Manuela Viola
Davide Vigetti
Evgenia Karousou
Barbara Bartolini
Anna Genasetti
Manuela Rizzi
Moira Clerici
Francesco Pallotti
Giancarlo De Luca
Alberto Passi

Department of Experimental and Clinical Biomedical Sciences, University of Insubria, Varese, Italy

Received November 26, 2007
Revised March 20, 2008
Accepted March 21, 2008

Research Article

New electrophoretic and chromatographic techniques for analysis of heparin and heparan sulfate

Heparin (HE) and heparan sulfated glycosaminoglycans are well-known mediators of tissue development, maintenance and functions; the activities of these polysaccharides are depending mainly on their sulfate substitutions. The HE structure is also a very important feature in antithrombotic drug development, since the antithrombin binding site is composed by sequences of a specific sulfation pattern. The analysis of disaccharide composition is then a fundamental point of all the studies regarding HE/heparan sulfate glycosaminoglycan (and thereby proteoglycan) functions. The present work describes two analytical methods to quantify the disaccharides constituting HE and heparan sulfate chains. The use of PAGE of fluorophore-labeled saccharides and HPLC coupled with a fluorescence detector allowed in one run the identification of 90–95% of HE disaccharides and 74–100% of rat kidney purified heparan sulfate. Moreover, the protocol here reported avoid the *N*-sulfation disaccharides degradation, which may affect *N*-sulfated/*N*-acetylated disaccharides ratio evaluation. These methods could be also very important in clinical treatments since they are useful for monitoring the availability kinetics of antithrombotic drugs, such as low-molecular-weight HEs.

Keywords:
2-Aminoacridone derivatization / Fluorescence / Glycosaminoglycan / Heparin / Polyacrylamide gel electrophoresis of fluorophore-labeled saccharides
DOI 10.1002/elps.200700855

1 Introduction

Heparin (HE) and heparan sulfate (HS) are sulfated glycosaminoglycans (GAGs) covalently bound to proteins in the proteoglycan (PG) molecules. HE/HS proteoglycans are widely expressed in tissues and display a variety of biological activities, recently reviewed by several authors [1, 2]. Biochemically, the HSPGs can be divided into two classes: the first being associated to the membrane and the second one present in extracellular matrix (ECM). As far as the PGs on cell membrane, the most abundant are the membrane-linked glypicans or the transmembrane syndecans [2], while minor forms include betaglycan [3] and the V-3 isoform of CD44 [4]. The secreted HSPGs commonly found in ECM are perlecan, agrin [5] and collagen XVIII [1], the last one especially associated with the basement membrane.

All GAGs are linear polysaccharide chains, and every GAG is defined by the repetition of specific disaccharide units, one hexosamine and one uronic acid (UA) or neutral hexose, with proper glycosidic bonds. The characteristic disaccharide composing HE and HS is UA-glucosamine. The UA is either iduronic or glucuronic (GlcA) acid and the glucosamine ($GlcNH_2$) substituted with *N*-acetyl or *N*-sulfonyl groups (GlcNAc or GlcNS): [-β(1,4)-UA-β(1,4)-GlcNβ1-]*n*. The biosynthesis of the HE/HS chain is a complex sequence of events inside the Golgi apparatus, in which a high number of enzymes takes part. Glycosyltransferases, sulfotransferases and epimerases are the major enzyme classes committed in this process that can be divided into the following steps: chain initiation with the bound of the linkage tetrasaccharide to the core protein (process shared with chondroitin/dermatan sulfate (CS/DS) proteoglycans); chain elongation by the stepwise addition of GlcA and GlcNAc residues carried out by glycosyltransferases; chain modification including epimerization of glucuronic to iduronic acid,

Correspondence: Professor Alberto Passi, Department of Experimental and Clinical Biomedical Sciences, University of Insubria, via Dunant, 5, I-21100 Varese, Italy
E-mail: alberto.passi@uninsubria.it
Fax: +39-0332-397119

Abbreviations: 2-AMAC, 2-aminoacridone; **CS/DS,** chondroitin/dermatan sulfate; **ΔDiHS,** unsaturated heparan sulfate disaccharide; **GAG,** glycosaminoglycan; **GlcN,** glucosamine; **GlcNAc,** glucosamine *N*-acetylated; **HA,** hyaluronan; **HE,** heparin; **HS,** heparan sulfate; **PAGEFS,** PAGE of fluorophore-labeled saccharides; **PG,** proteoglycan; **UA,** uronic acid

