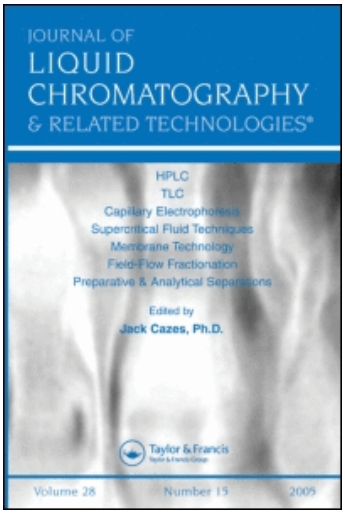

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF XYLITOLE AND HEXOSAMINITOLS PRESENT IN THE REDUCED TERMINAL OF GLYCOSAMINOGLYCANS

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ABSTRACT

A reversed phase isocratic high-performance liquid chromatographic method for the analysis of xylitole and hexosaminitols present in the reduced terminal of glycosaminoglycans is described. The glycosaminoglycans are covalently bound to protein forming proteoglycans via xylose, galactosamine or glucosamine. Alkaline treatment of the proteoglycan in the presence of $NaBH_4$ releases the glycosaminoglycan chains which contain in their reduced terminal xylitole, galactosaminitole or glucosaminitole. The obtained glycosaminoglycans are depolymerized with trifluoroacetic acid and the liberated neutral monosaccharides and xylitole are separated from the hexosamines and hexosaminitols on a DOWEX 50-X8 microcolumn. The obtained neutral monosaccharides are reduced to alditols by $NaBH_4$ and separated from xylitole as per-O-benzoylated derivatives on Supelcosil LC-18, using 70 % acetonitrile as eluent, at a flow rate of 1.0 ml/min. Per-O-benzoylated derivatives of hexosaminitols are separated from the hexosamines derivatives on the same

column, using 78 % acetonitrile as eluent, at a flow rate of 0.5 ml/min. Detection of the eluted derivatives is performed at 231 nm. The detection limit is 10 pmol for xylitole and 6 pmol for hexosaminitols. The reaction of per-O-benzoylation was shown to give linear calibration graphs up to 150 nmol (30 μg).

INTRODUCTION

Proteoglycans are macromolecules composed of a protein core onto which glycosaminoglycan chains are covalently bound [1,2]. The types of linkage between glycosaminoglycans and protein are (i) xylose to serine [3], (ii) N-acetylgalactosamine to serine or threonine [4] and (iii) N-acetylglucosamine to asparagine [4]. The O-glycosidic linkages of the first two types are labile under mild alkaline conditions (50 mM NaOH, 37-50 °C, 8-70h), in the presence or not of sodium borohydride [3-5], by which the polypeptide backbone is totally broken down [6]. In spite of the presence of sodium borohydride [6], the N-glycosidic linkage of the third type is more stable and no more than 6-20% of the N-linked carbohydrate chains are released from the protein [7,8]. When alkaline borohydride treatment [7] is used for the cleavege of O-glycosidic linkages between carbohydrate and protein, the obtained intact polysaccharide terminates to xylitole or hexosaminitol, *i.e.* only one residue of these is present per chain (Fig.1).

A previously described method utilizing amino acid analyser for the analysis of hexosamines and hexosaminitols [9] is time consuming, whereas the reported [10-12] high-performance liquid chromatographic methods are rapid and more sensitive. Methods developed for xylitole determination have utilized post-column deri-vatization by periodate oxidation [13] or scintillation counting as method of detection [14].

We have previously reported that uronic acids [15] and sialic acids [16] can be dètermined in picomole

