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Review

Advances in analysis of glycosaminoglycans: its application for the assessment of physiological and pathological states of connective tissues

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Abstract

Glycosaminoglycans are a class of biological macromolecules found mainly in connective tissues as constituents of proteoglycans, covalently linked to their core protein. Hyaluronan is the only glycosaminoglycan present under its single form and possesses the ability to aggregate with the class of proteoglycans termed hyalectans. Proteoglycans are localised both at the extracellular and cellular (cell-surface and intracellular) levels and, via either their glycosaminoglycan chains or their core proteins participate in and regulate several cellular events and (patho)physiological processes. Advances in analytical separational techniques, including high-performance liquid chromatography, capillary electrophoresis and fluorophore assisted carbohydrate electrophoresis, make possible to examine alterations of glycosaminoglycans with respect to their amounts and fine structural features in various pathological conditions, thus becoming applicable for diagnosis. In this review we present the chromatographic and electromigration procedures developed to analyse and characterise glycosaminoglycans. Moreover, a critical evaluation of the biological relevance of the results obtained by the developed methodology is discussed.

Keywords: Reviews; Glycosaminoglycans

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1. Introduction

Glycosaminoglycans (GAGs) are a specific class of biologic macromolecules distributed among all organisms. They are major structural components of proteoglycans (PGs), the main complex macromolecules of the extracellular matrix [1–3]. Evidence derived from studies during the last years in the fields of biochemistry, cell and developmental biology suggests that proteoglycans are not only structural components, but they participate in and regulate many cellular events and physiological processes, including cell proliferation and differentiation, cell–cell and cell–matrix interactions [4–8]. Their potential for being interactive in such a degree stems from the great structural diversity in their GAG chains, i.e., the GAG type, size and composition, as well as the degree of substitution and domain arrangement. At least six types of GAG chains have been identified so far in higher mammals.

GAGs are linear polysaccharides composed of variable number of repeating disaccharide units. Each disaccharide consists of one hexosamine (D-galactosamine or D-glucosamine) and one uronic acid (D-glucuronic acid, GlcA or L-iduronic acid, IdoA) or neutral hexose (D-galactose, Gal). According to the type of the monosaccharides and the glycosidic bonds between them, GAGs can be divided into four main categories: (1) hyaluronan (HA) (Fig. 1A), (2) chondroitin sulfate (CS) and dermatan sulfate (DS) (Fig. 1B), (3) heparan sulfate (HS) and heparin (Fig. 1C), and (4) keratan sulfate (KS) (Fig. 1D).

The repeating unit of HA is [→4GlcAβ1→3GlcNAcβ1→] and it is the only non-sulfated GAG, i.e., none of its hydroxyls is esterified with a sulfate group. It is synthesised by hyaluronan synthase in the plasma membrane, where the elongation occurs by the alternate addition of UDP-glucuronic acid and UDP-*N*-acetyl-glucosamine at the reducing end of the nascent chain [9]. Unlike all other GAGs, HA isolated from various tissues is not covalently bound to a protein core, although preparations of HA contain tightly bound small amounts of protein [10]. It is by far the largest GAG (>2500 disaccharide units) [11]. HA is found not only in connective tissues but also in all tissues and fluids of the body, seeming that most cells synthesise HA at some stage of their life [12] and it possess several functions. It interacts with a family of aggregating proteins, the hyaladherins, and these interactions are important in tissue morphogenesis, angiogenesis and tissue remodeling [12,13]. HA plays a particular role in cancer cell microenvironment. Cancer cells exhibit binding-sites (CD44, RHAMM) for HA and their adhesion to it can influence the cell motility; different factors may interplay to facilitate cell detachment from HA [14]. Increased amounts of HA are found in the affected tissues/fluids in many malignant cases, such as malignant mesothelioma, and in other diseases, such as atherosclerosis and exfoliation syndrome. Increased levels are also found in the serum of patients with inflammatory rheumatic diseases, chirrotic liver diseases or malignant mesothelioma. This is due to increased synthesis of HA in the tissues owed to stimulation of cells by different inflammatory mediators and growth factors, except in the case of liver chirrosis, where the removal of HA from the serum is reduced [15]. Thus, analysis of HA has been proposed to be useful for diagnosis especially of malignant mesothelioma (pleural fluid), rheumatoid arthritis and liver diseases (serum) [16–18].

All other GAGs are synthesised as PGs in the Golgi apparatus by elaborate biosynthetic machinery. Biosynthesis of chondroitin sulfate, dermatan sulfate, heparin and heparan sulfate is initiated by a tetrasaccharide linkage region (GlcA1β→3Gal1β→3Gal1β→4Xyl1β→), linked via an *O*-

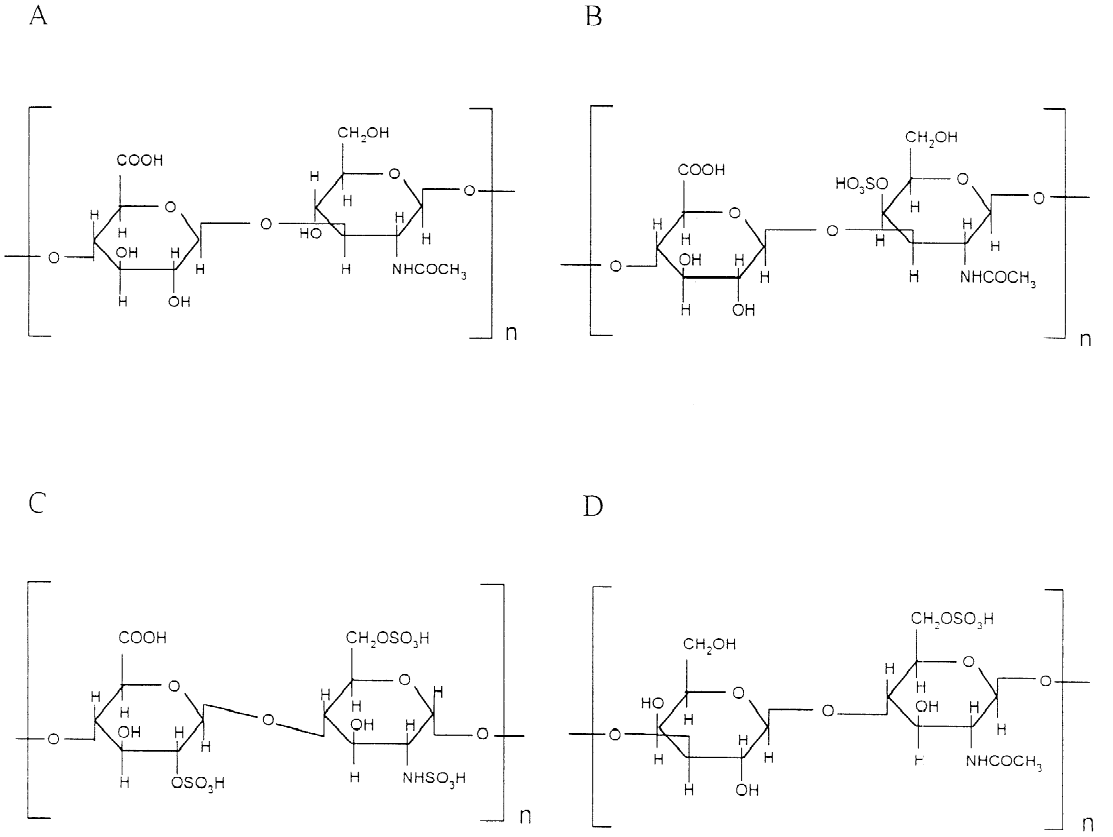

Fig. 1. Representative structures of hyaluronan (A), chondroitin sulfate (B), heparan sulfate (C) and keratan sulfate (D). Dermatan sulfate and heparin are hybrid structures containing GlcA and IdoA. Hexosamine in heparin/heparan sulfate may be either *N*-acetylated or *N*-sulfonylated.

glycosidic bond to certain serine or threonine residues in a specific protein, the protein core. The GAG chain is elongated on this tetrasaccharide by alternative additions to the non-reducing termini of GlcA and GalNAc, both derived from UDP-sugar donors. With 3′-phospho-adenosine-5′-phopshosulfate (PAPS) as a donor of sulfate group, a series of enzymic modifications of the growing polysaccharide chain determines the final structure of GAGs. Their molecular mass is usually about 20 000 (CS) and 30 000 (DS).

Chondroitin sulfate and dermatan sulfate chains are constructed by the repeating unit [→4GlcAβ1→3GalNAcβ1→], which is then sulfated at the C-4 or C-6 positions of GalNAc. Some, but little, of these positions remain unsulfated. In the case of DS, further enzymic modifications complete the final structure, such as C-5 epimerization of GlcA to IdoA, and *O*-sulfation at C-2 of IdoA. L-Iduronic acid imparts conformational flexibility to the dermatan sulfate chain. It further alters the shape and spatial orientation of sulfate residues, endowing the chain with higher negative charge content than the GlcA [19,20]. Although the principles of the biosynthetic process are not yet fully elucidated, it is well known that this process results in the generation of highly modified oligosaccharide domains within the polymer chain which are separated by regions of relatively low-degree structural modifications. Thus, the DS chain has a hybrid co-polymeric structure consisting of low modified (CS) and highly modified (DS) domains [21]. The IdoA-containing units are often sulfated at C-4 of the GalN, while sulfation at C-6 is frequently associated with GlcA-containing

disaccharides [22]. Twenty-three different CS/DS disaccharides have been identified so far [22]. The detailed structure of this GAG is modified during certain diseases, such as in all types of arthritis, atherosclerosis and cancer. The well described structural modifications involve changes in ratios of IdoA to GlcA and of 4-sulfated disaccharides to 6- and non-sulfated disaccharides. Changes in the size of chains are also described. Many of these alterations can be observed after analysis of CS/DS in urine of patients.