N-deacetylation/*N*-sulphation of GlcNAc residues into GlcNS or $GlcNH_2$, 2-*O*-sulphation of the UA, 6 and/or 3-*O*-sulphation of the hexosamine [6]. Because of the restrictions of the biosynthetic pathway due to the specific target of the involved enzymes, only few among the potential HE/HS disaccharides have been effectively identified in biological samples [6]. The analysis of the composition of the HE/HS chain is usually performed on unsaturated disaccharides (Δ-disaccharides) obtained after complete digestion of the GAG with HE lyases (I, II, III) in combination. After this treatment several studies reported the presence of only 12 Δ-disaccharides (see [7] for a comparison of literature data), which are present selectively in HE or heparan sulfate.

The importance of the knowledge of the GAG composition is actually due to the role of HE/HS in biological activities: these polysaccharides are involved in critical passages of cellular and tissue biology, both in physiological and pathological condition, but all their behaviors are dependent on their chemical structure, composition and sequence [2, 6]. After the determination of the antithrombin binding site of HE [8] the relationships between protein (such as growth factors) and polysaccharides (such as HE) were deeply investigated and since then a new concept of specificity was considered. In fact, it is noteworthy that in many cases and unexpectedly the overall organization of the GAG can be more important than the fine structure for the protein/GAG interaction [9]. Recently the studies about the comprehension of GAG composition have increased as with the knowledge of the role of these polysaccharides in development and cellular progression [10, 11]. Nevertheless, the structural analyses are not yet easy to perform, principally due to the absence of suitable tools. In fact the derivatization procedure by Calabro [12] was carried out for chondroitin sulfate and hyaluronan (HA) and the reported conditions were not suitable for HE/HS, due to the degradation of the *N*-sulfated disaccharides [13]. The aim of this work is to simplify the analysis of HE/HS composition using easy and convenient HPLC or electrophoretic methods in combination with the fluorescence labeling of the samples which improves the assay sensitivity. The methods developed in our laboratory and here described permit a complete separation of the known Δ-disaccharides HE/HS found in tissues in few and simple steps improving the similar techniques reported in the literature [7, 13].

2 Materials and methods

Standards of HE/HS disaccharides A and S series were from Seikagaku (Tokyo, Japan); standards of HE/HS disaccharides H series were from Sigma (St. Louis, MO, USA). Proteinase K (EC 3.4.21.64) was from Finnzymes (Espoo, Finland). Porcine Intestinal Mucosa Heparin was from Sigma. Heparinase I (E.C. 4.2.2.7), heparinase II (no number E.C.) and heparinase III (E.C. 4.2.2.8) are from Sigma–Aldrich (Steinheim, Germany). AMAC was obtained from Molecular Probes (Eugene, OR, USA) and $NaBH_3CN$ from Sigma–Aldrich. ACN HPLC grade was from Merck (Darmstadt, Germany). Acrylamide, *N,N′*-methylenebisacrylamide, TEMED and ammonium persulfate were obtained from BioRad (Richmond, CA). All other chemicals used were of analytical reagent grade.

2.1 Isolation and degradation of HS and HE GAGs

A fresh rat kidney sample was digested at 60°C for 2 h in 300 μL of 100 mM ammonium acetate buffer, pH 7.0, containing 20 U/mL of Proteinase K. The enzymatic treatment was terminated by boiling for 5 min. A 4 vol. of 96% ethanol *per* sample volume was added, and the GAGs in the mixture were precipitated at −20°C overnight. Ethanol-precipitated GAGs were centrifuged at 11 000 × *g* at 4°C for 30 min. The obtained pellets were dried and dissolved in 100 μL of 100 mM ammonium acetate, pH 7.0, containing a mix of heparinases I, II, III, 50 mU/mL each, and digested at 37°C for 16–18 h. A 4 vol. of 96% ethanol *per* sample volume was added, and the GAGs in the mixture were precipitated at −20°C overnight, while the unsaturated disaccharides (Δ-disaccharides) from HS were recovered in the supernatant and lyophilized.

