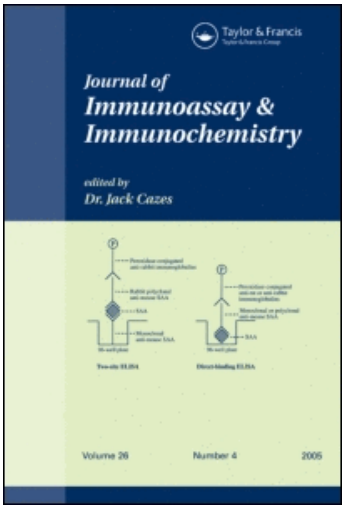

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A SOLID PHASE ASSAY FOR THE DETERMINATION OF HEPARAN SULFATE AND ITS APPLICATION TO NORMAL AND CANCEROUS HUMAN CARTILAGE SAMPLES

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ABSTRACT

A sensitive and accurate quantitative assay for the measurement of minor amounts of chondroitin/dermatan sulfate and heparan sulfate that does not require specific apparatus or reagents is described. The assay involves labeling of chondroitin sulfate A following reaction of carboxyl groups with biotin hydrazide in the presence of carbodiimide. ELISA plate wells were coated with glutaraldehyde and then spermine was coupled to it via a Schiff's base bond. In such activated wells, the biotinylated molecules were readily bound and

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detected after the interaction with avidin-peroxidase conjugates and the subsequent enzymic assay. Chondroitin/dermatan sulfate and heparan sulfate competed this interaction in a linear manner. Disaccharides derived from chondroitin sulfate A did not act as competitors, while heparan sulfate disaccharides showed significant competition. From the competition, before and after digestion with either chondroitinase ABC or heparitinases, the amounts of chondroitin sulfate and heparan sulfate in a sample could be calculated. The assay was applied for the determination of sulfated glycosaminoglycans in normal and cancerous human laryngeal cartilage samples. By using this procedure, the accurate determination, especially, of heparan sulfate in a mixture of glycosaminoglycans was achieved, which otherwise would require the use of very expensive technology.

INTRODUCTION

Glycosaminoglycans are heteropolysaccharides of varying size, involved in a wide range of different biologic processes. Seven different glycosaminoglycans have been recognized in mammalian tissues, namely hyaluronan, chondroitin sulfate A (chondroitin-4-sulfate), chondroitin sulfate B (dermatan sulfate), chondroitin sulfate C (chondroitin-6-sulfate), keratan sulfate, heparin, and heparan sulfate.(1–3) They are all linear polysaccharide chains of repeating disaccharide units, consisting of one hexosamine and one hexuronic acid or neutral hexose. Hexosamine is glucosamine in the case of glucosaminoglycans, such as hyaluronan, keratan sulfate, heparan sulfate and heparin and galactosamine in the case of galactosaminoglycans, such as chondroitin sulfate and dermatan sulfate. Hexuronic acid is glucuronic acid in the case of hyaluronan and chondroitin sulfate, while dermatan sulfate, heparan sulfate, and heparin contain both glucuronic and iduronic acids, the relative proportions of which depend on the tissue source. Keratan sulfate is the only glycosaminoglycan that contains a neutral hexose in its disaccharide unit instead of a hexuronic acid. Hyaluronan is the only unsulfated glycosaminoglycan, while all others contain variable number of sulfate ester groups in their hexosamine residues, being slightly smaller than one in the case of chondroitin/dermatan sulfate and higher than one in the case of heparin/heparan sulfate. All glycosaminoglycans, except hyaluronan, are present in the various tissues under the form of proteoglycans.(1–3)

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Laryngeal cartilage is a tissue characterized by a high content of chondroitin sulfate organized under the form of aggrecan, decorin, and biglycan, which have been proposed to be the major proteoglycans found in the tissue.(4,5) Minor amounts of fibromodulin(6) and syndecan(7,8) have also been identified. In malignant cases of laryngeal cartilage, alterations of proteoglycan and glycosaminoglycan content and composition have been reported to occur. Iirregular expression of hyaluronan,(9) loss of chondroitin-6-sulfate,(10) overexpression of mRNA of perlecan,(11) and loss of syndecan(8) are among these. It has also been reported that patients with head and neck tumors excrete a low sulfated chondroitin sulphate.(12) It seems, therefore, that quantitation of glycosaminoglycans and their alterations in a tissue specimen may provide evidence for the tissue status. However, the alterations are minimal in the onset of the malignancy and can not be identified by conventional analyses.