Heparin and HS have a distinctly different repeating disaccharide structure than the previous GAGs, that is [→4GlcAβ1→4GlcNAcα1→]. The distinction of these GAGs is confused, due to their structural similarity, however heparin is more negatively charged than HS. They follow different biosynthetic steps in different cells and in different core proteins. The glycosidic linkage between uronic acid and GlcN is β1→4 instead of β1→3, and that between GlcN and uronic acid is α1→4 instead of β1→4. The growing GAG polymer chain is *N*-deacetylated and *N*-sulfonylated at the glucosamine residues, yielding regions in the chain particularly available to further structural changes: C-5 epimerization of GlcA and O-sulfation mainly at C-2 of IdoA and C-6 of glucosamine [23]. Other more infrequent *O*-sulfations occur at C-2 of GlcA and C-3 of *N*-sulfonylated glucosamine. A few of the glucosamine amino groups may also remain unsubstituted. This process yields hybrid structures with hyper-variable, highly sulfated domains and poorly modified ones. Thirteen different disaccharide structures have been recognised. Heparin has the highest charge density of any known biological macromolecule, while HS is generally less sulfated and with lower IdoA content and, thus, presents greater structural variability [24,25]. Both GAGs are highly polydisperse molecules, depending on tissue origin and status [26–28]. Heparin exhibits diverse biologic functions, its hallmark being the antithrombotic activity. HS participates in a large number of interactions with other effective extracellular and cell membrane molecules, such as growth factors, integrins and thrombin/antithrombin [29–33]. Therefore, the analysis of heparin/HS, which may identify structural variations due to differential expression of the corresponding PGs and/or of the enzymes responsible for their biosynthesis, may provide evidence for tissue and cell status. Such structural changes are observed in cancer and correspond mainly to size changes and degree of epimerisation and of *O*- and *N*-sulfonylation of glucosamine. HS of various sizes is observed in the urine of patients of many of the known mucopolysaccharidoses (MPSs) due to deficiency in specific degradative enzymes.

The repeating disaccharide unit of KS [→3Galβ1→4GlcNAcβ1→] contains a galactose residue instead of uronic acid and the glycosidic bonds are reversed, compared to those in HA and CS/DS. Sulfate esters are present at the C-6 of one or both of the component monosaccharides, but any other hydroxyl group may carry an esterified sulfate group [34]. This GAG is synthesized on two different linkage oligosaccharide precursors, the structure of which depends on the type of glycosidic linkage on the protein core. *N*-glycosidic linkage, usually found in the cell-bound glycoproteins, is characteristic of the corneal KS (KS I) and *O*-glycosidic linkage, usually found in mucins, a specific type of glycoproteins, is characteristic of the cartilage KS (KS II). Sialic acids attached to this *O*-linked oligosaccharide regulate the synthesis of KS; KS elongation can occur by glycosyl-transferases and sulfotransferases only when sialic acids are not present in the non-reducing terminal of the chain. Once the sialic acids are added, the elongation of KS chains is terminated. The molecular mass of KS is usually small, and it increases with ageing [35]. The main tissue containing KS is cartilage and thus the determination of KS in serum, urine or synovial fluid is proposed as a marker for the measurement of cartilage damage in joint diseases.

GAGs may be implicated in a disease for a variety of reasons. There may be an in-built structural defect in the GAGs, as in the case of KS in corneal macular dystrophy, where, due to lack of sulfation, precipitates form and with time accumulate in the collagen lattice (which is less organised) of the stroma, leading to increased corneal opacity. Alternatively, defects may be present, for example, in matrix proteins with which GAG chains are involved, or deficiencies may be present in biosynthetic or catabolic steps of GAGs. Among the latter, characteristic examples are many types of cancer, where impairment in the biosynthesis of GAGs is observed,

Table 1
Examples of diseases in which GAG chains are implicated

Disease	Tissue affected	GAG altered in tissue	GAG altered in blood or urine
Gastrointestinal adenocarcinoma	Gastrointestinal tract	HA, CS/DS, HS	?
Degenerative joint diseases	Cartilage	HA, CS	HA, KS in serum
Macular dystrophy	Cornea	KS	?
Chirrosis	Liver	HA	HA in serum
Atherosclerosis	Blood vessels	HA, CS/DS	?
Mucopolysaccharidoses	Skeleton, nervous system, cornea	?	DS, HS, KS in urine, depending on the type
Marfan's syndrome	Cartilage	CS, KS	?
Metatropic dysplasia II	Cartilage	?	KS in urine
Exfoliated syndrome	Almost all eye tissues	HA	?
Brachymorphy	Cartilage	CS	?
Malignant mesothelioma	Pleural fluid	HA	HA in serum

and all MPSs, where an inherited deficiency of at least in one enzyme involved in GAG catabolism is found. Some characteristic diseases, the tissue and GAG affected are shown in Table 1.

2. Methods of analysis

In earlier years, isolation and characterisation of GAGs was achieved by solubilising the tissue with a proteolytic enzyme, usually papain, and then fractionating the mixture by either ion-exchange chromatography on Dowex or DEAE-cellulose or on CPC-cellulose columns [36]. The isolated GAGs were quantitated by using a colorimetric reaction and were further characterised by chemical analysis, solid infrared spectroscopy, electrophoresis and digestion with hyaluronidase (for a review, see Ref. [37] and references therein). In cases when no isolation of individual GAGs was required for their quantitation, or when the amounts of GAGs were very low, the samples were electrophoresed on cellulose acetate stained with toluidine blue, and quantified by scanning the strips in a densitometer. During the last years, specialised liquid chromatographic and electrophoretic instruments have been developed, together with sensitive detection systems, and therefore, very low amounts of GAGs can be separated and quantified accurately.

Currently, GAGs may still be isolated from a number of biological sources, tissues, cells, fluids either from PGs extracted with solutions containing chaotropic reagents and detergents or from direct protease digests of sample. For a more detailed description of procedures followed for the isolation of PGs and GAGs, the interested reader is referred to a published review article [38].

The analysis of glycosaminoglycans by the various separation techniques is performed by using either intact molecules/metabolic products or degraded molecules. A number of strategies may be used for GAG analysis according to the information desired and in all cases, similar methodology is used, with little variations.

2.1. Analysis of intact molecules or metabolic products

Analysis of intact polymers may give information on the charge polydispersity and the molecular size of GAG chains. The techniques proposed involve the direct separation of the GAGs under investigation followed by detection and quantitation or first the derivatisation of the molecules and then the subsequent separation. Chromatographic and electrophoretic techniques are used.

The detection and quantitation is usually accomplished by either far UV (210 nm) or after reaction with a cationic dye (alcian blue, dimethylmethylene blue, etc., and measurement of the colour formed in the appropriate wavelength) or by a colorimetric assay (borate–carbazole reaction). When the GAGs are first derivatised, a fluorophore such as 2-aminoacridone (2-AMAC) is usually introduced thus adding to the sensitivity of the detection.

2.2. Analysis of degraged molecules

GAG structure is traditionally studied by scission of the chains using specific enzymes and/or chemical treatments and analysis of the products using various analytical techniques, such as electrophoresis, high-performance liquid chromatography (HPLC), nuclear magnetic resonance (NMR) and mass spectroscopy [39–43]. The GAGs under investigation are first degraded with specific enzymes and then the degradation products are subsequently either separated using an appropriate procedure followed by detection and quantitation or derivatised first and then subjected to separation and quantitation.

The enzymes that are mainly used for the degradation of the GAGs belong to the family of chondro/dermato lyases and heparin- and HS-lyases. All act on the β(1→4) bond between hexosamine and uronic acid and produce disaccharides possessing a double bond between C-4 and C-5 of the uronic acid. The unsaturated uronic acid strongly absorbs ultraviolet light at 232 nm ($E=5500\ M^{-1}\ cm^{-1}$) and this wavelength is used for the quantitation of the products.

Chondroitinase ABC degrades HA, CS and DS, chondroitinase AC HA and CS, whereas chondroitinase B degrades only DS. A combination of enzymes may also be used for the characterisation of these GAGs in a biologic sample. When quantitation of GAGs is more significant than the identification of the various disaccharides present in a sample, chondro-4- and chondro-6-sulfatases are used to remove the sulfate ester groups from the disaccharides.

There are three different enzymes that degrade heparin/HS, known as heparin- and HS-lyases. They degrade heparin/HS according to the type of uronic acid and sulfation. Heparin lyase I (known also as heparinase, since it mainly degrades heparin) cleaves the GlcNS(±6S)(±3S)α(1→4)IdoA(±2S) bond, heparin lyase II (heparinase II) is catalytically active toward both types of uronic acid and therefore, possesses the ability to degrade both heparin and heparan sulfate regions of GAGs. Heparin lyase III (heparitinase I), which mainly degrades the HS chains, attacks the glycosidic bonds between GlcNAc or GlcNS that does not contain a sulfate ester group at C-6 and non-sulfated GlcA. As it has been reported [44], heparin- and HS-lyases can be used in combination when degradation of heparin and HS chains is required, since more than 92% of the polymers are degraded to Δ-disaccharides.

