

Improved method for analysis of glycosaminoglycans in glycosaminoglycan/protein mixtures: Application in Cohn–Oncley fractions of human plasma

Fabiola Cecchi^a, Marco Ruggiero^a, Renzo Cappelletti^a, Fabio Lanini^b, Simonetta Vannucchi^{a,*}

^a Department of Experimental Pathology and Oncology, University of Firenze, Viale Morgagni 50, 50134 Firenze, Italy

^b Medical Oncology Local Health Unit, Firenze, Italy

Received 30 June 2006; received in revised form 4 August 2006; accepted 4 August 2006

Available online 16 August 2006

Abstract

Background: Glycosaminoglycans are found in human tissues including plasma. They encompass chondroitin sulphates, heparan sulphate/heparin, hyaluronic acid, and keratan sulphate. Glycosaminoglycans, in particular heparan sulphate and heparin, are strongly associated with plasma proteins, so that their purification results quite difficult.

Methods: In order to study the distribution of glycosaminoglycans in plasma subfractions, we developed a novel method that allows their identification even if they were still associated with proteins or peptides. Plasma was fractionated following the procedure of Cohn–Oncley, and each fraction was treated with proteases. After centrifugation, glycosaminoglycan/protein complexes in the supernatant were analysed using a modified cellulose acetate electrophoresis which allowed identification of glycosaminoglycans in mixtures of glycosaminoglycans/proteins.

Results: Chondroitin sulphate was recovered in cryoprecipitate and in all Cohn–Oncley fractions. Glycosaminoglycans belonging to the class of heparan sulphate/heparin, however, were recovered in the cryoprecipitate and in fractions I and IV-1, and, in smaller amount, in fraction II+III.

Conclusions: Since the largest amount of plasma proteins is partitioned in Fractions II+III and V, these results demonstrate that heparan sulphate/heparin are not randomly distributed in Cohn–Oncley fractions and are associated with certain plasma proteins. This association might play a role in the physiological function of heparan sulphate/heparin, regulating hemostasis and atherogenesis.

© 2006 Elsevier B.V. All rights reserved.

Keywords: Electrophoresis; Glycosaminoglycan; Human plasma; Protein

1. Introduction

Glycosaminoglycans (GAGs) are large polyanionic molecules containing disaccharide repeating units. There are four major classes of GAGs classified on the basis of their chemical structure: hyaluronic acid (HA), keratan sulphate (KS), chondroitin sulphates (including chondroitin sulphate A, CSA; chondroitin sulphate C, CSC; and chondroitin sulphate B, CSB, also termed dermatan sulphate, DS) (the class of chondroitin sulphates is termed CSGAGs), and heparan

sulphate GAGs (HSGAGs) that include heparan sulphate (HS) and heparin (HP) [1]. With the exception of KS, they consist of alternating copolymers of uronic acid and hexosamine. HA is composed of alternate residues of the monosaccharide D-glucuronic acid and N-acetyl-D-glucosamine linked by β (1 → 3) bonds; it is unsulphated. CSGAGs are composed of alternate sequences of D-glucuronic acid (CSA and CSC) or D-glucuronic acid or L-iduronic acid (DS) and differently sulphated residues of N-acetyl-D-galactosamine linked by β (1 → 3) bonds. HP and HS (HSGAGs) are similar in structure, both consisting of alternate units of N-acetyl-D-glucosamine with D-glucuronic acid or L-iduronic acid; the disaccharide units can be N- and/or O-sulphated.

GAGs are found in all human tissues including plasma. Undersulphated CSGAGs are abundant in normal human plasma [2] and they could be easily purified by ion-exchange chromatography of protease-treated plasma [3,4]. In the past,

Abbreviations: CSA, chondroitin sulphate A; CSB, chondroitin sulphate B; CSC, chondroitin sulphate C; CSGAGs, class of chondroitin sulphates; DS, dermatan sulphate; GAG, glycosaminoglycans; HA, hyaluronic acid; HP, heparin; HS, heparan sulphate; HSGAGs, class of heparan sulphate and heparin; KS, keratan sulphate.

* Corresponding author. Tel.: +39 055 4598216; fax: +39 055 4598900.

E-mail address: simonetta.vannucchi@unifi.it (S. Vannucchi).

HSGAGs were described mainly associated with pathological conditions [5–7]. More recent studies demonstrated that HSGAGs could be found also in normal human plasma [3,4]. HSGAGs are associated with plasma proteins [8,9], and this association is so strong that even exhaustive proteolysis does not yield pure HSGAGs (i.e., protein-free) [4]. Little is known about endogenous HSGAGs binding proteins in normal human plasma [10]. In particular, it is not known whether HSGAGs specifically bind certain classes of proteins or whether they are randomly associated with basic plasma proteins [11]. In this study, we decided to fractionate human plasma using the Cohn–Oncley fractionation procedure. The Cohn–Oncley process is based on 1940s technology that has been modified through the decades, although the basic process remains unchanged [12,13]. It is a major primary purification process in use for commercial protein purification from human plasma [14]. Since it allows rapid and reproducible fractionation of plasma proteins, we thought that it was suitable to study the distribution of CSGAGs and HSGAGs in the obtained fractions with the aim of elucidating possible specific interactions that might play a role in HSGAGs function and regulation [1,11,15]. However, conventional methods for GAG extraction and purification lead to identification only of CSGAGs [9,16]. This happens because HSGAGs are so strongly associated with plasma proteins and peptides that only laborious, non-routinely used procedures allow their isolation [4]. In order to circumvent these limitations, in this paper, we describe the development of a novel and simple method, based on conventional cellulose acetate electrophoresis, that allows identification of all plasma GAGs (including HSGAGs) from mixtures of GAG/proteins recovered in Cohn–Oncley fractionation of human plasma.