Commercial HE was dissolved at 10 mg/mL in 100 mM ammonium acetate, pH 7.0 and treated as reported above for HS. The Δ-disaccharide digested products were then derivatized as described below.

2.2 Derivatization procedure

Derivatization of HE/HS standards was first done as described by Calabro *et al.* [12], using 5 nmol of each standard Δ-disaccharide. A 40 μL volume of 12.5 mM AMAC solution in glacial acetic acid/DMSO (3:17 v/v) was added, and samples were incubated for 10–15 min at room temperature. A 40 μL volume of a freshly prepared solution of 1.25 M $NaBH_3CN$ in water was added to each sample followed by an overnight incubation at 37°C. An appropriate dilution of these samples in ammonium acetate 100 mM pH 7.0 or the addition of 20% glycerol was used for the HPLC analysis or the PAGE of fluorophore-labeled saccharides (PAGEFS), respectively.

Alternatively the samples were labeled with AMAC as described by Militsopoulou *et al.* [7]; briefly, 10 μL of a 100 mM AMAC solution in glacial acetic acid/DMSO (3:17 v/v) and 10 μL of 100 mM $NaBH_3CN$ in water were added to the lyophilized samples, vortexed, centrifuged at 11 000 × *g* for 3 min and incubated at 45°C for 4 h. At the end of the labeling 60 μL of 50% DMSO was added and for PAGEFS analysis 20 μL of glycerol or, alternatively, 50% glycerol, 430 mM Tris-HCl, 4% NaOH [13].

2.3 HPLC analysis

Separation and analysis of AMAC-derivatives of Δ-disaccharides were modified from Karousou *et al.* [10] using an HPLC system coupled with a Jasco-Borwin chromatograph system with a fluorophore detector (Jasco FP-920, $\lambda_{ex} = 425$ nm and $\lambda_{em} = 525$ nm). Chromatography was carried out using an RP column (C-18, 4.6×150 mm^2, Bischoff 3.0 μm) at room temperature, equilibrated with 0.1 M ammonium acetate buffer, pH 7.0, filtered through a 0.22 μm membrane filter. A gradient elution was done using a binary solvent system composed of 0.1 M ammonium acetate buffer, pH 7.0 (eluent A), and ACN (eluent B). The flow rate was 1 mL/min, and the following program was used: gradient elution to 12.5% eluent B for 30 min, to 50% for 10 min, re-equilibration of the column with 100% eluent A for 10 min. Sample peaks were identified and quantified comparing the fluorescence spectra with standard Δ-disaccharides, using Jasco-Borwin software.

2.4 PAGEFS

A MiniProtean II or III cell vertical slab gel electrophoresis apparatus (BioRad) was used with 7.2-cm plates, 0.75 mm spacers, and wells of 0.5 cm. The stock solutions were 1.5 M Tris-HCl, pH 8.8, 500 mM Tris-HCl pH 6.8. Acrylamide solution T 50%/C 5% (% T refers to the total concentration w/v of acrylamide monomer (*i.e.* acrylamide plus methylenebisacrylamide); % C refers to the concentration w/w of cross-linker relative to the total monomer).

A 10 mL volume (for two gels) of T 30%/C 3%, 375 mM Tris-HCl pH 8.8 resolving gel buffer solution (final concentrations) was prepared and degassed. TEMED (5 μL) and a 50 μL of 10% w/v ammonium persulfate were added. The solution was carefully mixed before and placed between the glass plates; the gels had a length of at least 6 cm. The stacking gel was prepared in a volume of 5 mL (for two gels) of T 5%/C 0.5%, 120 mM Tris-HCl pH 6.8 gel buffer solution (final concentrations) with added 10 μL of TEMED and 50 μL of 10% w/v ammonium persulfate for the polymerization.

The running buffer was 25 mM Tris-HCl and 192 mM glycine pH 8.3.

The run was performed at 180 V for 45 min and at 400 V for 10–15 min or until disaccharides resolution. During the run the gel was kept at 4°C in the cold room.

A PAGEFS gel with Δ-disaccharides from commercial HE and rat kidney HS and a curve of standard unsaturated heparan sulfate disaccharide (ΔDiHS)-6S disaccharide was run and the gel was scanned in a UV-light box using a CCD camera (Gel Doc 2000 System). The identification and the quantification of the bands were done using ImageJ Software comparing migration and pixel density with standard disaccharides as reported by Karousou *et al.* [10].

