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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF EVEN- AND ODD-NUMBERED HYALURONATE OLIGOSACCHARIDES*

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SUMMARY

Even-numbered oligosaccharides derived from hyaluronate containing glucuronic acid or N-acetylglucosamine in a non-reducing position, as well as the corresponding odd-numbered oligosaccharides with N-acetylglucosamine or glucuronic acid at the non-reducing terminus, were separated by high-performance liquid chromatography and identified at 206 nm. Using an amino-modified silica gel column and 0.1 *M* KH_2PO_4 (pH 4.75) as the mobile phase, complete separation up to the octasaccharides was performed within 21 min. The effects of using various concentrations of acetonitrile in the eluent and of using various solvent pH values on the separation and retention data of the oligosaccharides were studied in detail.

INTRODUCTION

Degradation of hyaluronic acid is accomplished by the action of endoglycanohydrolases, such as bovine hyaluronidase (E.C. 3.2.1.35) or leech hyaluronidase (E.C. 3.2.1.36), which yield a homologous series of even-numbered oligosaccharides[1–3]. While bovine hyaluronidase cleaves β-1,4-N-acetylglucosaminidic bonds in hyaluronic acid resulting in the formation of even-numbered oligosaccharides with a terminal non-reducing glucuronic acid, the corresponding enzyme from leech produces even-numbered oligosaccharides with N-acetylglucosamine at the non-reducing terminus. These oligosaccharides are further degraded by sequential removal of the non-reducing monosaccharides by β-glucuronidase and β-N-acetylglucosaminidase, respectively[4–6].

In the course of our investigations on the biological functions and substrate specificities of glycosidases secreted by the parasite *Entamoeba histolytica* towards hyaluronate oligosaccharides, we became interested in methods permitting rapid and simultaneous estimation of these oligosaccharides, as well as of glucuronic acid and N-acetylglucosamine. Methods used so far for the estimation of oligosaccharides derived from hyaluronic acid involve time-consuming chromatographic procedures

* Dedicated to Eckhart Buddecke on the occasion of his 60th birthday.

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such as paper chromatography, gel filtration, ion-exchange chromatography and electrophoresis, followed by colorimetric reactions[7–10]. These methods all require relative large amounts of material. Recently, high-performance liquid chromatography (HPLC) was employed in separating even-numbered oligosaccharides obtained from hyaluronidase digests of hyaluronate, using a gel permeation technique[11]. However, odd-numbered oligosaccharides enzymatically formed from the even-numbered species had not so far been analysed by HPLC. Furthermore, for investigations concerning connective tissue metabolites it is necessary to carry out determinations of even- and odd-numbered oligosaccharides as well as of glucuronic acid and N-acetylglucosamine in the same sample.

This paper presents a new method for the analysis of small amounts of mixtures containing monosaccharides and oligosaccharides (up to octasaccharides) derived from hyaluronic acid. The technique permits the rapid checking of the purity of fractions obtained during large-scale chromatography of oligosaccharides. It can also be used for rapid activity assay of β-glucuronidase, β-N-acetylglucosaminidase and hyaluronidase using as substrates hyaluronate oligosaccharides and hyaluronic acid, respectively.

EXPERIMENTAL

All chemicals used were of analytical-reagent grade, with the exception of acetonitrile which was of HPLC specification.

Preparation of oligosaccharides

Even-numbered oligosaccharides with a terminal non-reducing glucuronic acid were prepared from hyaluronic acid of human umbilical cord by degradation with bovine testicular hyaluronidase (Serva, Heidelberg, G.F.R.) as reported previously[7]. Hepta-, penta- and trisaccharides with terminal non-reducing N-acetylglucosamine were obtained from the corresponding even-numbered oligosaccharides by removal of the non-reducing glucuronic acid moieties with β-glucuronidase (from *Patella barbara*; Roth, Karlsruhe, G.F.R.) that was devoid of β-N-acetylglucosaminidase. The reaction products were separated by chromatography on DEAE-Sephacel (Pharmacia, Freiburg, G.F.R.).

