# Isolation and Characterization of Human Heparin[†]

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ABSTRACT: Heparin was isolated from an unusually large human hemangioma that contained an elevated level of mast cells. Purification of multimilligram quantities of heparin from this tissue sample permitted a thorough examination of its structure and activity. Characterization of this human heparin included the following: one-dimensional and two-dimensional $^1$H-nuclear magnetic resonance spectral analysis; oligosaccharide mapping; saccharide compositional analysis; and in vitro assessment and anticoagulant activity. Oligosaccharide mapping and nuclear magnetic resonance spectroscopy showed that this human heparin is structurally similar to porcine intestinal mucosal heparin but distinctly different from bovine lung heparin. This human heparin also has substantially more in vitro anticoagulant activity than either of these pharmaceutical heparins.

Heparin is biosynthesized and stored in the granules of mast cells. It is usually prepared from tissues rich in mast cells such as porcine intestinal mucosa and bovine lung. The heparin prepared from these animal tissues is widely used as a pharmaceutical agent.

Heparin is a polydisperse, sulfated, linear copolymer of glucosamine and uronic acid (Casu, 1985). It has been used clinically as an anticoagulant for over a half-century (Linhardt, 1991; Linhardt & Loganathan, 1990; Lane & Lindahl, 1989; Casu, 1985). Heparin also has antiviral activity, binds to a variety of growth factors, inhibits complement activation and regulates angiogenesis (Linhardt, 1991; Linhardt & Loganathan, 1990; Lane & Lindahl, 1989; Casu, 1985). Heparin's angiogenic activity (Folkman et al, 1983; Taylor & Folkman, 1982) is of particular interest since this report describes the isolation and characterization of heparin from a hemangioma.

Capillary hemangiomas are vascular tumors that correspond to pure, uncontrolled capillary growth beginning shortly after birth and continuing for about a year in approximately 1% of infants (Mulliken & Young, 1988). Most of these lesions are infiltrated by mast cells at levels of up to 40-fold higher than those found in the surrounding skin (Glowacki & Mulliken, 1982). Patients with hemangiomas have normal clotting times, although blood coming directly from the lesion usually does not clot.

The purification of heparin from a particularly large human hemangioma rich in mast cells is described. The structure and activity of this human heparin are characterized.

## EXPERIMENTAL PROCEDURES

### *Materials*

Porcine mucosal heparin, sodium salt (145 units/mg), was from Hepar, Franklin, OH. Chondroitinase ABC, chondroitin ABC lyase (EC 4.2.2.4) having an activity of 110 units/mg, and heparitinase, heparin lyase III (EC 4.2.2.8) having an activity of 1.5 units/mg, were from Seikagaku America, Rockville, MD. Heparinase, heparin lyase I (EC 4.2.2.7) having an activity of 5 units/mg, was prepared and purified to homogeneity (Lohse & Linhardt, 1992) in our laboratory. Macroporous anion-exchange resin Lewatit MP500A was from Mobay Corp., Pittsburgh, PA. Spectropore 3500 dialysis membrane was from Spectrum Medical Industries, Los Angeles, CA. Alkalase was obtained from Novo, New York, NY. $^2H_2O$ (99.96 atom % $^2$H) and 3-(trimethylsilyl)-1-propionic acid sodium salt (TSP) were from Aldrich Chemical Co., Milwaukee, WI. HPLC was performed using a Shimadzu LC-7A dual-pump system and the detection system, consisted of an LKB Bromma 2141 variable-wavelength detector operating at 232-nm wavelength. The injector was a Rheodyne 7125 injector. GPC-HPLC used a Si300 Polyol column (9.5 mm × 50 cm, 3 μm) from Serva, Paramus, NJ. A Spherisorb 5-μm particle size 0.46- × 25-cm (analytical) strong anion-exchange SAX-HPLC column was from Phase Separations, Norwalk, CT. UV spectroscopy used a Shimadzu UV-160 spectrophotometer (Tokyo, Japan), and optical rotations were obtained on a Perkin Elmer 141 polarimeter (Norwalk, CT). The pH was measured on a solution analyzer (Model-450-3-A) from Amber Science, San Diego, CA. NMR was performed on a Bruker AMX spectrometer equipped with an X32 computer ($^1$H at 600 MHz and $^{13}$C at 150.9 MHz). Infrared spectrometry was done on a DXB FT-IR spectrometer from Nicolet Analytical Instruments, Madison, WI.

