Structural Characterization of Pharmaceutical Heparins Prepared from Different Animal Tissues

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Received 21 January 2013; revised 11 February 2013; accepted 15 February 2013

Published online 21 March 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.23501

ABSTRACT: Although most pharmaceutical heparin used today is obtained from porcine intestine, heparin has historically been prepared from bovine lung and ovine intestine. There is some regulatory concern about establishing the species origin of heparin. This concern began with the outbreak of mad cow disease in the 1990s and was exacerbated during the heparin shortage in the 2000s and the heparin contamination crisis of 2007–2008. Three heparins from porcine, ovine, and bovine were characterized through state-of-the-art carbohydrate analysis methods with a view profiling their physicochemical properties. Differences in molecular weight, monosaccharide and disaccharide composition, oligosaccharide sequence, and antithrombin III-binding affinity were observed. These data provide some insight into the variability of heparins obtained from these three species and suggest some analytical approaches that may be useful in confirming the species origin of a heparin active pharmaceutical ingredient. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 102:1447–1457, 2013

Keywords: bioanalysis; glycosaminoglycans; heparin; LC–MS; NMR; mass spectomery; surface plasmon resonance; antithrombin III

INTRODUCTION

Heparin and low-molecular-weight heparins are the most widely used clinical anticoagulants.[1] The structure of polysaccharide (~10–20 kDa) (Fig. 1) is predominantly made up of a major (>50%) repeating disaccharide unit, α-L-IdoA2S (1→4)-α-D-GlcNS6S (where IdoA is idopyranosyluronic acid, S is sulfo and GlcN is 2-deoxy, 2-amino glucopyranose).[1] The minor (<50%) disaccharide units have structure and sulfation pattern integral to its therapeutic value.[2] In particular, a pentasaccharide sequence present in heparin having the structure, →GlcNAc6S→GlcA→ GlcNS3S6S→IdoA2S→GlcNS6S→ (where GlcA is

Abbreviations used: HS–HP, heparan sulfate–heparin; LC–MS, liquid chromatography–mass spectrometry; SPR, surface plasmon resonance; AT, antithrombin III; API, active pharmaceutical ingredient; SEC, size-exclusion chromatography; NMR, nuclear magnetic resonance; HMQC, heteronuclear multiple-quantum coherence; HHCOSY, proton–proton correlation spectroscopy; CSA, chondroitin sulfate A; DS, dermatan sulfate; HILIC, hydrophilic interaction liquid chromatography; ESI, electrospray ionization; FT, Fourier transform.

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Journal of Pharmaceutical Sciences, Vol. 102, 1447–1457 (2013)

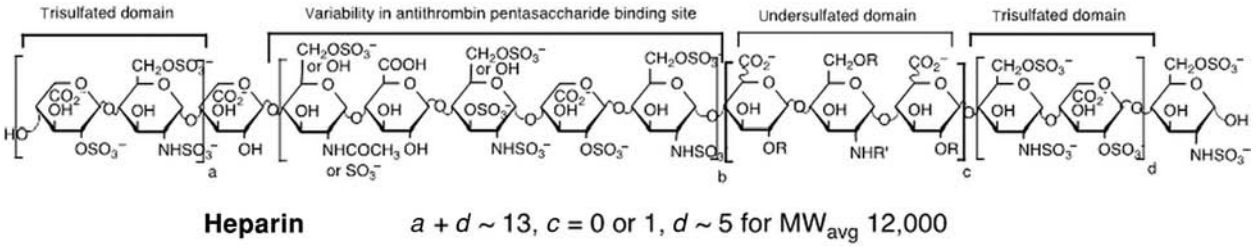

Figure 1. The structure of a representative heparin chain of approximately 12,000 molecular weight showing its different domains and the observed variability in the heparin binding site.

D-glucopyranosyluronic acid and Ac is acetyl), is responsible for its specific binding to the serine protease inhibitor antithrombin III (AT). Binding to this pentasaccharide sequence results in the conformational activation of AT leading to its inhibition of major coagulation cascade proteases, including thrombin [factor (F) IIa] and FXa.[1] There is a 3-*O*-sulfoglucosamine residue at the center of this AT-binding pentasaccharide sequence, and some structural variability of this site has been reported.[3]

Heparin is a highly sulfated, linear glycosaminoglycan that is biosynthesized in the Golgi of mast cells that are prevalent in specific tissues (i.e., intestine, lung, liver, etc.) of humans,[4] other mammals,[5] nonmammalian vertebrates,[3,5] and invertebrates.[3,6] Heparin active pharmaceutical ingredient (API) has been produced from the tissues of food animals, primarily pig intestine, sheep intestine, and beef lung. There are several drug safety concerns related to heparin products. The appearance of bovine spongiform encephalopathy in Europe in the 1990s and the concerns of prion contamination decreased the production and use of bovine (mad cow disease) and ovine (scrapies) heparin worldwide.[7] As a result, there was an increased demand on porcine intestinal heparin causing some shortages and increased costs for heparin API. Heparin is prepared from animal tissues collected at a slaughterhouse essentially through a two-step process that includes: (1) recovery of raw heparin from tissue and (2) purification of raw heparin to obtain heparin API.[8] A lack of control on Chinese production of raw heparin is believed to have resulted in its adulteration with oversulfated chondroitin sulfate in 2007–2008,[8] associated with numerous adverse reactions and multiple deaths.[9,10] There remains some regulatory concern that all heparin API may not be solely of porcine intestinal origin.

This paper takes a detailed look at physicochemical properties of heparins prepared from tissues of porcine, ovine, and bovine origin. These molecular weight, disaccharide composition, oligosaccharide mapping, and AT-binding affinity data provide a baseline value and an approach for the evaluation of the species source of heparin API.