3 Results and discussion

The importance of the arrangement of HE/HS chains in tissue development and maintenance is well known in specific literature. Therefore, the aim of this work is to set up a quick and sensitive method for defining the composition profile of the HE/HS GAG chains.

Basically, the analysis of GAG chain composition begins with the treatment that employs appropriate lyases to produce unsaturated disaccharides; in this case the digestion was carried out using heparinases I, II, III, three enzymes with different substrate specificity able to completely degrade the HE/HS chains to disaccharide units [14]. Depending on the selected method for the analysis, the digestion may be followed by the labelling with a fluorescent dye, for improvement of measurement sensitivity. The best label for charged molecules is 2-aminoacridone (2-AMAC) [15, 16]. After 2-AMAC labeling, the detection of the resolved GAG Δ-disaccharides allows measurement at an extraordinary sensitive level, with the fluorescence response linear from 10 to 75 pmol [10]. Some previous works with fluorescent HE/HS Δ-disaccharides (derivatized with 2-AMAC) were performed by Militsopoulou *et al.* [7] and Lauer *et al.* [13], using the CE and the gel electrophoresis technologies, respectively. The former paper describes a method to discriminate twelve Δ-disaccharides composing HE and HS, using a CZE with an LIF detector. Because of the high costs, this equipment is not very common. The study also reports the presence of only 12 Δ-disaccharides in mammal samples, highlighting the limited number of disaccharides actually composing the proteoglycan HE/HS chain. The latter study focuses on the analysis of HS from diabetic rat glomeruli, and uses a commercial patented gel electrophoresis kit leading to the separation of five or six of the most representative HE/HS disaccharides. The analysis allows the measurement of the ratio between *N*-acetylated and *N*-sulfated disaccharides obtained after HS digestion. Although the second method to analyse HE/HS is more convenient for common research laboratories, it discriminates only a limited number of HE/HS disaccharides present in mammals. Nevertheless, electrophoretic methods are very suitable for the quick and simple comparison of multiple samples within one gel and for a qualitative evaluation of the difference in composition of GAG chains, HA [10] and CS/DS [11], or HE/HS [13]. The differences in resolution were on the basis of buffers salts, acrylamide, bisacrylamide, TEMED concentration and pH value. The borate buffer is suitable for the electrophoresis separation of not sulfated Δ-disaccharides, *i.e.* the ones derived from HA and chondroitin chains [10] while the Tris-HCl buffer is to be preferred in the case of sulfated disaccharides, as reported in our previous work [11]. For these reasons, the analysis of Δ-disaccharides from HE/HS chains reported here is performed on a Tris-HCl-buffered polyacrylamide gel with a modified buffer composition, that is optimized for the separation of six most representative HE/HS Δ-disaccharides as reported in Figs. 1 and 2, both in single and in

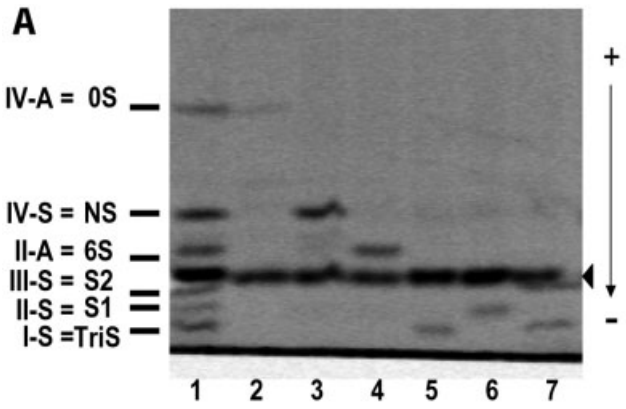

Figure 1. (A) PAGEFS of standard HE/HS disaccharides; 40 pmol of 2-AMAC-derivatized disaccharide of A and S series; lanes: 1, mix of I-S, II-S, III-S, IV-S, II-A and IV-A; 2, IV-A; 3, IV-S; 4, II-A; 5, I-S; 6, III-S; 7, II-S (for sulphation pattern see Tables 1 and 2). The arrow is pointed to an artefact due to a contaminant (for comments see Section 3). (B) 40 pmol of 2-AMAC disaccharide IV-A in two different conditions: with not freshly prepared or new running buffer, lanes 1 and 2, respectively.