### *Methods*

*Preparation of Human Heparin.* Human heparin was purified from an 11.4-g portion of 64.5 g of tissue, frozen immediately after surgical removal, ground, and suspended in sodium chloride (1%)/sodium bisulfite (1.5%) solution. After adding 150 mg of alkalase (a mixture of proteases acting at basic pH), the mixture was adjusted with sodium hydroxide to pH 9.3 and stirred at 60 °C for 48 h. The solubilized tissue was loaded on a column of 5 g of anion-exchange resin and eluted with 50 mL of 1% and 20 mL of 16% sodium chloride. The high-salt eluate was dialzyed (MWCO 3500) against

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water and freeze-dried. The sample was reconstituted in 400 μL of 16% sodium chloride, precipitated from 60% methanol, and vacuum dried. Crude heparin was reconstituted in 800 μL of 5 mM sodium phosphate buffer (pH 7) and treated with 20 milliunits of chondroitinase ABC, EC 4.2.2.4, and 20 milliunits of heparitinase, EC 4.2.2.8, to remove minor amounts of chondroitin, dermatan, and heparan sulfate impurities (Linhardt et al., 1988, 1990b). Gel permeation HPLC on a Si300 Polyol column (9.5 mm × 50 cm, 3 μm, Serva) removed low molecular weight contaminants. Four 50-μL injections were eluted with 0.5 M sodium chloride using ultraviolet detection (206 nm), and the column voids were collected, dialyzed against water, and freeze-dried. Pure human heparin weighing 8.25 mg was recovered. This same purification method yielded 220 μg of heparin/g of hog intestinal mucosa.

*Chemical and Spectroscopic Characterization of Human Heparin.* The molecular weight and polydispersity of the human heparin were compared to those of pharmaceutical porcine mucosal heparin (145 units/mg, Hepar) by gradient polyacrylamide gel electrophoresis (Edens et al., 1992). The GAG content prepared from hemangioma tissues were measured using a carbazole assay for uronic acids (Bitter & Muir, 1962).

NMR spectroscopic studies (at 600 MHz) relied on both $^{1}$H-NMR (one-dimensional and two-dimensional correlation spectroscopy) and $^{1}$H-detected $^{13}$C-NMR. Each sample was dissolved in $^{2}H_2O$ (99.96 atom %) and freeze-dried. This exchange was performed three times, after which the sample was dissolved in $^{2}H_2O$ (99.96 atom %) containing TSP (internal standard) for NMR studies. Heparin samples were at 1.1 mM, and all spectra were obtained at 29 °C. The 2D-NMR experiments were performed using standard Bruker software.

The FT-IR of human and porcine heparins were measured in a KBr pellet. The optical rotation ($[\alpha]_D$) of human and porcine heparins were measured by polarimetry at $c = 1$ mg of sodium heparin/mL of water and $l = 10$ cm.

*Oligosaccharide Mapping of Human Heparin.* Heparin (1 mg in 500 μL of 5 mM sodium phosphate, pH 7) was treated for 8 h at 30 °C with 15 milliunits of heparinase, after which it was frozen and stored at –70 °C.