MATERIALS AND METHODS

Materials

Porcine heparin sodium salt 195 U/mg was obtained from Celsus (Cincinnati, Ohio) and ovine sodium salt 182 U/mg and bovine heparin sodium salt 150 U/mg were obtained from Sigma Chemical Company (St. Louis, Missouri). Recombinant Flavobacterial heparin lyase 1, 2, and 3 were expressed in our laboratory using *E*scherichia *coli* strains, provided by Professor Jian Liu (University of North Carolina, College of Pharmacy, Chapel Hill, North Carolina).[11] Heparin oligosaccharides, from hexasaccharide to icosasaccharide, were used as calibrants for molecular weight determination by size-exclusion chromatography (SEC) and were purchased from Iduron (Manchester, UK). Unsaturated heparan sulfate–heparin (HS–HP) disaccharide standards (Di-0S, ΔUA-GlcNAc; Di-NS, ΔUA-GlcNS; Di-6S, ΔUA-GlcNAc6S; Di-UA2S, ΔUA2S-GlcNAc; Di-UA2SNS, ΔUA2S-GlcNS; Di-NS6S, ΔUA-GlcNS6S; Di-UA2S6S, ΔUA2S-GlcNAc6S; and Di-triS, ΔUA2S-GlcNS6S, where ΔUA is deoxy-α-L-threo-hex-4-enopyranosyl uronic acid) were obtained from Seikagaku Corporation (Chuo-ku, Tokyo, Japan).

SEC of Heparin for Molecular Weight Measurement

Size-exclusion chromatography was performed using TSK-GEL G3000PWxI size-exclusion column (Tosoh Corporation, Minato-Ku, Tokyo, Japan) with a sample injection volume of 20 μL and a flow rate of 0.6 mL/min on an apparatus composed of a Shimadzu LC-10Ai pump, a Shimadzu CBM-20A controller, and a Shimadzu RID-10A refractive index detector (Shimadzu, Kyoto, Japan).[12] The mobile phase consisted of 0.1 M $NaNO_3$. The column was maintained at 40°C with an Eppendorf column heater (Eppendorf, Hamburg, Germany) during the chromatography. The SEC chromatograms were recorded with the LC solution version 1.25 software (Shimadzu, Kyoto, Japan) and analyzed with its "GPC Postrun" function.

For molecular weight determination, heparin sodium oligosaccharide standards of different molecular weights (1612, 2687, 4300, and 5375), purchased

 DOI 10.1002/jps

from Iduron (Manchester, UK), were used as calibrants for the standard curve.

The number-average molecular weight ($\bar{M}_n$) is determined by measuring the molecular weight of n polymer molecules, summing the weights, and dividing by n.

$$\bar{M}_n = \frac{\Sigma_i N_i M_i}{\Sigma_i N_i}$$

where N_i is the number of molecules of molecular weight M_i.

The weight-average molecular weight (M_w) is calculated by

$$\bar{M}_w = \frac{\Sigma_i N_i M_i^2}{\Sigma_i N_i M_i}$$

where N_i is the number of molecules of molecular weight M_i.

Nuclear Magnetic Resonance Spectroscopy

Heparins were analyzed by ^{1}H,^{13}C nuclear magnetic resonance (NMR), and two-dimensional NMR spectroscopy. Heteronuclear multiple-quantum coherence (HMQC), proton–proton correlation spectroscopy (HHCOSY) and total correlation spectroscopy (TOCSY) were used to characterize their structures.[13,14] All NMR experiments were performed on Bruker Advance II 600 MHz spectrometer (Bruker BioSpin, Billerica, MA) with Topsin 2.1.6 software (Bruker). Samples were each dissolved in 0.5 mL D_2O (99.996%, Sigma Chemical Company) and freeze-dried repeatedly to remove the exchangeable protons. The samples were redissolved in 0.4 mL D_2O and transferred to NMR microtubes (outside diameter, 5 mm, Norell (Norell, Landisville, NJ)). The conditions for one-dimensional ^{1}H-NMR spectra were as follows: wobble sweep width of 12.3 kHz, acquisition time of 2.66 s, and relaxation delay of 8.00 s. Temperature was 298 K. The conditions for two-dimensional HMQC spectra were as follows: 32 scans, sweep width of 6.15 kHz, acquisition time of 0.33 s, and relaxation delay of 0.90 s. The conditions for two-dimensional HHCOSY spectra were as follows: 16 scans, sweep width of 7.46 kHz, acquisition time of 0.28 s, and relaxation delay of 1.50 s.

Enzymatic Digestion for Disaccharide Analysis and Tetrasaccharide Mapping

For disaccharide analysis, the heparin lyase 1, 2, and 3 (10 mU each) in 5 μL of 25 mM Tris, 500 mM NaCl, and 300 mM imidazole buffer (pH 7.4) were added to 10 μg heparin sample in 25 μL of distilled water and incubated at 35°C for 10 h to degrade heparin sample completely. The products were recovered by centrifugal filtration using a YM-10 microconcentrator (EMD Millipore, Billerica, MA), and the heparin disaccharides were recovered in the flow-through and freeze-dried. The digested heparin disaccharides were dissolved in water to concentration of 50–100 ng/2 μL for liquid chromatography (LC)–mass spectrometry (MS) analysis.[15,16]

For tetrasaccharides analysis, the heparin lyase 2 (40 mU) in 20 μL of 25 mM Tris, 500 mM NaCl, and 300 mM imidazole buffer (pH 7.4) were added to 50–100 μg heparin sample in 40 μL of distilled water and incubated at 35°C for 10 h. The degraded production was freeze-dried for further LC–MS analysis.[17]

Disaccharide Analysis and Tetrasaccharide Mapping Using LC–MS

Liquid chromatography–mass spectrometry analyses were performed on an Agilent 1200 LC/MSD instrument (Agilent Technologies, Wilmington, Delaware) equipped with a 6300 ion trap and a binary pump followed by a UV detector equipped with a high-pressure cell.[16] The column used was a Poroshell 120 C18 column (2.1 × 100 mm^2, 2.7 μm, Agilent). Eluent A was water–acetonitrile (85:15, v/v), and eluent B was water–acetonitrile (35:65, v/v). Both eluents contained 12 mM tributylamine and 38 mM ammonium acetate with pH adjusted to 6.5 with acetic acid. For disaccharides analysis, a gradient of solution A for 5 min followed by a linear gradient from 5 to 15 min (0%–40% solution B) was used at a flow rate of 150 μL/min.

