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Chemometric analysis of porcine, bovine and ovine heparins

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ABSTRACT

Heparin is a polysaccharide anticoagulant drug isolated from animal tissues. There have been concerns on the safety and security of the heparin supply chain since 2007-8 when a contamination crisis led to its disruption. The current study applies a suite of modern analytical techniques to porcine, bovine and ovine intestinal mucosal heparins. These techniques include structural analysis by nuclear magnetic resonance spectrometry, disaccharide compositional analysis, bottom-up analysis of tetrasaccharides corresponding to heparin's antithrombin III binding site. Chemometric analysis was then applied to understand how these structural differences to predict the animal/tissue source of heparin and to help detect blending of heparins from various sources.

1. Introduction

Heparin is a natural product derived from the tissues of food animals that is widely used in modern medicine to control blood coagulation [1]. Discovered over 100 years ago, heparins introduction as a clinical anticoagulant predated the establishment of the US Food and Drug Administration (FDA) in 1937 [2]. The first clinically used heparin was prepared from bovine organ tissue at Connaught Laboratories, Toronto [2], and bovine sourced heparin was widely used in addition to porcine heparin well into the 1980′s until a British outbreak of prion-based 'mad cow disease', or bovine spongiform encephalopathy (BSE) [3]. In the same time period, ovine heparin has niche applications in Oceana where sheep were prevalent. Since the 1990′s, porcine intestinal mucosa has been the major tissue source of heparin-based products (*i.e.,* unfractionated heparin, low molecular weight heparins, and heparinized medical devices) requiring over 100 metric ton production and commanding a ~10 B$ market [4,5]. Heparin is considered to be a critical drug and without an adequate supply of heparin products the practice of modern medicine would not be possible [6].

In 2007–2008, there was a heparin crisis in which adulterated porcine heparin, coming from China, entered the world market leading to the deaths of nearly 100 patients [6,7]. In response to this crisis, world regulatory agencies and compendial organizations, such as the US FDA and the US Pharmacopeia (USP), increased their diligence in the regulation and analyses of this critically important drug [8]. Recently, there has been growing concern about the stability, security and safety of the world's supply of heparin. There are three major areas of concern: 1. Heparin comes from a single animal species that has been subject to disease outbreaks impacting herd size, production levels and prices; 2. Over half of the world's supply is controlled by a single country [9]; and 3. This complex, animal-sourced, polycomponent/polypharamacological polysaccharide drug (Fig. 1) leaves it susceptible to future adulteration, the presence of viral/prion impurities, and potential blending with heparin sourced from other species [10–12]. Indeed, these recent concerns have been clearly expressed by the US FDA and US Congress [13] and the blending with heparin sourced from other species has recently resulted in the withdrawal of heparin from the market in Europe [14].

The current study extends a principal component analysis (PCA) approach, recently introduced to measure numerical differences

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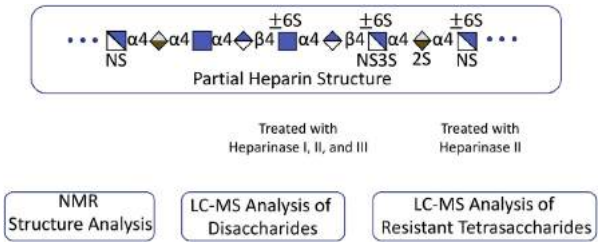


Fig. 1. Schematic illustration of heparin structure analysis strategy. ■ N-Acetylglucosamine, ◹ Glucosamine, ◆ Glucuronic acid, ◆ Iduronic acid, ◇ uronic acid, and S is sulfo.

between porcine, bovine and ovine heparins using ^{1}H-nuclear magnetic resonance (NMR) spectroscopy [15], to mass spectral disaccharide and tetrasaccharide analyses. Finally, visual data mining software [16] is applied to better understand the structural basis for these numerical differences between porcine, bovine and ovine heparins.

2. Materials and methods

2.1. Heparin samples

The API versions of bovine, ovine and porcine heparins were obtained in powdered form. The bovine heparins were obtained from Kin Master (Passo Fundo, Brazil) and exhibited the USP specific activity of 130–140 U/mg. The ovine heparin samples were obtained from Ronnsi Pharma (Suzhou, China) and exhibited a specific activity of 195–208 U/mg. The porcine heparins were obtained from Medefil Inc. (Glendale Heights, USA) and exhibited a USP potency range of 185–200 U/mg.

