. For example:

$$\begin{aligned} R_M(\text{HA-8}) &= C + (U4\text{-}OH) + 4\,(1\text{-}O\text{-}3) + 3\,(1\text{-}O\text{-}4) + 4\,(U) + \\ &\quad 4\,(N) + (N1\text{-}OH) \\ &= C + (U4\text{-}OH) + (U) + (U1\text{-}OH) + 3D + F \quad (13) \\ &= C + (N3\text{-}OH) + (N) + (N1\text{-}OH) + 3D + G \quad (14) \end{aligned}$$

According to eqn. 11–14, R_M(GlcUA) = R_M(HA-8)$-3D-F$ and R_M(GlcNAc) = R_M(HA-8)$-3D-G$. Similarly, the R_M values for GlcUA and GlcNAc were also calculated from each experimental value for the other oligosaccharides by using D–H.

RESULTS AND DISCUSSION

Relationships among group constants of hyaluronate oligosaccharides

R_M values derived from the TLC data in Table I and from log $[(t_R/t_0)-1]$ obtained by HPLC by Nebinger *et al.*[9], respectively, were plotted against the number of hexose units (Fig. 2A and B). As t_0 was not determined by the earlier authors, it was estimated on the hypothesis that the parallel plots found for each oligosaccharide series in TLC (Fig. 2A) should also be observed in HPLC. We noted from Fig. 2 that N-acetylglucosamine or glucuronic acid lies on the line for GlcNAc–(GlcUA-GlcNAc)$_n$ or GlcUA–(GlcNAc–GlcUA)$_n$, respectively. The only exception is glucuronic acid in Fig. 2A.

The structures of the hyaluronate oligosaccharides were subdivided as shown in Fig. 1 and the relationships among the group constants were calculated as described in Experimental. The results are summarized in Table II. The values of the

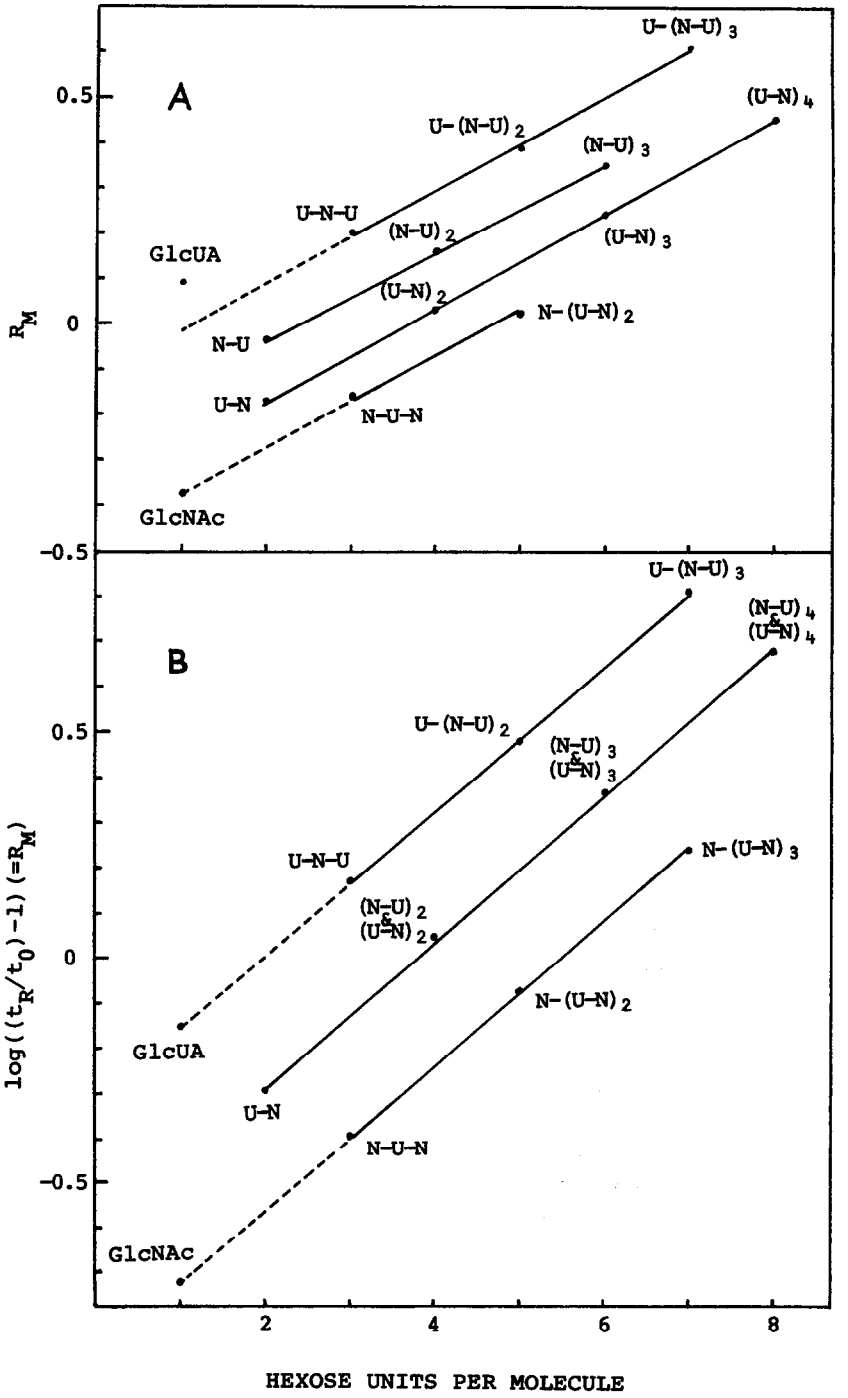

Fig. 2. Relationships between R_M and molecular size in TLC (A) and HPLC (B). U = Glucuronic acid; N = N-acetylglucosamine.