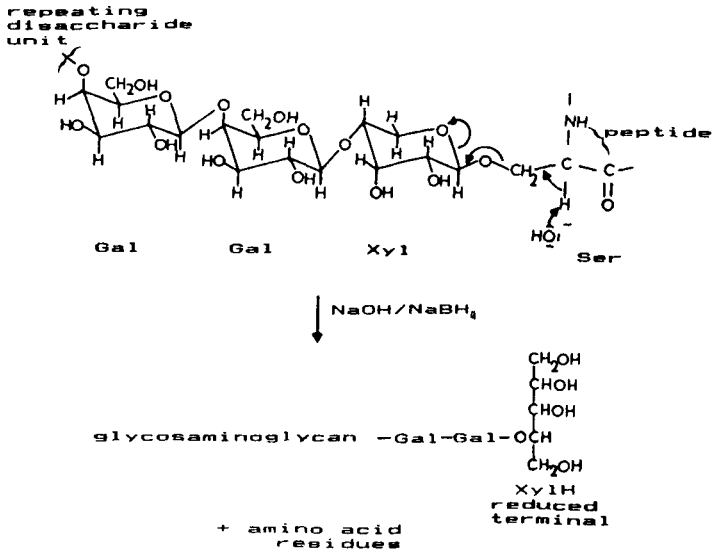

Figure 1.Alkaline treatment of a proteoglycan molecule in the presence of sodium borohydride. The obtained glycosaminoglycan contains xylitole (XylH) in the reduced terminal, while the protein is totally broken down to amino acids. Gal= galactose, Xyl=xylose.

amounts by per-O-benzoylation and subsequent isocratic HPLC separation. Hydroxyl groups of alditols and hexosaminitols could be per-O-benzoylated in the same way to form extremely stable and strongly UV-absorbing derivatives which are suitable for a highly sensitive UV detection. Although per-O-bezoylated methylglycosides have been previously used for the separation of alditols and hexosaminitols [11], this analysis is inhibited by aldoses which are coeluted with xylitole as well as hexosaminitols. This problem is mainly associated with glycosaminoglycans isolated from invertabrate tissues, where a significant number of neutral sugar branches onto the glycosaminoglycan chain are present [17-22].

The liberation of monosaccharides, necessary for their separation and subsequent determination, is performed by acid hydrolysis which is followed by simultaneous removal of N-acetyl groups present in the amino group of hexosamines and is associated with some destruction of the carbohydrates [15]. On the other hand, trifluoroacetic acid is an effective reagent which has been successfully used for the glycosaminoglycan depolymerization [23].

In this paper we report an isocratic highly sensitive HPLC method in which aldoses obtained after glycosaminoglycan depolymerization are reduced to alditols and completely separated from xylitole as per-O-benzoylated derivatives. Using the same chromatographic column per-O-benzoylated hexosaminitols are completely separated from hexosamines.

EXPERIMENTAL

Apparatus and Chemicals

An LDC system consisted of a LDC III pump, a UV-vis detector LDC 1204A set at 231 nm with 8-ul flow cell and a 20-µl loop injector was used. The analytical column is a Supelcosil LC-18, 5-µm, 250 x 4.6 mm I.D., stainless steel (Supelco, Bellfont, PA, U.S.A.) equipped with a RP-18 guard column, 30 x 4.6 mm I.D. (Brownlee Labs., Santa Clara, CA, U.S.A.). Sep Pak C_{18} cartridges were obtained from Milford, MA, U.S.A.

Glucosamine, galactosamine, xylose, mannose, glucose and galactose were from Sigma Chemical Co. Hexosaminitols and alditols were prepared by reduction of the corresponding hexosamines and aldoses with sodium borohydride [7,11]. The obtained products were crystallised from ethanol. Chondroitin sulfate (whale cartilage) was from Sigma, whereas non-sulfated chondroitin sulfate and dermatan sulfate were prepared from squid skin and sea

urchin respectively as previously described [24,25]. The molecular weights (M_r) of these glycosaminoglycans were 1.2 x 10^3, 8 x 10^4 and 1.65 x 10^4 respectively, were estimated by HPLC [27]. 2-acetamido-2-deoxy-3-O-β-D-galactopyranosyl-D-galactopy-ranose, abbreviated as β-D-Gal-[1->3]-D-GalNAc and 2-acetamido-2-deoxy-4-O-([4-O-β-D-galactopyranosyl]-β-D-galactopyranosyl)-D-glucopy-ranose, abbreviated as β-D-Gal-[1->4]-β-D-Gal-[1->4]-D-GlcNAc were from Sigma. Eluent components were HPLC-grade acetonitrile, obtained from Merck (Darmstadt, Germany) and glass-distilled water. All other chemicals used were of analytical reagent grade.

Chromatographic Conditions

The mobile phase used for the separation of xylitole from the other alditols was 70 % acetonitrile in water and for the separation of hexosaminitols from the hexosamines 78 % acetonitrile. The flow rate for the former separation was 1.0 ml/min and of the latter 0.5 ml/min. The detection was performed at 231 nm and the pressure was 1300 psi. The separations were performed at room temperature. The eluents used were degassed by vacuum fitration through a 0.2-μm membrane filter followed by aggitation in an ultrasonic bath.

