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Structural peculiarity and antithrombin binding region profile of mucosal bovine and porcine heparins

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ABSTRACT

The major compositional differences between bovine mucosal heparin (BMH) and the currently employed porcine mucosal heparin (PMH) have been reported to essentially consist of reduced 6-*O*-sulfation of the glucosamine residues in BMH and somewhat lower 2-*O*-sulfation of the iduronate residues in PMH. The present work is based on direct comparison of several BMH and PMH commercial preparations. A combined study by 2D (heteronuclear single quantum coherence, HSQC) NMR and ion-pair reversed-phase high performance liquid chromatography (IPRP-HPLC) coupled with electrospray ionization mass spectrometry (ESI-MS) on the heparins, extended to the analysis of their heparinases digests and fractions separated by affinity chromatography on antithrombin (AT), confirmed the previously reported lower degree of 6-*O*-sulfation and showed lower 3-*O*-sulfated glucosamine content in BMH. More detailed studies allowed the identification of structural variants of AT-binding region (ATBR) structural variants, showing higher content of the *N*-sulfated components in BMH than in PMH.

1. Introduction

For more than 70 years heparin has been used to treat or manage blood clots in the veins, arteries, or lungs. With the introduction of low molecular weight heparins (LMWHs), applications of heparin are primarily anticoagulant and antithrombotic treatment during cardiovascular surgery and renal dialysis. From the 1960s onwards unfractionated heparin (UFH), originally extracted from canine or bovine liver, then from bovine lung was obtained almost exclusively from bovine or porcine intestinal mucosa. However, the bovine heparin was withdrawn from the western markets in the 1990s following concerns of a potential risk of prion contamination due to the bovine spongiform encephalopathy (BSE) infection in Europe. Currently, the use of bovine heparin is limited to relatively few countries. The increased demand for porcine intestinal mucosal heparin, both as a drug and as a starting material for LMWHs, has required an increase in worldwide production, which today corresponds to about 100 ton per year of heparin active pharmaceutical ingredients (APIs) in China alone. The US Food and Drug Administration, considering also the demonstrated safety of current production methods, has recently proposed the re-introduction of bovine-derived heparin to the US market in an attempt to guarantee the supply of the drug by diversifying its sources.

Heparin consists of a linear backbone of alternating α-D-glucosamine (*GlcN*) residues 1,4-linked to α-L-iduronic acid (*IdoA*) or to β-D-glucuronic acid (*GlcA*). Variable substitution of its disaccharide subunits with *N*-sulfo, *O*-sulfo and *N*-acetyl groups gives rise to high sequence complexity. The prevalent highly sulfated sequences, comprising by the trisulfated disaccharide $IdoA_{2S}$-$GlcN_{NS,6S}$ repeating units, are separated from each other by *GlcA* and, less frequently, by *IdoA*-containing sequences, including some 6-*O*-desulfated glucosamine residues. *N*-acetylated domains, consisting predominantly of the non-sulfated disaccharide *GlcA*-*GlcNAc* repeating units, are also present [1]. These domains were proposed to occur almost exclusively close to the "linkage region" (LR) [2]. However, the molecular weight of fragments generated by heparin lyase III suggested that intact heparin chains can also include an internal GlcA-containing under-sulfated region [3,4]. Some of the heparin chains contain specific pentasaccharide sequences bearing a GlcA residue followed by the particular *N*-sulfo-3,6-*O*-di-sulfated-glucosamine (A*) the minimal antithrombin (AT) binding region

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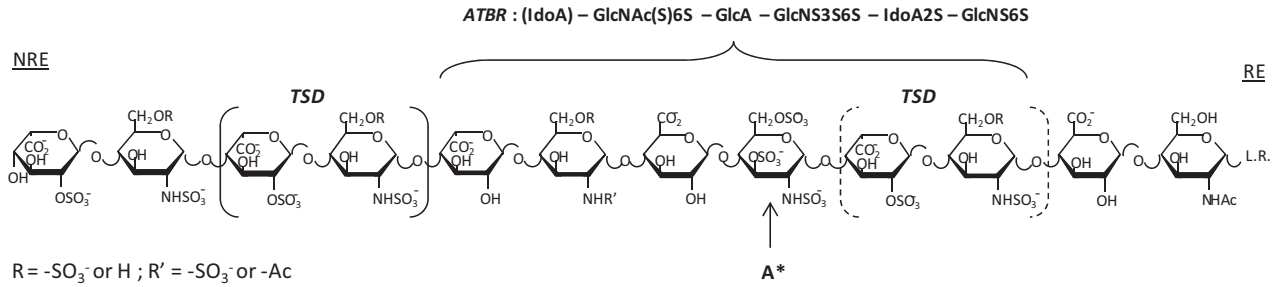

Fig. 1. Schematic representation of the structure of a heparin chain containing an ATBR sequence. A* = *GlcNS,3S,6S*, LR = linkage region at the "reducing" end (RE), NRE = "non reducing" end.

(ATBR, Fig. 1), that is considering essential for determining the high anticoagulant activity of heparin (reviewed in Ref. [5]).

Heparins from different animals and organs, especially from bovine lung, bovine and porcine intestinal mucosa, used in clinics over the years [6] are somewhat different from each other in terms of component units [7], and knowledge of their detailed structure and biochemical properties is a prerequisite for their pharmaceutical utilization.