Analysis of the various glycosaminoglycans is performed through a variety of procedures depending on the amount of glycosaminoglycans available. The colorimetric procedures initially proposed(13–16) determine accurately 2 μg of glycosaminoglycan, and in one of these, the dye binding assay,(14) the detection limit is scaled down to 250 ng in its semi-automated form.(17) Procedures even more sensitive that employ HPLC and HPCE analysis of either the disaccharides produced after chondroitinases and/or heparitinases treatment, or the individual sugar constituents after their liberation with acid hydrolysis, have been proposed, which decrease the detection limit down to the nanogram scale.(3,18) Another, very sensitive, assay that can be used for the determination of the sulfated glycosaminoglycans has been developed in our laboratory.(19–21) The detection limit of this assay is a few nanograms but it seems to be more applicable than HPLC or HPCE techniques, since it does not require any degradation step and over 90 samples can be analyzed at once. Other specific procedures have also been established for the determination of minor amounts of heparin, based on its anticlotting activity and for hyaluronan, based on its ability to bind cartilage link protein(22) or aggrecan G1 domain.(23) Another procedure as also been proposed to distinguish the various glycosaminoglycans when they are in a mixture, based on their different susceptibility in nitrite, because of the presence of IdoA residues.(24)

The quantitation of glycosaminoglycans is of great importance, especially when differences in the composition of a tissue sample or of a cell culture have to be detected for diagnostic purposes, such as cancer,(25–32) inflammatory diseases,(33–35) and degeneration or ageing.(36–39) In such cases, due to sample limitation, development of highly sensitive techniques

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with no further specific handling and/or sophisticated requirements is of great importance. The aim of the present work was, therefore, the development of a sensitive and accurate assay for the determination and identification of sulfated glycosaminoglycans by a solid-phase assay, which may be very useful for routine analytical purposes and the application of this procedure for the determination of glycosaminoglycans, with particular reference to heparan sulfate, in human normal and malignant laryngeal cartilage and the detection of any alterations in their content and composition.

EXPERIMENTAL

Chemicals and Biological Material

Polystyrene plates were obtained from Kima (Italy). Glutaraldehyde (GH), 70% (w/w) was purchased from Serva (Germany). Biotin hydrazide, avidin conjugated with peroxidase, chondroitin sulfate type II (equimolar mixture of chondroitin sulfate from shark and whale cartilage), papain, chondroitinase ABC, heparitinases I, II, and III, and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDAC) were obtained from Sigma Chemical Co. (USA). All other chemicals used were of the best available grade.

Chondroitin sulfate from pig laryngeal cartilage was isolated after alkaline treatment and DEAE-Sephacel ion exchange chromatography(40) and biotinylated as described.(21) Heparan sulfate from bovine intestinal mucosa was obtained from Sigma Chemical Co. (USA).

Human laryngeal cartilage was obtained from larynx after total laryngectomy for laryngeal carcinoma, classified in T4 stage. Two specimens were obtained from each patient, one from normal and the other from cancerous tissue.

Analytical Methods

Quantitation of the various glycosaminoglycans in solution was performed with the dimethylmethylene blue (DMMB)(17) assay. The concentration of the commercial preparation of glycosaminoglycans was calculated from the weight of glycosaminoglycan dissolved in known volume of double distilled water.

