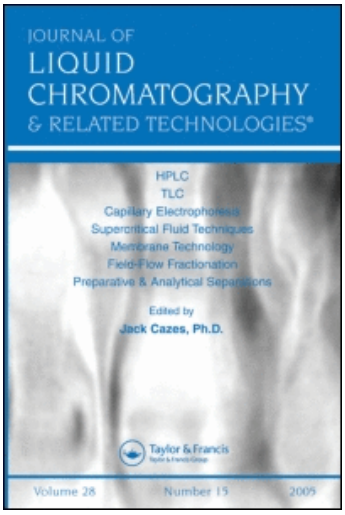

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Isolation and Quantitation of Hyaluronan Tetra- and Hexasaccharide by Anion Exchange HPLC

C. Bartolucci[a]; L. Cellai[a]; C. Corradini[b]; D. Corradini[b]; D. Lamba[a]; I. Velona[a]
[a] Istituto di Strutturistica Chimica "Giordano Giacomello" CNR, Monterotondo Stazione (Roma), Italy [b] Istituto di Cromatografia CNR, Monterotondo Stazione (Roma), Italy

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ISOLATION AND QUANTITATION OF HYALURONAN TETRA- AND HEXASACCHARIDE BY ANION EXCHANGE HPLC

C. BARTOLUCCI[1], L. CELLAI[1],
C. CORRADINI[2], D. CORRADINI[2]*, D. LAMBA[1],
AND I. VELONA[1]

[1]*Istituto di Strutturistica Chimica "Giordano Giacomello"*
[2]*Istituto di Cromatografia*
CNR - P.O. Box 10 -
00016 Monterotondo Stazione (Roma), Italy

ABSTRACT

A high performance liquid chromatographic method for the isolation and quantitation of the the tetra- and hexasaccharide derived from enzymatic depolymerization of hyaluronan by bovine testicular hyaluronidase (EC 3.2.135) is described in this paper.
This method is well suited for the HPLC analysis of depolymerization reactions of hyaluronan, either alone or in competition with chondroitin sulphate, another glycosaminoglycan which is also a substrate for the hyaluronidase. Using a polymeric-based DEAE column with 50 mM phosphate buffer (pH 3.5) as mobile phase, complete separation of hyaluronan tetra- and hexasaccharide was achieved both in analytical and preparative scale. Under these conditions oligomers higher than hyaluronan-hexasaccharide and the chondroitin sulphate depolymerization products were strongly retained and were not eluted. Their complete elution was ob-

* To whom correspondence should be addressed.

tained in a single desorption step with 1.0 M potassium chloride in the eluent. Changes of the ionic strength as well as of the pH value of the eluent were undertaken to study their influence on the chromatographic behaviour of the oligosaccharides.

INTRODUCTION

Hyaluronan (1) and chondroitin sulphate are glycosaminoglycans and are components of the extracellular matrix of connective tissues. Hyaluronan is composed of alternating β(1-3) D-glucuronic acid and β(1-4) N-acetyl-D-glucosamine residues and is believed to be the only glycosaminoglycan which naturally does not occur bound to proteins as proteoglycan. Hyaluronan acts as lubricant, has water binding capacity and regulates water flow in the tissues. Chondroitin sulphate consists of alternating β(1-3) D-glucuronic acid and β(1-4) N-acetyl-D-galactosamine residues and is sulphated at C-4 or C-6 of the N-acetylgalactosamine residue. Enzymatic digestion by bovine testicular hyaluronidase (EC 3.2.1.35) has been used to identify and quantify the isomeric composition of chondroitin and hyaluronan end-products. Bovine testicular hyaluronidase acts as endoglycanohydrolase by randomly hydrolyzing the β(1-4) linkages between N-acetyl-D-glucosamine and D-glucuronic acid residues in hyaluronan. This enzyme also hydrolyzes the β(1-4) glycosidic linkages between N-acetyl-D-galactosamine sulphate and D-glucuronic residues in chondroitin 4- and 6- sulphate. Extensive di-

gestions by bovine testicular hyaluronidase degrades hyaluronan mainly to tetra- and hexasaccharide with only minor amounts of disaccharide and of higher-molecular-weight oligomers (2).