System Suitability

The column was equilibrated separately with each mobile phase at flow rates of 1.0 ml/min and 0.5 ml/min for the analysis of xylitole and hexosaminitols, respectively. After a stable baseline was obtained, the standard and the sample solutions were injected into the column and the peaks appeared over the increased retention time. The resolution factors, R_s, were calculated between the chromatographic peak of xylitole and each individual peak of aldoses and alditols from the equation $R_s = 2\ (t_2-t_1)/(W_1+W_2)$, where t_2 and t_1 are the retention times of the two peaks and W_1, W_2 are the peak

TABLE 1

High-Performance Liquid Chromatographic Characteristics for the Determination of Xylitole.

Compound	Retention Time (t_R) [min]	Resolution (R_s)
Alditols		
Xylitole	34.0	-
Mannitole	49.0	1.45
Galactitole	49.3	1.47
Sorbitol	49.7	1.55
Aldoses [1]		
Xylose	20.5/21.5	1.56/1.66
Mannose	31.7/33.5	0.17/0.08
Galactose	33.0/35.0	0.07/0.09
Glucose	37.5/39.5	0.33/0.48

[1] Two values represent the anomeric forms of aldoses

widths at the base of the respective peaks. The same estimations were performed between the chromatographic peak of glucosaminitole and each peak of the hexosamines and galactosaminitole. The resolution factors, R_s, were more than 1.2 indicated complete separation between xylitole and the other alditols as well as between hexosaminitols and hexosamines, except the case of the separation between glucosaminitole and galactosaminitole. These are illustrated in Tables 1 and 2.

Sample Preparation

Glycosaminoglycans were liberated from proteoglycans by alkaline borohydride treatment with 50 mM NaOH-1M $NaBH_4$ at 45°C for 48 h [6]. The reduced glycosaminoglycans were isolated by gel chromatography on Sepharose CL-6B, hydrolysed with 2 M trifluoroacetic acid at 100°C for 8 h in screw capped polypropylene microtubes and the hydrolysates were subsequently freeze-dried. The

TABLE 2

High-Performance Liquid Chromatographic Characteristics for the Determination of Hexosaminitols.

Compound	Retention Time(t_R) [min]	Resolution (R_s)
Hexosaminitols		
Galactosaminitole	29	0.25
Glucosaminitole	29.8	-
Hexosamines [1]		
Glucosamine	17.5/20.2	1.6/1.2
Galactosamine	19.0/20.6	2.1/1.4

[1] As in Table 1.

dry residue was dissolved in 100-µl water and applied on a DOWEX 50-X8 (Na^+-form) microcolumn, 5 x 2.5 mm I.D. The column was eluted with 1-ml of water and 1-ml of 2M HCl. The first fraction contains the neutral monosaccharides and xylitole, while the second hexosamines and hexosaminitols. Both fractions were freeze-dried and the dry residue of the first fraction was subjected to reduction with 100-µl 1M $NaBH_4$ in 50 mM NaOH at 37°C for 30 min. The mixture was neutralized with glacial acetic acid and borate was removed by two sequential additions of 1-ml methanol and evaporation of the obtained mixtures. The dry residue was then taken for derivatization.

Derivatization Procedure

Per-O-benzoylation was performed by the addition of 100-µl of benzoylation mixture [10% (w/v) benzoic anhydride, 5% p-dimethylaminopyridine in pyridine] and heating at 80°C for 20 min. The reaction was stopped by the addition of 900-µl water followed by vigorous shaking on a vortex mixer. Excess of reagents was removed by passing the mixture through a Sep Pak C_{18} cartridge. The

cartridge was eluted with 5-ml of water and the per-O-benzoylated derivatives were recovered with 5-ml of acetonitrile. After evaporation, the dry residue was dissolved in 1-ml of the respective mobile phase, centrifuged at 10000 g for 5 min and aliquots of the supernatant were injected into the column.

Selectivity

Chromatography of the samples derived from hydrolysed glycosaminoglycans showed no endogeneous interferences at the retention times of xylitole as well as hexosaminitols. This indicated that the proposed method could be used in the determination of xylitole and hexosaminitols in the glycosaminoglycans without the use of internal standard.

