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Heparan sulfate disaccharide measurement from biological samples using pre-column derivatization, UPLC-MS and single ion monitoring

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ABSTRACT

Glycosaminoglycans are a heterogeneous family of linear polysaccharides comprised of repeating disaccharide subunits that mediate many effects at the cellular level. There is increasing evidence that the nature of these effects is determined by differences in disaccharide composition. However, the determination of GAG disaccharide composition in biological samples remains challenging and time-consuming. We have developed a method that uses derivatization and selected ion recording and RP-UPLCMS resulting in rapid separation and quantification of twelve heparin/heparin sulfate disaccharides from 5 μg GAG. Limits of detection and quantitation were 0.02–0.15 and 0.07–0.31 μg/ml respectively. We have applied this method to the novel analysis of disaccharide levels extracted from heparan sulfate and human cancer cell lines. Heparan sulfate disaccharides extracted from biological samples following actinase and heparinase incubation and derivatized using reductive amination with 2-aminoacridone. Derivatized disaccharides were analyzed used UPLC-MS with single ion monitoring. Eight HS disaccharide subunits were separated and quantified from HS and cell lines in eleven minutes per sample. In all samples the most abundant subunits present were the unsulfated ΔUA-GlcNAc, ΔUA-GlcNAc,6S and ΔUA,2S-GlcNS,6S. There was considerable variation in the proportions and concentrations of disaccharides between different cell lines. Further studies are needed to examine the significance of these differences.

Introduction

Glyosaminoglycans (GAGs) are long unbranched polysaccharide chains composed of repeating disaccharide subunits of alternating uronic acid and glycosamine (GlcN) residues. These molecules have a large net negative charge as a result of a complex arrangement of N- and/or O-linked sulfate binding on the sugar moieties. Heparin is the best known GAG and is expressed exclusively by mast cells [1], Other GAGs include heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS) and keratan sulfate (KS). These are ubiquitously expressed on the extracellular matrix and bound, via serine residues, to protein cores on many cell surfaces in the form of proteoglycans (PGs) [2].

Whilst heparin is well known for its anticoagulant properties, there is increasing evidence that heparin and other GAGs can influence cell functions through a diverse range of activities that include the regulation of cell survival, growth, differentiation and angiogenesis and invasion [3–5]. Although the molecular mechanism of the action of GAGs is not well understood, accumulating evidence suggests that the effects of heparan sulfate glycosaminoglycans may be attributable at least in part to their binding of the growth factors and regulating of their signaling [6,7].

GAGs show considerable variation in chain length and degree of sulfation on either sugar in the disaccharide subunit. In the case of HS, the disaccharide units are differentially sulfated on the C-6 carbon or N position of the GlcN, on C-2, or occasionally on C-3, of the GlcA/IdoA subunit [8]. The polysaccharide chain length coupled with the variation in the extent of sulfation, give rise to hundreds of potential polysaccharide structural combinations.

GAGs have been implicated in a wide variety of pathology including respiratory failure [9], placental malaria [10] and cancer [11]. HS and CS have been shown to be important for the initial attachment of Merkel cell polyomavirus (MCV) to cells while 6-O-

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sulfated and N-sulfated HS is important for the reporter vector entry [12] Determining the composition of these GAGs is important in understanding their role. Depolymerization of GAGs and analysis of their disaccharides is an important step in the characterization of GAGs. Depolymerization can be achieved using specific lyases which cleave the glycosidic linkage between a hexosamine and a hexuronic acid thus introducing an unsaturated bond (absorbing at 232 nm) between the C4 and C5 of the hexuronic acid. GAGs can also be depolymerized chemically by mild hydrazinolysis and subsequent deaminative cleavage with nitrous acid [13]. This method retains the original epimeric nature of the hexuronic acid but loses information about the N-sulfation or N-acetylation because the resulting disaccharide comprise a hexuronic acid linked to a 2,5-anhydrohexose bearing an aldehyde group [13].

Enzymatic depolymerization of heparin/heparan sulfate (hep/HS) with heparinises give rise to twelve disaccharides out of which only eight are commonly seen in normal organisms [14,15]. The other four (N-unsubstituted glucosamine) are rare and are believed to result from the loss of labile N-sulfate groups during isolation or incomplete modification (N-deactylase/N-sulfotransferase) during hep/HS biosynthesis [14]. These twelve hep/HS disaccharides are commercially available.

Previous techniques used for the compositional analysis of GAG disaccharides include liquid chromatography (LC) with UV [16], fluorescence [17], and MS detection. These GAG disaccharides are hydrophilic and hence will not be well retained on a reverse phase (RP) column. Underivatized GAG disaccharides can be analyzed directly using a variety of separation methods including hydrophilic interaction liquid chromatography (HILIC)-MS. However, co-elution of disaccharides has been reported [13]. To overcome these issues, GAG disaccharides may be derivatized with a hydrophobic fluorophore which increases their retention on RPLC and may improve MS sensitivity. Derivatizing reagents employed for this include 4,4-difluoro-5,7-dimethyl-4-bora-3a-diaza-s-indacene-3-propionic acid (BODIPY) and 2-aminobenzamide [14]. Retention of GAG disaccharides and oligosaccharides may also be improved using ion pair RPLC with reagents such as tetrabutylammonium [18], n-hexylamine, and n-pentylamine [19]. However, each of these has specific additional drawbacks that make analysis of GAG disaccharides problematic, particularly when analyzing biological samples. These require long run times and some of the non-volatile ion pairing reagents contaminate the MS interface [20]. Capillary electrophoresis with laser-induced fluorescent detection [21] and fluorophore-assisted carbohydrate electrophoresis (FACE) [22] have also been employed in GAG disaccharide analysis. However, both of these methods could suffer from poor reproducibility of migration times.