2.2. Nuclear magnetic resonance analysis

Nuclear magnetic resonance (NMR) analyses were performed on a Bruker Avance II 600 MHz spectrometer (Bruker BioSpin, Billerica, Massachusetts) with TopSpin 2.1.6 software (Bruker). Samples were prepared by dissolving approximately15 mg of heparin in 99.96% D_2O and lyophilizing the sample to remove exchangeable hydrogen from the backbone of the structure. This was repeated 3 times to ensure that the deuterium exchange was complete. The sample was then redissolved in 0.4 mL of 99.96% D_2O to minimize the HOD peak in the NMR spectrum. The conditions for 1-dimensional (1D) ^{1}H-NMR spectra were as previously reported. Ref x1 Briefly, a wobble sweep width of 12.3 kHz, acquisition time of 2.66 s, and relaxation delay of 8.00 s were used. Temperature was 298 K. Peaks are assigned based on our previous publication [17,18]. Each peak is normalized by the total value of all peaks (excluding the HOD peak).

2.3. Disaccharide analysis

Heparin disaccharides were prepared by exhaustive digestion using a mixture of heparinase I, II, and III (each lyase 0.1 IU/1.0 mg sample) in digestion buffer (50 mM ammonium acetate containing 2 mM calcium chloride adjusted to pH 7.0) at 37 °C for overnight. Enzymatic digestion was terminated by boiling samples for 10 min and removing the denatured enzymes with centrifugation at 10,000 rpm for 10 min. Supernatants were frozen dry and re-dissolved to 1 μg/μL for LC–MS analysis.

Online RPIP LC tandem Iontrap MS on an Agilent 1200 LC-Iontrap was used to analyze disaccharides from NACHs. LC was performed on an Agilent 1200 LC system at 45 °C using an Agilent Poroshell 120 EC-C18 (2.7 μm, 2.1 × 100 mm) column. Mobile phase A (MPA) was 38 mM ammonium acetate and 12 mM tributyl-ammonium acetate in 15% acetonitrile aqueous solution (pH = 6.5), and the mobile phase B (MPB) was 38 mM ammonium acetate and 12 mM tributyl-ammonium acetate in 65% acetonitrile aqueous solution (pH = 6.5). The mobile phase passed through the column at a flow rate of 100 μL/min. The gradient was: 0-8 min, 0% B; 8–15 min, 0–80% B; 15–25 min, 80% B; 25–26 min, 80-0% B; 26–40 min, 0% B. The injection volume is 2 μL. The MS parameters in negative mode as below: scan range 300–700 m/z, nebulizer 40 psi, dry gas 8 L/min, dry Temperature 350 °C, capillary 1 nA [19].

2.4. Tetrasaccharide analysis

Tetrasaccharides from heparins of different animal source were obtained by digestion only using a mixture of heparin lyases (0.1 IU/1.0 mg sample) in digestion buffer (50 mM ammonium acetate containing 2 mM calcium chloride adjusted to pH 7.0) at 37 °C for 2 h. Enzymatic digestion was terminated by boiling samples for 10 min and removing the denatured enzymes with centrifugation at 10,000 rpm for 10 min. Supernatants were frozen dry and re-dissolved to 2 μg/μL for LC–MS analysis

Online RPIP LC tandem Iontrap MS on an Agilent 1200 LC-Iontrap was used to analyze tetrasaccharides from heparins. LC–MS parameters and condition used same as disaccharide analysis described above, except flow rate was 120 μL/min and mobile phase gradient from 2% to 30% MPB in 40 min, then rose to 60% MPB in following 15 min.

2.5. K means clustering analysis

With visual data mining software Orange Canvas, [16] we utilized unsupervised learning technique as K-means clustering to group and depict the visualized and clustered data in figures. Projections were optimized by using an intelligent algorithm in Orange Canvas "Rank Projections" to find projections where instances where samples were are well separated. This algorithm scores feature-pairs by average classification accuracy and returns the top scoring pairs,which could separate clusters well from each other.