Even-numbered oligosaccharides with N-acetylglucosamine at the non-reducing terminus were separated from a leech hyaluronidase digest of hyaluronate by chromatography on Dowex 1X-8[2]. Odd-numbered oligosaccharides with a terminal non-reducing glucuronic acid were obtained from the even-numbered leech tetra-, hexa- and octasaccharide by digestion with β-N-acetylglucosaminidase A from bovine spleen[4]. The degradation products were isolated and separated on a preparative scale by chromatography on Dowex 1-X8 (formate form). Details of oligosaccharide preparation will be described elsewhere.

The purity and homogeneity of each oligosaccharide were controlled by chemical and enzymatic analysis, by paper chromatography and by following the ratio of total uronic acid to N-acetylglucosamine[12–15].

HPLC

Analyses were performed with a Beckman 110 A liquid chromatograph pump

equipped with an Altex injection valve and a 5-μl loop (Beckman, München, G.F.R.). Detection was carried out at 206 nm with a LKB Uvicord S detector (LKB, Gräfelfing, G.F.R.). The heights and areas of peaks as well as the retention times were measured with a Shimadzu C R 1 A chromatograph integrator (Beckman, München, G.F.R.).

Samples (2.5 nmol) were chromatographed at room temperature on a 40 × 4.6 mm Lichrosorb-NH_2 precolumn (Dr. Knauer, Bad Homburg, G.F.R.) and a 250 × 4.6 mm Ultrasil-NH_2 column (Beckman Instruments, München, G.F.R.). Elution was performed with different eluents consisting of acetonitrile and KH_2PO_4 in different proportions. The aqueous solutions were adjusted to the pH values mentioned in Table III. The flow-rate was 1 ml/min.

RESULTS AND DISCUSSION

Since even-numbered hyaluronate oligosaccharides of different chain length are separated by conventional ion-exchange chromatography[1,16,17], it should be expected that these molecules can be identified by HPLC using an ion-exchange column. Fig. 1 shows an isocratic HPLC chromatogram of a mixture of 17 μg of even- and odd-numbered oligosaccharides (2.5 nmol of each glycoside) obtained from hyaluronate by digestion with testes hyaluronidase and subsequent action of β-glucuronidase. Use of an Ultrasil-NH_2 column with 0.1 *M* KH_2PO_4 (pH 4.75) as eluent gave complete separation of all oligosaccharides. The elution diagram (Fig. 1) reveals that di-, tetra-, hexa- and octasaccharides with a terminal non-reducing glucuronic acid

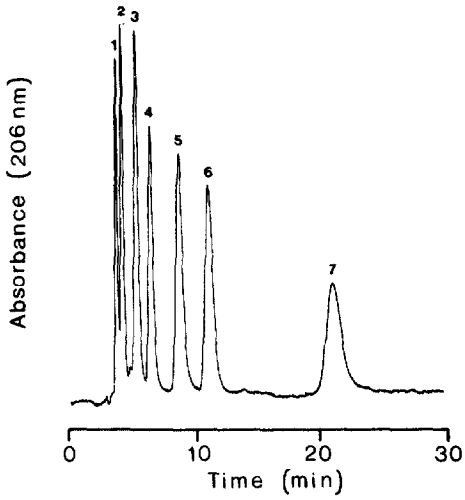

Fig. 1. Separation of a mixture of even-numbered oligosaccharides with glucuronic acid in non-reducing position and odd-numbered oligosaccharides with N-acetylglucosamine at the non-reducing terminus derived from hyaluronate. A 5-μl sample of a mixture containing 2.5 nmol of each oligosaccharide was applied to an Ultrasil-NH_2 column (250 × 4.6 mm) and eluted with 0.1 *M* KH_2PO_4 (pH 4.75) at a flow-rate of 1 ml/min. The separated products were detected with an UV detector at 206 nm. Peaks: 1 = βGlcNAc1–4βGlcUA1–3GlcNAc; 2 = βGlcUA1–3GlcNAc; 3 = βGlcNAc1–4(βGlcUA1–3βGlcNAc1–4)$_2$; 4 = (βGlcUA1–3βGlcNAc1–4)$_2$; 5 = βGlcNAc1–4(βGlcUA1–3βGlcNAc1–4)$_3$; 6 = (βGlcUA1–3βGlcNAc1–4)$_3$; 7 = (βGlcUA1 3βGlcNAc1–4)$_4$.

were retained according to increasing length of the molecules. Removal of the terminal glucuronic acid moieties from these oligosaccharides resulted in a strong decrease in the retention behaviour of the formed odd-numbered saccharides. As can be seen from Fig. 1, the odd-numbered oligosaccharides appeared at positions before the even-numbered species. No coincidence in the retention properties of even- and odd-numbered glycosides could be observed. This is confirmed by the retention data summarized in Table I.

