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Quantitative analysis of anions in glycosaminoglycans and application in heparin stability studies

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ABSTRACT

The sulfo groups of glycosaminoglycans contribute to their high charge densities, and are critical for the role they play in various physiological and pathophysiological processes. Unfortunately, the sulfo groups can be hydrolyzed to inorganic sulfate. Thus, it is important to monitor the presence of these sulfo groups. In addition, free anions, including chloride, sulfate and acetate, are often present in glycosaminoglycans as a result of multiple purification steps, and their presence also needs to be monitored. In this report, ion chromatography with conductivity detection is used to analyze the anions present in glycosaminoglycans, including heparin, heparan sulfate, chondroitin sulfate and dermatan sulfate. This method allows quantitation over a wide range of concentrations, affording a limit of quantitation of 0.1 ppm and a limit of detection of 0.05 ppm for most anions of interest. The stability of heparin was also studied, providing data on the formation of both sulfate and acetate anions.

1. Introduction

Glycosaminoglycans (GAGs) are linear, acidic polysaccharides found within cells, on cell surfaces, and in the surrounding extracellular matrix. Through their interaction with proteins, GAGs participate in and regulate many cellular events as well as physiological and pathophysiological processes, such as cell proliferation and differentiation, cell–cell and cell–matrix interactions, and viral infection (Wu, Zhang, Beeler, Kuberan & Rosenberg, 2002; Capila & Linhardt, 2002; Bernfield et al., 1999). GAGs are composed of repeating disaccharide units (Fig. 1), and they are divided into four main categories, hyaluronic acid (HA), chondroitin sulfate/dermatan sulfate (CS/DS), heparan sulfate/heparin (HS/Hp) and keratan sulfate (KS), based on their monosaccharide composition and the configuration and position of their glycosidic linkages. Differences in the specificity of interaction between GAGs and their binding proteins result from the structural diversity of GAGs, including type, size, saccharide composition, charge density, sequence and molecular weight (Taylor & Gallo, 2006; Sasisekharan, Raman & Prabhakar, 2006). Charge density, a critical factor for GAG–protein interaction specificity, often depends on the degree of sulfation of a GAG (Zsila & Gedeon, 2006). The impact of charge density on the proper functioning of a GAG is usually greater than the other structural properties of GAG (Yard, Lorentz, Herr & van der Woude, 1998; Naimy, Buczek-Thomas, Nugent, Leymarie & Zaia, 2011; Mummery, Mulloy & Rider, 2007). The amount of sulfation present in a GAG sample is not only related to its biological function but also provides information on the quality of that sample and on the presence of impurities or contaminants (Bo, Muschin, Kanamoto, Nakashima, & Yoshida, 2013; Nilasaroya, Poole-Warren, Whitelock, Martens, 2008). Thus, an assessment of the sulfo group content of a GAG is necessary for appropriate quality control.

Heparin, one of a few carbohydrate drugs, is widely used as an anticoagulant and has the highest sulfo group content of all GAGs (Liu, Zhang & Linhardt, 2009). Because of its high charge

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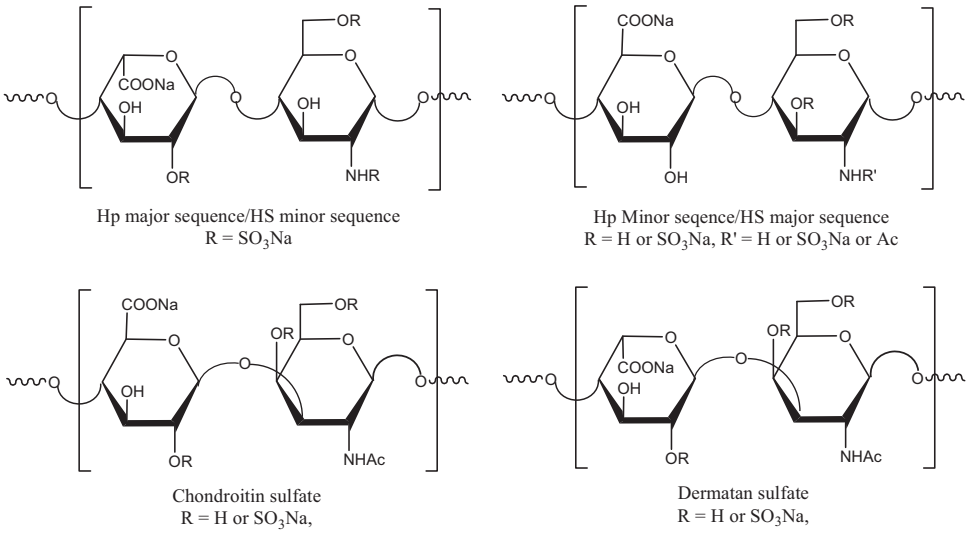

Fig. 1. Structures of heparin (Hp), heparan sulfate (HS), chondroitin sulfate (CS), and dermatan sulfate (DS).