For tetrasaccharide analysis,[17] a gradient of solution A for 2 min followed by a linear gradient from 2 to 40 min (0%–30% solution B) was used at a flow rate of 150 μL/min. The column effluent entered the source of the electrospray ionization (ESI)–MS for continuous detection by MS. The electrospray interface was set in the negative ionization mode with a skimmer potential of −40.0 V, a capillary exit of −40.0 V, and a source temperature of 350°C, to obtain the maximum abundance of the ions in a full-scan spectrum (200–1500 Da). Nitrogen (8 L/min, 40 psi) was used as a drying and nebulizing gas.

Hydrophilic Interaction Liquid Chromatography–ESI–Fourier Transform–MS Analysis of Intact Heparin

A top-down approach using hydrophilic interaction liquid chromatography (HILIC)–ESI–Fourier transform (FT)–MS-based method[18] was used to analyze the intact heparin sample from three different animal sources to obtain the structure information of small oligomers (degree of polymerization (dp4–dp18). Briefly, heparin samples were separated by a Luna HILIC column (2.0 × 150 mm^2, 200 A, Phenomenex, Torrance, California) and detected by an LTQ-Orbitrap XL FT–MS (Thermo Fisher Scientific, San-Jose, California) running at negative-ion mode. High-performance liquid chromatography (HPLC)

binary pump was used to deliver the gradient starting from 10%A to 35%A in 40 min and 35%A to 70%A in 60 min at a flow rate of 150 μL/min. Mobile phase A is HPLC grade water with 5 mM ammonium acetate. Mobile phase B is HPLC grade 98% acetonitrile with 2% water and 5 mM ammonium acetate. FT–MS detector operating at negative-ion mode with optimized parameters was used to prevent in-source fragmentation including spray voltage 4.2 kV, capillary voltage −40 V, tube lens voltage −50 V, capillary temperature 275°C, sheath flow rate 30, and auxiliary gas flow rate 6. External calibration of mass spectra was used to routinely produce a mass accuracy of better than 5 ppm. All FT mass spectra were acquired at a resolution 60,000 with 400–2000 Da mass range. Bioinformatics software (GlycReSoft 1.0, Boston University) was used to automatically assign structures within 5-ppm mass accuracy.[18]

Surface Plasmon Resonance Analysis

Biotinylated heparin prepared from Celsus heparin (Celsus) was immobilized to sensor chip SA (streptavidin) (GE Healthcare, Uppsala, Sweden), based on the manufacturer's protocol. Surface plasmon resonance (SPR) measurements were performed on a BIAcore 3000 (GE Healthcare) . Solution competition study between surface heparin and different soluble heparins to measure IC_{50} (the half-maximal inhibitory concentration) were performed using SPR.[19] AT (250 nM) mixed with different concentrations (range from 0, 6.25, 12.5, to 25 μg/mL) of heparin in HBS-EP buffer (10 mM 4-(2-hydroxyethyl-1-piperazineethanesulfonic acid), 150 mM sodium chloride, 3 mM ethylenediaminetetraacetic acid (EDTA), 0.005% polysorbate surfactant P20, pH 7.4) (GE Healthcare) were injected over heparin chip at a flow rate of 30 μL/min, respectively. At the end of the sample injection, the same HBS-EP buffer was flowed over the sensor surface to facilitate dissociation. After 2 min of dissociation time, the sensor was fully regenerated by injecting with 30 μL of 2 M NaCl. For each set of competition experiments on SPR, a control experiment (only protein without heparin) was performed to make sure the surface was completely regenerated and that the results obtained between runs were comparable. The response was monitored as a function of time (sensorgram) at 25°C.

RESULTS AND DISCUSSION

Molecular Weight Properties of Heparin Samples

The molecular weight properties of the heparin samples were assessed by SEC using individual heparin oligosaccharide standards (Table 1). The SEC chromatograms for heparins are shown in supplementary data. SEC analysis showed a range of $\bar{M}_n$ from 10.2 to 14.6 kDa, M_W from 16.6 to 24.9 kDa, and polydispersities ranging from 1.6 to 2.0. Porcine heparin had the highest molecular weight with ovine and bovine heparin having considerably lower molecular weight. Ovine heparin showed the greatest polydispersity with bovine heparin having the lowest polydispersity.

Table 1. Molecular Weight Measurement of Heparins Using SEM

Sample	$\bar{M}_n$	SD[a]	M_w	SD[a]	$M_w/\bar{M}_n$
Porcine	14,618	114	24,899	244	1.70
Porcine[b]	15,040	190	22,900	100	1.49
Ovine	10,228	120	20,023	167	1.96
Bovine	10,197	136	16,555	143	1.62

[a] Based on triplicate measurements.
[b] Published average values on seven heparin APIs prepared by different commercial manufacturers.[14]

NMR Spectroscopy

The one-dimensional ^{1}H- and ^{13}C-NMR spectra of porcine, ovine, and bovine heparins looked quite similar but with some notable differences (Figs. 2a and 3a). Porcine heparin contained considerably more *N*-acetylated glucosamine residues than either ovine or bovine heparins, as seen by the relative intensity of the peak at 1.96 and 21 ppm in the ^{1}H and ^{13}C spectra (Figs. 2a and 3a). A close examination of the ^{1}H-NMR spectra between 1.9 and 2.1 ppm can be used to assess the impurities of each (Fig. 2a). Porcine and bovine heparin contains dermatan sulfate (DS) as impurity, whereas ovine heparin contains chondroitin sulfate A (CSA) as impurity. The anomeric regions of ^{1}H (Fig. 2b) in conjunction with the partial and HMQC spectra (Fig. 2c) show a single peak for 3-*O*-sulfoglucosamine residue in porcine heparin but double peaks in ovine and bovine heparin samples. This observation suggests that the amount of 3-*O*-sulfo-*N*-acetylglucosamine and 3-*O*-sulfo-*N*-sulfoglucosamine residues might be useful for distinguishing between porcine heparin and both ovine and bovine heparin. The application of HMQC NMR (Fig. 3b) allows all of the signals to be fully assigned when combined with ^{1}H-NMR, ^{13}C-NMR, HHCOSY, and TOCSY. Spectral integration also affords the mol % of each type of saccharide residue present in a heparin (Table 2). Critical features in the GlcN residues, including *N*-sulfo, *N*-acetyl, 3-*O*-sulfo, and 6-*O*-sulfo, content vary a lot among porcine, ovine, and bovine heparins. Additionally, the relative amounts of *N*-acetylglucosamine, iduronic acid, and glucuronic acid residues appear to be highly dependent on the species sources of heparin (Table 2).