Oligosaccharide standards **1–8** were prepared by complete depolymerization of porcine mucosal heparin with heparinase and by preparative SAX[1]-HPLC as previously reported (Rice & Linhardt, 1989). The structures of **1–8** were also fully established (Merchant et al., 1985; Linhardt et al., 1986, 1988, 1992) and are as follows: **1**, ΔUAp2S(1→4)-α-D-GlcNp2S; **2**, ΔUAp2S(1→4)-α-D-GlcNp2S6S; **3**, ΔUAp2S(1→4)-α-D-GlcNp2S6S(1→4)-α-L-IdoAp2S(1→4)-α-D-GlcNp2S; **4**, ΔUAp2S(1→4)-α-D-GlcNp2S6S(1→4)-β-D-GlcAp(1→4)-α-D-GlcNp2S6S; **5**, ΔUAp2S(1→4)-α-D-GlcNp2S6S(1→4)-α-L-IdoAp2S(1→4)-α-D-GlcNp2S6S; **6**, ΔUAp2S(1→4)-α-D-GlcNp2S6S(1→4)-α-L-IdoAp(1→4)-α-D-GlcNpAc6S-(1→4)-β-D-GlcAp(1→4)-α-D-GlcNp2S6S; **7**, ΔUAp2S-(1→4)-α-D-GlcNp2S6S(1→4)-β-D-GlcAp(1→4)-α-D-GlcNp2S3S6S; and **8**, ΔUAp2S(1→4)-α-D-GlcNp2S6S(1→4)-α-L-IdoAp(1→4)-α-D-GlcNpAc6S(1→4)-β-D-GlcAp(1→4)-α-D-GlcNp2S6S.

[1] Abbreviations: ATIII, antithrombin III; IdoAp, idopyranosyluronic acid; GlcAp, glucopyranosyluronic acid; ΔUAp, 4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid; GlcNp, 2-deoxy-2-aminoglucopyranose; S, sulfate; TSP, 3-(trimethylsilyl)propionic acid sodium salt; SAX, strong anion exchange; PAGE, polyacrylamide gel electrophoresis; AUFS, absorbance units full scale; COSY, correlation spectroscopy; APTT, activated thromboplastin time; CaTT, calcium thrombin time; PT, prothrombin time.

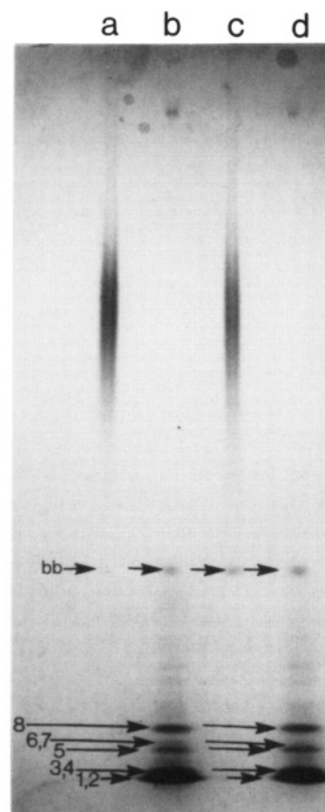


FIGURE 1: PAGE analysis of porcine mucosal heparin and purified human heparin before and after treatment with heparinase. The linear gradient PAGE gel shown [containing 20 μg of sample and bromophenol blue (bb) tracking dye in each lane] was stained with alcian blue. The lanes contained the following: (a) porcine heparin; (b) porcine heparin treated with 5 milliunits of heparinase (EC 4.2.2.7); (c) human heparin; (d) human heparin treated with 5 milliunits of heparinase. The major bands were identified by their comigration with standards and are labeled. Their structures are given in the Methods section.

Qualitative oligosaccharide maps were prepared by analyzing the oligosaccharide products by gradient PAGE (Linhardt et al., 1990a, 1988; Rice et al., 1987). Major bands were tentatively identified on the basis of their comigration with oligosaccharide standards. Quantitative oligosaccharide mapping (Linhardt et al., 1990a,b, 1988; Rice et al., 1987) was done by SAX-HPLC analysis of the heparinase-depolymerized heparins using a linear sodium chloride gradient at a flow rate of 1 mL/min. The major peaks were first assigned by the coinjection of oligosaccharide standards, and the peaks were integrated to obtain the oligosaccharide composition. The disaccharide composition was calculated from oligosaccharide composition with ΔUA2S being assigned to α-L-IdoAp2S consistent with the known specificity of heparinase (Linhardt et al., 1990b).

