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Journal of Chromatography B, 820 (2005) 131–135
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Simultaneous detection of submicrogram quantities of hyaluronic acid and dermatan sulfate on agarose-gel by sequential staining with toluidine blue and Stains-All

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Received 14 December 2004; accepted 30 March 2005
Available online 18 April 2005

Abstract

A new discontinuous agarose-gel electrophoresis in 0.05 M HCl/0.04 M barium acetate combined with the highly sensitive visualization technique using toluidine blue/Stains-All has been developed for the simultaneous assaying of hyaluronic acid (HA) and dermatan sulfate (DS) with a detection limit at submicrogram level greater than other conventional procedures. Furthermore, this procedure also separates and reveals chondroitin sulfate (CS). The densitometric analysis of bands resulted in a linear response between 0.01 and 0.5 μg of glycosaminoglycans (GAGs) with correlation coefficients greater than approximately 0.94. Hyaluronic acid and dermatan sulfate extracted and purified from the abdominal skin of six rats were separated and quantified in comparison with the evaluation made by treatment of chondroitin ABC lyase and separation of Δ-disaccharides from hyaluronic acid (ΔdiHA) and dermatan sulfate/chondroitin sulfate (Δdi4s and Δdi6s) by HPLC. The total amount of rat skin polysaccharides (hyaluronic acid and dermatan sulfate) was 1.24 ± 0.26 μg/mg of tissue by discontinuous agarose-gel electrophoresis and 1.20 ± 0.33 μg/mg by HPLC with hyaluronic acid and dermatan sulfate percentages of 50.32 ± 2.38 and 49.66 ± 2.53, respectively. The analyses also confirmed that hyaluronic acid and dermatan sulfate are the main rat abdominal skin polysaccharides with chondroitin sulfate present in trace amounts. This new agarose-gel electrophoresis could be particularly useful in the study of the distribution of glycosaminoglycans in the skin from different body sites of animals and normal human subjects and may be of importance in understanding the changes that occur in the skin, especially the metabolism of extracellular matrix constituents, in connective tissue disorders.

Keywords: Electrophoresis; Glycosaminoglycans; Hyaluronic acid; Dermatan; Chondroitin; Skin

1. Introduction

Conventional agarose-gel electrophoresis mainly permits the separation of sulfated polysaccharides, such as heparin with its two components, the more sulfated and with higher M_r slow-moving heparin and the less sulfated with lower M_r fast-moving heparin, heparan sulfate, dermatan sulfate (DS) and chondroitin sulfate (CS) [1]. These polysaccharides are characterized by the presence of variable contents of sulfated groups on the carbohydrate backbone (with a charge density ranging from approximately 0.5 to 2.5), and as a consequence they are normally stained by cationic dye, such as toluidine blue [2–5]. However, these conditions are not suitable for the visualization and detection of nonsulfated polysaccharides, such as hyaluronic acid (HA) [5]. Furthermore, we must consider that the common electrophoretic conditions used to separate mixtures of glycosaminoglycans (GAGs) in

Abbreviations: GAG, glycosaminoglycan; CS, chondroitin sulfate; DS, dermatan sulfate; HA, hyaluronic acid, hyaluronan; ΔdiHA, 2-acetamido-2-deoxy-3-*0*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose; Δdi4s, 2-acetamido-2-deoxy-3-0-(4-deoxy-α-L-threo-hex-4-enepyranosyluronic acid)-D-galactose 4-sulfate; Δdi6s, 2-acetamido-2-deoxy-3-0-(4-deoxy-α-L-threo-hex-4-enepyranosyluronic acid)-D-galactose 6-sulfate

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doi:10.1016/j.jchromb.2005.03.028

agarose-gel electrophoresis, such as barium acetate or 1,2-diaminopropane buffers, are not able to separate HA and DS from each other [5].