TABLE II

RELATIONSHIPS AMONG SOME GROUP CONSTANTS FOR HYALURONATE OLIGOSACCHARIDES IN TLC AND HPLC

The figures under no assumption, assumption I and assumption II are mean ± S.D. The relationship between *(1-O-4)* and *(1-O-3)* was calculated from each mean value for *E–H*. The relationship between *(U)* and *(N)* was also obtained by using these mean values.

Sum of group constants	*TLC*	*HPLC*
No assumption		
(1-O-4) + (U) + (1-O-3) + (N) = D	0.20 ± 0.01	0.32 ± 0.01
Assumption I [(N1-OH) = (U1-OH)]		
(1-O-4) + (U) = E	0.35 ± 0.02	0.44 ± 0.01
(1-O-3) + (N) = F	−0.15 ± 0.01	−0.12 ± 0.01
Assumption II [(N3-OH) = (U4-OH)]		
(1-O-3) + (U) = G	0.23 ± 0.02	0.44 ± 0.00
(1-O-4) + (N) = H	−0.02 ± 0.02	−0.12 ± 0.01
Assumptions I and II		
	(1-O-4) = (1-O-3) + 0.13	*(1-O-4) = (1-O-3)*
	(U) = (N) + 0.38	*(U) = (N)* + 0.56

quantity *D* [*(1-O-3)* , *(1-O-4)*, *(U)* and *(N)*], from TLC and HPLC, obtained by combination of contributions from the various kinds of hyaluronate oligosaccharides, were in good agreement. This will permit the estimation of an oligomer's R_M value by use of group constants. The quantities *E* and *F* determined using assumption I, and *G* and *H* using assumption II, also showed constancy and the data on *E* + *F* (*G* + *H*) were consistent with *D*. This indicates the validity of our assumptions, under which the relative values of *(1-O-3)* and *(1-O-4)*, and of *(U)* and *(N)*, can be found.

The relative magnitude of the group constants obtained under assumptions I and II can be regarded as a measure of their contributions to the mobilities of the parent oligosaccharides. As seen from the relationship between *(U)* and *(N)* in both kinds of chromatography (Table II), the higher value of *(U)* compared with *(N)* means that the *U* fragment has the lower mobility. The equality of the contributions from 1-O-3 and 1-O-4 in HPLC is reasonable, because the separation of hyaluronate oligosaccharides by this method is mainly attributed to ion exchange. Furthermore, the relationship mentioned above can elucidate the co-migration of two even-numbered samples of $(GlcUA–GlcNAc)_n$ and $(GlcNAc–GlcUA)_n$ with the same molecular size in HPLC (Fig. 2B). Contrary to HPLC, the TLC results indicated that the contribution from 1-O-4 was not identical to that of 1-O-3. By paper chromatography of dextran and amylose oligomers, French and Wild[8] also found different contributions from the oxygen atoms at the α-1,6- and -1,4-glycosidic linkages.

Calculated and observed R_M values for N-acetylglucosamine and glucuronic acid

Mean values (±S.D.) for R_M(GlcUA) and R_M(GlcNAc) calculated as described in Experimental were compared with the observed values. In the case of TLC, the calculated value of R_M(GlcNAc) −0.38 ± 0.01, was in good agreement with the

observed one (−0.37), whereas the calculated value of R_M(GlcUA), −0.01 ± 0.01, was significantly different from the experimental one (0.09), which reflects the deviation of glucuronic acid in Fig. 2A. This discrepancy remains to be elucidated. In the case of HPLC, both the calculated values, R_M(GlcNAc) = −0.71 ± 0.01 and R_M(GlcUA) = −0.15 ± 0.01, were in good agreement with the observed values (−0.72 and −0.15, respectively). From these results, the mobility of a given hyaluronate oligosaccharide in TLC or HPLC may be expressed in terms of the additive contributions of the constituent groups on the basis of the mobility of N-acetylglucosamine or glucuronic acid, with the exception of the latter monosaccharide in TLC.

REFERENCES

1 E. Shimada and G. Matsumura, *J. Biochem. (Tokyo)*, 96 (1984) 721.
2 E. C. Bate-Smith and R. G. Westall, *Biochim. Biophys. Acta*, 4 (1950) 427.
3 R. Consden, A. H. Gordon and A. J. P. Martin, *Biochem. J.*, 38 (1944) 224.
4 A. J. P. Martin, *Biochem. Soc. Symp.*, 3 (1949) 4.
5 F. Gütlbauer, *J. Chromatogr.*, 45 (1969) 104.
6 S. Marcinkiewicz, J. Green and D. Mchale, *J. Chromatogr.*, 10 (1963) 42.
7 E. Soczewiński and M. Bieganowska, *J. Chromatogr.*, 40 (1969) 431.
8 D. French and G. M. Wild, *J. Amer. Chem. Soc.*, 75 (1953) 2612.
9 P. Nebinger, M. Koel, A. Franz and E. Werries, *J. Chromatogr.*, 265 (1983) 19.
10 E. Shimada and G. Matsumura, *J. Biochem. (Tokyo)*, 88 (1980) 1015.
11 J. Green and S. Marcinkiewicz, *J. Chromatogr.*, 10 (1963) 35.