## RESULTS AND DISCUSSION

Pharmaceutical heparin is typically prepared from hog intestinal mucosa or beef lung (Linhardt, 1991; Linhardt & Loganathan, 1990; Lane & Lindahl, 1989; Casu, 1985). Human heparin has long been thought to be a component of mast cell granules because of metachromatic staining and the presence of anticoagulant activity (Linhardt, 1991; Linhardt & Loganathan, 1990; Lane & Lindahl, 1989). Heparin-like anticoagulants, in the circulation of both healthy and diseased patients, have also been identified by coagulation assays, ATIII affinity, radiolabeling, and electrophoresis (Tefferi et al., 1989; Snow et al., 1987; de Prost et al., 1987; Metcalfe et al., 1980).

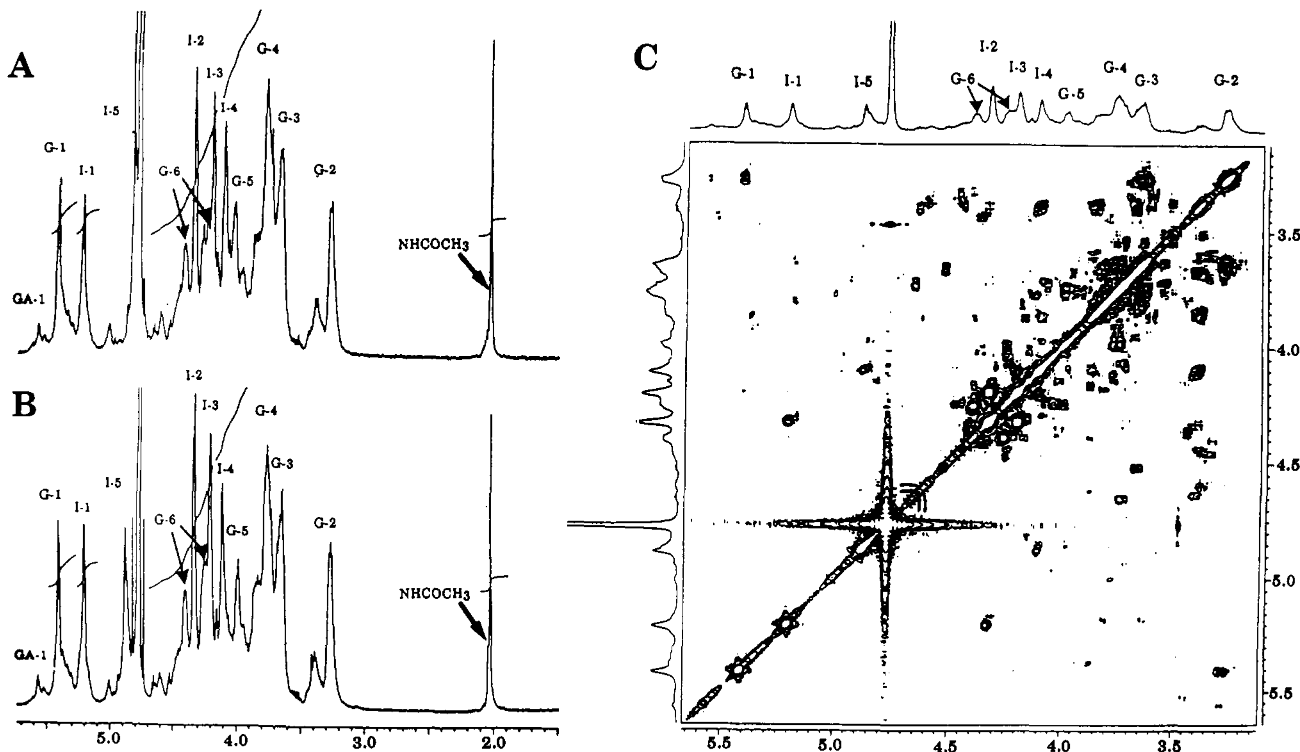


FIGURE 2: $^1$H-NMR spectra of human and porcine heparins. One-dimensional $^1$H-NMR spectra (600 MHz) of human heparin (A) and porcine heparin (B). Two-dimensional $^1$H-correlation spectroscopy (COSY) of human heparin (C). Chemical shifts are reported as ppm from TSP. Peaks are assigned as I = IdoAp, G = GlcNp, and GA = GlcAp with numbers corresponding to the substituted ring carbon.