When the even-numbered oligosaccharides having a terminal non-reducing N-acetylglucosamine and the corresponding odd-numbered glycosides with a non-reducing glucuronic acid were chromatographed under the same conditions as above, similar results were obtained. The elution diagram (Fig. 2) shows complete separation of all these molecules. Odd-numbered oligosaccharides obtained by sequential action of testes hyaluronidase (*endo*-β-N-acetylglucosaminidase) and β-glucuronidase, as well as such molecules that were formed by digestion of hyaluronic acid with leech hyaluronidase (*endo*-β-glucuronidase) and bovine β-N-acetylglucosaminidase, differ in their net charges and consequently can be separated under the above conditions. However, even-numbered oligosaccharides of the same chain length but produced by the different hyaluronidases contain equal amounts of glucuronic acid and N-ace-

TABLE I

RETENTION BEHAVIOUR ON AN ULTRASIL/NH_2 COLUMN OF (A) GLUCURONIC ACID AND N-ACETYLGLUCOSAMINE, (B) EVEN-NUMBERED OLIGOSACCHARIDES WITH NON-REDUCING TERMINAL GLUCURONIC ACID AND CORRESPONDING ODD-NUMBERED MOLECULES WITH TERMINAL NON-REDUCING N-ACETYLGLUCOSAMINE AND (C) EVEN-NUMBERED OLIGOSACCHARIDES WITH NON-REDUCING TERMINAL N-ACETYL-GLUCOSAMINE AND CORRESPONDING ODD-NUMBERED GLYCOSIDES WITH GLUCURONIC ACID AT THE NON-REDUCING TERMINUS

The eluent was 0.1 *M* KH_2PO_4 (pH 4.75) at a flow-rate of 1 ml/min.

Mixture	*Saccharides*	*Retention time (min)*
A	GlcNAc	4.16
	GlcUA	5.95
B	βGlcUA1-3GlcNAc	5.31
	(βGlcUA1-3βGlcNAc1-4)$_2$	7.45
	(βGlcUA1-3βGlcNAc1-4)$_3$	11.66
	(βGlcUA1-3βGlcNAc1-4)$_4$	20.27
	βGlcNAc1-4βGlcUA1-3GlcNAc	4.91
	βGlcNAc1-4(βGlcUA1-3βGlcNAc1-4)$_2$	6.47
	βGlcNAc1-4(βGlcUA1-3βGlcNAc1-4)$_3$	9.56
C	(βGlcNAc1-4βGlcUA1-3)$_2$	7.45
	(βGlcNAc1-4βGlcUA1-3)$_3$	11.66
	(βGlcNAc1-4βGlcUA1-3)$_4$	20.27
	βGlcUA1-3βGlcNAc1-4GlcUA	8.68
	βGlcUA1-3(βGlcNAc1-4βGlcUA1-3)$_2$	14.14
	βGlcUA1-3(βGlcNAc1-4βGlcUA1-3)$_3$	26.00