density, heparin also shows many other biological activities through its binding to various proteins. Heparin was contaminated with oversulfated chondroitin sulfate (OSCS) in 2007–2008 (Guerrini et al., 2008; Zhang et al., 2008, 2009). This contamination crisis was associated with the deaths of nearly 100 Americans (Liu, Zhang & Linhardt, 2009). The structure and activity of OSCS were so similar to heparin that this contaminant was difficult to detect by the standard pharmacopeial methods in place at the time of the heparin crisis. Other GAGs, such as DS, are often found in both crude heparin and heparin active pharmaceutical ingredient (API) as impurities, as a result of inefficient purification (Guerrini et al., 2008; Zhang et al., 2009). Some methods have been recently developed to detect and analyze these GAGs in heparin products (Trehy, Reepmeyer, Kolinski, Westengerger, & Buhse, 2009; Somsen, Tak, Torano, Jongen, & deJong, 2009; Limtiaco, Jones, & Larive, 2009; Guerrini et al., 2009; Fu et al., 2013) but most of these assays are not straightforward. Because the sulfo group contents are different for different GAGs, methods that quantitate sulfation levels can be used to evaluate the purity of heparin API.

Heparan sulfate (HS) is related to heparin and is the most structurally complex GAG (Sugahara & Kitagawa, 2002). Because of its structural heterogeneity, HS is an important regulator of signaling molecules in many physiological and pathophysiological processes (Princivalle & de Agostini, 2002; Edwards & Edwards, 2012; Kennedy, 2012). Characterization of its structural properties, such as degree of sulfation, can help explain its various activities (Poole, 1986).

Chondroitin sulfate (CS) has also been used for many years as a nutraceutical and in medicine (Bartus, James, Bosch, & Bradbury, 2012; Sharma, Wood, Richardson, Roberts, & Kuiper, 2007). CS is divided into CS-A (GlcA-GalNAc4S), CS-B (DS, IdoA-GalNAc4S), CS-C (GlcA-GalNAc6S), CS-D (GlcA2S-GalNAc6S) and CS-E (GlcA2S-GalNAc4S) based on differences in sulfation pattern and sugar composition. These CS GAGs are primarily extracted from animal tissues. The different applications for each CS often depends on their degree of sulfation (Barroca & Jacquinet, 2002).

The sulfo groups covalently linked to GAGs are labile and can be released as inorganic sulfate anions on prolonged storage or on storage under improper conditions (Zaia, 2013; Zaia & Costello, 2003). Different salts and buffers are often used in the commercial production of GAGs (Liu, Zhang & Linhardt, 2009). Thus, it is critical to monitor the presence of anions, introduced in the production of GAGs or in GAG decomposition, to ensure GAG purity and stability.

High performance anion exchange chromatography (HPAEC) has been developed to quantify free sulfate and other anions with high resolution and high sensitivity (Morales, de Graterol & Mesa, 2000; Cole & Evrovski, 1997; McPhee, Atkinson & Cole, 1990; Singh & Nancollas, 1988; Morris & Levy, 1988). Compared to combustion analysis, titration and colorimetric methods (Greweling, Bache & Lisk, 1972; Lambert & Ramasamy, 1975; Harenberg et al., 2009), quantitative analysis of sulfate by HPAEC is faster, requires less labor and sample consumption, can differentiate between free or covalently bound sulfate before and after hydrolysis, can provide information on other ions present, and can improve analytical sensitivity, precision, and accuracy. The recent US Pharmacopeia monograph on enoxaparin for injection requires sulfate group content testing by HPAEC (United State Pharmacopeial Convention, 2012).

In this paper, we compare free anions, including chloride, acetate, phosphate and sulfate in GAG products; analyze the degree of sulfation of heparin, HS, CS-A, and DS; monitor the sulfate and acetate group levels in heparin stability studies; and inspect the release of sulfate and acetate under various conditions.

2. Experimental

2.1. Materials

Two heparin standards were purchased from United State Pharmacopeia (USP, Rockville, MD) and Chinese National Institutes for Food and Drug Control (NIFDC, Beijing, PR China), respectively. Heparin, HS and DS were purchased from Celsus (Cincinnati, OH). Heparin, CS-A, HA and Certified Multi-anion Standard Solution PRIMUS (10 mg/kg ± 0.2% F^-, Cl^-, Br^-, NO_3^-, SO_4^{2-}, PO_4^{3-} of each anion) were purchased from Sigma–Aldrich (St. Louis, MO). Two lots of heparin were provided by a plant in China. High-purity water (resistivity ≥ 18.2 MΩ cm, 25 °C) was used throughout this study. All chemicals and reagents were of HPLC grade.

2.2. Standard and sample preparation

Standards—Five multi-anion standard solutions were prepared at a series of concentrations (0.1, 1, 2, 4, 5 ppm of each anion) in