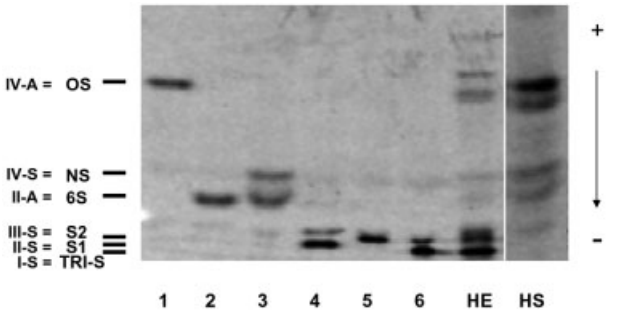

Figure 2. PAGEFS of HE/HS disaccharides; lanes 1–6 100 pmol of 2-AMAC-derivatized disaccharide of A and S series: lanes: 1, IV-A; 2, II-A; 3, IV-S; 4, II-S; 5, III-S; 6, I-S (for sulphation pattern see Table 1). HE: 1/100 of final derivatized volume of HE disaccharides; HS: 1/50 of final derivatized volume of rat kidney heparan sulfate disaccharides.

mixed preparation. The figures show a good separation of the most common disaccharides present in nature in HS and HE [7], allowing their analysis by semiquantitative measurements.

There are three HE/HS disaccharides series commercially available (see Tables 1 and 2), they all have specific *O*-sulfations (2 or 6), but they differ in hexosamine *N*-substitution. In fact, the H series is characterized by disaccharides containing a glucosamine, the S series a *N*-sulfated glucosamine and the A series a *N*-acetylglucosamine (this series is incomplete, since only two disaccharides are available). In Fig. 1, it is reported the separation of the A and S series Δ-disaccharides from HE/HS after 2-AMAC labelling. As the disaccharides of H commercial series are scarcely purified and poorly concentrated, even the sensitive PAGEFS technique was not able to detect fluorescent product bands.

Therefore, the PAGEFS analysis was then carried out only with A and S disaccharides, the most common and representative ones, as reported in Militsopoulou *et al.* [7]. The bands in the gel of Fig. 1A represent 2-AMAC HE/HS disaccharides standards; in the figure the arrow indicates a band which is an artefact due to impurities in the dye, in derivatization reagents (that should be freshly done immediately before use) or in the running buffer. In this context the buffer preparation plays a critical role, in fact the same sample, IV-A (ΔDiHS-0S) run with a not fresh running buffer caused the presence of the contaminant band, whereas in the separation of the same sample carried out with a freshly prepared running buffer, the contaminant disappeared (Fig. 1B). The buffer was notice to give a contaminant in the PAGEFS if used more than once or if let stored at 4°C for more than 1 wk. We did not further explore this aspect.

The stability of the reagent mixture is important for the whole derivatization procedure, in fact Lauer *et al.* [13] report the effect of acidic pH on the loss of *N*-sulfated disaccharides during the overnight derivatization procedure, a protocol usually used for HA and CS/DS disaccharides analysis [10, 11]. The derivatization of samples used for PAGEFS technique here described, was then modified in accordance with Militsopoulou *et al.* [7], where the addition of cyanoborohydride occurs immediately before the 2-AMAC solution, the derivatization time is shortened to 4 h (see Section 2) and the addition of glycerol is done with a buffered solution. Such procedure is suitable for keeping the sample at −80°C in order to maintain the amount of *N*-sulfated disaccharides unchanged.