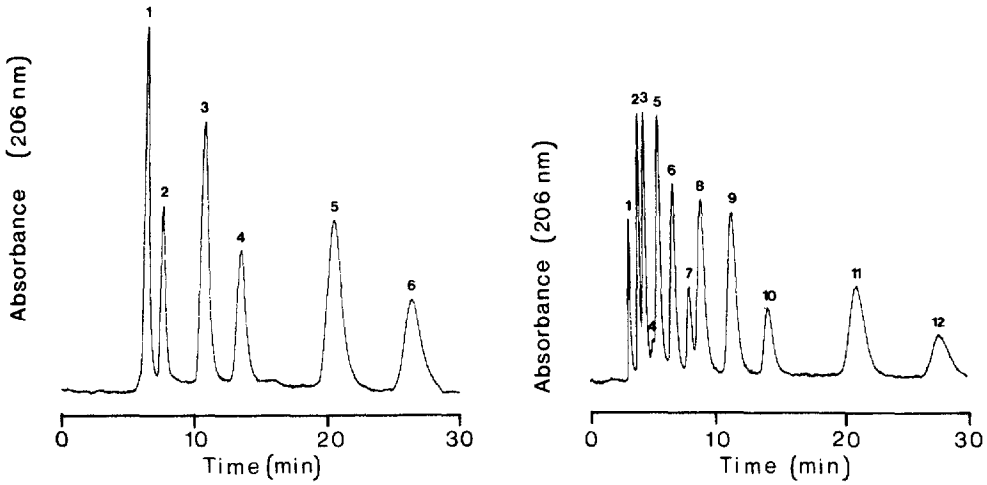

Fig. 2. Separation of a mixture of even-numbered oligosaccharides with N-acetylglucosamine in a non-reducing position and odd-numbered oligosaccharides with glucuronic acid at the non-reducing terminus. Chromatography details were as described in Fig. 1. Peaks: 1 = (βGlcNAc1-4βGlcUA1-3)$_2$; 2 = βGlcUA1-3βGlcNA1-4GlcUA; 3 = (βGlcNAc1-4βGlcUA1-3)$_3$; 4 = βGlcUA1-3(βGlcNAc1-4βGlcUA1-3)$_2$; 5 = (βGlcNAc1-4βGlcUA1-3)$_4$; 6 = βGlcUA1-3(βGlcNAc1-4βGlcUA1-3)$_3$.

Fig. 3. Simultaneous identification of even- and odd-numbered hyaluronate degradation products. A mixture of 1.25 nmol of each sugar was applied to the column and eluted as described in Fig. 1. Peaks: 1 = GlcNAc; 2 = βGlcNAc1-4βGlcUA1-3GlcNAc; 3 = βGlcUA1-3GlcNAc; 4 = GlcUA; 5 = βGlcNAc1-4(βGlcUA1-3βGlcNAc1-4)$_2$; 6 = (βGlcUA1-3βGlcNAc1-4)$_2$ or (βGlcNAc1-4βGlcUA1-3)$_2$; 7 = βGlcUA1-3βGlcNAc1-4GlcUA; 8 = βGlcNAc1-4(βGlcUA1-3βGlcNAc1-4)$_3$; 9 = (βGlcUA1-3βGlcNAc1-4)$_3$ or (βGlcNAc1-4βGlcUA1-3)$_3$; 10 = βGlcUA1-3(βGlcNAc1-4βGlcUA1-3)$_2$; 11 = βGlcUA1-3βGlcNAc1-4)$_4$ or (βGlcNAc1-4βGlcUA1-3)$_4$; 12 = βGlcUA1-3(βGlcNAc1-4βGlcUA1-3)$_3$.

tylglucosamine but in a different sequence. Because of their identical charge and molecular size, the oligosaccharides cannot be separated under these conditions. This is clearly demonstrated by the elution diagram of a mixture of all the saccharides (Fig. 3). The retention of hyaluronate oligosaccharides essentially depends on the composition of the eluent. Increasing amounts of organic solvents commonly yield prolonged retention times. Since acetonitrile has been described as influencing the retention behavior of oligosaccharides and shows a minimal absorbance at 206 nm, the effect of various acetonitrile concentrations in the eluent was examined for the separation and analysis of even- and odd-numbered glycosides with N-acetylglucosamine at the reducing terminus. Table II reveals that increasing the acetonitrile concentration in the eluent shifts the retention times of all saccharides to higher values. This is advantageous for separating smaller oligosaccharides, especially for the analysis of the di- and trisaccharide. On the other hand, the retention of the octasaccharide is increased by *ca.* 70%, leading to considerable peak broadening and thus complicating the integration of the peaks.