2. Materials and methods

2.1. Materials

Plasma samples were prepared from citrated blood of healthy donors provided by the Centro Trasfusionale Militare of the Istituto Farmaceutico Militare of Firenze, Italy. CSA (from bovine trachea), CSC (from shark cartilage), and CSB (from porcine intestinal mucosa) were purchased from Sigma Chem. Co. (St. Louis, MO, USA). HA (from human umbilical cord) was from Calbiochem (La Jolla, CA, USA). Unfractionated HP (MW, 12.4 kDa; sulphation degree: $\text{SO}_3^-/\text{COO}^-$, 2.15) was extracted from bovine intestinal mucosa and was provided by Opocrin, Modena, Italy. Fractions of heparin, termed slow-moving heparin 1927, fast-moving heparin 1020/25, and low-molecular-weight heparin (LMW 2123/850, MW, 4.5 kDa; sulphation degree: $\text{SO}_3^-/\text{COO}^-$, 2.19) were provided by Opocrin, Modena. HS was from Upjohn International Inc. (Kalamazoo, MI, USA). Chymotrypsin (EC 3.4.21.1), trypsin (EC 3.4.21.4), collagenase (EC 3.4.24.3), pepsin (EC 3.4.23.1), and chondroitinase ABC (EC 4.2.2.4) were from Sigma Chem. Co. (St. Louis, MO, USA). Papain (EC 3.4.22.2) was from Calbiochem (La Jolla, CA, USA). Titan III Zip Zone cellulose acetate sheets (60×76 mm) were purchased from Helena Laboratories (Beaumont, TX, USA). Alcian Blue was from

Fisher Scientific Co. (Pittsburg, PA, USA). Dialysis membranes (cut-off, 3.5 kDa) were from Spectrum (Breda, Holland). Human albumin, poly-L-lysine (kDa 84.0, Lot 031K5106) and all common other reagents were from Sigma Chem. Co. (St. Louis, MO, USA).

2.2. Improvement of conventional cellulose acetate electrophoresis

Conventional cellulose acetate electrophoresis was performed at pH 1.0 and at pH 5.0 by using two chambers containing 0.5 M barium acetate buffer, at pH 5.5, or 0.1 M HCl, pH 1.0, as described [17,18]. Then we introduced the following modification. An additional electrophoretic chamber, termed stacking chamber, containing 0.1 M EDTA buffer, at pH 11, was assembled. Titan III Zip Zone cellulose acetate plate was dipped for 2–3 s in 10 mM EDTA pH 10.5 (solution A) to a height of 1.5 cm. The opposite end was immersed in 4 M NaCl, 5 mM EDTA pH 10.5 containing 5% acetone (solution B) avoiding contact with the wet zone of the previous immersion in solution A, leaving a narrow dry band (2–3 mm large), between solutions A and B. Aqueous samples were loaded on the cellulose acetate electrophoresis sheet in the middle of the wet area previously immersed in solution A. The sheet was put in the stacking chamber and run for 2 m at 200 V. Then, the sheet was removed from the chamber and it was immersed in 3 mM EDTA, pH 10.0, for 30 s. Afterward, it was put again in the stacking chamber and run for 1 m at 200 V. The procedure described here (stacking) is different from that previously described [17] because it was performed at high pH. This modification was introduced in order to promote disassociation of GAGs from proteins to such an extent that, when the GAG/protein ratio was high enough (see Results), GAGs could migrate in cellulose acetate electrophoresis even if proteins were still in the samples.

Following the newly introduced stacking procedure, electrophoresis was performed either at pH 5.0 [17] or at pH 1.0 [18] in the respective chambers as described. In some experiments, samples were treated with nitrous acid according to Cappelletti et al. [19]. Staining of GAGs was obtained by immersing the sheet for 3–5 m in an aqueous solution of 0.1% Alcian Blue. The sheet was destained in 10% acetic acid, washed under running water for 5 m, and finally dried by heated air. Bands corresponding to GAGs, identified on the basis of comigration with standards, were analysed by densitometry for quantitative analysis using a dedicated software (Scion Image, Scion Co., MD USA). The results of quantitative analysis reported in Table 1 are referred to one experiment representative of three others that gave qualitatively identical results.

2.3. Plasma protein fractionation according to Cohn–Oncley

Plasma (400 ml) was frozen in a glass beaker at $-20\text{ }^\circ\text{C}$ for 24 h. Then, it was defrosted at $4\text{ }^\circ\text{C}$ for 24 h. Afterwards it was centrifuged at $15,000\times g$ for 10 min. We obtained a supernatant, termed cryosupernatant and a precipitate, termed cryoprecipitate. Next, we followed the Cohn–Oncley fractionation procedure as described [20].

Table 1
Distribution of glycosaminoglycans in Cohn–Oncley fractions of human plasma

	Total GAGs		HSGAGs (%)	CSGAGs (%)	Proteins		GAG/ protein (ratio)
	(ng)	(%)			(μ g)	(%)	
Cryoprecipitate	604.8	23.8	58.5	41.4	7.9	26.6	1/13.1
Fraction I	485.0	19.1	61.9	38.0	6.0	22.3	1/12.4
Fraction II+III	508.2	20.0	20.8	79.1	6.4	23.9	1/12.6
Fraction IV-I	522.8	21.6	37.2	62.7	2.0	7.4	1/3.8
Fraction IV-4	181.4	7.1	0.0	100.0	3.2	11.9	1/17.6
Fraction V	137.0	5.8	0.0	100.0	1.2	4.6	1/9.2

The indicated amounts of glycosaminoglycans (GAGs) and proteins are referred to 1 ml of the starting plasma samples. The results of quantitative analysis reported in this table are referred to one experiment representative of three others that gave qualitatively identical results.