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Enzymatic Digestions

Digestion of laryngeal cartilage with papain was performed overnight at 60°C in 0.1 M sodium acetate buffer, pH 6.5, containing 10 mM disodiumEDTA and 5 mM CysteineHCl, using 25 units of the enzyme and 10 ml of buffer per g of wet weight of tissue.(41) The digestion was terminated after boiling the solution for 5 min and then the digests were centifuged for 5 min at 6,000 rpm in an Eppendorff centrifuge to remove the papain precipitates and any undigested tissue. The clear supernatants were used for further experiments.

Digestion of the various glycosaminoglycans with either chondroitinase ABC or with a mixture of heparitinases I, II, and III was performed in 0.1 M Tris/HCl, pH 7.3, for 8 h at 37°C, using 0.5 units of the enzyme per 1 mg of substrate in 1 ml of buffer. In the case of heparitinases, calcium chloride was included in the incubation buffer at a final concentration of 50 nM. The enzymatic digestions were terminated after boiling the solutions for 5 min. The molecular weight of the products was determined by measuring the absorbance at 232 nm, where the double bonds formed by the action of enzymes absorb, and by using the molecular extinction coefficient $E_{232} = 5{,}500\ \mathrm{M}^{-1}\,\mathrm{cm}^{-1}$.(40)

Quantitation of Glycosaminoglycans and Oligosaccharides

The steps of the assay applied in the present study involved the coating of polystyrene plate wells with 100 μL of glutaraldehyde solution (1.25 mM) in sodium phosphate (pH 5.0; 0.1 M), followed by reaction with 100 μL of spermine (50 μM) in sodium phosphate (pH 9.0; 0.1 M).(19–21) Then, biotinylated chondroitin sulfate (b-CSA), in 10 mM sodium phosphate, pH 4.3, containing 0.14 M sodium chloride, 0.1% (w/v) bovine serum albumin and 0.1% (v/v) Tween-20 (PBS-T-BSA) was added onto the wells to bind electrostatically to spermine, together or not with reference glycosaminoglycans or samples. The latter competed the interaction of b-CSA and spermine to various extents according to their charge density.(21) After, biotin bound onto the wells was quantitated by avidin-peroxidase, diluted 1 : 5,000 in PBS-T-BSA, using o-phenylenediamine as substrate, and measuring the absorbance of the solutions at 492 nm. By plotting the per cent of competition measured against the concentration of each reference competitor used, almost linear curves were obtained. The curves were then used for the quantitative analysis of the respective competitor in samples.

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RESULTS

Quantitave Analysis of Sulfated Glycosaminoglycans

The interaction between spermine and b-CSA was highly specific and very sensitive. Very small amounts (30 ng) gave an absorbance value at 492 nm of 1.0 and by employing competitive binding using native glycosaminoglycans, it had been proposed that the assay could be a very sensitive tool for the determination of sulfated glycosaminoglycans.(21) In competitive binding experiments, however, different competition curves were obtained for reference chondroitin sulfate and heparan sulfate (Figure 1, A and B), suggesting that the determination of glycosaminoglycan content in a biologic specimen was not accurate, when both glycosaminoglycans were present. Digestion of the reference glycosaminoglycans with the respective enzymes showed that chondroitin sulfate oligosaccharides competed the interaction of b-CSA and spermine to smaller extent than the intact glycosaminoglycan. The competition depended on the size of oligosaccharides and reduced as the size of oligosaccharides reduced (Figure 1, A). Chondroitin sulfate disaccharides could not compete even at elevated concentrations (Figure 1, A). Heparan sulfate oligosaccharides behaved differently, and even disaccharides were strong competitors, comparable to the intact chain (Figure 1, B). This obsevation suggested that both glycosaminoglycans could be determined in a biologic sample following a pretreatment of the sample with the respective enzymes.

To establish this hypothesis, various mixtures of the reference glycosaminoglycans were prepared, and subjected directly to analysis following the procedure described above and outlined in scheme 1. It can be observed that the amounts and the ratios of the reference glycosaminoglycans determined in these synthetic mixtures were well in accordance with those used (Table 1). This finding suggested that the procedure could be used for the determination and the identification of the sulfated glycosaminoglycans in a biologic sample, especially when small amount of one of these was present.