3. Results and discussion

Heparin is a polydisperse, microhetergenous mixture of polysaccharides (Fig. 1) prepared from mast cell-rich tissues of food animals. The heparin content and its structural properties can be impacted by the species and organ source as well as animal age, health, genetics, and feed [5,20]. Thus, it is not surprising that over the years a number of studies have been aimed at understanding the structural and pharmacological differences between commercially available heparins [21–26]. Heparin has a complex structure. The GlcN residues in heparin can be modified with *N*-acetyl or *N*-sulfo groups and *O*-sulfo groups at positions 3 and 6. The UA residues in heparin can be D-glucuronic acid (GlcA) or L-iduronic acid (IdoA) that can be modified with *O*-sulfo groups at positions 2. The major disaccharide repeating unit in heparin its is →4) α-IdoA2S (1→4) α-GlcNS6S (1→, where S is sulfo [1]. Heparin's anticoagulant activity is primarily derived from the ability of specific sequences in heparin to bind to the serine protease inhibitor, antithrombin III (AT), and potentiate AT inhibition of blood coagulation proteases, such as thrombin [4]. A pentasaccharide of the structure →4) α-GlcNAc6S (1→4) β-GlcA (1→4) α-GlcNS3S6S (1→4) α-IdoAc2S (1→4) α-GlcNS6S (1→, where Ac is acetyl, is one such AT-binding site and some structural variation in the AT pentasaccharide binding site is possible while retaining its anticoagulant activity [27,28]. Early studies relied on relatively simple characterization of these heparins including their anticoagulant activities, molecular weight properties, and selected structural features. More recently, modern

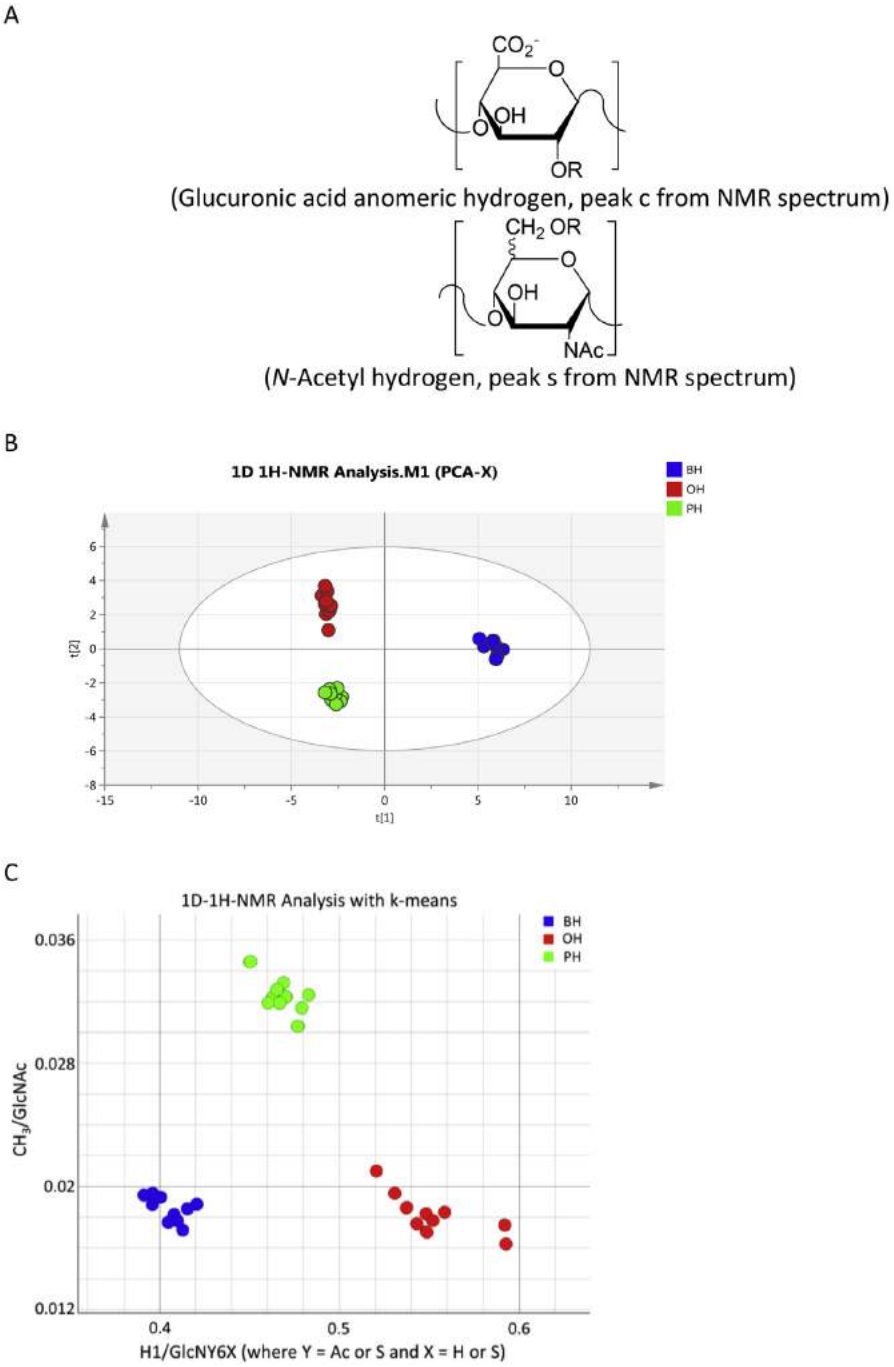
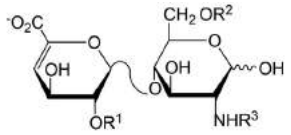