The compositional and anticoagulation profiles of PMH have been studied in sufficient detail to ensure its safe use. Heparin from bovine intestinal mucosa (BMH), used worldwide for several years and currently in use principally in Islamic countries, is one of the present candidates to reenter the heparin supply chain, in spite of its somewhat lower anticoagulant activity compared to PMH.

Comparison of the anticoagulant activity of PMH and BMH samples, showed a mean USP activity of 180 and 166 IU/mg, respectively [8], while their primary structure was characterized by higher 6-*O*-sulfation in PMH compared to to BMH (80% vs 60%), as determined by ^{13}C NMR [9]. This last finding was further confirmed by ^{1}H and two-dimensional (2D) TOCSY NMR and by HPLC disaccharide analysis of the heparinase I digests of both types of heparins from diverse producers [10]. Further quantitative analysis of 2D NMR (HSQC) spectra indicated that the molar content of A*, taken as a ATBR marker, was lower in BMH than in PMH (1.7 vs 5.9%), while an intermediate value (4.0%) was found in a bovine lung heparin (BLH) sample [9]. Previous and recent investigations on different but single BLH samples [11] in comparison with published data of diverse PMH samples [12], confirmed higher *N*- and *O*-sulfation in BLH (SOO_3^-/COO^- ratios around 2.5) [13]. An overall lower sulfation degree of BMH, mainly at the 6-*O* position of glucosamine residues, was recently confirmed through analysis of several commercial preparations of both BMH and PMH [14]. The above mentioned differences might contribute to the somewhat lower anticoagulant properties of BMH, depending also on the sequence chain distribution, sulfation patterns, apart for the ATBR content. This minor but important sequence, present in all the heparins so far analyzed, may exist in different variants, as shown in Table 1.

The *N*-acetylated pentasaccharide, structure **A** in Table 1 (preceded by a residue of non-sulfated *IdoA* residue) seems to be the major active ATBR in all the heparins of different origin, along with the minor active variant **B** (where the 3-*O*-sulfated *GlcNSO*$_3$ residue is non-6-*O*-sulfated) [15,16]. Iin contrast, another variant (not shown in Table 1) in which the *GlcNAc* preceding *GlcA* lacked 6-*O*-sulfation should not significantly contribute to the AT affinity [5]. The *N*-acetylated variant **C,** in which two 3-*O*-sulfated glucosamines are within the ATBR sequence, was found recently in an octasaccharide isolated from semuloparin, a very enoxaparin like LMWH and showed AT affinity more than ten times higher than that of the minimal sequence of the major ATBR **A** [17]. A comparable increase in AT affinity and aXa activity was reported for a synthetic variant of sequence **A** containing *N*-deacetyl-*N*-sulfated glucosamine and is shown in Table 1 as ATBR variant **D** [5]. The latter, one very minor component of PMHs, was more abundant in heparins of bovine origin, such as bovine lung heparin (BLH) known to be overall more *N*-and *O*-sulfated than PMH [18–20]. The full hexasaccharide including variant **E** containing a *IdoA2S* linked to the NRE *GlcN* of the ATBR sequence, has not been previously described, but its related tetrasaccharide, Δ*UA2S-GlcNS,6S-GlcA-GlcNS,3S,6S* (Δ*U*,4,5,0 (1)), was found in the BLH heparinases digest [19].

Owing to occasional inadequate analytical controls during the production of the crude material, the possibility of contamination of PMH has became evident, illustrated by the heparin crisis of 2008. There is a need for a continuous updating of even more efficient analytical tools to identify heparin contaminants and adulterations including with heparin from different sources. Recently mathematical elaboration using principal component analysis (PCA) has been applied to the ^{1}H NMR spectra of glycosaminoglycans [21]. Among the different methods proposed, two-dimensional correlation spectroscopic-filtering with iterative random sampling (2D-COS-firs) [22] was shown capable of revealing, with a high level of sensitivity, contamination from different origins in pharmaceutical heparins. Measurable differences between PMH and BMH in the content of some minor components can represent species specific markers and also explain the diverse level of activities (such as the USP test).

This paper describes in detail the physico-chemical properties (such as sulfate/carboxylate molar ratios, molecular weights) of samples of commercial BMH (nine) and PMH (five) samples, selected from a heparin data bank of a single producer, to avoid simply comparing differences induced by the extraction method. An in-depth structural investigation by a quantitative two-dimensional NMR study (HSQC) [23], on all the selected samples and the corresponding fractions obtained by affinity chromatography on ATIII, was also performed. A further LC–MS study was made on fragments obtained by digestion with heparin lyases from a sample of both the heparins and their ATIII affinity fractions. This high-resolution LC–MS approach, recently applied to heparins and their derivatives [24], provides information that is complementary to that provided by HSQC NMR, through identification and determination of the relative content, even of isomeric oligosaccharides that reflect the presence of different sequences in the original heparin chains.

2. Materials and methods

2.1. Reagents and starting materials

The heparin samples used for PCA were from 60 different batches of fully characterized, porcine API mucosal heparin, 5th and 6th International Standard candidates and other selected samples provided by several commercial manufacturers including Kin