It is known that lysosomal hyaluronidase, an enzyme similar to the commercial enzyme used in this study, is responsible for the degradation of hyaluronan in degenerative disorders of the joints and it has been hypothesized that chondroitin sulphate administered as a drug could compete for this enzyme and reduce the degradation process.

It was hence necessary to develop a method suitable for the characterization and for the quantitative analysis of the end products of the digestion by bovine testicular hyaluronidase. This would allow to study the kinetics of the digestion of hyaluronan alone and with chondroitin sulphate, present as a potential competitor.

Methods used for the estimation of oligosaccharides, derived from enzymatic depolymerization of hyaluronan by bovine testicular hyaluronidase, involve time-consuming chromatographic procedures such as thin-layer chromotography (3) paper chromatography, gel filtration, ion-exchange chromatography or electrophoresis, followed by colorimetric reactions (4-8).

High-performance liquid chromatography (HPLC) has been employed in separating oligosaccharides derived

from hyaluronidase digests of either hyaluronan (using size-exclusion (9) and amino-propyl (10) columns), or chondroitin sulphate (using anion-exchange columns (11)). However none of the HPLC methods for the analysis of hyaluronan or chondroitin sulphate cited above has proven useful for the separation and quantitative analysis of hyaluronan depolymerization products obtained from the digestion of hyaluronan in the presence of chondroitin sulphate.

This paper shows the results of a study of the chromatographic conditions for the separation and quantitation of the hyaluronan tetra- and hexasaccharide derived from hyaluronidase digests of both hyaluronan and mixtures of hyaluronan and chondroitin sulphate.

Higher molecular weight or sulphated oligomers do not interfere with the separation of the above mentioned oligosaccharides.

EXPERIMENTAL

Materials

The hyaluronan potassium salt from human umbilical cord was obtained from Fluka (Buchs, Switzerland).

Chondroitin C sodium salt (chondroitin 6-sulphate), from shark cartilage and the bovine testicular hyaluronidase, 800 IU/mg specific activity, were purchased from Sigma (St. Louis, MO, U.S.A.).

Partially depolymerized chondroitin sulphate C from shark cartilage was a gift from IRBI S.p.A. (Pomezia, Rome, Italy).

Reagent grade phosphoric acid, sodium hydroxyde potassium chloride and HPLC grade water were obtained from Carlo Erba (Milan, Italy).

Instrument

The experiments were performed with a Beckman (Berkeley, CA, U.S.A.) Model 342 liquid chromatograph consisting of two Model 114M solvent delivery pumps, a Model 420 system controller, a Model 210 sample injection valve with either a 20 ul or a 1.0 ml sample loop, a Model 340 dinamically-stirred high pressure mixer and a Model 163 variable UV detector.

Chromatograms were recorded and the peak areas measured by a Shimadzu (Kyoto, Japan) Model C-R5A chromatopac integrator.

Columns

Analytical separations were carried out with either a Bio-Gel TSK DEAE 5PW (75 x 7.5 mm) column (Bio-Rad, Richmond, CA, U.S.A.) or a Protein-Pak DEAE 5PW (75 x 7.5 mm) column (Waters, Milford, MA, U.S.A.). A Protein-Pak DEAE 5PW (150 x 21.5 mm) column was used for the preparative scale isolation of the hyaluronan tetra- and hexasaccharide.

Chromatographic Conditions

Chromatographic runs consisted of isocratic elutions. The flow-rate was either 1.0 ml/min or 8.0 ml/min for analytical and preparative scale separations, respectively. All solutions were filtered through a type HA 0.45 um membrane filter (Millipore, Bedford, MA, U.S.A.) and degassed by sparging with helium. The column effluent was monitored at 210 nm.

Enzymatic Depolymerization

The enzymatic reactions were carried out in 0.15 M phosphate buffer (pH 5.0) containing 0.15 M sodium chloride, at a constant temperature of 37 °C. Hyaluronan was dissolved in the reaction buffer and let overnight under stirring at 4 °C, to make a stock solution.