Cryoprecipitate and fractions I, II+III, IV-I, IV-4 and V, indicate the fractions obtained by Cohn–Oncley fractionation procedure. CSGAGs: class of chondroitin sulphates; HSGAGs: class of heparan sulphate and heparin.

2.4. GAG extraction from cryoprecipitate and Cohn–Oncley fractions for analysis on cellulose acetate electrophoresis

Cryoprecipitate and fractions I, II+III, IV-1, IV-4, and V were suspended in 0.05 M Tris–HCl pH 7.4, and treated with proteases as previously described [8]. Digested samples were centrifuged at 15,000 \times g for 10 min, and the supernatants were precipitated in 66% v/v ethanol for 24 h at -20 °C. Then samples were centrifuged at 15,000 \times g for 10 min, and precipitates were re-suspended in distilled water, dialysed against distilled water and lyophilised. The retentates were solubilised in distilled water and analysed on cellulose acetate electrophoresis at pH 5.0, and at pH 1.0. 1 μ l of each sample was loaded onto the cellulose acetate sheet for electrophoresis. Content of proteins in each sample was evaluated by the method of Bradford [21].

2.5. Enzymatic treatments

Treatment with chondroitinase ABC was performed adding 0.1 U of the enzyme to 5 μ l of the sample for 24 h at 37 °C, i.e., before electrophoresis [22].

3. Results

Cellulose acetate electrophoresis at pH 5.0 and at pH 1.0 separate GAGs on the basis of electrical charge and sensitivity to barium and ethanol precipitation at pH 5.0 [17] and of their different degree of sulphation and charge density at pH 1.0 [18]. This method is simple and convenient and has been successfully used for many years. However, this method is not suitable to study GAGs in the presence of proteins or peptides. Having demonstrated that plasmatic GAGs (and HSGAGs in particular) are strongly associated with proteins resistant to proteolysis [8,10], we introduced a modification to the above-mentioned methods in order to study the composition of GAG mixtures in plasma even in the presence of peptides remaining after exhaustive proteolysis. This modification consisted of the introduction of a preliminary electrophoretic step that we termed stacking procedure, aimed at dissociating proteins from GAGs. Before analysing the GAG content of plasma fractions, however, we validated this modification by evaluating the migration of GAGs previously subjected to the novel stacking procedure described in Materials and methods. Validation was performed observing the migration of a mixture of standard GAGs in cellulose acetate electrophoresis at pH 5.0, or at pH 1.0, in the absence (Fig. 1), or in the presence (Fig. 2) of different amounts of proteins/polypeptides. Fig. 1 (panel A) shows migration of standard GAGs, previously subjected to the stacking procedure, on cellulose acetate electrophoresis at pH 5.0. In lane 1 a mixture of GAGs (CSC, CSA, HA, CSB, HS and unfractionated HP, 250 μ g/ml each) was separated by electrophoresis. The results demonstrate that GAGs migrated as previously described [17]. We also determined the migration pattern of different HPs. In lane 2, unfractionated HP was loaded, whereas in lane 3 a slow-moving HP of high molecular weight and high degree of sulphation was loaded. In lane 4 a fast-moving HP of lower sulphation degree was loaded, and in lane 5, a low-molecular-weight HP. It is worth noting that the migration pattern of low-molecular-weight HP was superimposable to that of fast-moving HP of low sulphation degree.

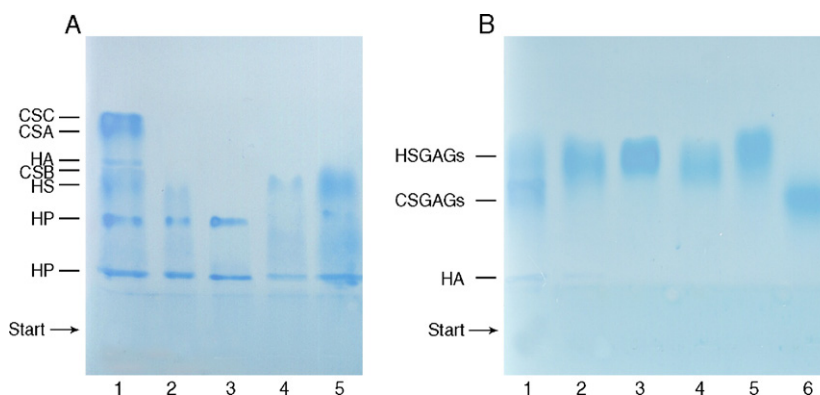


Fig. 1. Panel A: Cellulose acetate electrophoresis at pH 5.0. Lane 1: standard mixture containing 250 ng each of the following GAGs, listed from the top of the sheet: chondroitin sulphate C, CSC; chondroitin sulphate A, CSA; hyaluronic acid, HA; chondroitin sulphate B, CSB; heparan sulphate, HS; unfractionated heparin, HP. Lane 2: unfractionated HP. Lane 3: slow-moving heparin 1927. Lane 4: fast-moving heparin 1020/25. Lane 5: low-molecular-weight heparin. Panel B: Cellulose acetate electrophoresis at pH 1.0. Lane 1: standard mixture of GAGs as in lane 1 (panel A). Lane 2: unfractionated heparin. Lane 3: slow-moving heparin 1927. Lane 4: fast-moving heparin 1020/25. Lane 5: low-molecular-weight heparin. Lane 6: mixture of CSGAGs containing CSA, CSB and CSC.