Disaccharide Analysis by LC–MS

Heparin can nearly completely be (some resistant tetrasaccharides are obtained, see tetrasaccharide mapping section below) converted to disaccharides

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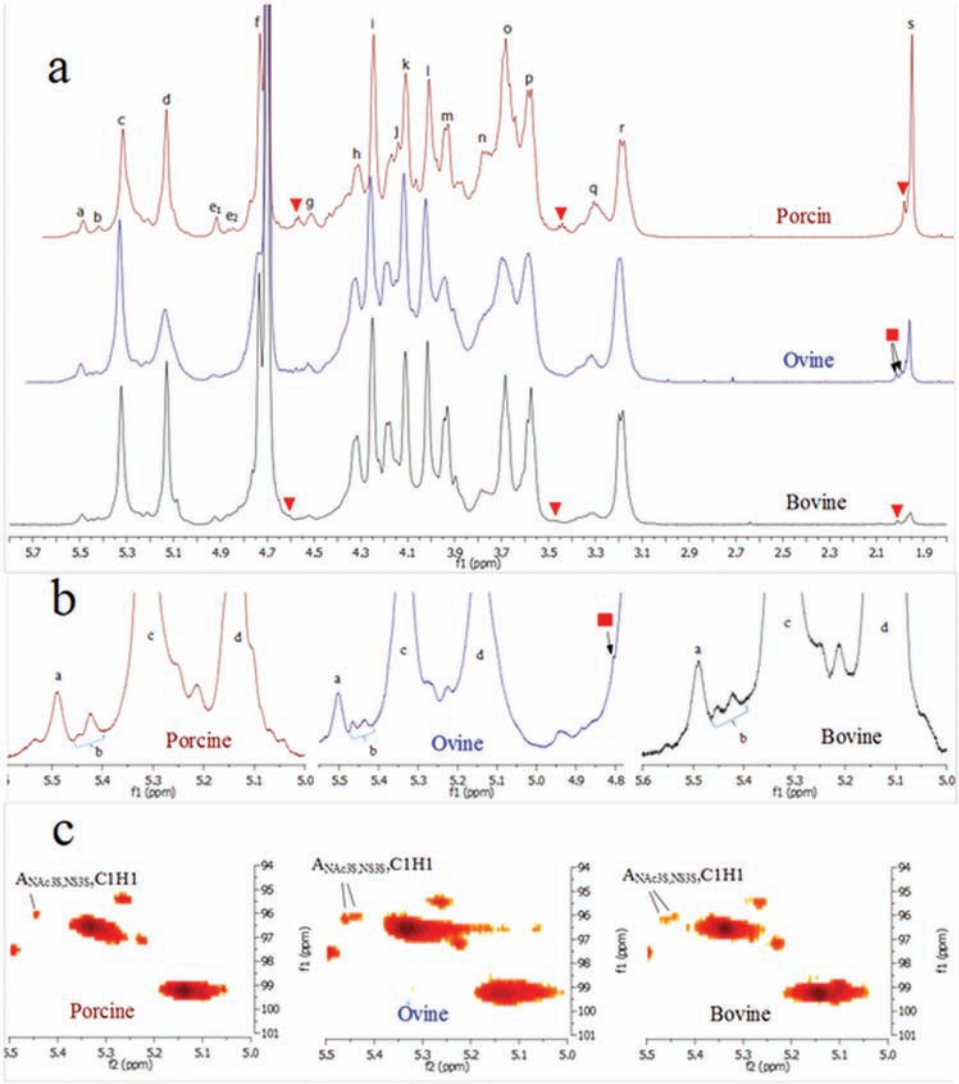

Figure 2. ^{1}H-NMR and HMQC spectra of three heparin samples from different sources. (a) Peak assignments and the compare for one-dimensional ^{1}H-NMR spectra of three samples: a, H1 A_{NS6X}-(G); b, H1 $A_{3S,3SNS}$; c, H1 A_{NS6X}-(I_{2S}) and H1 A_{NAc6X}-(G); d, H1 I_{2S}; e_1, H1 I-(A_{NS6S}); e_2, I-(A_{NS}); f, H5 I_{2S}; g, H1 G; h, H6 A_{NS6X}; i, H2 I_{2S}; j, H6' A_{NS6S}; k, H3 I_{2S}; l, H4, I_{2S}; m, H5 A_{NS6S}; n, H6, A_{NS}; o, H4 A_{NS6S}; p, H3 A_{NS6X}; q, H2 G and H2 $A_{3S,3SNS}$; r, H2 A_{NS6X}; s, acetyl CH3 (A, Glucosamine; I, Iduronic acid, G, glucuronic acid);▼, DS; ■, CSA. (b) The anomeric region in theone-dimensional ^{1}H-NMR spectra of three heparin samples. (c) The anomeric region in HMQC spectra of three samples.

through its treatment with heparin lyases 1, 2, and 3. Although disaccharide analyses of heparins, particularly porcine heparins, have been previously published,[14,15] the results of these analyses are dependent on the analytical method used. In the current study, the disaccharide analysis of porcine, ovine, and bovine heparin were performed back-to-back in triplicate (Table 3). The results, similar to those obtained using NMR (Table 2), show that porcine heparin has the lowest level and bovine heparin has the highest level of *N*- and *O*-sulfo groups. Ovine heparin appears to fit between the two extremes. The most remarkable difference appears to be in the amount of disulfated disaccharides, particularly ΔDiNS6S, which ranges from 15.6 mol % in porcine heparin to 1.81 mol % in bovine heparin. Again, caution needs to be taken in interpreting the disaccharide analysis as this is a complex multistep assay that often results in two to fivefold differences, when performed by different methods.