Each reaction tube contained 3.3 mg/ml hyaluronan, 0.2 mg (160 I.u.) bovine testicular hyaluronidase and in some experiments 3.3 mg/ml of chondroitin C, in a total volume of 1.0 ml reaction buffer. The enzymatic reactions were stopped by heating at 100 °C for 2 minutes. The tubes were then cooled in an ice-bath for 10 minutes, centrifuged at 3000 rpm for 5 minutes. The supernatant was filtered through a 0.45 um Acrodisc LC13 PVDF membrane (Dasit, Milan, Italy) and stored at -20 °C.

Quantitative Analysis

The most abundant depolymerization product of hyaluronan, the tetrasaccharide was isolated by HPLC on the preparative size Protein-Pak DEAE 5PW column, with 50 mM phosphate buffer (pH 3.5) as eluent. The collected fraction was lyophilized, desalted on a Bio-Rad (Richmond, CA, U.S.A.) Bio-Gel P2 column (900 x 2.9 mm) and lyophilized again. A hyaluronan tetrasaccharide stock solution was prepared by dissolving the purified oligosaccharide in the eluent. The concentration of this solution was 1.40 umole/ml.

Working standard solutions were prepared by subsequent dilutions of the stock solution with the 50 mM phosphate buffer (pH 3.5).

All solutions were prepared on the day of use. Each working standard was injected onto the analytical column in triplicate and chromatographed isocratically with the above eluent.

The calibration curve was obtained by plotting the area of the chromatographic peaks detected at 210 nm as a function of the oligosaccharide concentration in the working standards.

RESULTS AND DISCUSSION

Hyaluronan was depolymerized by using bovine testicular hyaluronidase at various incubation times ranging from 2 to 48 hours.

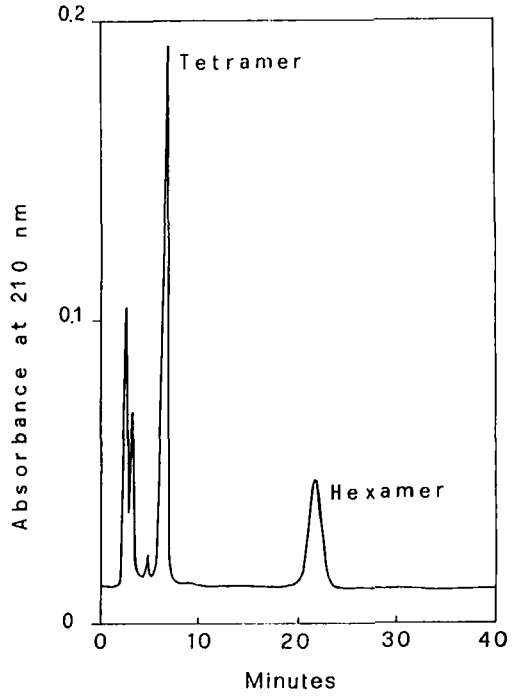


Figure 1. Separation of hyaluronan depolymerization mixture incubated for 24 hours with bovine testicular hyaluronidase. Column, Protein-Pak DEAE 5PW (75 x 7.5 mm I.D.); eluent, 50 mM phosphoric acid adjusted to pH 3.5 with 0.1 M sodium hydroxyde; Flow rate, 1.0 ml/min; temperature 20 °C.

The separation and the isolation of the oligosaccharides was achieved by using HPLC columns packed with a large pore size (1000 Å) polymeric-based DEAE anion-exchanger. Fig. 1 shows the analytical separation of the hyaluronan depolymerization mixture incubated for 24 hours. This separation was obtained on the Protein Pak DEAE 5PW column (75 x 7.5 mm) by eluting isocrati-

cally with 50 mM phosphoric acid, adjusted to pH 3.5 with sodium hydroxyde.

The chromatogram was obtained by monitoring the column effluent at 210 nm.

The chromatographic peaks with retention factor smaller than 0.9 were also detected by either monitoring the column effluent at 280 nm or injecting the enzymic solution (submitted to the same treatment as the enzymatic reaction mixture, but lacking the polysaccharide) and monitoring the column effluent at either 280 or 210 nm.

