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Quantitative determination of the glycosaminoglycan Δ-disaccharide composition of serum, platelets and granulocytes by reversed-phase high-performance liquid chromatography

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ABSTRACT

Seven Δ-disaccharide standards from heparan sulfate/heparin (HS/H) and nine Δ-disaccharide standards from chondroitin/dermatan sulfate (CS/DS) and hyaluronic acid (HA) were derivatized with the fluorophore 2-aminoacridone (AMAC) and separated in two runs each by reversed-phase HPLC with baseline separation and very short run times. This novel method facilitates the separation of the largest number of Δ-disaccharides from both CS/DS/HA and HS/H with one column and buffer system after fluorophore labeling in two runs at present. For the first time nine glycosaminoglycan (GAG) Δ-disaccharides from CS/DS/HA were separated after fluorophore labeling in one run. The limits of quantification (LOQs) were below 0.2 pmol for CS/DS/HA and HS/H Δ-disaccharides. We demonstrated applicability of our method for biological samples. Furthermore, normal ranges of the GAG Δ-disaccharide compositions from platelets and granulocytes were determined for the first time.

1. Introduction

Glycosaminoglycans (GAGs) are negatively charged linear heteropolysaccharides which are classified into several groups on the basis of structure. GAGs are found mainly in connective tissue as constituents of proteoglycans, covalently linked to the core protein. Proteoglycans are localized at both the extracellular and cellular levels and participate in and regulate several cellular events and physiological processes [1]. Their potential for being interactive to such a degree results from the great structural diversity in their GAG chains, i.e. GAG type, size and composition. GAGs are composed of a variable number of repeating disaccharide units. The disaccharide units differ in their uronic acid (D-glucuronic acid, D-iduronic acid or galactose), their hexosamine unit (D-galactosamine or D-glucosamine) and their number (zero to three) and position of sulfate groups. According to their composition, GAGs can be divided into different groups of disaccharides: (1) hyaluronic acid (HA), (2) chondroitin sulfate (CS) and dermatan sulfate (DS), (3) heparan sulfate (HS) and heparin (H), (4) keratan sulfate (KS) [2]. For compositional analysis GAGs are enzymatically digested into their disaccharide units, which can be separated and quantified by chromatography, electrophoresis and mass spectrometry. Δ-Disaccharides strongly absorb at 232 nm (molar absorption coefficient of 5500 M^{-1} cm^{-1}) due to the double bonds in unsaturated uronic acid [3]. Nevertheless Δ-disaccharides are derivatized with fluorescent molecules to significantly increase detection sensitivity and specificity of HPLC [4], capillary electrophoresis and polyacrylamide gel electrophoresis. Derivatization may also change the disaccharide properties to enable or improve resolution. AMAC is a fluorescent hydrophobic molecule that has been successfully used for the derivatization and separation of Δ-disaccharides. Derivatized Δ-disaccharides show superior stability compared to most other fluorescent derivatives and remain stable even after one month at room temperature [5]. In recent years laser-induced fluorescence capillary electrophoresis methods have been published for the analysis of AMAC derivatized Δ-disaccharides [3,6,7] with a superior detection limit of less than 1 amol. Nevertheless, no baseline resolution between the CS-derived and the HA-derived nonsulfated standard Δ-disaccharides could be shown [7]. Unsaturated disaccharides have also been separated with various HPLC methods for more than 25 years. In contrast to laser-induced fluorescence capillary electrophoresis apparatus, standard HPLC chromatography apparatus is present in almost every laboratory/research facility.

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GAGs from blood serum or urine have been analyzed in many studies investigating whether diseases like diabetes or pseudoxanthoma elasticum influence GAG amount and composition [8,9]. Changes in GAG content and distribution are believed to play an important role in the development of diabetic complications [10]. Since it has been known that GAGs are involved in cell surface interactions, surface proteoglycans and GAGs of many types of blood cells have been purified and investigated. The main proteoglycan of human platelets is a macromolecule of M_r 136,000 containing four C-4-sulfate chains, each with M_r of 28,000 [11]. This proteoglycan has been revealed as a serglycin proteoglycan [12]. Serglycin is crucial for the storage of specific secretory proteins in mast cells, neutrophils and cytotoxic T-cells. It has been demonstrated that platelet serglycin is necessary for platelet function and thrombus formation [13]. CS GAGs on the surface of neutrophils, which are the main fraction of granulocytes, have been shown to bind to platelet factor 4 [14]. Platelet factor 4 is a platelet-derived α-chemokine that binds to and activates human neutrophils for specific functions like adhesion or exocytosis. Whereas proteoglycans and GAGs of granulocytes and platelets have been investigated [10,15,16], there is no information about the normal range of their GAG disaccharide composition in human whole blood. To investigate the GAGs of platelets and granulocytes, we improved existing purification methods. For the purification of granulocytes we achived improved purities with regard to platelet contamination to an existing, more expensive method using antibodies for purification [17]. We also improved the method for purification of GAGs, extracting and purifying GAGs from many kinds of biological samples with good recovery. Here we present a RP-HPLC method for the separation of either seven Δ-disaccharide standards from HS/H or nine Δ-disaccharide standards from CS/DS/HA, derivatized with the fluorophore AMAC. The developed method is the first HPLC method that facilitates the separation of either seven Δ-disaccharides from HS/H or nine Δ-disaccharides from CS/DS/HA with one column and buffer system when fluorophore labeled. This is also the first HPLC method which facilitates the separation of all nine GAG Δ-disaccharides from CS/DS/HA after fluorophore labeling in one run. The applicability of the GAG purification protocol and the HPLC separation for biological samples was demonstrated for serum and blood cells. We developed a stable, sensitive and fast HPLC method for the separation of a large number of GAG Δ-disaccharides by standard HPLC equipment, that can be used in every research lab.