HA is quite distinct from the other natural polysaccharides, because of the absence of sulfated groups [6,7]. HA is typically found in the connective tissues and biological fluids of vertebrates, and tissues such as the skin, umbelical cord, skeleton and supporting tissues, muscles and synovial fluids are characterized by a high HA content [6]. DS, also known as CS B, covalently linked to core proteins to form various proteoglycans (reviewed in [8]), is largely present in several tissues and organs, such as skin, cartilage, cornea, bone. Furthermore, these two GAGs are synthesized by most kinds of vertebrate and also invertebrate cells [6–8] and this feature can be suppressed or activated in changing circumstances, as in the case of pathological conditions. Due to their ubiquitous presence and their fundamental biological functions [7–9], HA and DS may be implicated in many different kinds of disease. Deficiencies may be present, for example, in certain biosynthetic or catabolic steps of these GAGs. Regarding the latter, characteristic examples are many types of cancer, where impairment in HA and DS biosynthesis is observed, and all mucopolysaccharidoses, where an inherited deficiency of at least one enzyme involved in GAG catabolism is found [10]. As a consequence, a specific and sensitive analytical technique to detect simultaneous quantitative modifications of these two polymers in tissue and biological fluid extracts is necessary.

In a previous paper, we developed a new improved submicrogram visualization procedure involving the cationic dye toluidine blue followed by a Stains-All staining step able to detect complex sulfated GAGs (i.e. heparin, heparan sulfate, CS, DS) and also nonsulfated polysaccharides [5] not stained with toluidine blue alone. In this study, we have extended the previous reported methodology and we propose to describe a new agarose-gel electrophoresis approach with a submicrogram visualization procedure involving staining with the cationic dye toluidine blue followed by the Stains-All step for the simultaneous detection of HA and DS.

2. Materials and methods

2.1. Materials

DS having an M_r of about 28,200 and a charge density of 1.09 was purified from beef mucosa [5]. Chondroitin sulfate from bovine trachea with an M_r of about 23,760 and a sulfate-to-carboxyl ratio of 0.93 and HA from bovine trachea with a M_r of about 1,000,000 were obtained from IBSA (Institut Biochimique SA, Lugano, Switzerland). Proteinase K from *Tritirachium album* [E.C. 3.4.21.64], >500 units/ml, and chondroitin ABC lyase from *Proteus vulgaris* [E.C. 4.2.2.4] were obtained from Sigma-Aldrich (St. Louis, USA). QAE Sephadex A-25 was from Amersham Pharmacia Biotech (Piscataway, USA). Dialysis Spectra/Por 3 membrane were from Spectrum (Rancho Dominguez, USA). 5 μm Spherisorb SAX 150 × 4.6-mm stainless-steel column (trimethylammoniopropyl groups $Si{-}CH_2{-}CH_2{-}CH_2{-}N^+(CH_3)_3$ in Cl^- form) was from Phase Separations Limited, Deeside Industrial Park, Deeside Clwyd, UK. Nonsulfated unsaturated HA disaccharide [ΔdiHA, 2-acetamido-2-deoxy-3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose] and the variously sulfated unsaturated CS/DS disaccharides [Δdi4s, 2-acetamido-2-deoxy-3-0-(4-deoxy-α-L-threo-hex-4-enepyranosyluronic acid)-D-galactose 4-sulfate and Δdi6s, 2-acetamido-2-deoxy-3-0-(4-deoxy-α-L-threo-hex-4-enepyranosyluronic acid)-D-galactose 6-sulfate] were obtained from Seikagaku Corporation, Tokyo, Japan. High purity agarose and barium acetate were from Bio-Rad (Hercules, USA). 1,2-Diaminopropane (PDA) was from Merck, Darmstadt, Germany. Cetylpyridinium chloride was from Aldrich, Steinheim, Germany. Toluidine blue and Stains-All (3,3′-dimethyl-9-methyl-4,5,4′,5′-dibenzothiacarbocyanine) were purchased from Sigma-Aldrich. All the other reagents were analytical grade.