Sulfated Glycosaminoglycan Content of Normal and Cancerous Human Laryngeal Cartilage

Human laryngeal cartilage, obtained after total laryngectomy, was cleaned from the surrounding tissues and the perichondrium and digested with papain. The sulfated glycosaminoglycan content of the digests was determined according to the DMMB assay and the solid phase assay proposed previously(21) for the determination of total glycosaminoglycans

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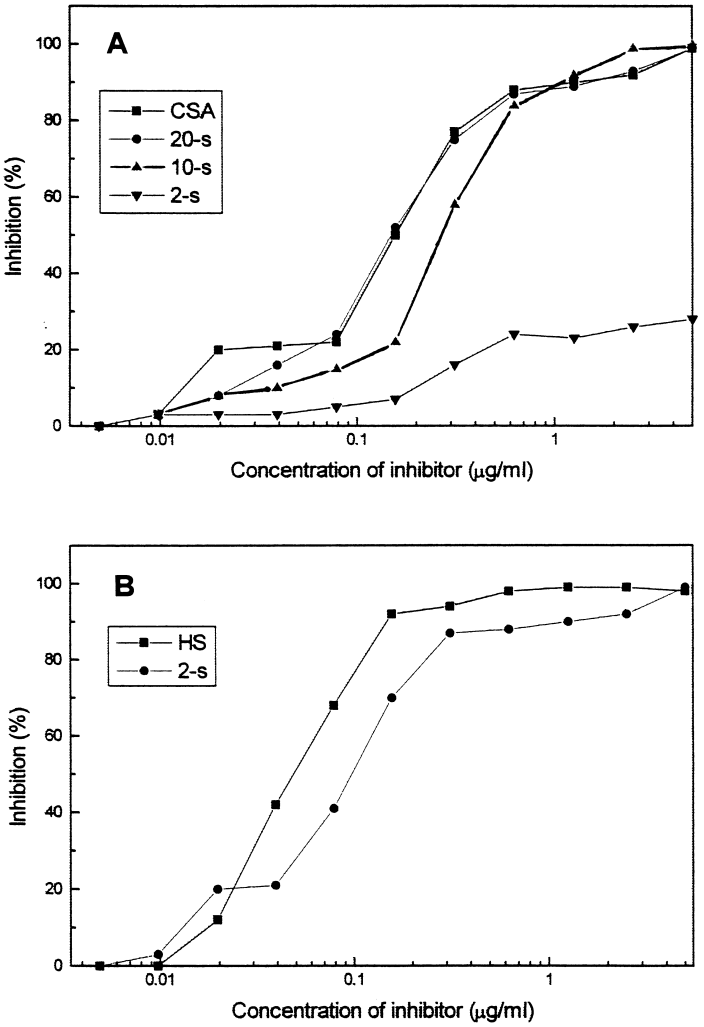

Figure 1. Competition of reference chondroitin sulfate and heparan sulfate in the interaction of biotinylated chondroitin sulfate with spermine immobilized onto ELISA plate wells. In activated wells 20 ng of biotinylated chondroitin sulfate mixed with various amounts of chondroitin sulphate (A) or heparan sulphate (B) or oligosaccharides thereof in 0.1 ml of buffer were added and the amount bound onto the wells was determined. Reference wells where no competitor was added were used to calculate the competition obtained in each concentration of competitor used. The competition was finally plotted against the corresponding concentration of competitor. (For details, see text.).

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(Table 2). The two assays gave slightly different results. The glycosaminoglycan content of normal samples calculated from the DMMB assay was found to be higher than the respective from the solid phase assay and this could be attributed to the amounts of keratan sulfate present in the samples, which could not be determined by the solid phase assay.(21) On the other hand, the glycosaminoglycan content of the cancerous samples calculated

Table 1. Determination of Chondroitin Sulfate/Heparan Sulfate Ratios in Various Synthetic Mixtures by the Competitive Binding Assay Using the Procedure Outlined in Scheme 1*

Initial Amount of CS Relative to HS	Determined Amount of CS Relative to HS
100	99.950
10	9.880
1	0.992
0.1	0.104
0.01	0.011

*The synthetic mixtures were prepared by mixing known amounts of pig laryngeal chondroitin sulfate and commercial heparan sulfate, the concentrations of which were determined by the dye binding assay.(17) The final glycosaminoglycan concentration of the synthetic mixtures used throughout the experiment was 40, 80, 120 and 160 ng/ml.