The oligosaccharides eluted with retention factor of 2.0 and 9.2, represented together 80% of the total area of the chromatographic peaks. The identification of those two depolymerization products as the tetramer (K'=2.0) and the hexamer (K'=9.2) was achieved by TLC (3), 1H-NMR and FAB-MS after collecting and lyophilizing the peak fractions eluted from a preparative size (150 x 21.5 mm) Protein-Pak DEAE 5PW column.

In order to find the optimal conditions a study of the influence of pH and ionic strength of the mobile phase on the chromatographic behaviour of the oligosaccharides was undertaken.

The effect of the eluent pH on the retention of the hyaluronan tetra- and hexasaccharide on the DEAE column was investigated in the pH range from 3.0 to 6.0. The chromatograms in Fig. 2 show that the reten-

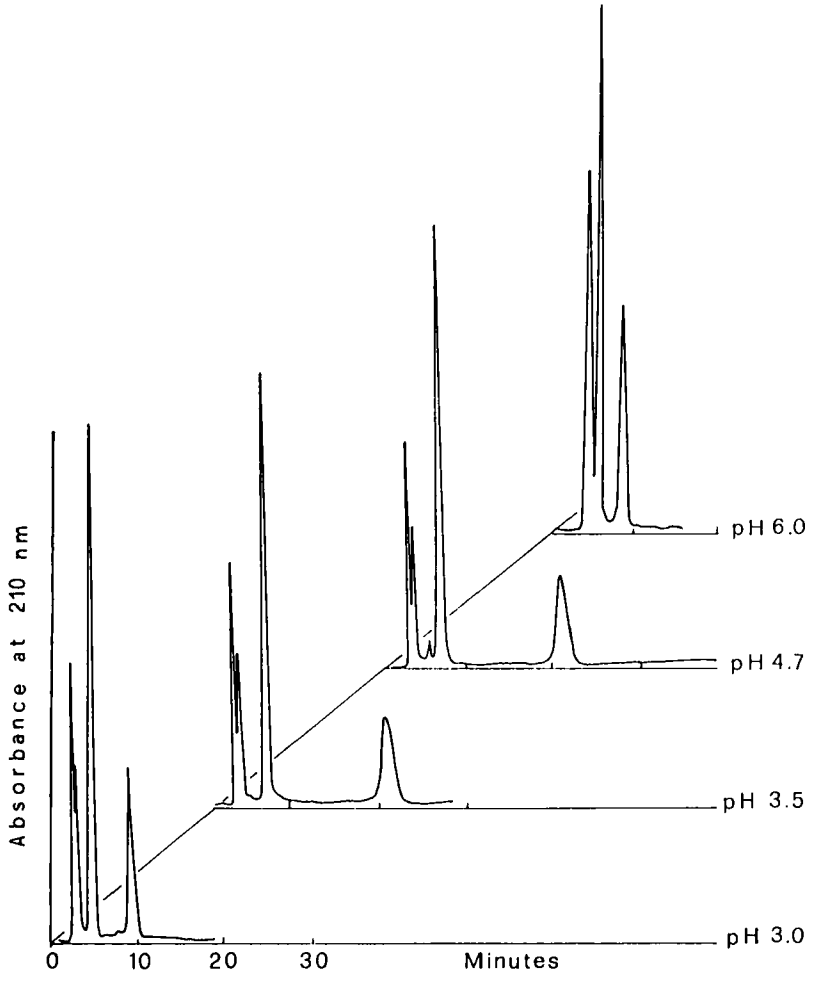


Figure 2. Separation of hyaluronan depolymerization mixture incubated for 24 hours with bovine testicular hyaluronidase with eluents of different pH. Column, Bio-Gel DEAE 5PW (75 x 7.5 mm I.D.); other conditions as in Fig. 1 except the pH of the 50 mM phosphoric acid was varied.