2. Experimental

2.1. Equipment

The equipment for RP-HPLC consisted of an Shimadzu Prominence LC-20A system, with an RF 10 AXL fluorescence detector. The used column was a Waters X-Bridge Shield (3.5 μm, 100 mm × 4.6 mm I.D.). The cell number of blood cells was measured by two consecutive readings for each sample by the CellDyn 4000 (Abbott).

2.2. Reagents

GAG disaccharide standards were obtained from MoBiTec (Göttingen, Germany). Vivaspin 500 Ultrafilter 3.000 MWCO PES were obtained from Sartorius (Göttingen, Germany). Heparin lyase 1, 2 and 3 were obtained from Grampian enzymes (Orkney, UK). Ficoll was obtained from Biotest (Dreieich, Germany). Chondroitinase ABC, CS sodium salt from shark cartilage, AMAC and percoll, all of the purest grade available, were obtained from Sigma–Aldrich (Schnelldorf, Germany). HPLC gradient grade acetonitrile and water was obtained from Roth (Karlsruhe, Germany). All other reagents were of analytical reagent grade.

2.3. Serum

All blood samples were obtained from healthy blood donors. All subjects gave their informed consent. Serum samples were obtained from 27 healthy blood donors. Serum from 15 ml of coagulated blood was obtained by centrifugation at 3300 × *g* for 10 min.

2.4. Purification of red blood cells (RBCs)

RBC samples were taken from 6 RBC concentrates. The cells were washed three times with HEPES buffer [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.85% NaCl, 1 mM EDTA, pH 7.4] to eliminate plasma traces.

2.5. Purification of platelets

Platelets from 35 healthy blood donors were purified. 15 ml of EDTA anticoagulated blood was centrifuged at 110 × *g* for 10 min. The upper phase was centrifuged through a solution of percoll (1.063 g/ml, 0.15 M NaCl) at 350 × *g* for 10 min with the brake off. The upper band of platelets was diluted with an equal volume of HEPES buffer and centrifuged (950 × *g*, 20 min). The pelleted platelets were resuspended in 6 ml HEPES buffer, centrifuged (20 min, 650 × *g*), resuspended again and finally pelleted (850 × *g*, 20 min) to eliminate plasma traces.

2.6. Purification of granulocytes

Granulocytes were purified from 56 healthy blood donors. 15 ml of EDTA anticoagulated blood was diluted by an equal volume of hepesbuffer. The diluted blood was centrifuged through an equal volume of a solution of percoll (1.095 g/ml, 0.15 M NaCl) at 800 × *g* and 20 °C for 25 min with the brake off. The cell layer between plasma and percoll was taken and centrifuged (450 × *g*, 15 min) after dilution in HEPES buffer. The cell pellet was resuspended in 5 ml buffer and carefully layered over 5 ml of ficoll. After centrifugation (400 × *g*, 25 min, 20 °C, brake off) the pelleted granulocytes were washed three times with HEPES buffer (400 × *g*, 10 min).

2.7. Extraction of GAGs

Vacuum-dried sample pellets were treated with a solution of 1 M sodium borohydride in 0.5 M sodium hydroxide at 37 °C overnight, to release all GAG chains from proteins. Samples were neutralized with HCl and proteins were precipitated on ice after TCA was added to a final concentration of 10%. After centrifugation (10,000 × *g*, 10 min, 4 °C) the supernatant was collected and mixed with four volumes of 5% (w/v) potassium acetate in 99% ethanol. After precipitation overnight at −20 °C and centrifugation (10,000 × *g*, 10 min, 4 °C) the GAG pellet was vacuum dried and dissolved in 0.5 ml HPLC-water. GAGs were desalted by ultrafiltration steps (Vivaspin 500) at 14,800 × *g*, adding three times 0.4 ml water after centrifugation. The retentate was split up into three equal parts (1 = CS/DS/HA, 2 = HS/H and 3 = control) and dried. The first sample part was reconstituted in 45 μl 50 mM ammonium acetate, pH 8 and 5 μl 1 U/ml chondroitinase ABC and incubated overnight at 37 °C. The second part was reconstituted in 35 μl buffer (20 mM ammonium acetate, pH 7.1, 4 mM calcium acetate) and 5 μl of each of heparin lyase 1, 2 and 3 (0.1 IU/ml) were added for digestion overnight at 35 °C. All three sample parts were vacuum dried and derivatized with AMAC. Derivatization was performed as described by Jackson [18] and modified by Kitagawa et al. [19] and Lamari