The commercial availability of the above-mentioned lyases has made possible their use by a number of different laboratories, applying modified digestion protocols. The conditions normally used are as follows. Simultaneous digestions of the galactosaminoglycans (GalAGs) with chondroitinases ABC or AC and chondro-4- and -6-sulfatases (0.1 units of each per mg polysaccharide) are performed in 50 μl solution of 50 m*M* Tris–HCl, pH 7.5, at 37 °C for 90 min. Digestion with chondroitinase B is carried out in 50 m*M* Tris–HCl, pH 8.0, at 37 °C for 60 min. Digestions of HS and heparin are performed in 100 μl of 20 m*M* acetate buffer, pH 7.0, containing 1 μmol calcium acetate and 0.3 units of each of heparin lyase I and heparinases II and III per mg polysaccharide dry mass at 37 °C for 90 min. The end point of all enzymic depolymerisations is recorded by a stable value at 232 nm. The reactions are terminated by heating the solutions at 100 °C for 3 min. Following centrifugation in a microfuge (11 000 *g*) for 5 min, aliquots are used for direct analysis of variously sized and sulfated Δ-saccharides.

Streptomyces hyalurolyticus hyaluronidase is another useful enzyme in the analysis of GAGs. It splits exclusively the *N*-acetylglucosaminidic linkages in HA, yielding unsaturated tetrasaccharides and hexasaccharides as end-products. Other hyaluronidases, which are used in many procedures, cannot be used for analytical purposes, since they give products of varying size, some owed to transglycosylation reactions, and all lack double bond in the uronate residues due to the hydrolytic nature of the enzymes.

Apart from the various lyases, some hydrolases, such as keratanases I and II can be used for the specific degradation of keratan sulfate. Keratanase I catalyses the hydrolytic cleavage of β(1→4) galactosidic linkages between non-sulfated galactose and *N*-acetylglucosamine-6-sulfate of KS and related glycans. Keratanase II catalyses the cleavage of β(1→3) glucosaminidic linkages of *N*-acetylglucosamine-6-sulfate, the major products being

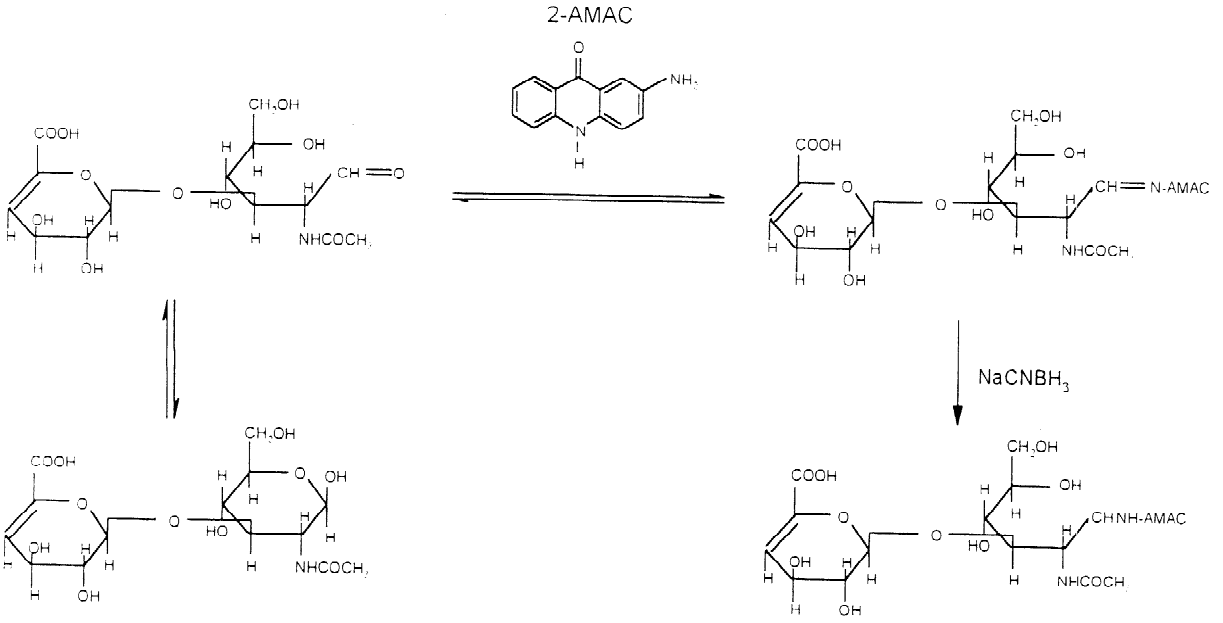

Fig. 2. Reaction of reducing sugar residue with 2-aminoacridone (2-AMAC).

mono- and disulfated disaccharides. Oversulfated regions remain unaffected by the enzymes.

Labelling of glycosaminoglycans or their degradation products with 2-AMAC is a very simple reaction (Fig. 2), which can be carried out in any conventional laboratory. The steps involved are as follows [45,46]: (1) preparation of a 0.1 *M* AMAC solution in dimethylsulfoxide–acetic acid (85:15, v/v). (2) Mixing of the dried samples with 5 μl of AMAC solution and incubation at 37 °C for 5–15 min. (3) Addition of 5 μl of a 1 *M* solution of sodium cyanoborohydride in water and incubation at 37 °C for 16 h. (4) Cooling to room temperature and mixing with 30 μl of 25% (v/v) glycerol. The derivatised samples can be used directly or stored at −80 °C for up to 6 months.

3. Chromatographic methods

Chromatographic procedures are widely used for the separation and characterisation of GAGs. Ion-exchange and gel permeation are the earliest types of chromatography applied, because charge density and molecular size are thought to be characteristic for each GAG and any alteration observed in a sample would reflect the metabolic status of the tissue from which it is obtained. However, since polydispersity is an inherent phenomenon of GAG preparations, other, more or less sophisticated procedures have been introduced for the characterisation of this family of macromolecules. Enzymic degradation is the step that is generally accepted as a prerequisite before any separation procedure is employed. Specific enzymic cleavage of a GAG using chondroitinases or heparin lyases, depending on the type of GAG the analysis is required for in a papain digest of a tissue, permits the identification of the cleaved GAG and its subsequent characterisation. Δ-Disaccharide analysis cannot be performed by the traditional chromatographic systems and therefore, HPLC methods have been developed and successfully used. Although HPLC methods do not generally provide complete information on the sequence of the GAG chains, nevertheless they become popular because they reveal the pattern of sulfation and therefore, the degree of modifications. Up to now, this has been the only available source of information that enabled an insight as to how GAG structure is modified during the progress of normal biological events or in certain

diseases, such as atherosclerosis and cancer [47–49]. Moreover, all GAGs present in a sample may be sequentially degraded by appropriate specific enzymes and thus, the full characterisation of all GAG chains present may be accomplished. Alternatively, the intact GAG(s) in the initial sample may be directly characterised, if a specific physical property, such as molecular size and charge density, modified during the development and progress of a certain disease can be detected.

3.1. HPLC disaccharide analysis of GAGs

Over the years various HPLC modes, including normal-phase, reversed-phase, ion-pair and ion-exchange chromatography, have been used for compositional disaccharide analysis of GAGs [43,50]. In general, analysis of unsaturated disaccharides is simple and rapid, avoiding any time-consuming derivatisation reactions. There is no need for special equipment, such as fluorescence detectors, and the sensitivity, provided by the absorption of unsaturated disaccharides at 232 nm, is satisfactory enough and comparable with the sensitivity obtained by derivatisation [21,44,51]. Derivatisation, however, may be useful for the sensitive detection of saturated saccharides, such as various normal metabolic products of GAGs. Pre-column derivatisation with 2-AMAC [52], 2-aminopyridine [53], and 2-aminobenzamide [54] has been reported. Recently, Volpi [55] reported that derivatisation with dansylhydrazine offers high sensitivity. Toyoda et al. [56] have used post-column derivatisation with 2-cyanamide. The majority of the separations have been performed on positively charged (amino) columns and elutions were carried out either by salt gradients or isocratic systems often at elevated temperatures.

Ion-pair reversed-phase HPLC has proved quite useful in GAG compositional analyses and the determination of oligosaccharide size [44]. Binding of tetrabutylammonium hydrogen sulfate (TBA) cation to the anionic charges of the Δ-saccharides (the carboxyl and sulfate groups) results in hydrophobic interactions of the TBA–saccharide complex with the C_{18} bonded phase of the stationary phase (Fig. 3). Thus, the increased number of anionic charges, owed to either the increased number of sulfates and/or the size of the saccharide, results in higher retention times. Due to this property, all non-, mono-, di- and trisulfated GAG-derived Δ-disaccharides with a sulfate/carboxyl ratio ranging from 0 to 3 were completely separated by ion-pair HPLC. In the case of GalAGs, Δdi-mono2S can also be resolved from Δdi-mono4S and Δdi-mono6S, and Δdi-di(4,6)S from Δdi-di(2,4)S and Δdi-di (2,6)S, as well. Consequently, the presence of Δdi-mono2S and Δdi-di(4,6)S in these digests can be demonstrated without further HPLC/HPCE analysis. In the case of heparin/HS-derived Δ-disaccharides, all known non-, mono-, di- and trisulfated Δ-disaccharides, bearing *N*-sulfonylated, *N*-acetylated or non-substituted glucosamine, are completely resolved by ion-pair

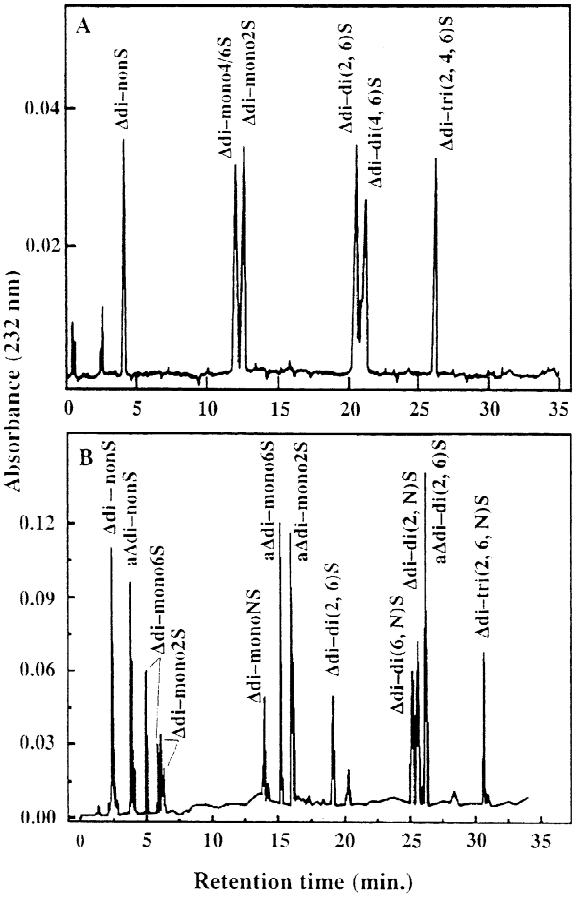

Fig. 3. Typical chromatograms showing the resolution of variously sulfated Δ-disaccharides of CS/DS (A), and heparin/heparan sulfate (B). Chromatography was performed on a reversed-phase column (Supelcosil LC-18) and elutions as described in Ref. [42].