2.2. Extraction and purification of skin GAGs

Approximately 1 g of abdominal skin from male Sprague–Dawley rats (13–14 weeks old) was cut into small slices and digested with 7.0 mg proteinase K in 10 ml 20 mM Tris-buffer (pH 7.0) at 55 °C for 12 h (then boiled for 5 min). After centrifugation, 180 ml acetone were added to the surnatant and the tubes were stored at −20 °C for 12 h. After precipitation, the tubes were centrifugated at 4000 rpm for 5 min and the precipitate was dried at 50 °C overnight. The dried material was dissolved in 10 ml distilled water and introduced into glass columns containing 9 ml of the anion-exchange resin (Sephadex QAE). After washing the resin with 2 × 10 ml 100 mM NaCl, pH 4, the GAGs were eluted with 15 ml 2.5 M NaCl, pH 4. The eluate was transferred to centrifugation tubes and again precipitated with 180 ml acetone at −20 °C for 12 h. After precipitation, the tubes were centrifugated at 4000 rpm for 5 min and the precipitate dried at 50 °C for further 12 h. The dried material with the purified GAGs was then dissolved in 10 ml water, transferred to a dialysis bag and dialyzed against distilled water for 24 h. After lyophilization, the purified skin GAGs were ready for agarose-gel analysis.

2.3. Agarose-gel electrophoresis of glycosaminoglycans

A Pharmacia Multiphor II (from Pharmacia LKB Biotechnology, Uppsala, Sweden) electrophoretic cell instrument was used. Agarose-gel was prepared at a concentration of 0.5% in 0.04 M barium acetate buffer pH 5.8. Plates with a thickness of about 4–5 mm were prepared. Samples of 2–20 μl dissolved in distilled water were layered by micropipets. The run was in 0.05 M HCl for 180 min at 200 mA and in 0.04 M barium acetate (buffered at pH 5.8 with acetic acid) for 60 min at 100 mA. After migration, the plate

was soaked in a solution of 0.2% cetylpyridinium chloride. After drying, the plate was stained with freshly prepared toluidine blue (0.2% in ethanol–water–acetic acid 50:49:1) for 30 min, and destained with ethanol–water–acetic acid 50:49:1. Plates were further stained with Stains-All (25 mg in 500 ml ethanol–water 50:50 overnight in the dark and destained with water) to reveal HA [5].

Quantitative analysis of GAGs was performed with a densitometer composed of a Macintosh IIsi computer interfaced with Microtek Color Scanner from Microtek International Inc., Hsinchu, Taiwan. The plates were scanned in the RGB mode and saved in gray scale. Image processing and analysis program, Ver. 1.41 from Jet Propulsion Lab., NASA, Florida, USA, was used for densitometry.

2.4. Disaccharide analysis of HA and DS by HPLC

Ten microliters of samples (10 mg/ml) were treated with 5 mU of chondroitin ABC lyase in 50 μl of 100 mM Tris/150 mM sodium acetate buffer pH 8.0 at 37 °C for 12 h. The reaction was blocked by boiling the solutions for 1 min. The unsaturated disaccharides generated from HA (ΔdiHA) and DS/CS (Δdi4s and Δdi6s) purified from skin samples after enzymatic treatment were analyzed by strong anion-exchange (SAX)-HPLC separation and detection at 232 nm, as reported [3]. Isocratic separation was from 0 to 5 min with 0.05 M NaCl, pH 4.00 and linear gradient separation was from 5 to 90 min with 100% 0.05 M NaCl, pH 4.00 to 100% 1.2 M NaCl, pH 4.00. Flow rate was 1.2 ml/min. HPLC equipment was from Jasco, Tokyo, Japan (pump mod. PU-1580, UV detector mod. UV-1570, Rheodyne injector equipped with a 100 μl loop, software Jasco-Borwin rel. 1.5). The amount of each identified disaccharide, in particular the unsaturated HA disaccharide ΔdiHA and the 4-sulfated unsaturated DS disaccharide Δdi4s, was determined by purified standards and reported as weight.