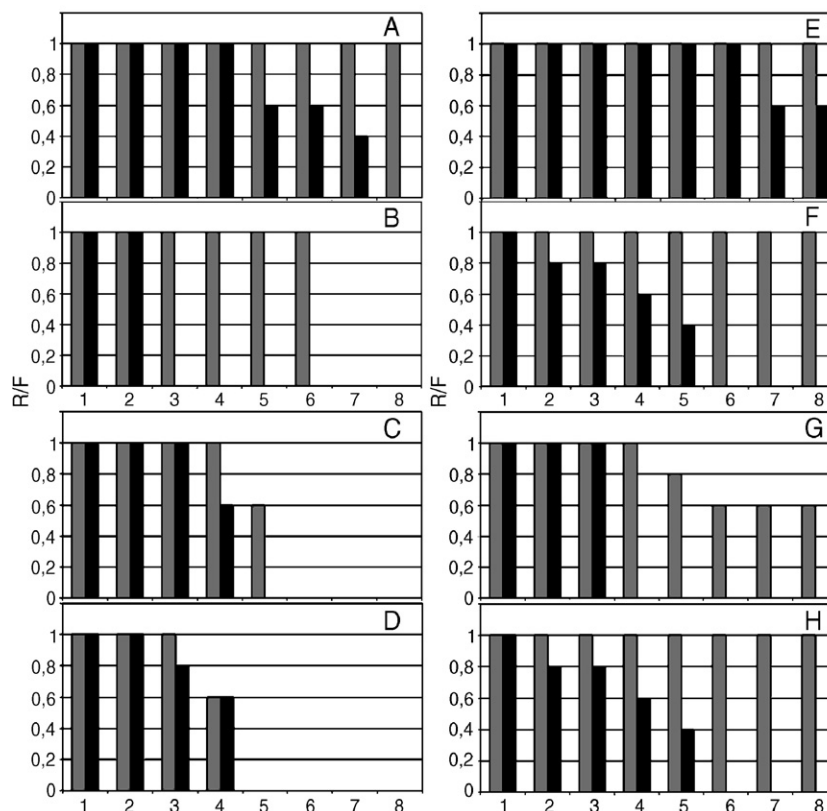


Fig. 2. Effects of stacking procedure on impairment of GAG migration in cellulose acetate electrophoresis by proteins. Cellulose acetate electrophoresis at pH 5.0 (panels A, C, E, G), and at pH 1.0 (panels B, D, F, H) of 1 μ l of standard HSGAGs (500 μ g/ml) (panels A–D) and CSGAGs (500 μ g/ml) (panels E–H) in the absence of proteins (Column 1 in all the panels), or in the presence of different amounts of albumin (panels A, B, E, F), or poly-L-lysine (panels C, D, G, H), with stacking (grey bars), and without stacking (black bars). R/F indicates the distance of migration from the start in arbitrary units. Columns 2–8: ratio GAG/albumin: 1/1.5, 1/3.1, 1/6.25, 1/12.5, 1/25, 1/50, 1/100 in panels A, B, E, F. Columns 2–8: ratio GAG/poly-L-lysine: 1/0.15, 1/0.3, 1/0.625, 1/1.25, 1/2.5, 1/5, 1/10 in panels C, D, G, H. Absence of bars indicates lack of migration or staining with Alcian Blue.

The same samples were analysed by cellulose acetate electrophoresis at pH 1.0 (Fig. 1, panel B), i.e., a method that allows separation of GAGs on the basis of charge density. At pH 1.0, HA remained at the bottom of the sheet because it has no negative charges (lane 1), whereas all types of HPs (lanes 1–5) migrated above CSGAGs (lanes 1, 6) because of higher degree of sulphation. Consistent, low-molecular-weight HP, encompassed with higher charge density, migrated higher than other HP species (lane 5). Taken together, these results demonstrate that introduction of the stacking procedure described in Materials and methods did not impair the ability of cellulose acetate electrophoresis at pH 5.0 and at pH 1.0 to separate standard GAGs in the absence of proteins/peptides.

Indeed, proteins impair GAG migration in conventional cellulose acetate electrophoresis. In order to demonstrate that introduction of the stacking procedure allowed migration of GAGs even in the presence of proteins, we performed the following experiment (Fig. 2): fixed amount of HSGAGs, or CSGAGs, was mixed with different amounts of a basic polypeptide, i.e., poly-L-lysine, or with albumin.

The mixtures were then subjected or not to the stacking procedure. Then, the mixtures were subjected to cellulose acetate electrophoresis at pH 5.0, or at pH 1.0. The migration of GAGs in the presence of different amounts of proteins were

recorded as ratio of front (RF). Fig. 2, panel A, shows that introduction of the novel stacking procedure allowed migration in cellulose acetate electrophoresis at pH 5.0, of HSGAGs mixed with albumin even when the ratio HSGAG/albumin was 1/100 (column 8). At this HSGAG/albumin ratio, HSGAGs did not migrate at all in the absence of the novel stacking procedure. A similar pattern was observed when HSGAG/albumin mixtures were subjected to cellulose acetate electrophoresis at pH 1.0 (Fig. 2, panel B). In the absence of the novel stacking procedure, when the ratio HSGAG/albumin was 1/3 (column 3), HSGAGs did not migrate.

It is well known that albumin does not specifically bind HSGAGs; however, at acidic pH and with excess amount of albumin, the total basic charge of the protein was so high that binding with HSGAGs was strong enough to have these GAGs migrating together with albumin toward the cathode.

However, introduction of the stacking procedure allowed migration at ratios HSGAG/albumin 1/6, 1/12, 1/25 (columns 4, 5, 6). In other words, introduction of the novel stacking procedure allowed identification of HSGAGs even in large excess of albumin, i.e., in conditions where conventional cellulose acetate electrophoresis could not allow identification of HSGAGs. Panels C and D show the results concerning mixtures of HSGAG/poly-L-lysine. At pH 5.0 (panel C),

introduction of the novel stacking procedure allowed migration of HSGAGs even when the ratio was 1/1 (column 5). At this HSGAG/poly-L-lysine ratio, absence of the novel stacking procedure did not allow any migration of HSGAGs. Improvement was less pronounced with cellulose acetate electrophoresis at pH 1.0 (panel D). When the ratio HSGAG/poly-L-lysine was 1/0.3 (column 3), introduction of the stacking procedure allowed better migration of HSGAGs. However, when the amount of poly-L-lysine increased (columns 5–8), no migration of HSGAGs could be observed even with the stacking procedure.