Detection Limit

The detection limits for xylitole and hexosaminitols were estimated as the quantity of these monosaccharides producing a signal of the peak height twice the baseline noice. The minimum detectable amount in pmol injected into the column was estimated to be 10 pmol (1.5 ng) for xylitole and 6 pmol (1.1 ng) for hexosaminitols.

Standard Calibration Graphs

All monosaccharides tested were accurately weighed and dissolved in water to give stock solutions of 10 ng/µl each. Standard xylitole and hexosaminitole solutions of 0.1, 0.2, 1.0, 2.0, 5.0, and 10.0 ng/µl were prepared by appropriate dilutions of the stock solutions. Aliquots of 10-µl were taken for derivatization and analysis. The calibration curves were constructed by plotting the peak heights of xylitole, galactosaminitole and glucosaminitole signals against their concentration.

RESULTS AND DISCUSSION

The retention times of xylitole and hexosaminitols were reproducible, under the chromatographic conditions

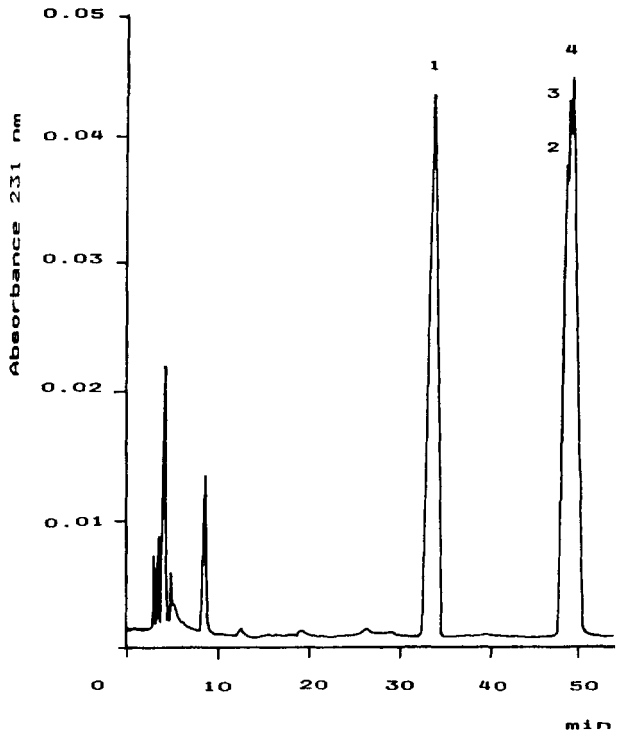

Figure 2.Typical HPLC chromatogram of xylitole and alditols. Peaks: 1= xylitole, 2=mannitole, 3=galactitole and 4=sorbitol.

used, with a relative standard deviation less than 0.5%. The mobile phases used, enabled a good column performance for long periods of time and the derivatives used were stable for at least several months when kept at 4°C.

The per-O-benzoylαted derivatives of xylitole, hexosaminitoles and the other alditols gave rise to only one single peak, (Figures 2 and 3), while those of aldosed and hexosamines two peaks (Tables 1 and 2). The latter is due to mutarotation of aldoses and hexoses since only these monosaccharides contain an aldehyde

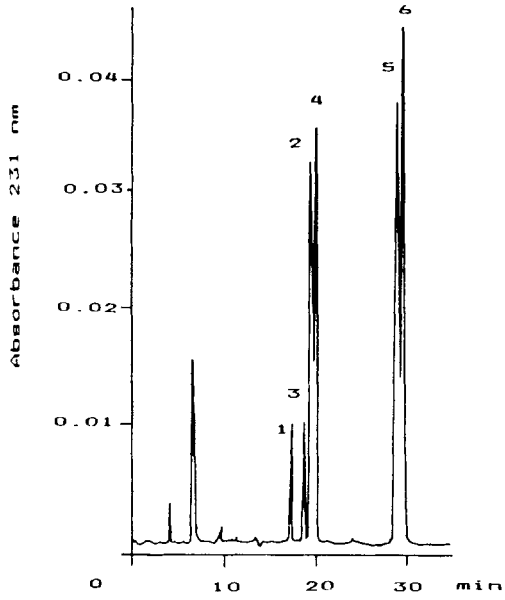

Figure 3.High-performance liguid chromatogram of hexosaminitols and hexosamines. Peaks: 1, 2=α, β-anomeric forms of glucosamine, 3,4=α, β-anomeric forms of galactosamine, 5= galactosaminitole and 6=glucosaminitole.