3. Results and discussion

Fig. 1A illustrates the discontinuous agarose-gel electrophoresis separation in 0.05 M HCl/0.04 M barium acetate of increasing amounts, from 1 to 4 μg, of HA and DS standard. After staining with toluidine blue, the DS appeared as a purple–blue band due to the metachromasia typical of sulfated GAGs while no HA was detected. After treatment with Stains-All, also HA appeared as a strong blue band typical of nonsulfated polysaccharides [5]. As evident from Fig. 1A, a good resolution between these two polysaccharides was achieved, with HA and DS having a mobility of 1.29 and 2.14 cm/(A h), respectively. This was further confirmed by densitometric scanning (Fig. 1B). Furthermore, the densitometric analysis of HA and DS bands enabled us to give quantitative values depending on their concentration, from 0.01 to 2 μg (Fig. 2), according to previous data obtained by conventional agarose-gel separation stained with toluidine blue and

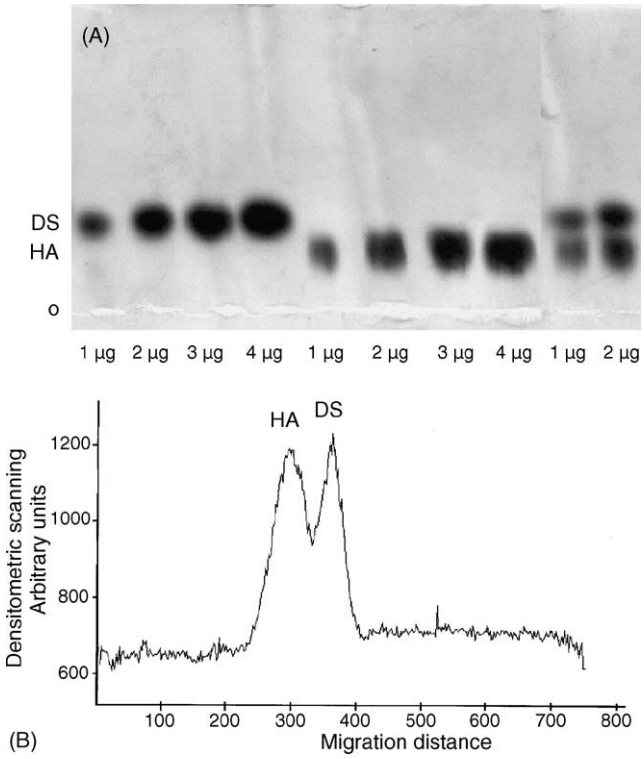

Fig. 1. (A) Discontinuous agarose-gel electrophoresis in 0.05 M HCl/0.04 M barium acetate combined with the visualization technique using toluidine blue/Stains-All of increasing amounts of dermatan sulfate (DS) and hyaluronic acid (HA), from 1 to 4 μg. Origin (o). (B) Densitometric scanning of the last line in (A) was performed by using a densitometer composed of a Macintosh IIsi computer interfaced with a Microtek Color Scanner from Microtek International Inc., Hsinchu, Taiwan. The agarose-gel was scanned in the RGB mode and saved in gray scale. Image processing and analysis program, Ver. 1.41 from Jet Propulsion Lab., NASA, Florida, USA, was used for densitometry.

Stains-All [5]. However, linear response was found only between 0.01 and 0.5 μg of GAGs (Fig. 2 upper panel, showing the linear regression calculated for HA and DS from 0.01 to 0.5 μg from Fig. 2) with correlation coefficients greater than approximately 0.94.