Fig. 2, panel E shows that introduction of the novel stacking procedure allowed migration of CSGAGs in cellulose acetate electrophoresis at pH 5.0, even when they were mixed with albumin at the ratio CSGAG/albumin 1/100 (column 8). CSGAGs did not migrate at CSGAG/albumin ratio of 1/50 in the absence of the novel stacking procedure. A similar pattern was observed when CSGAG/albumin mixtures were subjected to cellulose acetate electrophoresis at pH 1.0 (Fig. 2, panel F). In the absence of the novel stacking procedure, when the ratio CSGAG/albumin was 1/25 (column 6), CSGAGs did not migrate. However, introduction of the stacking procedure allowed migration at ratio CSGAG/albumin of 1/100. In other words, introduction of the novel stacking procedure allowed identification of CSGAGs even in large excess of albumin, i.e., in conditions where conventional cellulose acetate electrophoresis could not allow identification of CSGAGs. Panels G and H show the results concerning mixtures of CSGAG/poly-L-lysine. At pH 5.0 (panel G), introduction of the novel stacking procedure allowed migration of CSGAGs even when the ratio was 1/10 (column 8). Absence of the novel stacking procedure did not allow migration of CSGAGs at CSGAG/poly-L-lysine ratio of 1/0.6. Improvement was also pronounced with cellulose acetate electrophoresis at pH 1.0 (panel H). When the ratio CSGAG/poly-L-lysine was 1/0.3 (column 3), introduction of the stacking procedure allowed migration of CSGAGs. However, when the amount of poly-L-lysine increased (columns 4–8), migration of CSGAGs could be still observed with the stacking procedure, whereas no migration of CSGAGs could be observed without the stacking procedure. Taken together, the results shown in Fig. 2 demonstrate the following points: introduction of the novel stacking procedure allowed migration (and identification) of HS- and CSGAGs even in the presence of a large amount of albumin, chosen as a non-specific GAG-binding protein. This phenomenon was particularly evident with the cellulose acetate electrophoresis at pH 1.0. Poly-L-lysine binds HS- and CSGAGs; the binding of poly-L-lysine with HSGAGs is stronger than that with CSGAGs [23]. Consistent introduction of the novel stacking procedure produced a slight improvement in HSGAG migration in the presence of low amount of poly-L-lysine, whereas it caused a significant improvement when CSGAG/poly-L-lysine mixtures were analysed.

Having demonstrated that we could now identify GAGs even in the presence of variable amount of proteins, we decided to study the distribution of GAGs in Cohn–Oncley fractions of human plasma.

Cohn–Oncley fractionation of plasma yields a cryoprecipitate and five fractions termed fraction I, fraction II+III, fraction IV-1, fraction IV-4, and fraction V. Each fraction contains a number of proteins that are described in [24].

Fig. 3 shows migration of GAGs recovered in Cohn–Oncley fractionation of plasma, using the stacking procedure described above followed by cellulose acetate electrophoresis in barium acetate at pH 5.0. In cryoprecipitate, we detected GAGs co-migrating with CSGAGs, HA, and HSGAGs (HS and HP). As far as HP was concerned, we detected bands co-migrating with unfractionated HP. In fraction I, we detected a pattern of GAG migration analogous to that observed in cryoprecipitate. In fraction II+III, however, we could detect large amount of GAGs co-migrating with CSGAGs and only trace of HSGAGs. In fraction IV-1, we detected GAGs co-migrating with CSGAGs, HA, and HSGAGs. Also, in this fraction, we detected bands co-migrating with unfractionated HP. In fraction IV-4 and V, we could detect GAGs co-migrating with CSGAGs and HA. In summary, plasma GAGs co-migrating with CSGAGs were recovered in cryoprecipitate and in all Cohn–Oncley fractions, whereas plasma GAGs co-migrating with HSGAGs were recovered only in cryoprecipitate and fractions I, II+III and IV-1.

In order to ascertain whether the plasma GAGs described in Fig. 3 actually belonged to CSGAGs or HSGAGs, samples were subjected to chondroitinase ABC or to nitrous acid treatment. Chondroitinase ABC treatment was performed in order to selectively degrade all types of CSGAGs [22], whereas nitrous acid treatment was performed in order to selectively degrade HSGAGs [25]. It is worth noting that excess chondroitinase ABC, as used in this study, also degrades HA [22]. As far as HSGAGs are concerned, we decided to use nitrous acid instead of enzymatic digestion with different enzymes (heparinases I, II, and III), because this treatment degrades all types of HSGAGs (HS and HP) in one single step. Fig. 4 (panel A) shows that chondroitinase ABC treatment removed all the bands co-migrating with CSGAGs in all Cohn–Oncley fractions, thus demonstrating that the bands described in Fig. 3 actually belonged to this class of plasma GAGs. Nitrous

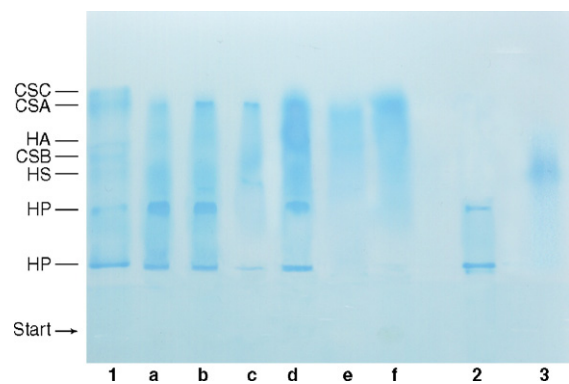


Fig. 3. Cellulose acetate electrophoresis of cryoprecipitate and Cohn–Oncley fractions at pH 5.0. Lane 1: GAG mixture (see Fig. 1). Lane 2: unfractionated HP. Lane 3: HS. Lane a: cryoprecipitate. Lane b: fraction I. Lane c: fraction II+III. Lane d: fraction IV-1. Lane e: fraction IV-4. Lane f: fraction V.