Tetrasaccharide Mapping Using LC–MS

When heparin is exhaustively treated with heparin lyase 2 in addition to the disaccharides formed, some lyase-resistant tetrasaccharides are observed (Fig. 4) because of the presence of 3-*O*-sulfo containing

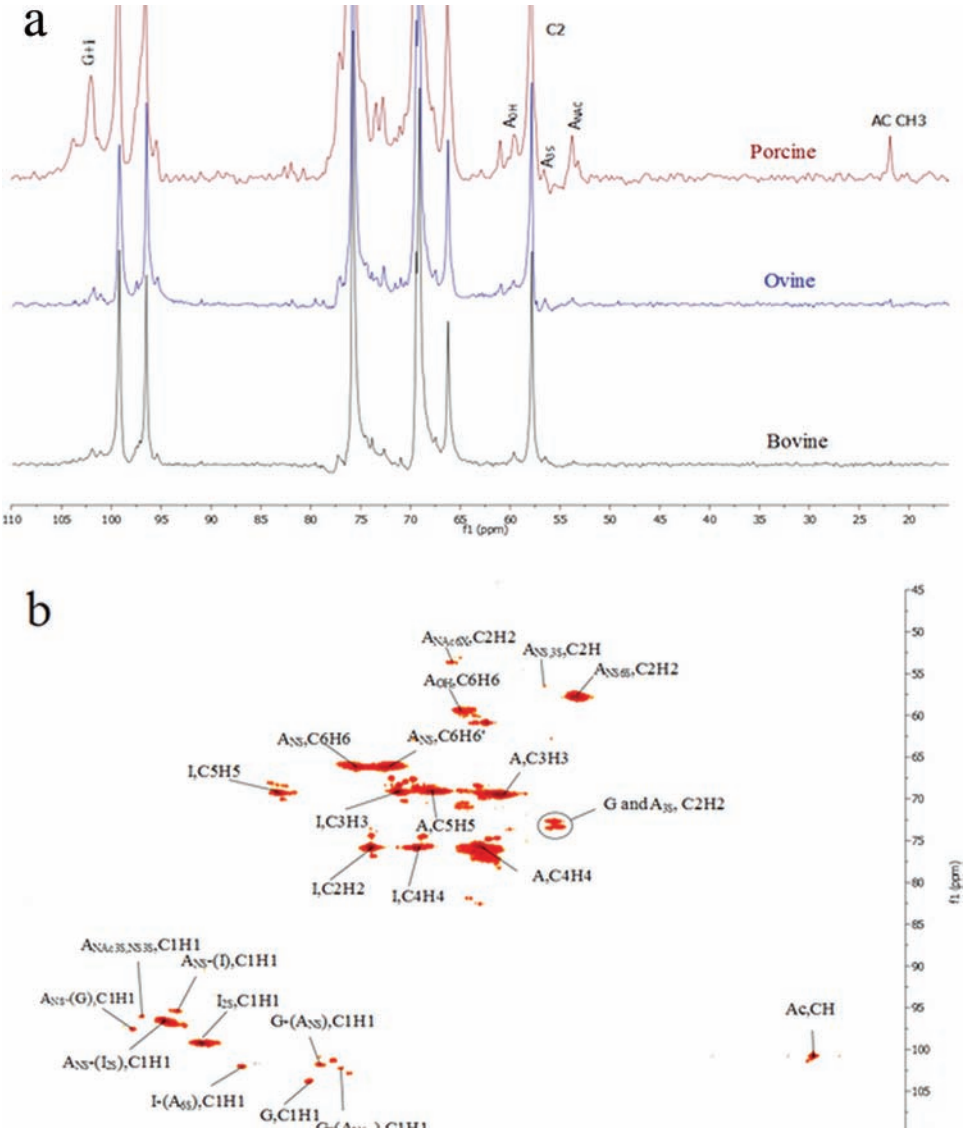

Figure 3. ^{13}C-NMR spectra of three heparin samples from different sources and the full HMQC spectra of porcine heparin. (a) ^{13}C-NMR spectra of three heparin samples and (b) HMQC of porcine heparin.

glucosamine residues.[15] Five of these heparin lyase 2-resistant tetrasaccharides (T1–T5) have been identified (Table 4). Their molecular ratio provides a fingerprint of the heparin from which they are derived as well as an insight into the structural diversity of the AT-binding pentasaccharide sequence within heparin (Fig. 1). Using tetrasaccharide standards,[15] standard curves were constructed to calculate the relative distribution of these sites. Porcine heparin had the highest mass percent of heparin lyase 2-resistant tetrasaccharides (T1–T5) at 5.1% followed by bovine heparin at 4.6% and ovine heparin at 4%. It is

Table 2. Percentage of Substitution of Glucosamine (A) and Uronic Acids (I and G) for Three Heparin Samples from Different Sources

Sample	A_{NS} (%)	A_{NAC} (%)	A_{3S} (%)	A_{6S}[a] (%)	I_{2S} (%)	I (%)	G (%)	Impurity Observed
Porcine	76.6	14.4	9.0	84.3	62.2	12.9	24.9	DS
Porcine[a]	80 (0.9)	13 (0.5)	6.7 (0.7)	83.4(0.6)	61 (2.6)	10.5 (0.7)	28.4 (2)	N.D.
Ovine	90.3	4.2	5.5	91.2	80.6	4	15.3	CSA
Bovine	88.8	2.7	8.5	83.4	87.5	9	3.5	DS

[a]Published value on seven heparin APIs prepared by different commercial manufacturers with A_{6S} values obtained by the integration of ^{13}C spectra. SD shown in parentheses.[14]
N.D., not detected.