The new electrophoresis procedure also allowed us to separate and reveal CS possessing a mobility of 2.43 cm/(A h) (Fig. 3A) with a good resolution from DS as also confirmed by the densitometric scanning (Fig. 3B).

GAGs from abdominal skin were extracted and purified from several rats and submitted to qualitative and quantitative evaluation by discontinuous agarose-gel electrophoresis separation in 0.05 M HCl/0.04 M barium acetate. As illustrated in Fig. 4, HA and DS from the skin of six rats were separated and quantified in comparison with the quantitative evaluation of skin GAGs performed by treatment with chondroitin ABC lyase and separation of Δ-disaccharides from HA (ΔdiHA) and DS/CS (Δdi4s and Δdi6s) by HPLC (Fig. 5). The total amount of rat skin GAGs (HA + DS) was found to be 1.24 ± 0.26 μg/mg of tissue by discontinuous agarose-gel electrophoresis and 1.20 ± 0.33 μg/mg of tissue by HPLC with HA and DS percentages of 50.32 ± 2.38 and

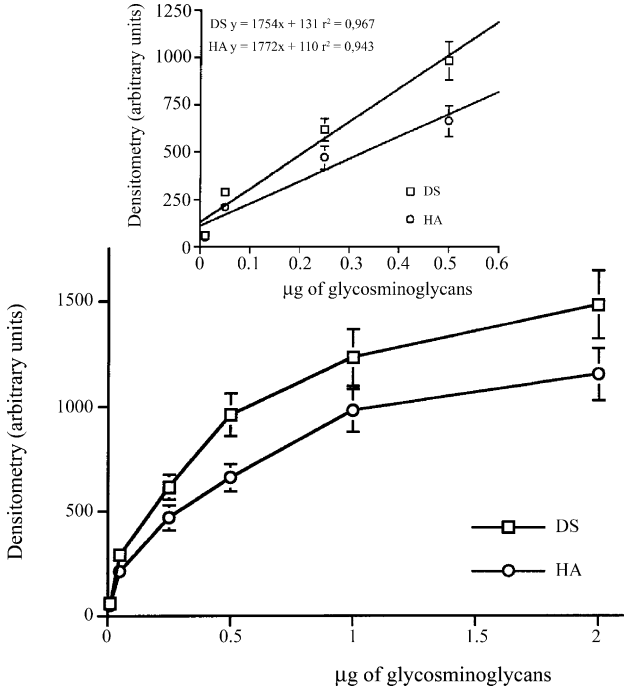

Fig. 2. Quantitation of sulfated glycosaminoglycans (from 0.01 to 2 μg) after discontinuous agarose-gel electrophoresis stained with toluidine blue and Stains-All. Upper panel: linear correlation between 0.01 and 0.5 μg for dermatan sulfate (DS) and hyaluronic acid (HA). The equations and the correlation coefficients are reported.