Table 2. Determination of Glycosaminoglycan Amounts of Normal and Cancerous Human Laryngeal Cartilage by the Dye Binding (DB) and by the Competitive Binding (CB) assays

	DB (mg/g Wet Weight of Tissue)		CB (mg/g Wet Weight of Tissue)	
Patient No.	Normal	Cancerous	Normal	Cancerous
1	12.986 ± 0.043	13.088 ± 0.054	12.787 ± 0.132	13.264 ± 0.138
2	13.073 ± 0.075	13.158 ± 0.064	12.907 ± 0.087	13.475 ± 0.116
3	13.156 ± 0.085	13.267 ± 0.093	13.004 ± 0.127	13.582 ± 0.143
4	13.228 ± 0.064	13.335 ± 0.088	13.117 ± 0.101	13.615 ± 0.154
5	13.029 ± 0.057	13.128 ± 0.073	12.902 ± 0.156	13.504 ± 0.178
6	13.124 ± 0.045	13.189 ± 0.058	12.995 ± 0.154	13.572 ± 0.169

Pig laryngeal cartilage chondroitin sulfate was used as reference.
Total glycosaminoglycans were measured in papain digests of the tissues.

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Table 3. Determination of Chondroitin Sulfate (CS) and Heparan Sulfate (HS) Content of Normal and Cancerous Human Cartilage by the Competitive Binding Assay

	Normal		Cancerous	
Patient No.	CS (mg/mL)	HS (mg/mL)	CS (mg/mL)	HS (mg/mL)
1	12.680 ± 0.199	0.088 ± 0.026	12.728 ± 0.245	0.556 ± 0.078
2	12.797 ± 0.145	0.126 ± 0.022	12.732 ± 0.238	0.748 ± 0.077
3	12.882 ± 0.156	0.134 ± 0.016	12.825 ± 0.262	0.785 ± 0.083
4	12.914 ± 0.176	0.113 ± 0.023	12.856 ± 0.237	0.704 ± 0.056
5	12.785 ± 0.168	0.104 ± 0.030	12.828 ± 0.214	0.638 ± 0.072
6	12.843 ± 0.115	0.147 ± 0.021	12.787 ± 0.218	0.798 ± 0.081

The samples were first digested exhaustively with chondroitinase ABC and the amount of HS was calculated from the reference curve. The amount of CS was calculated after exhaustive digestion of the samples with heparitinases and subtraction of the amount belonging to HS disaccharides.

from the solid phase assay was found to be higher than the respective from the DMMB assay. This result could be explained only by the presence of an oversulfated glycosaminoglycan molecule in the cancerous samples.(21) In addition, both assays gave an increase in the total glycosaminoglycan content of the cancerous samples compared to the respective normal ones.

The samples were also subjected to analysis using the assay proposed in the present study, following the calculations outlined in scheme I after digestion with either chondroitinase ABC or heparitinases I, II and III (Table 3). The analytical results in this case were more accurate, since each glycosaminoglycan was determined separately, and revealed a substantial increase in the absolute amount of heparan sulfate in the cancerous cartilage, being almost six times. Chondroitin sulfate amount seemed not to be altered compared to that of the normal tissue.