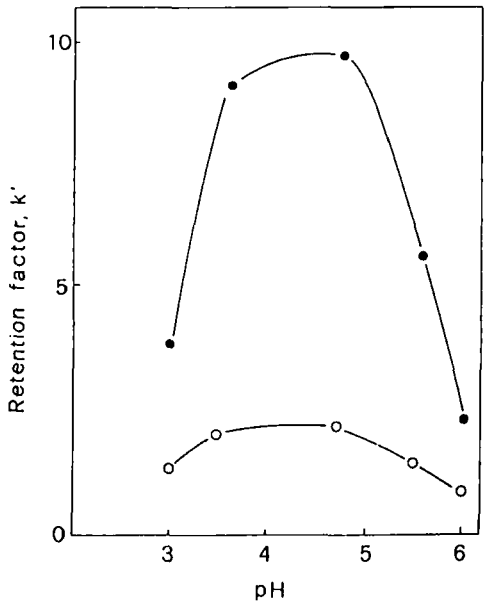


Figure 3. Plots of retention factors of hyaluronan tetra- (o) and hexasaccharide (•) against the pH of the eluent. Conditions as in Fig. 2.

tion of both hyaluronan tetra- and hexasaccharide is particularly pH dependent. The resulting plots of the retention factors versus the pH of the eluent are shown in Fig. 3. For both oligosaccharides, the retention first increased and then decreased with increasing pH and reached a maximum between pH 4.0 and 5.0. The increase in retention when going from pH 3.0 to pH 4.7 is caused by dissociation of the carboxylic group in the glucuronic moiety, whereas the decrease in retention with increasing pH could be due to a gradual de-

protonation of the amino groups on the surface of the stationary phase above pH 5.0.

The retention of the hyaluronan tetra- and hexasaccharide on the DEAE stationary phase strongly depended on the ionic strength of the mobile phase and dramatically decreased by adding salt to the eluent in the pH range studied. By adding 0.05 M potassium chloride to the eluent the retention factors of hyaluronan tetra- and hexasaccharide decreased from 2.0 and 9.2 to 0.4 and 0.9, respectively and with 0.2 M potassium chloride both oligosaccharides were eluted as unretained.

These findings suggest that the hyaluronan-disaccharide, present in the depolymerization mixture, was eluted faster than the tetrasaccharide, probably together with the non-saccharidic components of the reaction mixture detected at 280 nm.

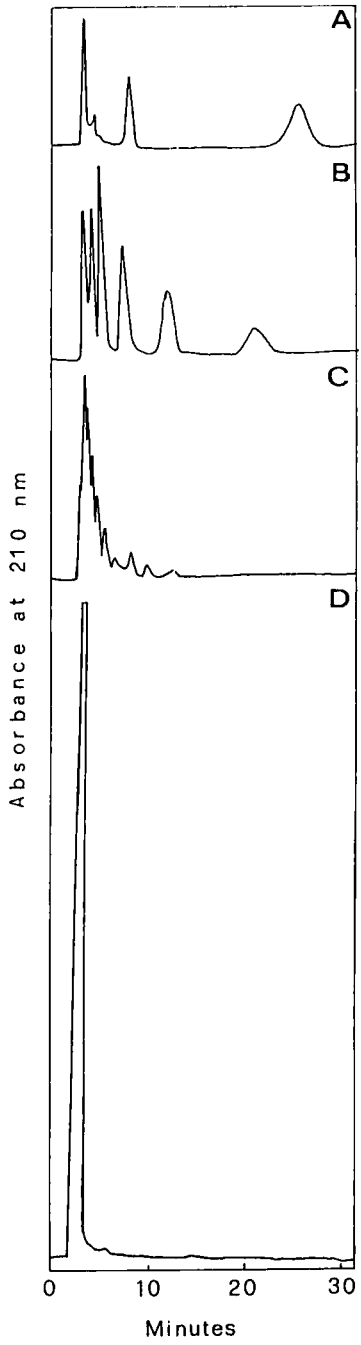
The effect of the potassium chloride concentration in the eluent on the chromatographic behaviour of the higher-molecular-weight oligosaccharides was also investigated. Fig. 4 reports the chromatograms of the depolymerization mixture incubated for 4 hours. It was chromatographed on the Bio-Gel DEAE 5PW (75 x 7.5 mm) column with 50 mM phoshate buffer (pH 3.5) containing no salt (panel A), and potassium chloride at three different concentrations (panels B, C, and D). Without any

addition of salt the oligosaccharides higher than the hexamer were strongly retained and were not eluted (panel A, Fig. 4). They were however eluted by using mobile phases containing salt (panel B and C), and the retention decreased with increasing potassium chloride concentration. With 0.2 M potassium chloride in the eluent none of them was retained on the DEAE column.