HPLC [44]. For the determination of the exact sulfation pattern of GalAG-derived Δ-disaccharides, Karamanos et al. [22] have described an HPLC method, by which 23 different disaccharides in GalAGs and the HA-derived disaccharide can be determined on the same analytical column (Econosphere NH_2) through a series of three elutions. The column is eluted isocratically with: (1) 5 m*M* sodium dihydrogenorthophosphate, pH 2.55, for nonsulfated Δ-disaccharides, (2) 50 m*M* sodium dihydrogenorthophosphate, pH 2.50, for monosulfated, and (3) 50 m*M* sodium sulfate–10 m*M* sodium acetate, pH 5.0, for the separation of di- and trisulfated Δ-disaccharides. This method is very useful for disaccharide analysis, since monosulfated disaccharides at either C-2 or C-3 of the uronic acid moieties and mono-, di- and trisulfated disaccharides, as well as non-, mono-, and oversulfated disaccharides derived from IdoA can be identified.

3.2. Gel permeation chromatographic characterisation of GAGs

A recent development, alternative to Δ-disaccharide analysis by HPLC, is the gel chromatography behaviour of a GalAG-containing mixture before and after treatment with specific enzymes. Quantitative determination of the GalAG content in each chromatography fraction before and after the enzymic treatment enables one to determine the amount of the digested GalAG. In addition, from the difference in GalAG content in each fraction from both chromatographies, the elution profile of the digested molecules may be derived. By applying this methodology, the absolute content and the size distribution of the digested GalAG is obtained using a precalibrated column [57]. This procedure has been successfully applied to determine the net amounts and size distribution of CS and DS in samples from various cartilages [57] and from normal and cancerous tissues [58].

3.3. Ion-exchange chromatographic separation of GAGs

This type of chromatography, although it has been used for gross separation of various GAGs, it is not normally applied for fine characterisation of GAGs. Because of the natural structural variations of GAGs, this method is not sensitive enough to determine net alterations found in many pathological conditions. Ion-exchange chromatography, i.e., on DEAE-Sephacel, is mainly used for the enrichment of a tissue protease digest in GAGs or for the separation of glycopeptides and HA from the sulfated GAGs and their subsequent characterisation after enzymic treatment and HPLC or another analytical technique.

4. Electromigration methods

Electromigration methods include a variety of techniques involving separation on a solid support of biomolecules under analysis according to their charge density and/or molecular size; GAGs being highly negatively charged molecules are, therefore, separable by this technique. Separation, however, is accomplished by the combination of GAG size and charge density, which reflect structural differences between these molecules. The known electromigration methods are gel (polyacrylamide or agarose) electrophoresis, capillary electrophoresis (CE) and fluorophore-assisted carbohydrate electrophoresis (FACE). Capillary electrophoresis is a technique developed during the last decade. CE is a unique technique since it utilises the principles of separation of both electrophoresis and liquid chromatography, giving much more resolution and sensitivity and uses more sophisticated apparatus. Because of the various CE modes developed for the analysis of GAG-derived Δ-disaccharides and the increasing rate of evidence on separations by the use of capillary electrophoresis, this technique is reviewed separately.

4.1. Intact molecules

Intact GAGs or metabolic products thereof can be analysed by cellulose acetate, agarose or polyacrylamide gel electrophoresis. In all cases, the identification of bands is performed after reaction mainly with toluidine blue, although many other staining techniques are used, such as alcian blue, stainsall and silver nitrate. Quantitation of the resolved bands may be obtained by using image analysis systems, band density or digital scanning.

The conditions proposed for polyacrylamide gel electrophoresis permit the separation of GAGs mainly by their molecular size. However, the bands are highly diffuse and this does not allow detection of small molecular mass changes without the use of sophisticated gels, such as complicated polyacrylamide gradient gels. On the other hand, the so far applied cellulose acetate electrophoresis procedures permit the separation of the known GAGs based exclusively on their charge density. Once again, small molecular size changes cannot be detected. The most common conditions involve a monodimensional electrophoresis in bivalent cation buffers or pyridine–formate buffer. However, no such a system can separate all the known GAGs in one run and therefore, two-dimensional methods have been proposed with the disadvantage to examine only one sample per run. Comparison of the electrophoresis profiles before and after selective enzymic treatment allows characterisation of the major GAGs in a given sample and quantitative estimation.

4.2. Degraded molecules

4.2.1. Polyacrylamide gel electrophoresis

Because of the difficulties in detecting structural differences in intact GAGs, all attempts are focused on analyses of degradation products, especially Δ-disaccharides thereof. Degraded GAGs may be analysed either by polyacrylamide gel electrophoresis or capillary electrophoresis, with or without derivatisation of the molecules. The pioneer work from John Gallagher's laboratory [59–61] concerning the identification of radiolabelled heparan sulfate oligosaccharides after polyacrylamide gel electrophoresis has widely been regarded as a powerful tool for the separation and structural characterisation of GAG-derived oligosaccharides [39,62,63]. The advantage of this methodology lies on the ability of the electrophoretic system to detect not only Δ-disaccharides of different structures but also possibly less degraded regions of the molecules due to unusual structures. Employing fluorotagging of the enzymic products with 2-AMAC, the sensitivity of the detection system reaches down to pmol of product. This methodology is especially applicable for studies when small amounts of tissue or GAGs are available. Each lyase product contains a free reducing end that can be stoichiometrically coupled to 2-AMAC (or whatever fluorescent tag) via reductive amination and the labelled products can be resolved into discrete bands by electrophoresis on high percentage polyacrylamide gels. No specialised equipment is required for the application of such a procedure, which, in addition, does not involve preparative steps that can lead to losses of products.

When only the analysis of Δ-disaccharides or of Δ-oligosaccharides of small size is required less complicated gels than initially applied may be used. Turnbull [64] has proposed such a system for sequencing heparin/HS-derived saccharides of specific functional structures. Regardless of the separational system applied, fine differences in the structure of the digestion products, found in cancerous tissues, corneal macular dystrophy or osteoarthritis, can be easily revealed [45,65,66].

4.2.2. Capillary electrophoresis

The separation principles of the various HPCE operating modes, the ways of detection commonly used in carbohydrate analysis and applications of HPCE in the structural characterisation of GAG moieties of PGs have been extensively presented in the literature [67–78] (Fig. 4). The commonest and simplest mode of CE is capillary zone electrophoresis (CZE). Upon voltage application, the negatively charged wall of the uncoated fused-silica capillary causes electroosmotic flow (EOF) of buffer species, which moves all analytes towards the negative electrode (cathode). The separation of solutes is accomplished by the vector sum of EOF and their electrophoretic mobility (EM); the latter depending on the charge to mass ratio of the molecules. In the case of GAG-derived saccharides, which are inherently negatively charged, EOF and EM counteract each other, and the apparent migration depends on the net difference between these two driving forces. At acidic pH, EOF is so low that it cannot overwhelm the EM of anions. In this case, the polarity is reversed.

Micellar electrokinetic capillary chromatography (MECC) mimics reversed-phase HPLC conditions, since analytes interact with micelles in the operating buffer, which is also known as pseudostationary phase. MECC can easily be used for separation of

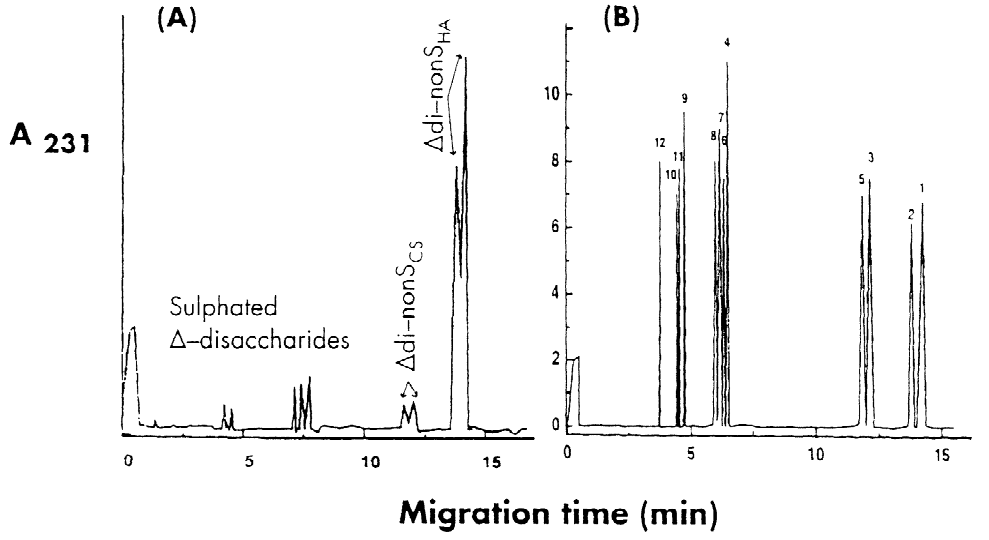

Fig. 4. (A) Typical CE separation of the non-sulfated disaccharides from HA and CS. Conditions are as described in Ref. [78]. (B) Typical electropherogram showing the complete resolution of all 12 known heparin- and HS-derived Δ-disaccharides. Analysis was performed at 25 °C, using a 50 m*M* phosphate buffer, pH 3.5, with reversed polarity at 30 kV and 25 °C using a HP^{3D}CE (Agilent Technologies) instrument with a built-in diode array detector set at 255 nm. Peaks: 1=aΔdi-nonS, 2=Δdi-nonS, 3=aΔdi-mono6S, 4=Δdi-mono6S, 5=aΔdi-mono2S, 6=Δdi-mono2S, 7=Δdi-monoNS, 8=aΔdi-di(2,6)S, 9=Δdi-di(2,6)S, 10=Δdi-di(2,N)S, 11=Δdi-di(6,N)S, 12=Δdi-tri(2,6,N)S. (From Ref. [142]).