 DOI 10.1002/jps

Table 3. HS–HP Disaccharide Composition Analysis by LC–MS

	HS–HP Disaccharides Composition							
Samples	ΔDi-0S	ΔDi-NS	ΔDi-6S	ΔDi-2S	ΔDi-NS6S	ΔDi-NS2S	ΔDi-6S2S	ΔDi-TriS
Porcine	8.7 ± 0.3	4.1 ± 0.1	5.8 ± 0.5	0.3 ± 0.5	15.6 ± 1.1	4.2 ± 0.3	1.2 ± 0.2	60.1 ± 0.2
Porcine[a]	0.3–3.2	0.8–2.9	1.2–3.2	0.8–1.6	5.4–10.8	3.7–11.6	0.8–2.6	67–85
Ovine	3.8 ± 0.3	0.2 ± 0.8	0.7 ± 0.6	1.1 ± 0.4	3.6 ± 0.9	0.9 ± 0.5	0.3 ± 0.2	89.4 ± 0.1
Bovine	0.72 ± 0.8	0.17 ± 0.5	0.08 ± 0.8	0.06 ± 0.6	1.81 ± 1.2	0.96 ± 0.4	–	96.2 ± 0.3

[a]Range of published values on seven heparin APIs prepared by different commercial manufactures.[14]

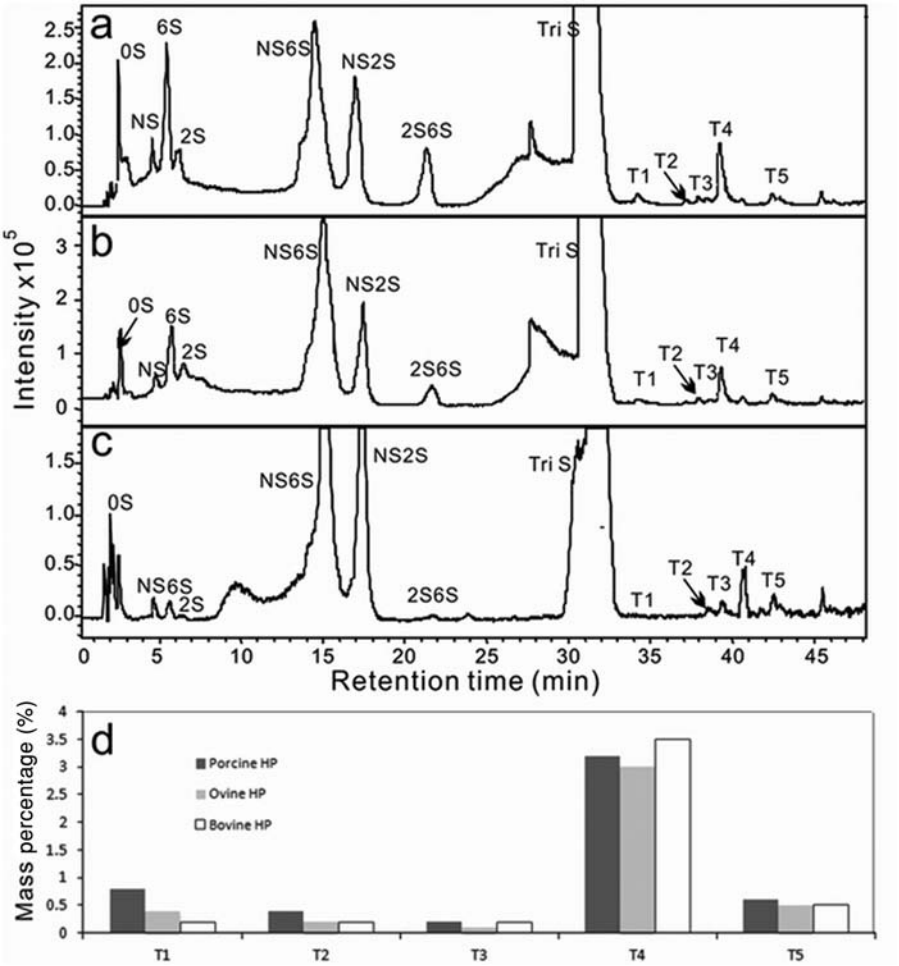

Figure 4. Tetrasaccharide mapping data for three heparin samples from different sources. Extracted ion chromatography (EIC) of (a) porcine heparin, (b) ovine heparin, (c) bovine heparin, and (d) the relative mass percentages of the heparin lyase 2-resistant tetrasaccharides are shown in the bar graph for porcine (black), ovine (gray), and bovine (white) heparins. T1, ΔUA-GlcNAc6S-GlcA-GlcNS3S; T2, ΔUA-GlcNS-GlcA-GlcNS3S6S; T3, ΔUA-GlcNS6S-GlcA-GlcNS3S6S; T4, ΔUA-GlcNAc6S-GlcA-GlcNS3S6S; T5, ΔUA-GlcNS6S-GlcA-GlcNS3S6S.

Table 4. Composition and Molecular Mass of Tetrasaccharides for Tetrasaccharide Mapping

Fractions	*m/z*	Calculated Mol Mass	Theoretical Mol Mass	Sequence
T1	$[477.4]^{2-}$	956.8	956.1	ΔUA-GlcNAc6S-GlcA-GlcNS3S
T2	$[496.6]^{2-}$	994.4	994.0	ΔUA-GlcNS-GlcA-GlcNS3S6S
T3	$[496.6]^{2-}$	994.4	994.0	ΔUA-GlcNS6S-GlcA-GlcNS3S
T4	$[517.4]^{2-}$	1036.8	1036.0	ΔUA-GlcNAc6S-GlcA-GlcNS3S6S
T5	$[536.3]^{2-}$	1074.6	1074.0	ΔUA-GlcNS6S-GlcA-GlcNS3S6S