Non-depolymerized hyaluronan, present in the depolymerization mixture incubated for a short time, was strongly retained under the conditions used to elute the hyaluronan tetra- and hexasaccharide and was washed out from the column, together with the higher oligosaccharides in a single desorption step with 1.0 M potassium chloride in the eluent.

Under these conditions non-depolymerized or partially depolymerized hyaluronan co-eluted as unretained peak, which was detected at 210 nm.

To evaluate the mass recovery the DEAE column was replaced with an empty capillary tube having an internal volume equivalent to the elution volume of the unretained peak on the DEAE column. The same volume of sample injected onto the DEAE column was injected onto the empty tube. The mass recovery of the sample from the DEAE column was calculated as the variation percent of the peak area recorded with and without the column. By using this method the mass recovery of either unde-



polymerized or partially depolymerized hyaluronan from the DEAE column was found to be greater than 98%.

Hyaluronan tetra- and hexasaccharide are the most abundant end-products of the depolymerization reaction by bovine testicular hyaluronidase, the tetrasaccharide alone accounting for 85-90% (2).

Using the above mentioned chromatographic system we analyzed hyaluronan mixtures incubated for 2-48 h for hyaluronan tetra- and hexasaccharide content since these are representative of the whole kinetic profile. Fig. 5 shows that the formation of the tetra- and hexasaccharide is time dependent.

The calibration curve of the tetrasaccharide was obtained by the procedure described in the experimental section and was linear in the concentration range 5-25 ug, with correlation coefficient of 0.994. This calibration curve was used to evaluate the rate of formation of both hyaluronan tetra- and hexasaccharide as a function of the enzymatic digestion.

Figure 4. Chromatograms of hyaluronan depolymerization mixture incubated for 4 hours . Column Bio-Gel DEAE 5PW (75 x 7.5 mm I.D.); eluent, 50 mM phosphoric acid adjusted to pH 3.5 with 0.1 M sodium hydroxyde, containing no salt (panel A), and potassium chloride at the following concentrations: 0.05 M (panel B), 0.1 M (panel C), and 0.2 M (panel D). Other conditions as in Fig. 1.