Fig. 2. Statistical analysis of bovine, ovine, and porcine heparin structures based on ^{1}H NMR information. (A) The structure units of heparin that caused major variance in NMR analysis. (B) PCA score plot of two first principle components of the NMR data sets. The first component explains 72.9% of the variation and the second component 21.5%. Samples were grouped by different animal source. (C) K-means clustering analysis of the NMR data, an example of top scoring feature pair is shown in panel c, when only using peak c H1/GlcNY6X (where Y = Ac or S and X=H or S) (Fig. S1) and paired with peak s CH3/GlcNAc, samples could clearly grouped into different animal sources. Bovine heparin (BH, blue), ovine heparin (OH, red) and porcine heparin (PH, green) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

sophisticated analytical methods including NMR, mass spectrometry (MS), and hyphenated techniques such as bottom-up analysis [29] have facilitated very detailed mapping of a heparin's structural features.

We undertook to establish a suite of methods to examine structural differences between three currently used heparins, porcine intestinal mucosal heparin, bovine intestinal mucosal heparin and ovine intestinal mucosal heparin (Fig. 1). Three analytical approaches, applied to 10 heparin samples from each animal source, were: 1. ^{1}H-NMR analysis of intact heparin; 2. disaccharide compositional analysis of heparin lyase 1, 2, 3-treated heparin using high performance liquid chromatography (HPLC)-MS; and 3.

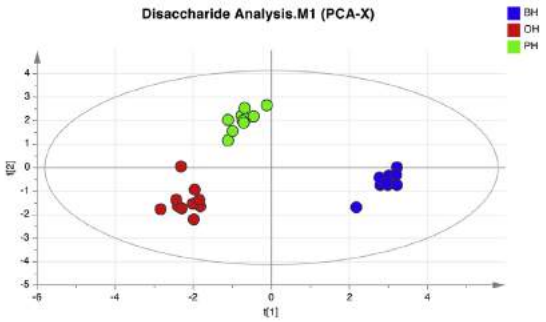
A



Heparan Sulfate Disaccharide Structure

HS dp2	R^1	R^2	R^3
0S	H	H	H
2S	SO_3H	H	H
6S	H	SO_3H	H
NS	H	H	SO_3H
2S6S	SO_3H	SO_3H	H
NS2S	SO_3H	H	SO_3H
NS6S	H	SO_3H	SO_3H
2S6SNS	SO_3H	SO_3H	SO_3H