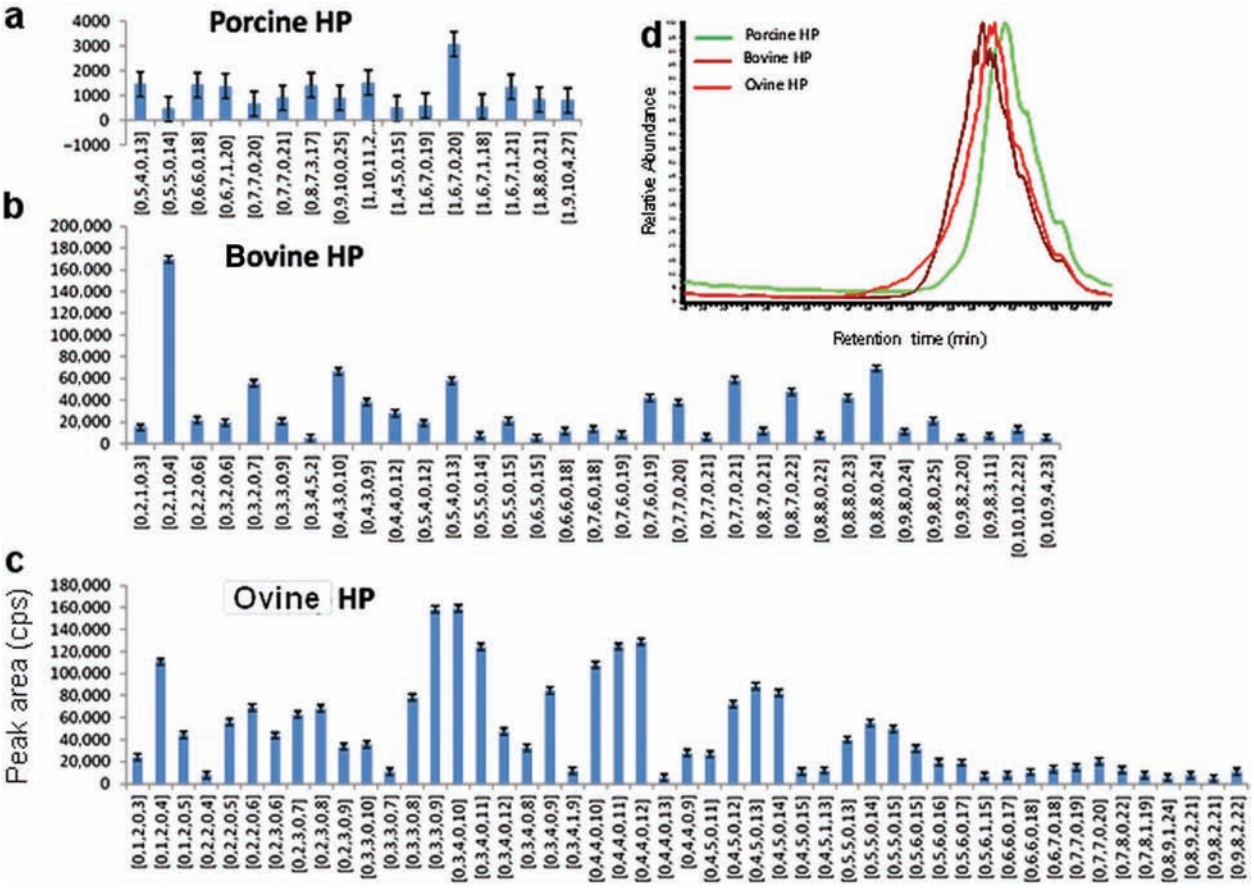

Figure 5. Top-down approach using HILIC LC–FT–ESI–MS to characterize different heparins. (a) Detected porcine heparin oligosaccharides. The oligosaccharides are labeled with the number of [ΔHexA, HexA, GlcN, Ac, SO_3] moieties present in each structure. For example, [0,5,4,0,14] corresponds to an oligosaccharide having 0 ΔHexA, 5 HexA, 4 GlcN, 0 Ac, and 14 SO_3. (b) Detected bovine heparin oligosaccharides. (c) Detected ovine heparin oligosaccharides. (d) TIC (total ion current) of porcine heparin, bovine heparin, and ovine heparin indicated porcine heparin has the highest average molecular weight, whereas bovine heparin has the smallest average molecular weight. Ovine heparin has the widest molecular weight distribution among three heparins tested.

difficult to correlate the total amount of tetrasaccharide to the measured units of anticoagulant activity of these three heparins because their listed anticoagulant activities were determined using an United States Pharmacopeia (USP) clotting assay, which is dependent on thrombin binding and AT binding to heparin. The major tetrasaccharide in all three heparins was the highly sulfated *N*-acetylated tetrasaccharide, T4. Porcine heparin has the highest level of T1, corresponding to an AT binding site pentasaccharaide with a low level of sulfation containing an *N*-acetylglucosamine residue. Further studies will be needed to understand the reliability and repeatability of these mapping data. In addition, there are still unassigned tetrasaccharide peaks observed in these maps that might shed additional light on the structural variability of heparin.

Hydrophilic Interaction LC–ESI–FT–MS Analysis of Intact Heparin

With top-down approach using HILIC LC–ESI–FT–MS, we can profile oligosaccharides and small polysaccharide chains (dp3–dp20 for heparin).[18] Major oligosaccharides and small polysaccharide chains in three different sources of heparin were profiled using HILIC LC–ESI–FT–MS (Fig. 5). Porcine heparin has lowest amount of oligosaccharides (dp3–dp20) and largest average molecular weight, whereas the ovine heparin has the largest number of oligosaccharides detected with relative smaller average molecular weight than porcine heparin. Bovine heparin has the smallest average molecular weight with an intermediate level of oligosaccharides (dp3–dp20). And also, ovine heparin has the widest molecular