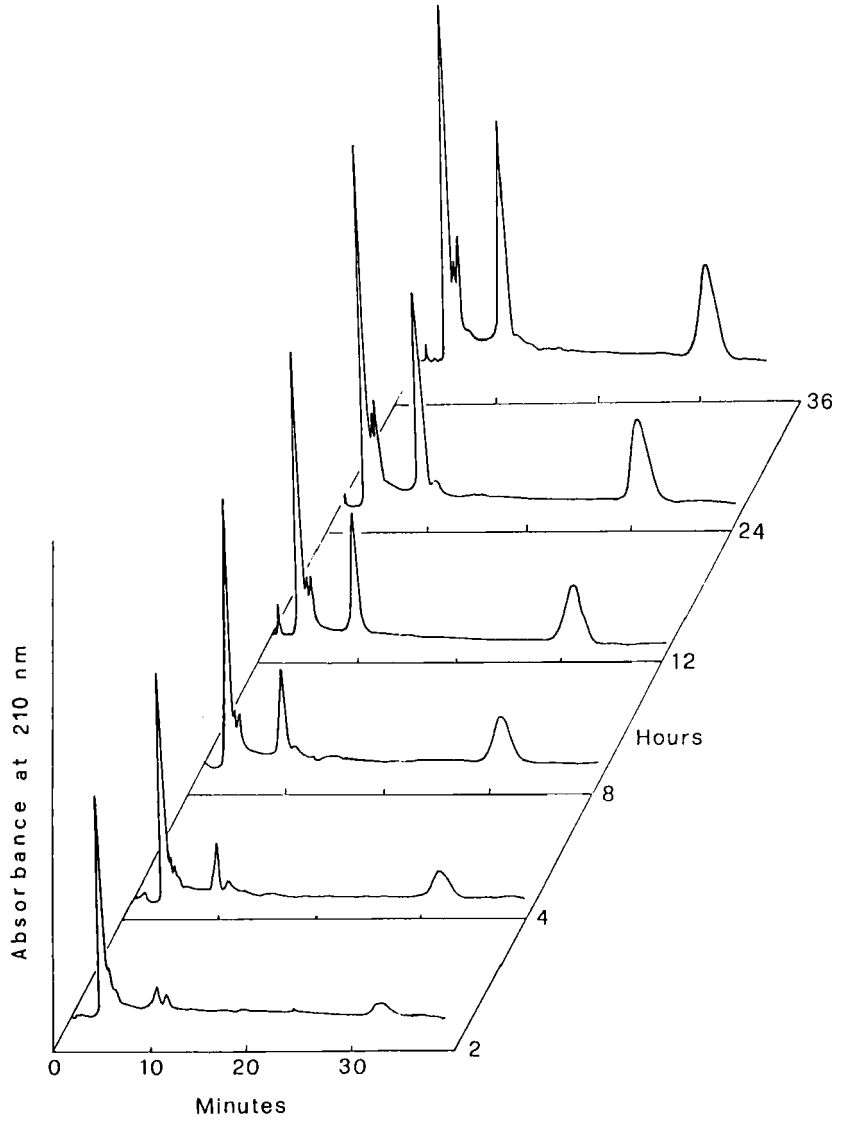
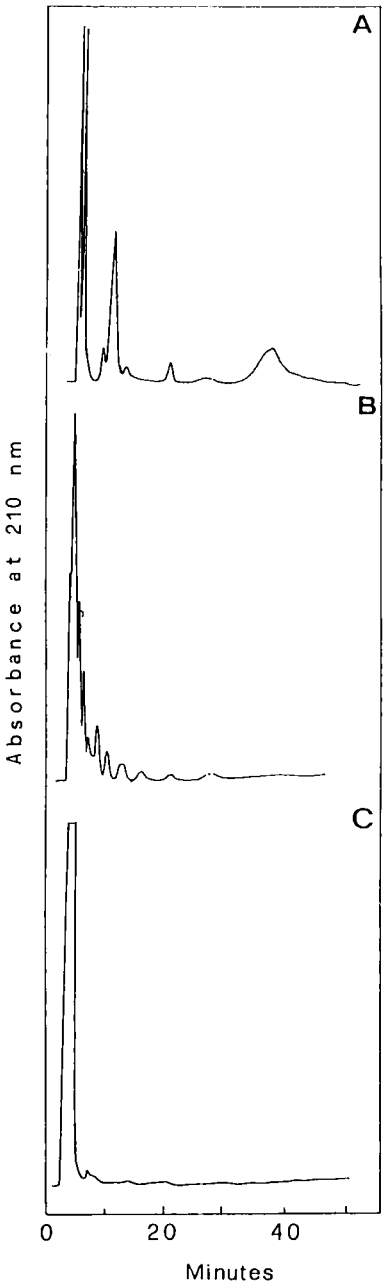


Figure 5. Separation of hyaluronan depolymerization mixtures incubated for periods ranging between 2 and 36 hours. Column Bio-Gel DEAE 5PW; conditions as in Fig 1.

The results obtained by the HPLC method and by the Reissig method (12) were sufficiently well correlated.