B



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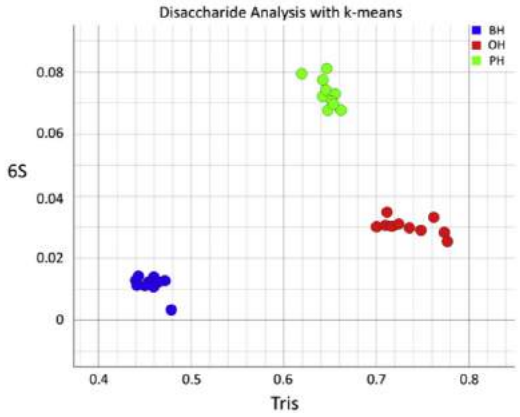

Fig. 3. Statistical analysis of bovine, ovine, and porcine heparin structures based on LC—MS disaccharide analysis. (A) The structure of eight standard heparan sulfate disaccharide. (B) PCA score plot of two first principle components of the disaccharide data sets. The first component explains 60.9% of the variation and the second component 30.9%. Samples were grouped by different animal source. (C) K-means clustering analysis of the disaccharide data, an example of top scoring feature pair is shown in panel c, when only using TriS and paired with 6S, samples could clearly grouped into different animal sources. Bovine heparin (BH, blue), ovine heparin (OH, red) and porcine heparin (PH, green) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

bottom-up resistant tetrasaccharide analysis of heparin lyase 2-treated heparin using HPLC-MS. The analytical data obtained on each of the 30-heparin samples were then analyzed using PCA and by data mining using K-means to assess the numerical clustering of data and to understand the structural features behind this clustering.

One-dimensional 600 MHz ^{1}H-NMR analysis of the thirty heparin samples were obtained (Fig. S1). These spectra were assigned based on previously published two-dimensional spectra for selected porcine heparin (PH), bovine heparin (BH) and ovine heparin (OH) [18,26]. Twenty-four signals having distinctive chemical shifts were assigned (Fig. S1) and these were integrated as the percentage of each total integrated spectrum and the resulting data was chemometrically analyzed (Fig. 2). As anticipated, the 10 PH, BH and OH heparins showed distinct non-intersecting clusters in the PCA analysis (Fig. 2b). The application of K-means analysis not only provided a similar degree of resolution between the heparins from each source but also identified the structural elements in each set that were different (Fig. 2b). Two of the assigned and resolved peaks, C at 5.34 ppm and S at 1.95 ppm, corresponding to the GlcNY6X (where Y = Ac or S and X = H or S) and the CH_3 sig-