There is therefore a need to develop a more rapid, selective and sensitive method to quantify levels of GAG disaccharides in biological samples. In this article, we report a method of rapid separation and detection of twelve heparin/heparan sulfate disaccharides and an internal standard in 11 min. This method demonstrates excellent reproducibility, which does not require the specialized separation techniques needed for the separation of native disaccharides and has been successfully applied to the comparison of disaccharide expression in a variety of human cancer cell lines. Derivization-based methods frequently report the use of highly toxic reducing agents such cyanoborohydride. The method described uses the less toxic 2-methylpyridine borane. This analysis employs an optimized selected ion recording (SIR) precolumn RP-UPLC-MS method based on 2-aminoacridone derivatization without the need to remove excess unreacted reagent. We have applied this method to the analysis of disaccharide levels in a variety of human cell lines.

Materials and methods

Materials

Actinase (from *Streptomyces griseus*), 2-aminoacridone (AMAC), 2-methylpyridine borane complex, heparan sulfate (sodium salt from bovine kidney), anion exchange spin columns, 3 kDa, and 10 kDa molecular weight cut off filters were purchased from Sigma-Aldrich, (Poole, UK). Twelve unsaturated heparin/HS disaccharides (ΔUA,2S - GlcNS,6S; ΔUA,2S-GlcNS; ΔUA,2S - GlcNAc,6S; ΔUA-GlcNS,6S; ΔUA-GlcNS; ΔUA-GlcNAc; ΔUA,2S- GlcNAc; ΔUA-GlcNAc,6S; ΔUA,2S - GlcN; ΔUA,2S-GlcN, 6S; ΔUA-GlcN, 6S; ΔUA−GlcN) and an internal standard (ΔUA,2S - GlcNCOEt,6S) were purchased from Iduron (Manchester, UK). Heparinases (from *Flavobacterium heparinum*) I, II, and III were also purchased from Iduron (Manchester, UK). Acetic acid, LC-MS grade dimethyl sulfoxide (DMSO), LC grade ammonium acetate, methanol, and UPLC water were purchased from Fisher Scientific, (Loughborough, UK).

Cell culture

GAGs were extracted from a variety of human cell lines grown in our laboratory. These included cell lines from ovarian adenocarcinoma (OVCAR-3), metastatic breast adenocarcinoma (MDA-468), acute myeloid leukaemia (MOLM-13), choriocarcinoma (BeWo) and a colon cancer cell line (HCT116). Cells were incubated at 37 °C in media containing 10% fetal bovine serum, 1% penicillin and streptomycin (Thermofisher, Hemel Hempsted, UK) in an atmosphere containing 5% CO_2. All cells were cultured using RPMI 1640 medium except HCT116 cells which were cultured using DMEM (Sigma) and BeWo cells were cultured using DMEM:F12. All cell lines were purchased from LGC standards (Teddington, UK) with the exception of MOLM-13 cells (DSMZ, Braunschweig, Germany) and were cultured for at least two passages prior to GAG analysis.

Extraction and enzymatic depolymerization of heparin/HS disaccharides from cells

We used an adaptation of the extraction method reported by Han et al. [23] and Volpi et al. [24] for the extraction of heparin/HS from cells. Briefly, cells grown to confluence were scraped gently from the flask and washed in 100 mM phosphate buffered saline. They were then incubated with actinase (20 mg/mL) for 18 h to proteolyse intracellular and membrane bound proteins. The resulting solution was filtered to remove particulates, and the peptides removed using a 10 kDa molecular weight cut-off filter. The retenate was lyophilized and re-suspended in 8 M urea with 2% CHAPS. Anion exchange spin columns were used to extract GAGs from this mixture, washed with 200 mM NaCl and subsequently eluted with 16% NaCl. This solution was desalted using a 3 kDa molecular weight cut-off filter, lyophilized, and re-suspended in 50 mM phosphate buffer (pH 7.0). Time-controlled enzymatic depolymerization of the GAGs was achieved by the addition of 3 mIU of heparinase I, II, and III at 37 °C for 48 h as described above. The solution was then heated in boiling water for 10 min and centrifuged at 12,000x*g* for 10 min to remove denatured proteins. The supernatant was collected and 0.5 μL of internal standard (ΔUA,2S - GlcNCOEt,6S, 1 mg/mL) was added. This was then lyophilized, derivatized with AMAC and analyzed using UPLC-MS.

In order to determine the recovery of disasccharides from this extraction procedure, heparan sulfate standards (5 and 10 μg) were extracted and disaccharides quantified as described above (extracted HS). In addition, the same amount of HS was directly digested with heparinases without prior extraction and then