The developed method was also succesfully applied to study the competitive effect of chondroitin C on the digestion of hyaluronan by bovine testicular hyaluronidase. For this study the enzymatic depolymerization of hyaluronan was carried out in the presence of variable amounts of commercial chondroitin C and depolymerized chondroitin C also substrates for bovine testicular hyaluronidase. Both chondroitins C are mixtures of chondroitin 4- and 6-sulphate in a ratio approximately 30-70 as seen by 13C NMR (13). While chondroitin C from Sigma has an average MW of 18.000 Da, chondroitin C from IRBI has an average MW of 7500 Da, as measured by the intrinsic viscosity method (14). The incubation mixtures, containing hyaluronan and chondroitin C depolymerization products were stopped at the selected incubation time and analyzed for hyaluronan tetra- and hexasaccharide content, using the above chromatographic method. Both types of chondroitin C gave similar competitive effects, thus reducing the formation of hyaluronan tetra- and hexasaccharide proportionally to the amount of CS present in solution, without any apparent influence of the CS average MW.

In order to investigate the interferences of the chondroitin depolymerization products on the HPLC ana-



lysis of hyaluronan tetra- and hexasaccharide, samples of chondroitin C were depolymerized by bovine testicular hyaluronidase at incubation periods ranging between 2 and 48 hours and chromatographed on the DEAE column. With the eluent used for the analytical separation of hyaluronan tetra- and hexasaccharide, (50 mM phosphate buffer, pH 3.5), all the chondroitin sulphate depolymerization products were strongly retained and were not eluted. They were eluted with mobile phases containing potassium chloride at concentrations higher than 0.1 M. The retention decreased, increasing the salt concentration in the eluent. With 0.5 M potassium chloride all the chondroitin sulphate depolymerization products were eluted as unretained (Fig. 6).

These findings are in agreement with the expectation that the chondroitin sulphate depolymerization products, which besides the carboxylic groups are also bearing sulphate groups, are more acidic than the corresponding hyaluronan depolymerization products, and consequently, have stronger electrostatic interactions

Figure 6. Chromatograms of chondroitin C depolymerization mixture incubated for 24 hours with bovine testicular hyaluronidase. Column, Protein-Pak DEAE 5 PW (75 x 7.5 mm I.D.); eluent, 50 mM phosphoric acid adjusted to pH 3.5 with 0.1 M sodium hydroxyde, containing potassium chloride at the following concentrations: 0.15 M (panel A), 0.2 M (panel B), and 0.5 M (panel C). Other conditions as in Fig. 1.

TABLE I
Competitive Effect of Chondroitin C (CsC) from IRBI on the Digestion of Hyaluronan (Hy).

TIME	TETRAMER (umoles)		HEXAMER (umoles)	
(h)	Hy	Hy + CsC	Hy	Hy + CsC
2	0.48	0.18	0.84	0.29
4	0.39	0.34	0.45	0.41
8	0.59	0.49	0.60	0.51
12	0.60	0.89	0.89	0.59
24	1.69	0.90	1.55	0.77
36	1.68	1.09	1.33	0.86
48	2.00	1.25	1.55	0.81

with the positively charged DEAE stationary phase. Furthermore, the experimental data show that even the lower oligosaccharide derived from hyaluronidase digest of chondroitin sulphate are more strongly retained than any hyaluronan oligosaccharide. As a consequence the presence in the samples of the chondroitin sulphate depolymerization products did not interfere with the HPLC separation and quantitation of the hyaluronan oligosaccharides in hyaluronidase digests of hyaluronan and chondroitin sulphate mixtures.

Therefore, this method allows to study the digestion of hyaluronan also in the presence of chondroitin sulphate since it is selective for hyaluronan tetra-

and hexasaccharide. The comparison of the rate of formation of hyaluronan tetra- and hexasaccharide, allows for the evaluation of the competitive effect of chondroitin sulphate on the digestion of hyaluronan as shown in Table I.

Our study has shown that anion exchange HPLC with the wide pore polymeric based DEAE 5PW columns is an effective method for separating the depolymerization products of hyaluronan, either alone or in the presence of chondroitin sulphate.

At the lowest ionic strength of the eluent (no salt added) only the hyaluronan oligomers lower than the hexasaccharide are eluted, without interferences from higher or sulphated oligomers.

However, depending on the desired separation, conditions can be varied to adjust the retention of the depolymerization products of partially digested mixtures, to favour the elution of higher molecular weight hyaluronan oligomers.

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