both charged and neutral solutes bearing either hydrophobic or hydrophilic characteristics. In GAG analysis, the benefits of MECC have been exploited by the addition of detergents and other additives, such as sodium dodecyl sulfate (SDS) and cetyltrimethylammonium bromide (CTAB) in the alkaline borate buffers commonly used. Microemulsion electrokinetic capillary chromatography (MEEKC) is another recently introduced CE operation mode in which neutral and ionized species can be resolved according to their partitioning into oil droplets moving in the operating buffer. The alteration of the structure of the saccharides and of the CS-derived Δ-disaccharides using pre-column derivatisation with 2-AMAC has been exploited for the separation of the derivatised saccharides by MEEKC [79].

By and large, CZE analysis of different types of variously sulfated Δ-disaccharides involves two operating systems: one of reversed polarity at low pH and another of normal polarity at high pH. Triethylamine as an additive in alkaline borate buffer (pH ranging from 8.8 to 10.4) suppresses EOF and EM, and efficient separations of eight CS- and 11 HS-derived Δ-disaccharides have been obtained at normal polarity [80].

Analysis in acidic phosphate buffers (low pH) and reversed polarity provides a rapid resolution of all variously sulfated GAG-derived disaccharides [81–83]. The only problem that has been observed in this system is the lack of reproducibility of the migration times of non-sulfated disaccharides, which may be attributed to the stability of the capillary used [83,84]. Eight GalAG-derived Δ-disaccharides and the non-sulfated HA disaccharide are separated in a phosphate buffer at pH 3.0 [81]. All known 12 HS/heparin-derived Δ-disaccharides are separated at a pH of 3.5 [83]. The same separation conditions can be applied to AMAC-derivatives of these Δ-disaccharides [82].

The real benefits of CE are demonstrated by both the ease and speed of separations (an excellent resolution of all resultant disaccharides is accomplished in one run at short times), the trace amounts of samples required, and the high sensitivity. Ultraviolet detection at 232 nm of the Δ-disaccharides is quite satisfactory, as demonstrated by both the mass

detection limits (in the order of attomol) and the concentration detection limits (in the order of a few p*M*) [81]. Pre-column derivatisation with AMAC and detection with an Ar-ion laser-induced fluorescence (LIF) detector resulted in sensitivity 100- and 10-times higher than those obtained by ultraviolet detection at 232 nm of underivatised Δ-disaccharides and at 254 nm of those derivatised with aminoacridone, respectively [82]. It has been reported [85] that disaccharides derivatised with 7-amino-naphthalene-1,3-disulfonic acid (ANDSA) were detected by a He–Cd LIF detector and the detection limit was at the nanomolar level, three orders of magnitude lower than the limits of detection of underivatised disaccharides at 232 nm.

5. Hyphenation procedures

The establishment of the fine structure of the various GAGs can be facilitated by using a variety of spectroscopic techniques, such as NMR spectroscopy and mass spectrometry (MS). Highly purified GAG preparations or fragments thereof are required, and such samples have been used to identify specific structural characteristics and to detect unusual structures, especially in KS (for a review, see Ref. [86] and reference therein). In all cases, a separational system is firstly used to isolate and/or subfractionate the specific GAG product followed by characterisation of the isolated specific structures by these techniques. Coupling of separational modes to spectrometric detection instruments, although it has been extensively used for a variety of natural products and drugs, its use in studies of GAGs is up to now very limited [87,88]. In early studies of GAGs, however, gas chromatography (GC) coupled to MS was applied for the determination of fine structure of oligosaccharide-linkage region of GAGs [89]. Although this methodology is highly applicable for studying structures of neutral oligosaccharides, its application for structural elucidation or analysis of GAGs is limited, because uronic acids are vulnerable to such treatments.

6. Sensitivity and validation

The various procedures developed for the analysis of glycosaminoglycans aim to be rapid, sensitive and accurate. However, due to the complex structure of the molecules and their similarities in chemical structure it is difficult to have an one-step analytical procedure. Therefore, any multistep procedure used should have high recoveries, low sample consumption and also to give accurate and reproducible results. The analytical procedures developed during the last years were focused on increasing the analytical sensitivity so that they could be applied for the analysis of samples available in very low amounts, such as tissue biopsies.

HPLC is the mostly studied technique, since its application on the separation of GAG disaccharides is studied during the last 25 years. The more recently proposed HPLC analytical techniques are shown in Table 2. Detection of GAG-derived disaccharides is

Table 2
Detection limits of derivatised and non-derivatised GAG products in more recent HPLC methodologies

Type of GAG	Type of column	Detection limit (μmol l^{-1})	Ref.
HA and GalAGs	Amino	ca. 2.4[a]	[21]
HA and GalAGs	Amino	ca. 5[b]	[55]
GalAGs	Amino	ca. 5[c]	[54]
GalAGs	AS4A anion exchange	ca. 0.3[d]	[53]
GalAGs	Amino	ca. 0.1[e]	[54]
HS and heparin	C_{18}	ca. 0.16[a]	[44]
HS and heparin	Sulfonated styrene–divinylbenzene	ca. 2.4[a]	[51]
HS and heparin	Amino	ca. 5[c]	[52]
HS and heparin	Amino	ca. 0.1[e]	[54]
HS and heparin	Amino	ca. 0.5[b]	[55]
HS and heparin	Amido-bonded silica	ca. 0.2[f]	[56]

[a]: Double bonds, [b–f]: derivatives with dansylhydrazine, 2-aminoacridone, 2-aminopyridine, 2-aminobenzamide, 2-cyanoacetamide, respectively.

Table 3
Detection limits of FACE methodologies

Type of GAG	Detection limit (μmol l^{-1})	Ref.
HA and GalAGs	ca. 3[a]	[43]
KS	ca. 3[a]	[44]
HS and heparin	ca. 0.2[b]	[64]
HS and heparin	ca. 0.02[c]	[91]

Derivatives of: [a]: 2-aminoacridone; [b]: 2-aminobenzoic acid; [c]: 7-amino-naphthalene-1,3-disulfonic acid.

usually carried out by measuring the absorption at 232 nm, because of the double bond of uronate [21,44,90] or by using fluorescent labelling before [52–55] or after [56] separation. The detection limits range from 0.1 to 5 μmol l^{-1} and this reflects the high sensitivity of the HPLC procedures.

Separation of GAGs, GAG-oligosaccharides and disaccharides by gel or cellulose acetate electrophoresis is mainly used for their qualitative analysis. The detection limits are rather high (more than 20 μmol l^{-1}). Derivatisation of GAG disaccharides with a fluorophore makes feasible their quantitative analysis. FACE, due to the use of specific fluorescent derivatives, seems to be more sensitive than the HPLC utilising detection of Δ-disaccharides at 232 nm. The proposed FACE procedures can be applied for the detection of HA, GalAGs, KS and heparin/HS. The limits of their detection depend on the fluorescent label (Table 3), being 3 μmol l^{-1} using 2-AMAC, 0.5 μmol l^{-1} using 2-aminobenzoic acid and 0.05 μmol l^{-1} using ANDSA [45,46,64–66,91].

CE separational analysis of intact GAG, GAG-derived oligosaccharides and disaccharides is the most recently developed and rapidly expanding miniaturised methodology. The developed methods offer high resolution and extremely high sensitivities. Some characteristic CE separations of GAG disaccarides are shown in Table 4. The sensitivity of these assays is very high reaching down to the pmol level (underivatised samples) [79,80,83,90,93–104] or to the amol level (derivatised samples) [52,82,85].

7. Critical evaluation of biological relevance of GAG analyses

GAGs are the polysaccharides that are found in all tissues and body fluids. Under normal conditions, their concentration depends on the tissue, i.e., it varies from 20 g l^{-1} (cartilage) to less than 1 mg l^{-1} (body fluids), and depends on the type of GAG. Local changes in the concentration of GAGs have been reported to occur with ageing, but more intense changes are associated with almost all diseases.