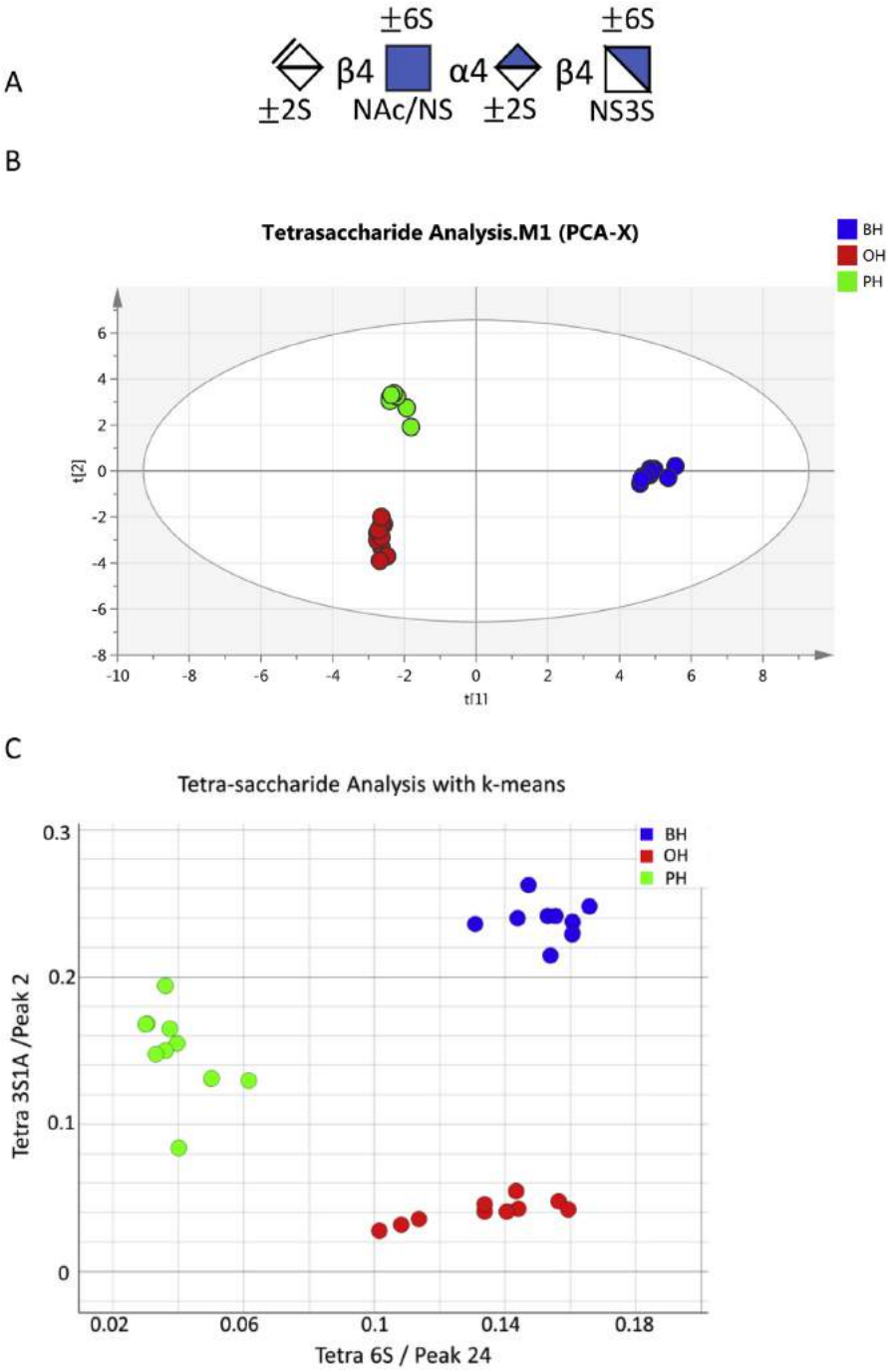

Fig. 4. Statistical analysis of bovine, ovine, and porcine heparin structures based on LC—MS tetrasaccharide analysis. (A) The general structure of tetrasaccharide. (B) PCA score plot of two first principle components of the tetrasaccharide data sets. The first component explains 59.4% of the variation and the second component 29.7%. Samples were grouped by different animal source. (C) K-means clustering analysis of the tetrasaccharide data, an example of top scoring feature pair is shown in panel c, when only using TriS and paired with 6S, samples could clearly grouped into different animal sources. Bovine heparin (BH, blue), ovine heparin (OH, red) and porcine heparin (PH, green). See figure legend 1 for definition of structural symbol nomenclature. The uncolored diamond is UA and Ac is acetyl (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

nal of GlcNAc, respectively, are diagnostic for porcine, bovine and ovine intestinal mucosal heparins.

Disaccharide compositional analysis of 30-heparins, using reverse-phase ion-pairing (RPIP) HPLC-MS, showed the presence of all 8 commonly reported heparin disaccharides but in different amounts (Fig. S2). Again, PCA analysis shows distinct non-intersecting clusters for the data analyzed for porcine, bovine and ovine heparins (Fig. 3b) and K-means analysis showed a similar data separation that was primarily dependent on the concentration of the TriS and 6S disaccharides.