In addition, the influence of the buffer pH on the retention time and resolution was studied. Table III shows that a pH of 4.75 gave optimal results, while at higher pH values, retention on the Ultrasil-NH_2 column decreased and peaks overlapped. At pH 6.0, tetra- and hexasaccharide appeared at the same position.

TABLE II

RETENTION DATA OF HYALURONATE OLIGOSACCHARIDES AS A FUNCTION OF THE ACETONITRILE CONCENTRATION IN THE ELUENT ON AN ULTRASIL-NH_2 COLUMN

The mobile phase was a mixture of 0.1 *M* KH_2PO_4 and acetonitrile of various composition at a flow-rate of 1 ml/min.

Ratio of 0.1 M KH_2PO_4 to acetonitrile (pH 4.75)	*Retention time (min)*						
	βGlcUA1–3-GlcNAc	*(βGlcUA1–3-βGlcNAc1–4)$_2$*	*(βGlcUA1–3-βGlcNAc1–4)$_3$*	*(βGlcUA1–3-βGlcNAc1–4)$_4$*	*βGlcNAc1–4-βGlcUA1–3-GlcNAc*	*βGlcNAc1–4-(βGlcUA1–3-βGlcNAc1–4)$_2$*	*βGlcNAc1–4-(βGlcUA1–3-βGlcNAc1–4)$_3$*
60:40	8.37	12.57	19.74	34.60	8.22	10.90	16.19
70:30	7.65	10.73	16.73	29.06	7.86	9.29	13.86
80:20	6.66	9.34	14.62	25.39	6.16	8.13	12.13
90:10	5.92	8.30	13.00	23.00	5.49	7.21	10.77
100:0	5.31	7.45	11.66	20.27	4.91	6.47	9.56

The HPLC system for high-resolution separation of hyaluronate oligosaccharides described here, extends the list of HPLC procedures used so far for quantitative assays of oligosaccharides containing neutral sugars[18,19] or acidic monosaccharides[20–22], as well as for the estimation of oligosaccharides derived from chitin[23]. A system described recently[11] for the determination of even-numbered hyaluronate oligosaccharides using the gel permeation technique does not lead to a satisfactory separation of the saccharides and cannot be adapted for the separation of the odd-numbered oligosaccharides from the even-numbered molecules.

The system we have developed permits a precise simultaneous analysis of even- and odd-numbered oligosaccharides as well as of glucuronic acid and N-acetylglucosamine on an amino-modified stationary phase. Since, by use of an isocratic development, an excellent separation of all glycosides was obtained within at least 30 min, gradient elution could be omitted.

The detection limit of the hyaluronate oligosaccharides was 0.01 nmol per sample when detection was carried out at 206 nm. The sensitivy was twice as high

TABLE III

RETENTION DATA OF HYALURONATE OLIGOSACCHARIDES IN RELATIONSHIP TO pH OF THE ELUENT ON AN ULTRASIL-NH_2 COLUMN

The mobile phase was 0.1 *M* KH_2PO_4 at a flow-rate of 1 ml/min.

pH of the eluent	*Retention time (min)*				
	βGlcUA1 3GlcNAc	*(βGlcUA1–3-βGlcNAc1–4)$_2$*	*(βGlcUA1–3-βGlcNAc1–4)$_3$*	*βGlcNAc1–4-βGlcUA1–3-GlcNAc*	*βGlcNAc1 4-(βGlcUA1 3-βGlcNAc1 4)$_2$*
4.75	5.30	7.45	11.64	4.90	6.47
5.50	4.96	6.08	7.05	4.73	5.66
6.00	4.52	5.06	5.08	4.31	4.76

as at 206 nm when a variable-wavelength detector was used at 195 nm. It should be mentioned that because of the very high sensitivity detection this system does not require any derivatization of the samples. As samples are applied directly to the column, one must ensure that they do not contain citrate or acetate, since these compounds show strong absorbances at the wavelength used. Phosphate has been shown to be the favoured buffer. Using a RI detector, according to the ratio of glucuronic acid to N-acetylglucosamine in the sample, the sensitivity of detection was 10–100-fold lower than at 206 nm using an UV detector.

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