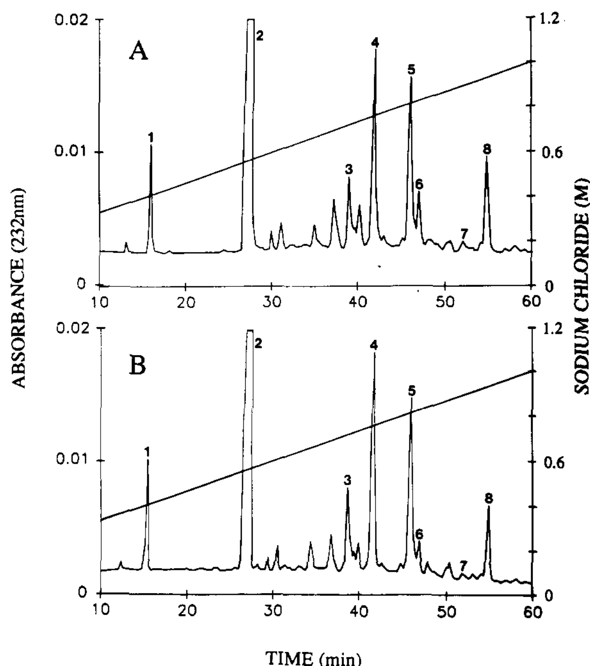


FIGURE 3: SAX-HPLC of heparinase-treated human heparin and porcine heparin. Each heparin (100 μg) was treated with 5 milliunits of heparinase, and 20 μg was analyzed by SAX-HPLC. (A) depolymerized human heparin; (B) depolymerized porcine heparin. The major peaks, assigned by coinjection with standards. Their structures are given in the Methods section.

Table I: Disaccharide Composition of Human and Porcine Heparins[a]

| disaccharide sequence | human mol % | porcine mol % |
|---|---|---|
| →4)-α-L-IdoAp2S(1→4)-α-D-GlcNp2S6S(1→ | 71.00 | 72.80 |
| →4)-β-D-GlcAp(1→4)-α-D-GlcNp2S6S(1→ | 7.62 | 7.32 |
| →4)-α-L-IdoAp2S(1→4)-α-D-GlcNp2S(1→ | 4.39 | 4.29 |
| →4)-α-L-IdoAp(1→4)-α-D-GlcNpAc6S(1→ | 4.29 | 3.10 |
| →4)-β-D-GlcAp(1→4)-α-D-GlcNp2S3S6S(→ | 3.03 | 2.36 |
| total mol % (measured in oligosaccharides **1**–**8**) | 90.30 | 89.80 |

[a] Analysis was from a single SAX-HPLC chromatogram detected at 232 nm using dual attenuations of 0.02 and 0.2 AUFS. The mol % of each oligosaccharide was % total absorbance corresponding to each peak. The oligosaccharides detected and their mol % in human and porcine mucosal heparins are as follows: **1**, 2.76, 2.59; **2**, 63.21, 65.15; **3**, 3.11, 2.95; **4**, 7.99, 8.16; **5**, 7.16, 6.65; **6**, 2.20, 1.28; **7**, 0.52, 0.32; and **8**, 3.54, 2.73. Their structures are given in the Methods section. Heparinase acts on the →4)-α-D-GlcNp2S(6*S* and/or 3*S*)(1→4)-α-L-IdoAp2S(1→ glycosidic linkage (Linhardt et al., 1990b). The oligosaccharides each contain a ΔUAp2S at their reducing termini that is used for their detection. The ΔUAp2S residue is assumed to arise from an α-L-IdoAp2S residue on the basis of the specificity of heparinase.

Heparin has recently been isolated in very small amounts from human tissues. Human mastocytoma yielded 700 μg of heparin (71 units/mg of anticoagulant activity)/g of tissue (Thunberg et al., 1980) while human lung had 14 μg of heparin (108 units/mg of activity)/g of tissue (Metcalfe et al., 1979). However, these human heparins were obtained in insufficient amounts for the detailed characterization of their structure and biological activities.