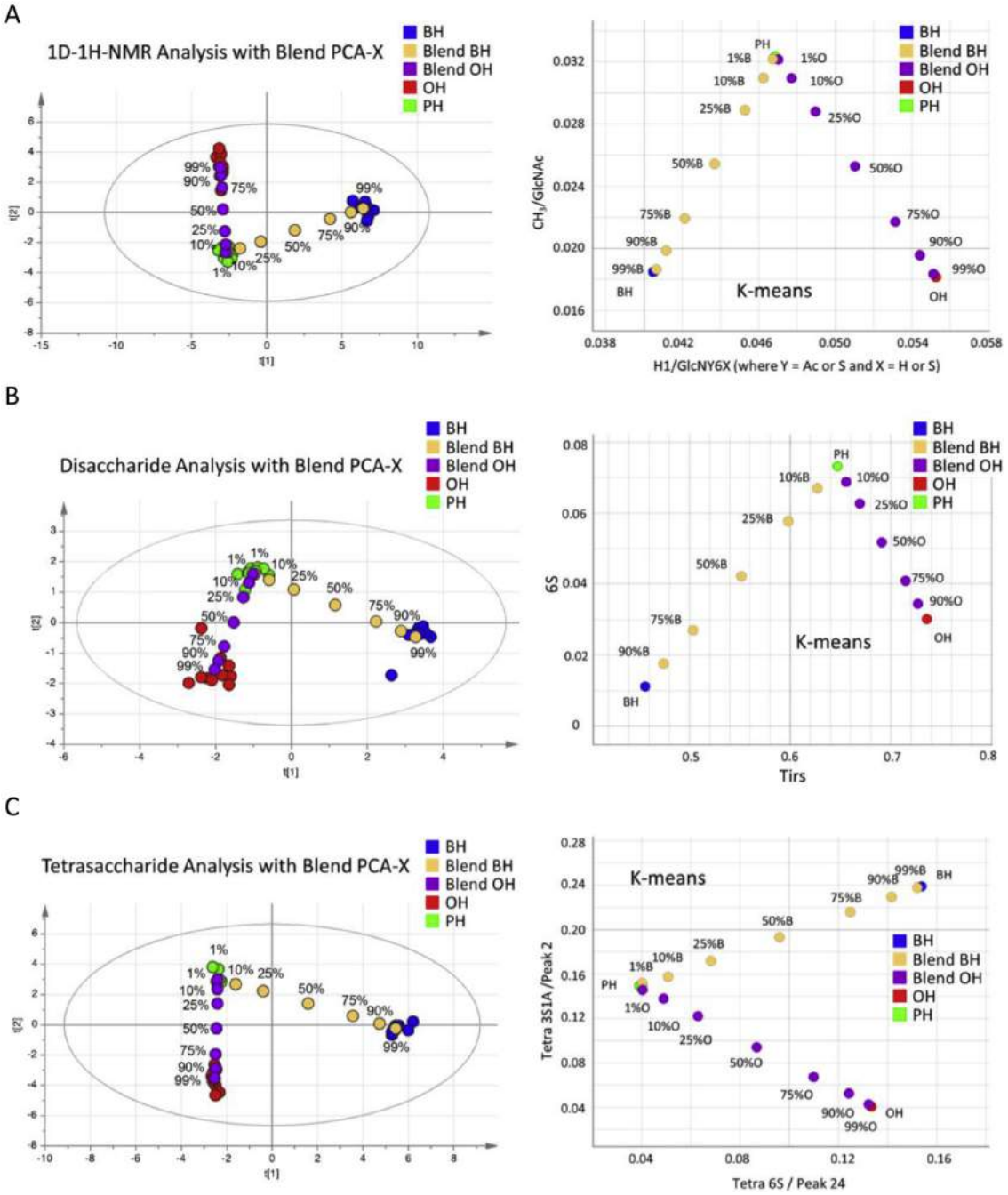

Fig. 5. The scatter plot of principal component analysis(left) and k-means (right) for porcine heparin blending bovine or ovine heparin based on (A) ^{1}H NMR analysis, (B) disaccharide analysis, and (C) tetrasaccharide analysis. Bovine heparin (BH, blue), ovine heparin (OH, red) and porcine heparin (PH, green) and blends of pH and BH (yellow) and pH and OH (purple) are shown (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

The glycosidic linkages between glucosamine and uronic acid at a disaccharide residue adjacent (on the non reducing side) to a 3-*O*-sulfoglucosamine, *N*-sulfoglucosamine residue containing disaccharide unit are known to be resistant to heparin lyase 2 [30]. This 3-*O*-sulfo, *N*-sulfoglucosamine residue is the critical, invariant central residue in heparins AT-binding site, thus, when anticoagulant heparin is exhaustively treated with heparin lyase 2 it gives rise to resistant tetrasaccharides containing half of heparins AT-binding site with a reducing terminal 3-*O*-sulfo, *N*-sulfoglucosamine residue. The structures of these resistant tetrasaccharides give critical information on the structural variability of a heparin and the quantity and distribution of these resistant tetrasaccharides can be correlated to the anticoagulant activity of a heparin or LMW heparin [28,31]. Thus, a bottom-up analysis of the 30-heparin samples was undertaken with the goal of examining heparin lyase 2-resistant tetrasaccharides to better understand the structural differences in porcine, bovine and ovine heparins having the largest impact on their anticoagulant activity.