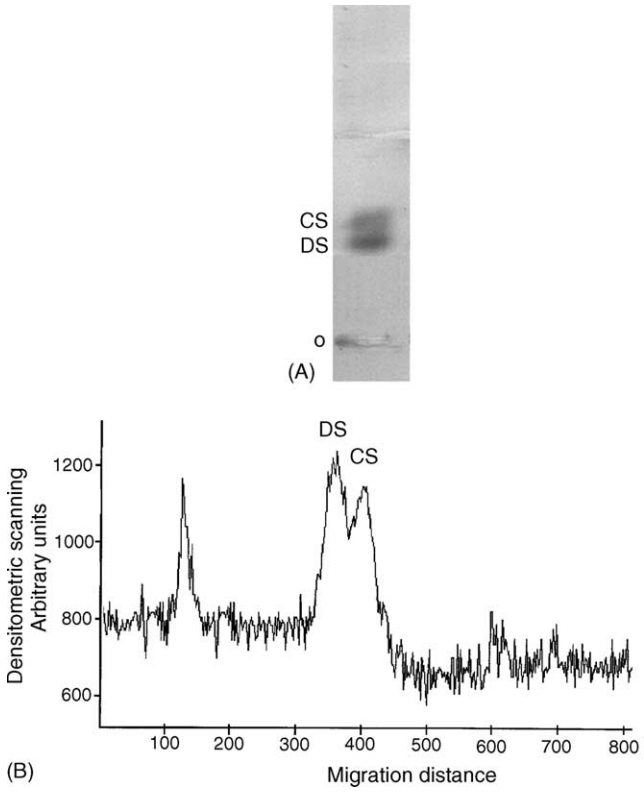

Fig. 3. (A) Separation of 0.5 μg of chondroitin sulfate (CS) and 0.5 μg of dermatan sulfate (DS) by discontinuous agarose-gel electrophoresis in 0.05 M HCl/0.04 M barium acetate combined with the visualization technique using toluidine blue/Stains-All. Origin (o). (B) Densitometric scanning of the gel.

49.66 ± 2.53, respectively, compared with the data obtained by HPLC (Table 1). Furthermore, the analyses confirmed that HA and DS are the main rat skin GAGs with CS present in trace amounts as detected by HPLC.

Along with heparin, heparan sulfate, CS and keratan sulfate, HA and DS are the main natural polysaccharides distributed among all organisms [10]. Apart HA, these biological molecules are major structural components of proteoglycans, the main macromolecular complex of the extracellular matrix [11,12] but also localized at cellular level, as intracellular components [13] and distributed on the cellular membrane [14]. HA and DS/CS are present in great amounts in the extracellular environment and constitute the main GAGs of connective tissues, such as skin, bone, cartilage, muscle [6–8], where they participate in and regulate many physiological processes [15]. Furthermore, modification in the skin quantity and type of these polysaccharides have been observed in pathological conditions, for example in scleroderma [16]

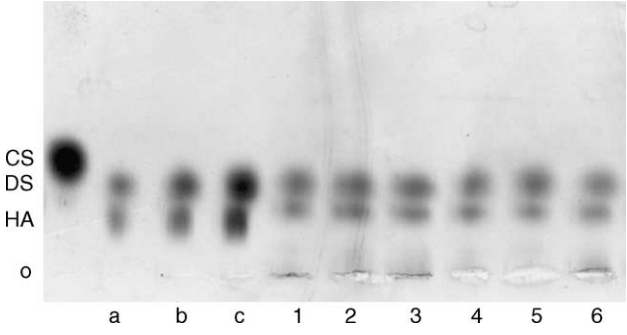

Fig. 4. Discontinuous agarose-gel electrophoresis separation of GAGs extracted and purified from the skin of several rats (from one to six). Purified GAGs were dissolved in distilled water at a concentration of 1 mg/ml and 15 μl were layered on the agarose-gel plate. Increasing amounts (a: 0.5 μg; b: 1.0 μg; c: 1.5 μg) of HA and DS standard, and 3 μg of CS standard.

Table 1
Total GAGs amount of rat skin and the relative percentage of hyaluronic acid (HA) and dermatan sulfate (DS) calculated by HPLC and agarose-gel electrophoresis stained with toluidine blue and Stains-All

	Agarose-gel	HPLC
Total GAGs (μg/mg tissue)	1.24 ± 0.26 (CV = 21.9%)	1.20 ± 0.33 (CV = 27.5%)
% HA	50.32 ± 2.38 (CV = 4.8%)	52.42 ± 3.95 (CV = 7.5%)
% DS	49.66 ± 2.53 (CV = 5.1%)	47.58 ± 3.95 (CV = 8.3%)

Data are reported as mean ± standard deviation from six animals. CV: coefficient of variation.