The separation of the Δ-disaccharides occurs on the basis of sulphation grade, since higher sulfated Δ-disaccharides run faster than the ones with lower sulphation. Likely depending on a different geometry of the sulfate groups, disaccharides with the same sulfate content showed different migration (for example IV-S and II-A or II-S and III-S). The Δ-disaccharides standard resolved by PAGEFS account to the 90% of total HE disaccharides, as well as the 74% of total HS disaccharides, as reported in literature [13]. Unlike the ready to use kit used by Lauer *et al.* [13] the PAGEFS technique described in this study is always able to separate all the available disaccharides of A and S series; moreover, the procedure allows the researcher to monitor continuously the separation of Δ-disaccharides and therefore to adapt the technique to a specific condition and sample characteriza-

Table 1. PAGEFS *versus* HPLC analysis of Δ-disaccharides from commercial HE and rat kidney HS

Δ-Disaccharide	Structure	HS from rat kidney		Commercial HE	
		HPLC	PAGEFS	HPLC	PAGEFS
I-S (ΔDiHS-triS)	α-ΔUA-2S-[1→4]-GlcNS-6S	–	–	76.7	71
II-S (ΔDiHS-diS1)	α-ΔUA-[1→4]-GlcNS-6S	5.9	15	5.4	6
III-S (ΔDiHS-diS2)	α-ΔUA-2S-[1→4]-GlcNS	5.8	20	–	–
IV-S (ΔDiHS-NS)	α-ΔUA-[1→4]-GlcNS	24.7	11	11.2	5
IV-A (ΔDiHS-0S)	α-ΔUA-[1→4]-GlcNAc	55.4	54	2.6	7
II-A (ΔDiHS-6S)	α-ΔUA-[1→4]-GlcNAc-6S	0.6	–	4.2	11

Table 2. HPLC analysis of Δ-disaccharides from commercial HE and rat kidney HS

Δ-Disaccharide	Structure	HS from rat kidney (%)	Commercial HE (%)
I-H	α-ΔUA-2S-[1→4]-GlcN-6S	24.7	16.1
II-H	α-ΔUA-[1→4]-GlcN-6S	–	–
III-H	α-ΔUA-2S-[1→4]-GlcN	28.1	6.5
IV-H	α-ΔUA-[1→4]-GlcN	–	10.2
I-S (ΔDiHS-triS)	α-ΔUA-2S-[1→4]-GlcNS-6S	–	51.5
II-S (ΔDiHS-diS1)	α-ΔUA-[1→4]-GlcNS-6S	3.0	3.6
III-S (ΔDiHS-diS2)	α-ΔUA-2S-[1→4]-GlcNS	3.0	–
IV-S (ΔDiHS-NS)	α-ΔUA-[1→4]-GlcNS	12.6	7.5
IV-A (ΔDiHS-0S)	α-ΔUA-[1→4]-GlcNAc	28.3	1.7
II-A (ΔDiHS-6S)	α-ΔUA-[1→4]-GlcNAc-6S	0.3	2.8

tion. The application of this technique to GAG analysis is shown in Fig. 2. The analysis was carried out on preparations of commercial HE and on HS GAGs purified from normal rat kidney. The samples present a very different patterns, in facts HE shows an high amount of di- or trisulfated Δ-disaccharides, whereas in rat kidney the analysis shows the presence of HS disaccharides with a minor degree of sulfation [7]. In order to confirm PAGEFS data, we develop a new HPLC method to evaluate the 2-AMAC labeled HE/HS disaccharides. For this purpose we used RP chromatography in an HPLC system equipped with a fluorescence detector. This method was modified from a technique already described for the analysis of HA [10] and CS/DS GAGs [11]. The modification of the elution gradient for highly charged Δ-disaccharides here reported, is able to obtain a complete separation of HE/HS disaccharides. The sample preparation for HPLC and PAGEFS analysis is the same, therefore the combination of these procedures is able to overcome the possible presence of contaminants in PAGEFS. In Table 1, the analysis of Δ-disaccharides from commercial HE and rat kidney HS is reported in comparison with the results obtained by PAGEFS. The two methods present some differences in the quantification of the disaccharides that might be due to the impossibility to identify the H series Δ-disaccharides with PAGEFS technique. In fact, the HPLC analysis leads to the separation of all Δ-disaccharides standards: A, S and H series (Fig. 3C) with high resolution and reproducibility, permitting to resolve all the 10 Δ-disaccharides known in the GAG chain of HS and almost 95% of the HE disaccharides [7]. The HPLC separation of standard disaccharides, commercial HE and rat kidney HS is reported in Fig. 3, where the reproducibility and sensitivity of this HPLC-based technique applied to different biological samples is evident. In Table 2, the HPLC quantification of all Δ-disaccharides contained in commercial HE and rat kidney HS is reported. As reported for PAGEFS, HE demonstrated an elevated concentration of highly charged disaccharides, up to 52% of trisulfated ones, and very low amounts of mono- or disulfated ones. Concerning unsulfated disaccharides, the presence of a minimal amount (1.7%) of IV-A (OS-GlcNAc disaccharide) it is noteworthy and more interestingly is an about 10% amount of IV-H (OS-GlcNAc disaccharide). HS presents a lower sulfate concentration and an increased variety of sulphation pattern, with the prevalence of not- or monosulfated disaccharides, a 31% of disulfated residues without any trisulfated disaccharides.