Table 4
Running conditions for the separation of GAG products using CE methodologies

Type of GAG	Conditions	Ref.
HA- and CS-derived Δ-disaccharides	UFS, normal polarity, high pH	[90]
HA- and CS-derived Δ-disaccharides	UFS, normal polarity, high pH	[92]
HA- and CS-derived Δ-disaccharides	UFS, normal polarity, high pH+additives	[93]
HA- and CS-derived Δ-disaccharides	UFS, normal polarity, high pH+additives	[94]
HA- and CS-derived Δ-disaccharides	UFS, reversed polarity, low pH	[95]
CS-derived Δ-disaccharides	UFS, normal polarity, high pH+additives	[96]
CS-derived Δ-disaccharides	UFS, normal polarity, high pH+additives	[97]
CS-derived Δ-disaccharides	CFS, normal polarity, low pH	[85]
CS-derived Δ-disaccharides	UFS, reversed polarity, low pH	[82]
CS- and HS-derived Δ-disaccharides	UFS, reversed polarity, low pH	[52]
CS-, HS- and heparin-derived Δ-disaccharides	UFS, reversed polarity, low pH	[98]
DS- and HS-derived Δ-disaccharides	UFS, normal polarity, high pH+additives	[82]
HS-derived Δ-disaccharides	UFS, normal polarity, high pH+additives	[99]
HS-derived Δ-disaccharides	UFS, normal polarity, high pH+additives	[100]
HS-derived Δ-disaccharides	UFS, reversed polarity, low pH	[83]
HS- and heparin-derived Δ-disaccharides	UFS, reversed polarity, low pH	[101]
Heparin-derived Δ-disaccharides	UFS, reversed polarity, low pH	[79]
Heparin-derived Δ-di- and oligosaccharides	UFS, reversed polarity, low pH	[102]
Heparin-derived Δ-di- and oligosaccharides	UFS, reversed polarity, low pH	[103]
Acetylated heparin-derived disaccharides	UFS, normal polarity, high pH+additives	[104]

These changes usually reflect changes of specific GAG(s). Typical examples are many malignant cases, where a significant increase of HA [15,16,58,105–112] and/or CS [111,113,114] concentration occurs, and the various MPSs, where increased amounts of either DS, HS or KS are excreted in the urine due to lysosomal enzyme deficiencies [115]. However, an increase of the concentration of a specific GAG in a tissue or body fluid may be observed in more than one cases, i.e., diabetes, renal failure, systemic diseases, meningococcal septicemia, emphysema, mercury poisoning, causing an increase of GAGs or their products in urine [116–122]. In these cases, additional information is required to distinguish them. Therefore, separational techniques must be applied to delineate possible alterations of GAGs, such as changes in molecular size and fine chemical structure, and thus to use them for the diagnosis of the pathophysiological status of a specific tissue or of a patient.

The choice of the type of separational technique that should be used, depends upon the information required. When the metabolic products of a tissue have to be examined, the separational technique should provide evidence on the size of the molecules and thus a gel permeation chromatographic or a gel electrophoretic procedure is recommended. On the other hand, when the detailed chemical structure of a GAG is required, the separational technique should be able to provide information on the structure of the disaccaride repeating units, and therefore, the use of HPLC, CE or FACE becomes unavoidable. In some cases, where fine biochemical characterisation is needed, the combination of HPLC with CE may be necessary. It should be emphasised that high sensitivity of the analytical system is an additional prerequisite for its use for diagnostic purposes.

The analytical procedures described so far have their own merits and disadvantages in determining GAGs, metabolic products thereof or their respective disaccarides obtained after specific enzymic degradation. However, since similar alterations of GAGs content and composition may be observed in more than one cases, emphasis will be given in those pathological conditions, where the results of the separational procedures can be directly applied for the diagnosis of a disease.

The most characteristic example is that referring to the analysis of HA in malignant cases, where increased amount of HA is detected in biopsy samples. In the case of malignant mesothelioma, especially, the net increase of HA is high enough to be used for diagnostic purposes. Here, the analysis of HA is better performed after its degradation with chondroitinases ABC and AC and the quantitation of Δ-disaccharide by HPLC or CE, instead of using any other technique. From measurements of HA in a large number of pleural fluids, it was concluded that values below 25 mg of HA-uronic acid per litre should be considered as normal. People having such values belong to low risk for development of malignant mesothelioma (normal population). High-risk individuals have HA concentration values between 25 and 75 mg l^{-1}, and values over 75 mg l^{-1} are taken to mean presence of malignant mesothelioma [16,17,123]. These analytical results, together with the pathological findings are used for differential diagnosis of malignant mesothelioma. In other types of cancer, where the net increase in HA content is quite small, and, since variations in HA concentrations may be due to other causes, such as ageing or other diseases, i.e., rheumatoid arthritis or certain liver diseases, HA values alone are not conclusive for the diagnosis. However, in a number of malignant cases, additional changes in the other GAGs are reported, such as increase in CS [111,113,114,124] with a concomitant increase in Δdi-mono6S in various gastrointestinal adenocarcinomas. Increase of Δdi-nonS is found in most cancers [58,111,112,114], and the presence of undersulfated chondroitin in urine is reported [125]. It is not only the fine chemical structure, but also the size of the GAGs that changes in many carcinomas, possibly due to altered GAG biosynthesis by the cells [58]. Thus, the combined results regarding the fine chemical structure and size alterations of all GAGs should be consider when applied for cancer diagnosis. On the other hand, the significant increase of HA concentration alone in a tissue sample, has been proposed as a marker of metastatic potential of some tumours [124,126,127].

Characteristic examples of urinary GAG analysis are used for the various types of MPS, where the presence of specific GAGs in the urine correlates with the clinical findings [128–134]. The GAGs found are mainly DS and/or HS, and in some cases

KS. They can be identified after degradation with specific enzymes and subsequent separation of the products using HPLC, CE or FACE. The normal population excretes in urine predominantly CS, and therefore, the amounts of the other GAGs, which may be up to 500-times greater than those of CS, can help to describe the severity of the diseases, although in some cases of mild MPSs there is no substantial excretion of GAGs. The biochemical findings must be suitably evaluated taking into account the clinical observations. The latter is of crucial importance, since other diseases, such as renal diseases [118,135], stone-forming [136], diabetes [116,117] and systemic diseases [119] may be the cause for the excreted GAGs.

Fine structural differences of GAGs isolated from tissue specimens are observed in many pathological conditions, with cancer being the most characteristic example. Those observed in various joint diseases, where the ratios of Δdi-mono6S to Δdi-mono4S in synovial fluid is altered [137–139], are of similar significance among other alterations, such as the elevated amounts of KS in serum [140] or the presence of neoepitopes in PGs due possibly to metalloproteinase action [141]. Therefore, the analysis of the Δ-disaccharides, together with other biochemical parameters and the clinical findings, may contribute to the diagnosis of the type of joint disease.

The application of a certain method for the detection of any alterations of GAGs in a tissue sample or a body fluid is depending on the data required. Analysis of HA is better performed after its degradation with chondroitinases and subsequent separation and quantitation of the released Δ-disaccharide. This procedure is recommended because other GAGs that might be present will contribute to direct colorimetric determination of HA (measured as uronic acid) or the possible presence of non-sulfated chondroitin sulfate will also affect its quantitation if cellulose acetate electrophoresis is used, because it comigrates with HA. Since HA Δ-disaccharide is eluted first from HPLC ion-exchange columns or from CE procedures and completely separates from Δdi-nonS_{CS}, its quantitation can be easily and accurately achieved using either HPLC or CE. The same analytical steps can be performed when both amounts of GAGs and HA are required. On the other hand, when quantitation and characterisation of other GAGs is required, FACE methodologies may be used with similar accuracy and sensitivity as HPLC or CE. FACE, in addition, seems to be the most applicable methodology when the characterisation of GAGs metabolic products is required.

From the accumulated data until now it becomes apparent that GAGs may be useful as biochemical markers for many diseases. However. as it happens with other biochemical markers, conclusive diagnosis may be achieved when the analytical findings are in harmony with the clinical findings. Analysis of HA, at least for malignant cases, may be useful for reliable diagnosis. More studies are required to establish the usefulness of the analysis of other GAGs, such as the CS/DS content and ratio, the amount of Δdi-nonS_{CS}, and the ratio of Δdi-6S to Δdi-4S, for direct or differential diagnostic purposes. However, from the available information and experience, their analysis may be reliable for monitoring follow up of patients after treatment.

8. Nomenclature

CE	Capillary electrophoresis
CS	Chondroitin sulfate
DS	Dermatan sulfate
FACE	Fluorophore assisted carbohydrate electrophoresis
GAG	Glycosaminoglycan
GalAG	Galactosaminoglycan
HA	Hyaluronan
HPLC	High-performance liquid chromatography
HS	Heparan sulfate
KS	Keratan sulfate
MPS	Mucopolysaccharidosis
PG	Proteoglycan

References

[1] R.V. Iozzo (Ed.), Proteoglycans: Structure, Biology and Molecular Interactions, Marcel Dekker, New York, 2000.
[2] H. Kresse, H. Hauser, E. Schönherr, Experientia 49 (1993) 861.
[3] R.V. Iozzo, Annu. Rev. Biochem. 67 (1998) 609.
[4] R.V. Iozzo, Crit. Rev. Biochem. Mol. Biol. 32 (1997) 141.
[5] N. Schwartz, Front. Biosci. 5 (2000) D649.