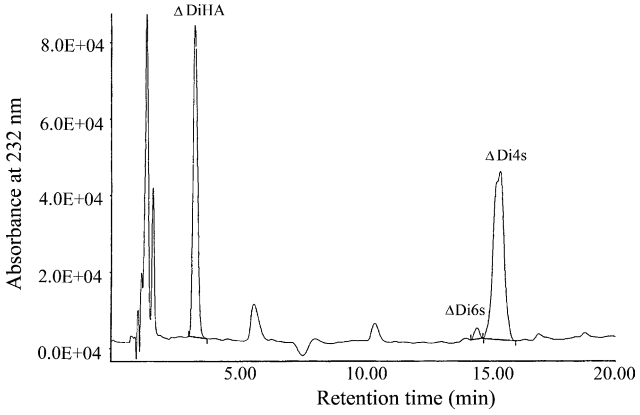

Fig. 5. HPLC separation of Δ-disaccharides from HA (ΔdiHA) and DS/CS (Δdi4s and Δdi6s) derived from the treatment of rat skin GAGs performed by chondroitin ABC lyase. ΔdiHA, 2-acetamido-2-deoxy-3-*0*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-glucose; Δdi4s, 2-acetamido-2-deoxy-3-0-(4-deoxy-α-L-threo-hex-4-enepyranosyluronic acid)-D-galactose 4-sulfate; Δdi6s, 2-acetamido-2-deoxy-3-0-(4-deoxy-α-L-threo-hex-4-enepyranosyluronic acid)-D-galactose 6-sulfate.

or under ultraviolet light irradiation [17], during pharmacological treatment [18] and fetal development [19] but also depending on diet [17,20] and ageing [19].

The most common analytical methods for the determination of skin GAGs are electrophoretic approaches, such as on cellulose acetate [21] or nitrocellulose [22] strips, and/or HPLC. This last technique permits the quantitative evaluation of HA, DS and CS by measuring the corresponding unsaturated disaccharides (ΔdiHA for HA, Δdi4s for DS and Δdi6s for CS) after treatment with chondroitinase ABC or other specific lyases [10]. In fact, the iduronic acid-containing units, monosaccharide mainly belonging to DS, are often sulfated at C-4 of the *N*-acetyl-galactosamine, while sulfatation at C-6 is frequently associated with glucuronic acid-containing disaccharides, mainly present in CS chains [10]. By using HPLC we confirm the new discontinuous agarose-gel electrophoresis presented in this paper as a suitable approach for the quantitative evaluation of skin HA/DS/CS. In fact, according to other literature data, rat skin GAGs (and also healthy human skin [16]) are mainly constituted by HA and DS with CS present in trace amounts [17,18,23]. However, we must consider that the percentage of these two GAGs in the skin strongly depends on the different body sites as determined in humans [16]. Furthermore, discontinuous agarose-gel electrophoresis in 0.05 M HCl/0.04 M barium acetate was found to possess a greater sensitivity (detection limit of approximately 0.01 μg) than HPLC (detection limit of approximately 1 μg) but also than electrophoresis on nitrocellulose (detection limit of approximately 0.1–0.5 μg [22]) and on cellulose acetate (detection limit of approximately 0.01–1 μg depending on the GAGs [21]) due to the very sensitive staining procedure performed by toluidine blue/Stains-All [5].

4. Conclusions

In conclusion, a new discontinuous agarose-gel electrophoresis in 0.05 M HCl/0.04 M barium acetate combined with the highly sensitive visualization technique using toluidine blue/Stains-All has been established and shown to be a valuable method for the simultaneous assaying of HA and DS/CS with a detection limit at submicrogram level greater than other conventional procedures. Furthermore, the reported electrophoretic approach is simple and rapid as it uses the conventional procedure usually utilized for staining polyanions after agarose-gel separation followed by a unique step to improve the sensitivity of the method. Finally, this new agarose-gel electrophoresis could be particularly useful to study GAG distribution in the skin from different body sites in animals and normal human subjects also considering possible changes occurring in the skin extracellular matrix at the GAG level in various connective tissue disorders.

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