 DOI 10.1002/jps

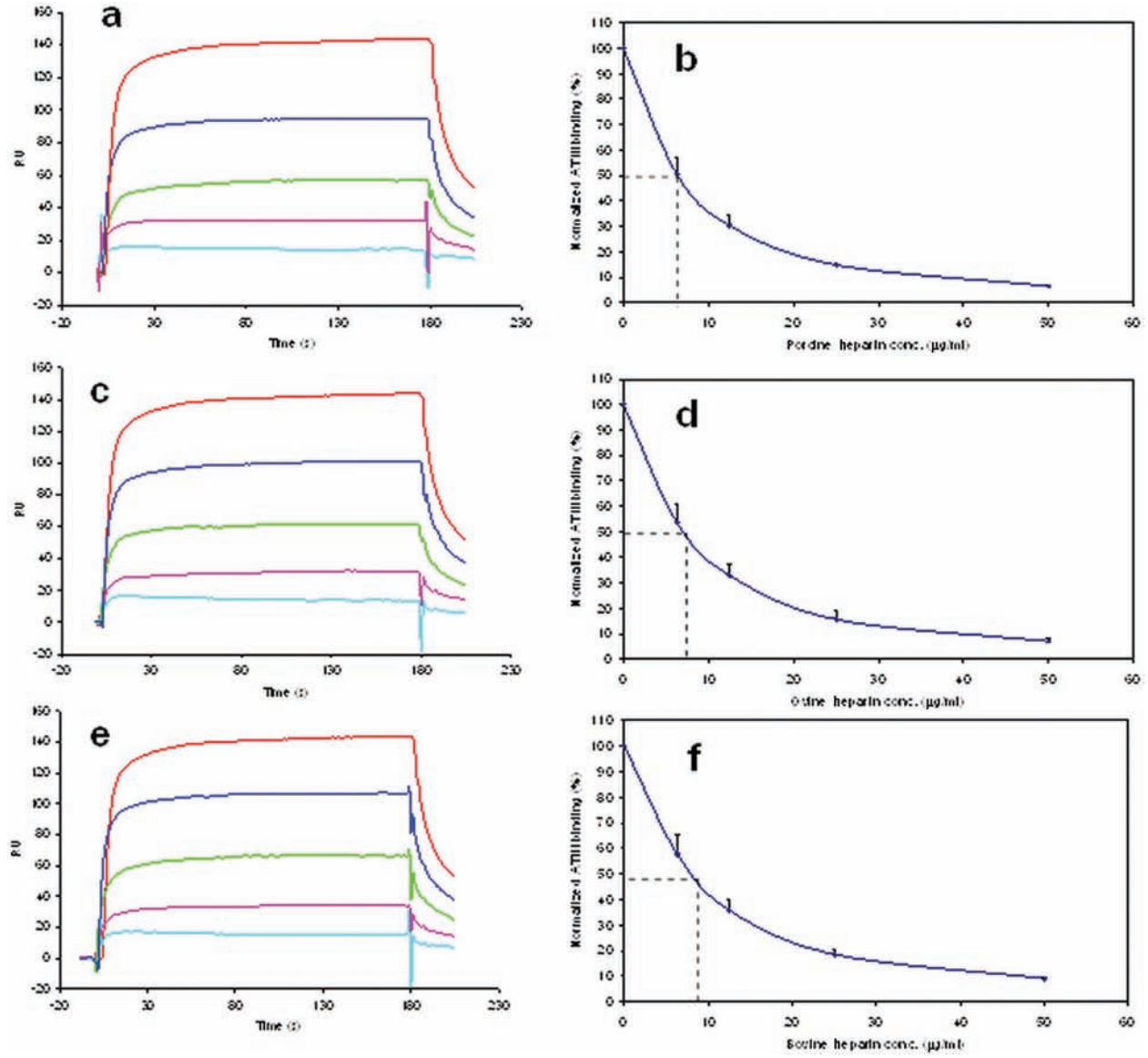

Figure 6. SPR sensorgrams and IC_{50} measurement of heparins using surface competition SPR. AT concentration was 250 nM, and concentrations of heparin in solution (from top to bottom on the sensorgrams) were 0, 6.25, 12.5, 25 μg/mL, respectively. (a) Competition SPR sensorgrams of AT–heparin interaction (solution heparin/surface heparin competition) using porcine heparin. (b) IC_{50} measurement for porcine heparin. (c) Competition SPR sensorgrams of AT–heparin interaction using ovine heparin. (d) IC_{50} measurement for ovine heparin. (e) Competition SPR sensorgrams of AT–heparin interaction using bovine heparin. (f) IC_{50} measurement for bovine heparin.

weight distribution among three heparin tested. From the detected oligosaccharides, we can also see that bovine heparin has oligosaccharides with the greatest number of sulfo groups per disaccharides, which corresponds well with the results of the disaccharide analysis. These detailed HILIC LC–FT–MS structure information either suggests that the three heparin samples have inherent differences or that different manufacturing, extraction, and purification processes may have been used in their preparation.

SPR Analysis

The AT-binding affinity of a heparin is critical for its anticoagulant activity. Recently, our laboratory developed a competitive SPR binding assay for heparins.[19] Using this assay, solution/surface competition experiments were performed to examine the relative binding affinity of different heparins to AT interaction. AT (250 nM) mixed with different of concentrations of heparin in HBS-EP buffer were injected over heparin chip. Once the active binding sites on AT molecules

were occupied by heparin in the solution, the binding of AT to the surface-immobilized heparin should decrease resulting in a reduction in SPR (Fig. 6). The IC_{50} values (concentration of competing analyte resulting in a 50% decrease in response units) can be calculated from the plots [AT binding signal (normalized) versus heparin concentration in solution]. The calculated IC_{50} values for porcine, ovine, and bovine are 6.3 (±0.85) μg/mL, 7.2 (±1.0) μg/mL, and 7.9 (±1.1) μg/mL, respectively. These data are consistent with the view that porcine heparins bind best to AT, whereas bovine heparins that bind less tightly to ATup. Bovine heparin can bind thrombin with higher affinity because these chains may have a greater number of sulfo groups per disaccharide repeating unit. Again, ovine heparin appears to fall between porcine and bovine heparin in its AT-binding affinity.

In conclusion, a thorough study of the physicochemical properties was performed on heparin API from three different species. The results of this study suggest that there are a number of subtle structural features that distinguish these heparins. The variability of porcine intestinal heparin is possible to assess because of its current widespread use. There is remarkable similarity between porcine intestinal heparins prepared by different manufacturers from around the world.[14] In contrast, there is considerably fewer numbers of commercial ovine and bovine heparins available for analyses, and we need to rely on historical samples. Because of this, the inherent variability of ovine and bovine heparins is not well understood. One can only assume that the production processes are similarly well optimized as for porcine intestinal heparin and that these products would show the same level of variability as porcine intestinal heparin.

The regulatory control of the species source of heparin API currently relies on record keeping. Clearly, there is a need to develop analytical approaches that can distinguish the species origin of heparin API. The current study offers a starting point by providing the thorough, side-by-side characterization of a single porcine, ovine, and bovine heparin using state-of-the-art carbohydrate analysis. Further work will be required to assess the reliability of these analytical methods, the natural variability of heparins from each species, and the variability of heparins obtained using different commercial processes. It is clear, however, that the reliance of regulatory agencies on record keeping alone is not sufficient to secure the safety of these critical anticoagulant drugs.

ACKNOWLEDGMENTS

This work was supported by grants from the National Institutes of Health HL101721 and HL096972.

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