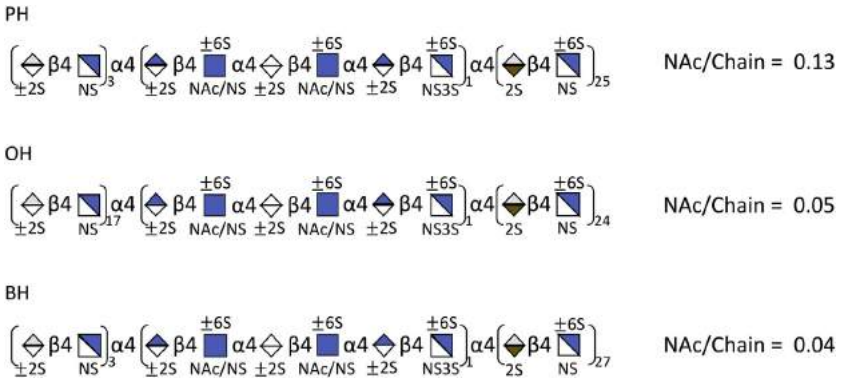

Fig. 6. General structure for porcine, ovine, and bovine heparin. Estimated *N*-acetyl group per heparin chain determined by NMR.

Based on the known specificity of heparin lyase 2 and the presence of at least two sulfo groups in each tetrasaccharide there are 2^5 permutations suggesting 32 possible resistant tetrasaccharide structures (Fig. 4A). Treatment of each of these 30 heparins with heparin lyase 2, followed by RPIP HPLC-MS gave remarkably distinctive distributions of tetrasaccharides dependent on species source (Fig. S3). Porcine heparin, as previously reported [31], shows a relatively simple mixture of resistant tetrasaccharides dominated by 5–6 structures. Ovine heparin shows a slightly more complex mixture with 8–9 prominent resistant tetrasaccharide structures. Bovine heparin shows a much more complex pattern with as many as 24 prominent resistant tetrasaccharide structures, suggesting it contains almost all of the tetrasaccharides possible based on the known heparin biosynthetic pathway [32] and the known specificity of heparin lyase 2. PCA analysis shows tightest clustering of non-intersecting data for porcine, bovine and ovine heparins (Fig. 3b) and K-means analysis showed a similar data separation that was primarily dependent on the integration of peak 2 and peak 24, corresponding to tetrasaccharides having 3 sulfo groups and 6 sulfo groups, respectively (Table S1).

The tight clustering of the PH, BH and OH samples allows for optimal detection of blended products. The expected performance of PCA and K-means analysis using NMR, disaccharide compositional and bottom-up tetrasaccharide analysis on mixtures of pH and OH or BH heparins at ratios ranging from 90:10 to 10:90 is shown Fig. 5. All data sets can be used to detect 25% blends but these data suggest that bottom-up tetrasaccharide analysis might be capable of detecting 10% blends.

Finally, these data offer particularly detailed structural information on porcine bovine and ovine intestinal mucosal heparins. Indeed using the data presented together with previously published molecular weight data allow us to construct general molecular structures of each commercial heparin product (Fig. 6).

4. Conclusions

Analysis of 30 commercial heparin samples relying on NMR structural data obtained on intact heparins, disaccharide compositional data prepared using heparin lyase 1, 2, 3 treatment followed by HPLC-MS analysis, and heparin lyase 2-resistant bottom-up HPLC-MS tetrasaccharide analysis was acquired. Chemometric analysis of these data by PCA and K-means showed the well-resolved separation of commercial intestinal mucosal heparins derived from three animal species, pig, cow and sheep. The data from bottom-up HPLC-MS tetrasaccharide analysis showed the best clustering offering an opportunity to detect blending of as little as 10% bovine or ovine heparin into porcine heparin. These data and their analyses also affords an improved insight into the structural properties of each of these heparins and in particular the structural variability of their AT-pentasaccharide binding sites, primarily responsible for the anticoagulant activity.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jpba.2018.10.052.

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