DISCUSSION

The present study was undertaken to establish a new methodology for the determination of the various sulfated glycosaminoglycans in a biologic sample, when small amounts of one of them are present. In addition, to apply this procedure for the identification and the determination of the sulfated glycosaminoglycans in normal and cancerous human laryngeal cartilage. The methodology exploited the sensitivity of biotin-avidin

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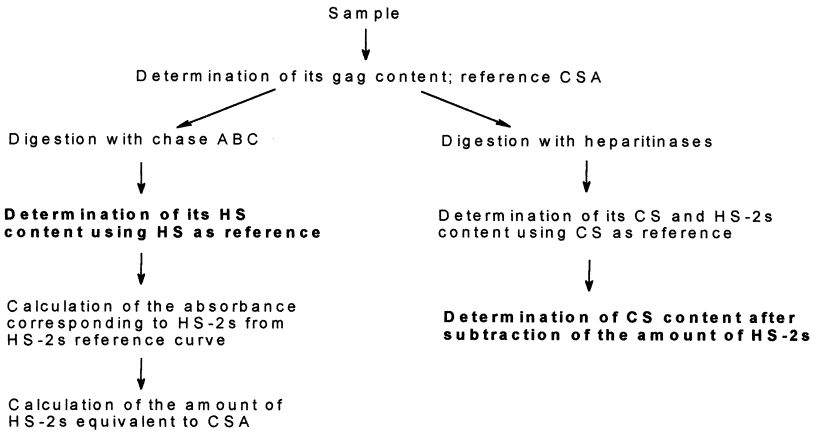
complex formation and the ability of analysis of large number of samples on the same time by using ELISA plates. It was a competitive binding assay, where the glycosaminoglycan under analysis competed the interaction of b-CSA with spermine bound onto the plate wells, and its amount was calculated from a reference curve. By applying this methodology for the quantitative analysis of the glycosaminoglycans in a synthetic mixture, it was found that very little amounts of one glycosaminoglycan in the presence of huge amounts of another could be determined following specific enzymatic treatment. The digestion of chondroitin sulfate with chodroitinase ABC produced disaccharides that could not compete with b-CSA in its interaction with immobilized spermine. Thus, after this treatment, the competition measured would be attributed to only heparan sulfate and its amount could be determined from the reference curve. The amount of chondroitin sulfate could be determined directly after subtraction of the amount of heparan sulfate from the total amount of the glycosaminoglycans measured. However, when large amount of chondroitin sulfate is present in a biologic sample, as in the case of laryngeal cartilage, it is preferred to apply the analytical procedure exactly as it is described in Scheme 1.

The proposed procedure was very simple and with high accuracy. It was very sensitive and it could be used for the determination of as little as 1.3 ng of heparan sulfate or 13 ng of chondroitin sulfate, due to the biotinylation step. Its major advantage was the use of ELISA plates, which permitted the simultaneous determination of large number of samples.

A small number of studies have been directed to human laryngeal cartilage and the knowledge regarding the glycosaminoglycans of this



Scheme 1.

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type of cartilage has come mainly from studies of pig laryngeal cartilage. From these studies it is known that laryngeal cartilage contains the three interstitial proteoglycans, namely aggrecan, biglycan and decorin, which are substituted with chondroitin sulfate chains.(4,5) In human samples, minor amounts of the heparan sulfate proteoglycans syndecan and perlecan are present. In the case of cancer of human laryngeal cartilage, alterations in the content of syndecan and perlecan it is reported to occur,(7,10,11,42) which would affect the heparan sulfate content of the tissue. These alterations should be quite small because of the cell surface localisation of syndecan and perlecan. Therefore, using convenient methodology it was difficult to quantitate accurately the glycosaminoglycan composition of human laryngeal cartilage and to detect any alterations due to various diseases such as cancer. By applying the procedure presented here, it was possible to quantitate accurately the amount of each sulfated glycosaminoglycan being present in both normal and cancerous tissue.

The inability of the procedure presented here to quantitate hyaluronan was not a disadvantage, since this polysaccharide can be quantitated from the difference of the result from the borate-carbazole reaction and that of the competitive assay, or directly by other assays.(3,11,22,23) Moreover, other, dye-based assays,(15–17) applied to glycosaminoglycan quantitation, can not detect hyaluronan. Similarly, the amount of keratan sulfate in a glycosaminoglycan mixture can be quantitated by specific immunoassays.(43–45)

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