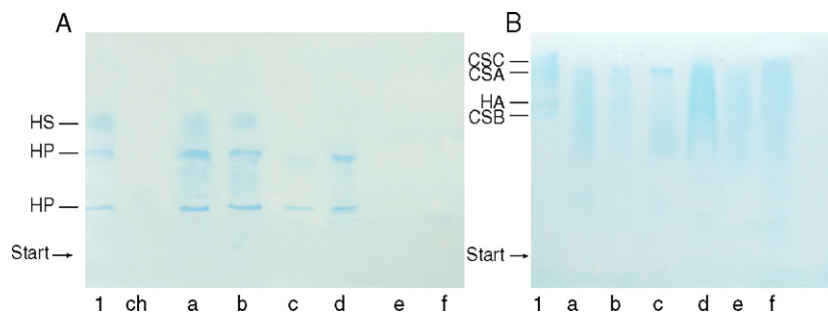


Fig. 4. Panel A: Cellulose acetate electrophoresis of cryoprecipitate and Cohn–Oncley fractions at pH 5.0 following chondroitinase ABC treatment. Lane 1: GAG mixture (see Fig. 1) following chondroitinase ABC treatment. Lane ch: chondroitinase ABC. Lane a: cryoprecipitate following chondroitinase ABC treatment. Lane b: fraction I following chondroitinase ABC treatment. Lane c: fraction II+III following chondroitinase ABC treatment. Lane d: fraction IV-1 following chondroitinase ABC treatment. Lane e: fraction IV-4 following chondroitinase ABC treatment. Lane f: fraction V following chondroitinase ABC treatment. Panel B: cellulose acetate electrophoresis of cryoprecipitate and Cohn–Oncley fractions at pH 5.0 following nitrous acid treatment. Lane 1: GAG mixture (see Fig. 1) subjected to nitrous acid. Lane a: cryoprecipitate subjected to nitrous acid. Lane b: fraction I subjected to nitrous acid. Lane c: fraction II+III subjected to nitrous acid. Lane d: fraction IV-1 subjected to nitrous acid. Lane e: fraction IV-4 subjected to nitrous acid. Lane f: fraction V subjected to nitrous acid.

acid treatment (Fig. 4, panel B) removed the bands co-migrating with HSGAGs in cryoprecipitate and in fractions I and IV-1, thus demonstrating that these bands actually belonged to the class of HSGAGs.

Next, we sought to determine the charge density of CSGAGs and HSGAGs recovered in cryoprecipitate and Cohn–Oncley fractions. Indeed, samples were submitted to stacking procedure and electrophoresis at pH 1.0, before (Fig. 5 panel A) and after (Fig. 5 panel B) treatment with nitrous acid. Fig. 5 (panels A and B) shows that HSGAGs sensitive to nitrous acid, recovered in cryoprecipitate and fractions I, II+III and IV-1, co-migrated with standard HP of high degree of sulphation. Consistent with results showed in Figs. 3 and 4 (panel A), CSGAGs, resistant to nitrous acid, were represented by bands co-migrating with standard CS and by slower bands (undersulphated CS). These CSGAGs were observed in cryoprecipitate and in all Cohn–Oncley fractions; undersulphated CS was observed in higher amount in cryoprecipitate and in fractions II+III and IV-1.

Having described the qualitative distribution of CS- and HSGAGs in Cohn–Oncley fractions of human plasma, then we evaluated the amount of GAGs in each fraction by densitometric analysis of GAG spots identified in cellulose acetate

electrophoresis at pH 5.0, performed after the stacking procedure. We also determined the amount of proteins (GAG-binding proteins) still present in each fraction after the treatment described above. We were particularly interested in the ratios GAG/protein since we had demonstrated that it was critical for GAG migration in cellulose acetate electrophoresis. Recovery of total (i.e., CSGAGs+HSGAGs) GAGs from cryoprecipitate and all Cohn–Oncley fractions after the extraction procedure described above was 2.5 μg of GAGs from each milliliter of starting plasma sample. Of these GAGs, 62% was represented by CSGAGs sensitive to chondroitinase ABC and resistant to nitrous acid treatment. 38% was represented by HSGAGs sensitive to nitrous acid treatment and resistant to chondroitinase ABC. Table 1 shows the distribution of CS-, HSGAGs, and proteins in cryoprecipitate and each Cohn–Oncley fraction. Protein distribution here refers to the proteins remaining after the procedure described to extract GAGs, i.e., proteolysis, centrifugation, ethanol precipitation of the supernatant, dialysis and lyophilization. In other words, the proteins referred to are those resistant to proteolysis that were described in [10]. Total amount of protein recovered was 26.8 μg for each milliliter of starting plasma sample. Table 1 shows that most proteins were