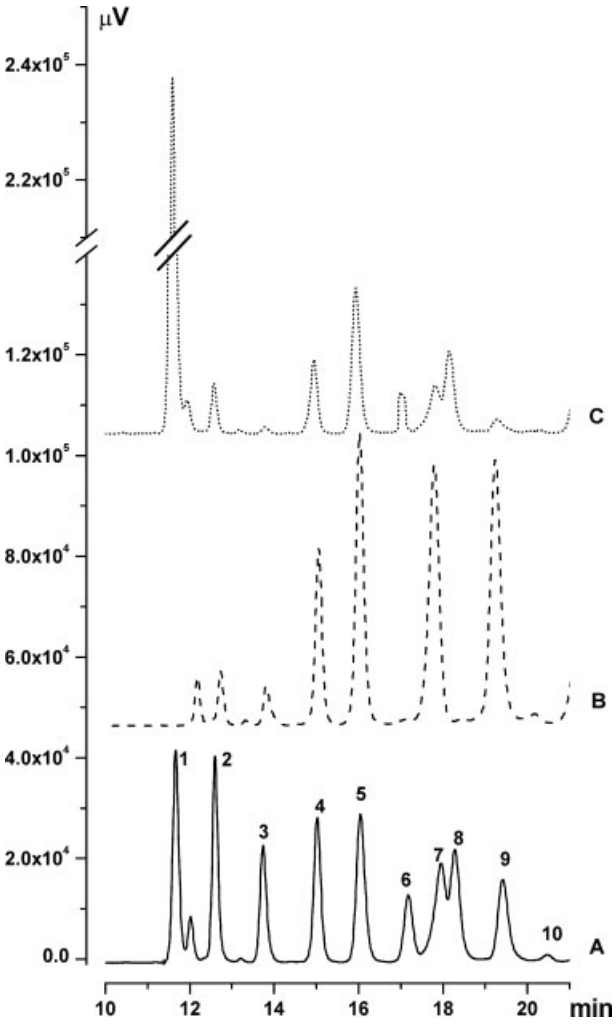

Figure 3. HPLC chromatograms of (A) mix of standard HE/HS disaccharides 100 pmol each from A, H and S series. Peaks: 1, I-S; 2, II-S; 3, III-S; 4, IV-S; 5, I-H; 6, II-A; 7, III-H; 8, IV-H: 9, IV-A; 10, II-H (see Tables 1 and 2); (B) 1/1000 of final derivatized volume of rat kidney heparan sulfate disaccharides; (C) 1/1000 of final derivatized volume of commercial HE disaccharides.

4 Concluding remarks

In the present work, we describe a new ultrasensitive method for the analysis of HE/HS disaccharides. This is remarkably important considering that recent studies about the PGs reveal a new point of view on glycoscience: the composition of the saccharidic moieties is not a static component of the molecules, but GAGs are the true variable portion of the PGs and play a pivotal role during physiological events such as ageing [17] and in several pathologies [11, 13]. The awareness of the adaptability of the GAG portion of the PGs and the critical role in binding growth factors underline the need of tools for defining the exact composition of GAGs in a sensitive and easy way to perform. On the basis of our results the PAGEFS method seems to be more suitable for fast qualitative analysis, even the quantification of samples is possible using standard curves obtained from commercial disaccharides, whereas HPLC approach showed a longer procedure but a more powerful analytic capacity as it was able to separate all disaccharides.