group at C_1. Xylitole was cleanly separated from other alditols (Fig.2).Similarly hexosaminitols were cleanly separated from hexosamines (Fig.3) in the LC-18 column. The first peaks, eluted before the compounds of interest, were residual benzoic anhydride, benzoic acid or under-benzoylated derivatives (Figures 2 and 3). The greater retention times of the alditols, galactitole, sorbitol and mannitole than that of xylitole (Fig.2) were compatible with the number of sites for benzoylation. Xylitole carries five benzoyl groups, while the other alditols six and therefore the latter are more hydrophobic than the former and eluted latter in the reversed phase conditions used. Hexosamines and

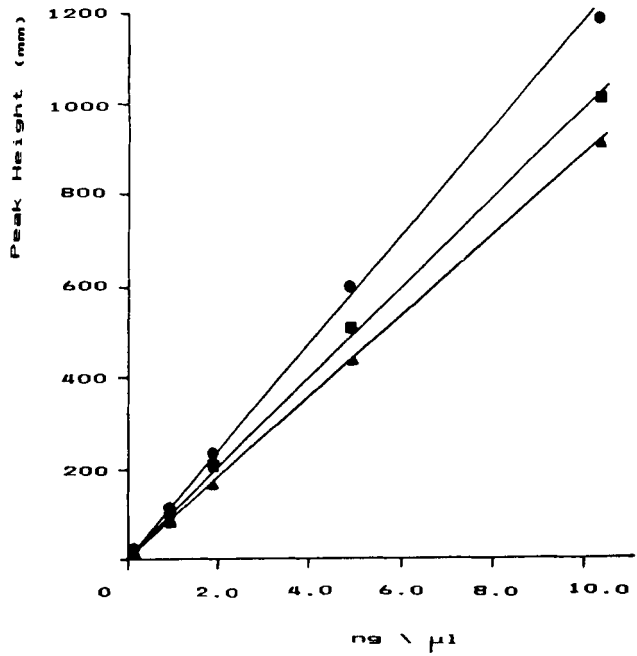

Figure 4.Calibration graphs obtained by injecting various amounts of per-O-benzoylated derivatives of xylitole (●), galactosaminitole (■) and glucosaminitole (▲).

hexosaminitols carry four and five benzoyl groups respectively and therefore the former eluted earlier than the latter.

Both the sensitivity and the linearity of the method were tested with the use of standard mixtures of various concentrations (Fig.4). The obtained peak heights for the compounds of interest were found to be linear related to concentrations up to 150 nmol, *i.e.*, when 30-µg of each monosaccharide injected into the column (Fig.4). The precision of the method was determined by six repeated determinations of each monosaccharide. When 15 nmol of each monosaccharide were measured, the relative standard

TABLE 3

Chemical Composition of the Reduced Terminal of Various Glycosaminoglycans

Sample	M_r	Xylitole[1]	Galactosa-minitole[2]	Glucosa-minitole[2]
Chondroitin sulfate (*whale*)	1.2×10^3	4.12	nd	nd
Chondroitin (*squid skin*)	8×10^4	0.48	nd	nd
Dermatan sulfate (*sea urchin*)	16.5×10^3	3.27	nd	nd
β-D-Gal-[1->3]-D-GalNAc	383.4	nd	186.7	nd
β-D-Gal-[1->4]-β-D-Gal-[1->4]-D-GlcNAc	545.5	nd	nd	191.2

1: µmol/100 µmol galactosamine
2: µg/µmol of compound analysed
nd: not detected

deviation was 2.5% for xylitole and 2.3% for hexosaminitols, and with 5 nmol the corresponding values were 2.9% and 2.6% respectively.

Applications

The described method was employed to analyse various glycosaminoglycans which are completely liberated from their proteoglycans by alkaline borohydride treatment. The xylitole contents of the various glycosaminoglycans of known M_r are presented in Table 2. The results are in good agreement with the theoretically expected amount of xylitole present per glycosaminoglycan chain. For the determination of hexosaminitoles, commercial preparations of a disaccharide and a trisaccharide were used. The

amounts of hexosaminitols determined were also in accordance to those theoretically estimated (Table 3).

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