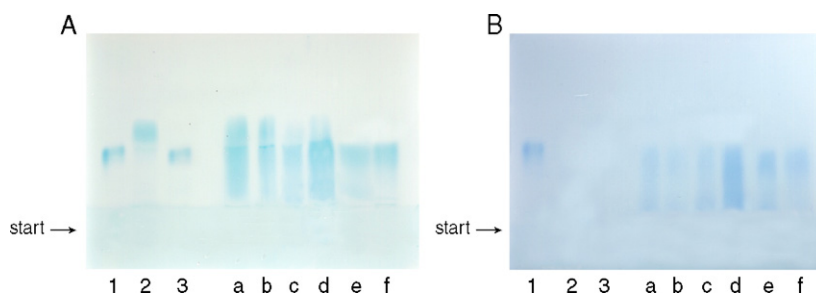


Fig. 5. Panel A: cellulose acetate electrophoresis of cryoprecipitate and Cohn–Oncley fractions at pH 1.0. Lane 1: standard mixture containing 250 ng each of the following chondroitin sulphates: CSA, CSB, and CSC. Lane 2: unfractionated HP. Lane 3: HS. Lane a: cryoprecipitate. Lane b: fraction I. Lane c: fraction II+III. Lane d: fraction IV-1. Lane e: fraction IV-4. Lane f: fraction V. Panel B: cellulose acetate electrophoresis of cryoprecipitate and Cohn–Oncley fractions subjected to nitrous acid at pH 1.0. Lane 1: standard mixture containing 250 ng each of the following chondroitin sulphates: CSA, CSB, and CSC subjected to nitrous acid. Lane 2: unfractionated HP subjected to nitrous acid. Lane 3: HS subjected to nitrous acid. Lane a: cryoprecipitate subjected to nitrous acid. Lane b: fraction I subjected to nitrous acid. Lane c: fraction II+III subjected to nitrous acid. Lane d: fraction IV-1 subjected to nitrous acid. Lane e: fraction IV-4 subjected to nitrous acid. Lane f: fraction V subjected to nitrous acid.

recovered in cryoprecipitate and in fractions I and II+III. As far as total GAGs are concerned, they were evenly distributed in cryoprecipitate, fractions I, II+III, and IV-1 (about 20% of total GAGs in each of these fractions). CS- and HSGAGs, however, showed a different distribution. Indeed, HSGAGs were recovered mostly in cryoprecipitate, fractions I and IV-1, and, although in smaller amount, in fraction II+III. CSGAGs, however, were recovered in cryoprecipitate and in all Cohn–Oncley fractions, including fractions IV-4 and V, where they represented the only recovered GAGs. The highest amounts of CSGAGs were recovered in fractions II+III and IV-1, where they represented about 80% and 60%, respectively, of all recovered GAGs. The ratio GAG/protein in cryoprecipitate and Cohn–Oncley fraction ranged from 1/3.84 (fraction IV-1) to 1/17.69 (fraction IV-4). These ratios were consistent with those shown in Fig. 2, thus demonstrating that the amount of proteins was such that GAGs could not have migrated without the newly introduced stacking procedure.

4. Discussion

It is believed that GAGs play a role in different physiological and pathological conditions [1,11]. In recent years, we focussed our attention on plasmatic HSGAGs, with particular emphasis on those HSGAGs endowed with anticoagulant activity [4]. We and others demonstrated a strong association between plasma proteins and HSGAGs, but the role of this association is still largely unknown [8–10,16]. In this study, we show for the first time the distribution of plasmatic GAGs in Cohn–Oncley fractions. In order to achieve this goal, we chose to update a relatively simple technique, i.e., cellulose acetate electrophoresis, by introducing the so-called stacking procedure. This decision was based upon recent observation demonstrating that plasmatic GAGs, in particular HSGAGs, are so strongly associated with proteins that complete purification of HSGAGs from healthy subjects is a quite long and difficult process, unsuitable for routine analysis of plasmatic HSGAGs [4]. Because of this, we opted for analysing plasmatic GAGs even in the presence of proteins remaining after proteolysis [10]. It is worth noting that HSGAGs behaved differently than CSGAGs. Thus, CSGAGs were recovered in all fractions and cryoprecipitate, whereas HSGAGs were recovered in cryoprecipitate, fractions I and IV-1, and, in smaller amount, in fraction II+III. Since the bulk of plasma proteins is partitioned in fractions II+III and V (i.e., those fractions containing immunoglobulins and albumin, respectively), these results demonstrate that HSGAGs are not randomly associated with plasma proteins. It is worth noting that most coagulation factors and proteins involved in the control of hemostasis are concentrated in cryoprecipitate and in fractions I and IV-1 [26–28]. This observation lends further credit to the hypothesis that endogenous plasmatic HSGAGs could play a role in the hemostatic balance. Fraction IV-1 is also rich in lipoproteins and lipids [29]. It is well known that HP (administered as a drug) has a strong hypolipidemic effect, and we demonstrated that HP formed supramolecular complexes with phosphatidylcholine [30]. Thus, the recovery of HSGAGs in fraction IV-1 could indicate that these GAGs might play a role in lipid metabolism and might be involved in atheroprotective effects [31].

Acknowledgements

This study was supported by grants from Ente Cassa di Risparmio di Firenze and University of Firenze. We appreciate the help of Mr. Tommaso Sansoni for technical assistance.