In particular, our understanding of the HE/HS importance to control biological effects on cells and tissues has greatly improved starting from the discovery of antithrombotic activity of HE [8]. Nowadays, the relationship between HS chain alteration and the onset of pathologies (see for example [18] and [1]) is a cornerstone of glycobiology research. The GAGs analysis including CZE, PAGEFS and HPLC for qualitative and quantitative measurement of disaccharides from HA, CS/DS is now available also for HE/HS. In fact, the present report shows for the first time the separation of HE/HS fluorescent disaccharides by PAGEFS and HPLC, improving dramatically the sensitivity of the methods. Moreover, the PAGEFS and HPLC methods are reproducible, rapid and useful for laboratory research, leading the quantification of most of HE and HS disaccharides, representing a useful tool for glycobiology research. For clinical chemistry, the HPLC methods could be a proper, rapid and simple procedure for monitoring the amount of low-molecular-weight HE used as antithrombotic drug in several pathologies, and the quantitative and qualitative analysis allowed by our method can improve dramatically the specificity of HE determination. In fact, the amount of HE or HE fragments in plasma of patients is a critical point for the treatment effectiveness [19], and the detection of I-S disaccharides in plasma may be a powerful tool for a more tuned therapy.

The authors are in debt to Andrea Moriondo for the rat kidney specimen. The authors are grateful to "Centro di Servizi Grandi Attrezzature per la Ricerca Biomedica dell'Università dell'Insubria" for instruments availability.

The authors have declared no conflict of interest.

5 References

[1] Whitelock, J. M., Iozzo, R. V., *Chem. Rev.* 2005, *105*, 2745–2764.

[2] Bernfield, M., Gotte, M., Park, P. W., Reizes, O. *et al.*, *Annu. Rev. Biochem.* 1999, *68*, 729–777.

[3] Andres, J. L., DeFalcis, D., Noda, M., Massague, J., *J. Biol. Chem.* 1992, *267*, 5927–5930.

[4] Jackson, D. G., Bell, J. I., Dickinson, R., Timans, J. *et al.*, *J. Cell Biol.* 1995, *128*, 673–685.

[5] Groffen, A. J., Ruegg, M. A., Dijkman, H., van de Velden, T. J. *et al.*, *J. Histochem. Cytochem.* 1998, *46*, 19–27.

[6] Esko, J. D., Selleck, S. B., *Annu. Rev. Biochem.* 2002, *71*, 435–471.

[7] Militsopoulou, M., Lamari, F. N., Hjerpe, A., Karamanos, N. K., *Electrophoresis* 2002, *23*, 1104–1109.

[8] Lindahl, U., Backstrom, G., Thunberg, L., Leder, I. G., *Proc. Natl. Acad. Sci. USA* 1980, *77*, 6551–6555.

[10] Karousou, E. G., Militsopoulou, M., Porta, G., De Luca, G. *et al.*, *Electrophoresis* 2004, *25*, 2919–2925.

[11] Viola, M., Karousou, E. G., Vigetti, D., Genasetti, A. *et al.*, *J. Pharm. Biomed. Anal.* 2006, *41*, 36–42.

[12] Calabro, A., Hascall, V. C., Midura, R. J., *Glycobiology* 2000, *10*, 283–293.

[13] Lauer, M. E., Hascall, V. C., Wang, A., *J. Biol. Chem.* 2007, *282*, 843–852.

[14] Karamanos, N. K., Vanky, P., Tzanakakis, G. N., Tsegenidis, T., Hjerpe, A., *J. Chromatogr. B* 1997, *765*, 169–179.

[15] Robbe, C., Capon, C., Flahaut, C., Michalski, J. C., *Electrophoresis* 2003, *24*, 611–621.

[16] Oonuki, Y., Yoshida, Y., Uchiyama, Y., Asari, A., *Anal. Biochem.* 2005, *343*, 212–222.

[17] Grzesik, W. J., Frazier, C. R., Shapiro, J. R., Sponseller, P. D. *et al.*, *J. Biol. Chem.* 2002, *277*, 43638–43647.

[18] Fuster, M. M., Wang, L., Castagnola, J., Sikora, L., Reddi, K. *et al.*, *J. Cell Biol.* 2007, *177*, 539–549.

[19] Mousa, S. A., Zhang, F., Aljada, A., Chaturvedi, S. *et al.*, *J. Clin. Pharmacol.* 2007, *47*, 1508–1520.