[6] K. Sugahara, H. Kitagawa, Curr. Opin. Struct. Biol. 10 (2000) 518.
[7] D. Spillmann, U. Lindahl, Curr. Opin. Struct. Biol. 4 (1994) 677.
[8] M. Bernfield, M. Gotte, P.N. Park, O. Reizes, M.L. Fitzgerald, J. Lincecum, M. Zaka, Annu. Rev. Biochem. 68 (1999) 729.
[9] P. Prehm, Biochem. J. 220 (1984) 597.
[10] C.P. Tsiganos, D.H. Vynios, D.L. Kalpaxis, Biochem. J. 235 (1986) 117.
[11] T.C. Laurent, U.B. Laurent, J.R. Fraser, Immunol. Cell Biol. 74 (1996) A1.
[12] J.R.E. Fraser, T.C. Laurent, in: D. Evered, J. Whelan (Eds.), The Biology of Hyaluronan, Wiley, Chichester, 1989, p. 41.
[13] P. Heldin, T.C. Laurent, C.H. Heldin, Biochem. J. 258 (1989) 919.
[14] B. Delpech, N. Girard, P. Bertrand, M.N. Courel, C. Chauzy, A. Delpech, J. Intern. Med. 242 (1997) 41.
[15] A. Engstrom-Laurent, in: D. Evered, J. Whelan (Eds.), The Biology of Hyaluronan, Wiley, Chichester, 1989, p. 233.
[16] M. Nurminen, A. Dejmek, G. Martensson, A. Thylen, A. Hjerpe, Clin. Chem. 40 (1994) 777.
[17] N.K. Karamanos, A. Hjerpe, J. Chromatogr. B 697 (1997) 277.
[18] F. Lamari, J. Katsimpris, S. Gartaganis, N.K. Karamanos, J. Chromatogr. B 709 (1998) 173.
[19] P.N. Sanderson, T.N. Huckerby, I.A. Nieduszynski, Biochem. J. 243 (1987) 175.
[20] B. Casu, M. Petitou, M. Provasoli, P. Sinay, Trends Biochem. Sci. 13 (1988) 221.
[21] N.K. Karamanos, A. Syrokou, P. Vanky, M. Nurminen, A. Hjerpe, Anal. Biochem. 221 (1994) 189.
[22] N.K. Karamanos, P. Vanky, A. Syrokou, A. Hjerpe, Anal. Biochem. 225 (1995) 220.
[23] M. Salmivirta, K. Lidholt, U. Lindahl, FASEB J. 10 (1996) 1270.
[24] J.T. Gallagher, Biochem. Soc. Trans. 25 (1997) 1206.
[25] U. Lindahl, Haemostasis 29 (Suppl. S1) (1999) 38.
[26] R.V. Iozzo, J. Clin. Invest. 108 (2001) 165.
[27] R.V. Iozzo, J.D. SanAntonio, J. Clin. Invest. 108 (2001) 349.
[28] Z. Shriver, D. Liu, R. Sasisekharan, Trends Cardiovasc. Med. 12 (2002) 71.
[29] M. Maccarana, B. Casu, U. Lindahl, J. Biol. Chem. 268 (1993) 23898.
[30] S. Faham, R.E. Hileman, J.R. Fromm, R.J. Linhardt, D.C. Rees, Science 271 (1996) 1116.
[31] U. Lindahl, K. Lidohlt, D. Spillmann, L. Khellen, Thromb. Res. 75 (1994) 1.
[32] P. Duchaussoy, G. Jaurand, P.A. Driguez, P. Lederman, M.L. Ceccato, F. Gourvenec, J.M. Strassel, P. Sizun, M. Petitou, J.M. Herbert, Carbohydr. Res. 317 (1999) 85.
[33] S. Guimond, M. Maccarana, B.B. Olwin, U. Lindahl, A. Rapraeger, J. Biol. Chem. 268 (1993) 23906.
[34] J.L. Funderburgh, Glycobiology 10 (2000) 951.
[35] D.A. Theocharis, D.L. Kalpaxis, C.P. Tsiganos, Biochim. Biophys. Acta 841 (1985) 131.
[36] C.A. Antonopoulos, S. Gardell, J.A. Szirmai, E.R. de Tyssonsk, Biochim. Biophys. Acta 83 (1964) 1.
[37] J.F. Kennedy (Ed.), Studies in Organic Chemistry, Proteoglycans—Biological and Chemical Aspects in Human Life, Vol. 2, 1979.
[38] R.V. Iozzo (Ed.), Proteoglycan Protocols, Humana Press, Totowa, NJ, 2001.
[39] L.A. Fransson, B. Havsmark, I. Silverberg, Biochem. J. 269 (1990) 381.
[40] C. Ueka, S. Nadanaka, N. Seno, K.H. Khoo, K. Sugahara, Glycoconj. J. 6 (1999) 291.
[41] A. Calabro, M. Benavides, M. Tammi, V.C. Hascall, R.J. Midura, Glycobiology 10 (2000) 273.
[42] A. Calabro, V.C. Hascall, R.J. Midura, Glycobiology 10 (2000) 283.
[43] T. Imanari, T. Toida, I. Koshiishi, H. Toyoda, J. Chromatogr. A 720 (1996) 275.
[44] N.K. Karamanos, P. Vanky, G.N. Tzanakakis, T. Tsegenidis, A. Hjerpe, J. Chromatogr. A 765 (1997) 169.
[45] A.H. Plaas, L. West, R.J. Midura, V.C. Hascall, Methods Mol. Biol. 171 (2001) 117.
[46] A.H. Plaas, L.A. West, R.J. Midura, Glycobiology 11 (2001) 779.
[47] A.D. Theocharis, I. Tsolakis, T. Tsegenidis, N.K. Karamanos, Atherosclerosis 145 (1999) 359.
[48] A. Syrokou, G. Tzanakakis, T. Tsegenidis, A. Hjerpe, N.K. Karamanos, Cell Prolif. 32 (1999) 85.
[49] T.N. Mitropoulou, A.D. Theocharis, K.D. Stagiannis, N.K. Karamanos, Biochimie 83 (2001) 529.
[50] F. Lamari, N.K. Karamanos, manuscript in preparation.
[51] K. Murata, A. Murata, K. Yoshida, J. Chromatogr. B 670 (1995) 3.
[52] H. Kitagawa, A. Kinoshita, K. Sugahara, Anal. Biochem. 232 (1995) 114.
[53] A.H. Plaas, V.C. Hascall, R.J. Midura, Glycobiology 6 (1996) 823.
[54] A. Kinoshita, K. Sugahara, Anal. Biochem. 269 (1999) 367.
[55] N. Volpi, Anal. Biochem. 277 (2000) 19.
[56] H. Toyoda, T. Nagashima, R. Hirata, T. Toida, T. Imanari, J. Chromatogr. B 704 (1997) 19.
[57] D.A. Theocharis, N. Papageorgakopoulou, D.H. Vynios, S.T. Anagnostides, C.P. Tsiganos, J. Chromatogr. B 754 (2001) 297.
[58] A.D. Theocharis, N. Papageorgakopoulou, D.H. Vynios, D.A. Theocharis, manuscript in preparation.
[59] J.E. Turnbull, J.T. Gallagher, Biochem. J. 251 (1988) 597.
[60] J.E. Turnbull, J.T. Gallagher, Biochem. J. 277 (1991) 297.
[61] J.E. Turnbull, J.J. Hopwood, J.T. Gallagher, Proc. Natl. Acad. Sci. USA 96 (1999) 2698.
[62] F. Cheng, K. Yoshida, D. Heinegard, L.A. Fransson, Glycobiology 2 (1992) 553.
[63] F. Cheng, D. Heinegard, A. Malmstrom, A. Schmidtchen, K. Yoshida, L.A. Fransson, Glycobiology 4 (1994) 685.
[64] J.E. Turnball, Methods Mol. Biol. 171 (2001) 129.
[65] A. Calabro, R. Midura, A. Wang, L. West, A. Plaas, V.C. Hascall, Osteoarthritis Cartilage 9 (Suppl. A) (2001) S16.
[66] A.H. Plaas, L.A. West, E.J. Thonar, Z.A. Karcioglu, C.J. Smith, G.K. Klintworth, V.C. Hascall, J. Biol. Chem. 276 (2001) 39788.