References

- [1] Raman R, Sasisekharan V, Sasisekharan R. Structural Insights into biological roles of protein–glycosaminoglycans interactions. *Chem Biol* 2005;12:267–77.
- [2] Volpi N, Maccari F. Microdetermination of chondroitin sulfate in normal human plasma by fluorophore-assisted carbohydrate electrophoresis (FACE). *Clin Chim Acta* 2005;356:125–33.
- [3] Volpi N, Cusmano M, Venturelli T. Qualitative and quantitative studies of heparin and chondroitin sulfates in normal human plasma. *Biochim Biophys Acta* 1995;1243:49–58.
- [4] Ruggiero M, Melli M, Parma B, Bianchini P, Vannucchi S. Isolation of endogenous anticoagulant N-sulfated glycosaminoglycans in human plasma from healthy subjects. *Pathophysiol Haemost Thromb* 2002;32:44–9.
- [5] Palmer RN, Rick ME, Rick PD, Zeller JA, Gralnick MD. Circulating heparan sulfate anticoagulant in a patient with a fatal bleeding disorder. *N Engl J Med* 1984;310:1696–9.
- [6] Horne MK, Chao ES, Wilson OJ, Scialla SJ, Lynch MA, Kragel PJJ. A heparin-like anticoagulant as a part of global abnormalities of plasma glycosaminoglycans in a patient with transitional cell carcinoma. *J Lab Clin Med* 1991;118:250–60.
- [7] Wages DS, Staprans I, Hambleton J, Bass NM, Corash L. Structural characterization and functional effects of a circulating heparan sulphate in a patient with hepatocellular carcinoma. *Am J Hematol* 1999;58:285–92.
- [8] Pasquali F, Oldani C, Ruggiero M, Magnelli L, Chiarugi V, Vannucchi S. Interaction between endogenous circulating sulphated-glycosaminoglycans and plasma proteins. *Clin Chim Acta* 1990;192:19–27.
- [9] Calatroni A, Vinci R, Ferlazzo AM. Characteristics of the interaction between acid glycosaminoglycans and proteins in normal human plasma as revealed by the behaviour of the protein polysaccharides complexes in ultrafiltration and chromatographic procedures. *Clin Chim Acta* 1992;206:167–80.
- [10] Chevanne M, Caldini R, Manao G, Ruggiero M, Vannucchi S. Heparin binding peptides co-purify with glycosaminoglycans from human plasma. *FEBS Lett* 1999;463:121–4.
- [11] Munoz EM, Linhardt RJ. Heparin-binding domains in vascular biology. *Arterioscler Thromb Vasc Biol* 2004;24:1549–57.
- [12] Cohn EJ, Strong LE, Hughes WL, et al. Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. *J Am Chem Soc* 1946;68:459–75.
- [13] Oncley JL, Malin M, Richert DA, Cameron JW, Gross PM. The separation of antibodies, isoagglutinins, prothrombin, plasminogen and B-plasma. Lipoproteins into the sub-fractions of human plasma. *J Am Chem Soc* 1946;71:541–50.
- [14] Martin TD. IGIV: contents, properties, and methods of industrial production-evolving closer to a more physiologic product. *Int Immunopharmacol* 2006;6:517–22.
- [15] Handel TM, Johnson Z, Crown SE, Lau EK, Sweeney M, Proudfoot AE. Regulation of protein function by glycosaminoglycans as exemplified by chemokines. *Ann Rev Biochem* 2005;74:385–410.
- [16] Staprans I, Felts JM. Isolation and characterization of glycosaminoglycans in human plasma. *J Clin Invest* 1985;76:1984–91.
- [17] Cappelletti R, Del Rosso M, Chiarugi V. A new electrophoretic method for the complete separation of all known glycosaminoglycans in a monodimensional run. *Anal Biochem* 1979;99:311–5.
- [18] Wessler E. Electrophoresis of acidic glycosaminoglycans in hydrochloric acid: a micro method for sulfate determination. *Anal Biochem* 1971;41:67–9.
- [19] Cappelletti R, Del Rosso M, Chiarugi V. A new method of characterization of N-sulfated glycosaminoglycans by a rapid and multisample nitrous acid treatment during an electrophoretic run and its application to the analysis of biological samples. *Anal Biochem* 1980;105:430–5.

- [20] Green AA, Hughes WL. Protein fractionation on the basis of solubility in aqueous solutions of salts and organic solvents. *Methods Enzymol* 1955; 1:67–90.
- [21] Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 1976;72:248–54.
- [22] Yamagata T, Saito H, Habuchi O, Suzuki S. Purification and properties of bacterial chondroitinases and chondrosulfatases. *J Biol Chem* 1968;243: 1523–35.
- [23] Schodt KP, Gelman RA, Blackwell J. The effect of changes in salt concentration and pH on the interaction between glycosaminoglycans and cationic polypeptides. *Biopolymers* 1976;15:1965–77.
- [24] Cohn EJ. The separation of blood into fractions of therapeutic value. *Ann Intern Med* 1947;26:341–52.
- [25] Shively JE, Conrad HE. Nearest neighbor analysis of heparin: identification and quantitation of the products formed by selective depolymerization procedures. *Biochemistry* 1976;15:3943–50.
- [26] Farrugia A, Giangrande P. Choice of replacement therapy for hemophilia-cryoprecipitate issues: rebuttal. *J Thromb Haemost* 2004;2:1022–3.
- [27] Blomback M, Blomback B. Plasma fractions for the treatment of hemophilia. 1. On the preparation and use of fraction I-O. *Thromb Diath Haemorrh Suppl* 1969;35:21–5.
- [28] Wunderwald P, Schrenk WJ, Port H. Antithrombin BM from human plasma: an antithrombin binding moderately to heparin. *Thromb Res* 1982;25:177–91.
- [29] Abdelnoor AM, Harvie NR, Johnson AG. Neutralization of bacteria and endotoxin induced hypotension by lipoprotein-free human serum. *Infect Immun* 1982;38:157–61.
- [30] Vannucchi S, Ruggiero M, Chiarugi V. Complexing of heparin with phosphatidylcholine. A possible supramolecular assembly of plasma heparin. *Biochem J* 1985;227:57–65.
- [31] Deepa PR, Veralakshima P. Atheroprotective effect of exogenous heparin-derivative treatment on the aortic disturbances and lipoprotein oxidation in hypercholesterolemic diet fed rats. *Clin Chim Acta* 2005;355:119–30.