[67] N.K. Karamanos, A. Hjerpe, Electrophoresis 19 (1998) 2561.
[68] N.K. Karamanos, F. Lamari, Biomed. Chromatogr. 13 (1999) 501.
[69] N.K. Karamanos, A. Hjerpe, Biomed. Chromatogr. 13 (1999) 507.
[70] J. Grimshaw, Electrophoresis 18 (1997) 2408.
[71] M.V. Novotny, J. Chromatogr. B 689 (1997) 55.
[72] A. Paulus, A. Klockow, J. Chromatogr. A 720 (1996) 353.
[73] R.J. Linhardt, A. Pervin, J. Chromatogr. A 720 (1996) 323.
[74] Z. El Rassi, Y. Mechref, Electrophoresis 17 (1996) 275.
[75] A. Pantazaki, M. Taverna, C. Vidal-Madjar, Anal. Chim. Acta 383 (1999) 137.
[76] S. Suzuki, S. Honda, Electrophoresis 19 (1998) 2539.
[77] R. Kuhn, Capillary Electrophoresis: Principles and Practice, Springer Verlag, London, 1993.
[78] Z. El Rassi, High-Performance Capillary Electrophoresis of Carbohydrates, Beckman Instruments, Fullerton, CA, 1996.
[79] V. Ruiz-Calero, L. Puignou, M.T. Galceran, M. Diez, J. Chromatogr. A 775 (1997) 91.
[80] L. Scapol, E. Marchi, G.C. Viscomi, J. Chromatogr. A 735 (1996) 367.
[81] N.K. Karamanos, S. Axelsson, P. Vanky, G.N. Tzanakakis, A. Hjerpe, J. Chromatogr. A 696 (1995) 295.
[82] F. Lamari, A.D. Theocharis, A. Hjerpe, N.K. Karamanos, J. Chromatogr. B 730 (1999) 129.
[83] N.K. Karamanos, P. Vanky, G.N. Tzanakakis, A. Hjerpe, Electrophoresis 17 (1996) 391.
[84] T.N. Mitropoulou, F. Lamari, A. Syrokou, A. Hjerpe, N.K. Karamanos, Electrophoresis 22 (2001) 2458.
[85] Z. El Rassi, J. Postwait, Y. Mechref, G.K. Ostrander, Anal. Biochem. 244 (1997) 283.
[86] T.N. Huckerby, Prog. NMR Spectr. 40 (2002) 35.
[87] L. Silvestro, I. Viano, A. Naggi, G. Torri, R. Da Col, C. Baiocchi, J. Chromatogr. 591 (1992) 225.
[88] R. Da Col, L. Silvestro, A. Naggi, G. Torri, C. Baiocchi, D. Moltrasio, A. Cedro, I. Viano, J. Chromatogr. 647 (1993) 289.
[89] L.S. Lohmander, S. DeLuca, B. Nilsson, V.C. Hascall, C.B. Caputo, J.H. Kimura, D. Heinegard, J. Biol. Chem. 255 (1980) 6084.
[90] A. Al-Hakim, R.J. Linhardt, Anal. Biochem. 195 (1991) 132.
[91] K.J. Drummond, E.A. Yates, J.E. Turnball, Proteomics 1 (2001) 304.
[92] S. Honda, T. Ueno, K. Kakehi, J. Chromatogr. 608 (1992) 289.
[93] E. Payan, N. Presle, E. Lapicque, J.Y. Jouzeau, K. Bordji, S. Deither, G. Miralles, D. Mainard, D. Netter, Anal. Chem. 70 (1998) 4780.
[94] S.L. Carney, D.J. Osborne, Anal. Biochem. 195 (1991) 132.
[95] N.K. Karamanos, S. Axelsson, P. Vanky, G.N. Tzanakakis, A. Hjerpe, J. Chromatogr. A 696 (1995) 295.
[96] S. Hayase, Y. Oda, S. Honda, K. Kakehi, J. Chromatogr. A 768 (1997) 295.
[97] S. Michaelsen, M.B. Schroder, H. Sorensen, J. Chromatogr. A 652 (1993) 503.
[98] A. Pervin, A. Al-Hakim, R.J. Linhardt, Anal. Biochem. 221 (1994) 182.
[99] U.R. Desai, H. Wang, S.A. Ampofo, R.J. Linhardt, Anal. Biochem. 213 (1993) 120.
[100] S.A. Ampofo, H.M. Wang, R.J. Linhardt, Anal. Biochem. 199 (1991) 249.
[101] T. Toida, H. Yoshida, H. Toyoda, I. Koshiishi, T. Imanari, R.E. Hileman, J.R. Fromm, R.J. Linhardt, Biochem. J. 322 (1997) 499.
[102] R.E. Hileman, A.E. Smith, T. Toida, R.J. Linhardt, Glycobiology 7 (1997) 231.
[103] A. Pervin, C. Gallo, K.A. Jandik, X.J. Han, R.J. Linhardt, Glycobiology 5 (1995) 83.
[104] R.J. Kerns, I.R. Vlahov, R.J. Linhardt, Carbohydr. Res. 267 (1995) 143.
[105] W. Knudson, C. Biswas, X.Q. Li, R.E. Nemec, B.P. Toole, Ciba Found. Symp. 143 (1989) 150.
[106] B.P. Toole, in: E. Hay (Ed.), Cell Biology of Extracellular Matrix, 2nd ed., Plenum Press, New York, 1991, p. 305.
[107] J. Entwistle, C.L. Hall, E.A. Turley, J. Cell Biochem. 61 (1996) 569.
[108] P.K. Auvinen, J.J. Parkkinen, R.T. Johansson, U.M. Agren, R.H. Tammi, M.J. Eskelinen, V.M. Kosma, Int. J. Cancer 74 (1997) 477.
[109] K. Ropponen, M. Tammi, J. Parkkinen, M. Eskelinen, R. Tammi, P. Lipponen, U. Agren, E. Alhava, V.M. Kosma, Cancer Res. 58 (1998) 342.
[110] M.A. Anttila, R.H. Tammi, M.I. Tammi, K.J. Syrjanen, S.V. Saarikoski, V.M. Kosma, Cancer Res. 60 (2000) 150.
[111] A.D. Theocharis, M.E. Tsara, N. Papageorgakopoulou, D. Karavias, D.A. Theocharis, Biochim. Biophys. Acta 1502 (2000) 201.
[112] Th.A. Papadas, M. Stylianou, N.S. Mastronikolis, N. Papageorgakopoulou, S. Skandalis, P. Goumas, D.A. Theocharis, D.H. Vynios, Acta Otolaryng. 122 (2002) 330.
[113] R.V. Iozzo, Lab. Invest. 53 (1985) 373.
[114] M.E. Tsara, N. Papageorgakopoulou, D.D. Karavias, D.A. Theocharis, Anticancer Res. 15 (1995) 2107.
[115] L. Sweetman, in: S.J. Soldin, N. Rifia, J.M.B. Hicks (Eds.), Biochemical Basis of Pediatric Disease, AACC Press, Washington, DC, 1992, p. 359.
[116] Y.M. Unlucerci, S. Bekpinar, G. Seferoglu, Acta Diabetol. 37 (2000) 71.
[117] R.A. Cadaval, O. Kohlman, Y.M. Michelacci, Glycobiology 10 (2000) 185.
[118] Y.M. Michelacci, R.A. Cadaval, R.M. Rovigatti, O. Kohlman, Exp. Nephrol. 9 (2001) 40.
[119] P. De Muro, R. Faedda, M. Formato, F. Re, A. Satta, G.M. Cherchi, A. Carcassi, Clin. Exp. Rheumatol. 19 (2001) 125.
[120] E.E. Oragui, S. Nadel, P. Kyd, M. Levin, Crit. Care Med. 28 (2000) 3002.
[121] C.H. van de Lest, E.M. Versteeg, J.H. Veerkamp, J.H. Berden, J. van den Born, L. Heunks, J.W. Lammers, C.L. van Herwaarden, P.N. Dekhuijzen, T.H. van Kuppevelt, Am. J. Respir. Crit. Care Med. 154 (1996) 952.
[122] K. Olczyk, E.J. Kucharz, A. Glowacki, Cent. Eur. J. Public Health 2 (1994) 77.
[123] A. Thylen, A. Hjerpe, G. Martensson, Cancer 92 (2001) 1224.

[124] A.D. Theocharis, D.A. Theocharis, Biomed. Chromatogr. 16 (2002) 157.
[125] J.R. Martins, M.E. Gadelha, S.M. Fonseca, L.O. Sampaio, P.A. De L Pontes, C.P. Dietrich, H. Nader, Otolaryngol. Head Neck Surg. 122 (2000) 115.
[126] A. Herrera-Gayol, S. Jothy, Int. J. Exp. Pathol. 82 (2001) 193.
[127] R.L. Marquet, M.P. van der Tol, J. Jeekell, Br. J. Surg. 88 (2001) 246.
[128] S. Byers, T. Rozaklis, L.K. Brumfield, E. Ranieri, J.J. Hopwood, Mol. Genet. Metab. 65 (1998) 282.
[129] M.P. Gallegos-Arreola, A. Gonzalez-Noriega, G.M. Zuniga-Gonzalez, S.E. Flores-Martinez, M.C. Moran Moguel, L.E. Figuera, J. Sanchez-Corona, Arch. Med. Res. 28 (1997) 91.
[130] S.C. Chuang, W.L. Hwu, C.C. Wu, J.W. Hou, T.R. Wang, Zhonghua Min Guo Xiao Er Ke Yi Xue Hui Za Zhi 37 (1996) 320.
[131] L. Toma, C.P. Dietrich, H.B. Nader, Lab. Invest. 75 (1996) 771.
[132] M.P. Gallegos-Arreola, M.V. Machorro-Lazo, S.E. Flores-Martinez, G.M. Zuniga-Gonzalez, L.E. Figuera, A. Gonzalez-Noriega, J. Sanchez-Corona, Arch. Med. Res. 31 (2000) 505.
[133] M. Bhaumik, V.J. Muller, T. Rozaklis, L. Johnson, K. Dobrenis, R. Bhattacharyya, S. Wurzelmann, P. Finamore, J.J. Hopwood, S.U. Walkley, P. Stanley, Glycobiology 9 (1999) 1389.
[134] N. Alif, K. Hess, J. Straczek, S. Sebbar, Y. Belahsen, N. Mouane, A. Abkari, P. Nabet, M.A. Gelot, Arch. Pediatr. 7 (2000) 597.
[135] H. Yokoyama, K. Sato, M. Okudaira, C. Morita, C. Takahashi, D. Suzuki, H. Sakai, Y. Iwamoto, Kidney Int. 56 (1999) 650.
[136] F. Harangi, Z. Gyorke, B. Melegh, Pediatr. Nephrol. 10 (1996) 555.
[137] T. Iwase, Y. Hasegawa, N. Ishiguro, T. Ito, S. Iwasada, S. Kitamura, H. Iwata, J. Rheumatol. 25 (1998) 527.
[138] T. Shibata, K.I. Murakami, E. Kubota, H. Maeda, J. Oral Maxillofac. Surg. 56 (1998) 209.
[139] C. Belcher, R. Yaqub, F. Fawthrop, M. Bayliss, M. Doherty, Ann. Rheum. Dis. 56 (1997) 299.
[140] E.J.-M.A. Thonar, M.E. Lenz, G.K. Klintworth, B. Caterson, L.M. Pachman, P. Glickman, R. Katz, J. Huff, K.E. Kuettner, Arthr. Rheum. 28 (1985) 1367.
[141] B. Caterson, C.R. Flannery, C.E. Hughes, C.B. Little, Matrix Biol. 19 (2000) 333.
[142] M. Militsopoulou, F. Lamari, A. Hjerpe, N.K. Karamanos, Electrophoresis 23 (2002) 1104.