The blood coming directly from hemangiomas usually does not clot. These hemangiomas are heavily infiltrated with mast cells, and we hypothesized that the 1–4 pg of heparin, believed to be in each mast cell (Tefferi et al., 1989), was responsible for this localized, protamine-neutralizable, anticoagulant effect. The purification of heparin, from a particularly large (64.5-g) hemangioma obtained from an 8-month-old baby, was undertaken, because (1) this tissue represented a potentially rich source of human heparin and (2) demonstrating the presence of heparin in such a lesion might have important implications in the development and regression of vascular growth (Folkman et al., 1983; Taylor & Folkman, 1982).

Human heparin (649 μg/g of tissue) was recovered from 64.5 g of hemangioma tissue that had been frozen immediately after surgical removal. This same purification method yielded 220 μg of heparin/g of hog intestinal mucosa. The molecular

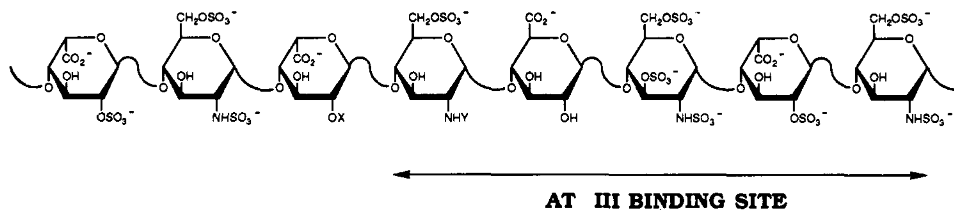


FIGURE 4: ATIII binding site in heparin is shown together with three flanking sugar residues on its nonreducing end. The predominant structure of the ATIII binding site in human heparin and porcine mucosal heparin is X = H and Y = $C(O)CH_3$, while the ATIII binding site in bovine lung heparin is X = $SO_3^-$ and Y = $SO_3^-$.

weight and polydispersity of the human heparin was examined by gradient PAGE (Edens et al., 1992) and was nearly identical to pharmaceutical porcine mucosal heparin (Figure 1).

The $^1$H NMR spectra of human and porcine heparin (Figure 2, panels A and B) were assigned as follows: residue IdoAp2S, δ 5.20 (br s, H-1), 4.30 (br s, H-2), 4.12 (br s, H-3), 4.02 (br s, H-4), 4.75 (br s, H-5); residue GlcNp2S δ 5.38 (br s, H-1), 3.30 (br q, H-2), 3.60 (H-3), 3.70 (H-4), 4.00 (H-5), 4.21 and 4.39 (m, 2 H, H-6); residue GlcAp δ 5.55 (br s, H-1); residue GlcNpAc, δ 2.02 (s, $COCH_3$). $^1$H-detected $^{13}$C-NMR showed human heparin to be free of impurities (Neville et al., 1989). The $^{13}$C NMR spectrum was assigned as follows: residue IdoAp2S, δ 101.5 (C-1), 78.2 (C-2), 71.2 (C-3), 78.1 (C-4), 71.3 (C-5); residue GlcNp2S, δ 98.5 (C-1), 60.1 (C-2), 72.0 (C-3), 78.5 (C-4), 71.0 (C-5), 67.0 (C-6). The FT-IR spectrum of human heparin was identical to that reported for porcine mucosal heparin. Human heparin had an optical rotation of $[\alpha]_D = +40°$ ($c$ = 1 mg/mL) identical to the optical rotation observed for porcine mucosal heparin. These spectral analyses indicated that this human heparin was free of impurities. It is nearly identical to porcine mucosal heparin but distinctly different from bovine lung heparin, which contains a reduced level of *N*-acetylglucosamine residues (Loganathan et al., 1990).

Qualitative oligosaccharide maps (Linhardt et al., 1990a, 1988; Rice et al., 1987) were prepared by depolymerizing each heparin with heparinase (EC 4.2.2.7) and then analyzing the oligosaccharide products by gradient PAGE (Figure 1). These maps were nearly identical. No oligosaccharides above molecular weight 3000 were observed, demonstrating the absence of contaminating glycosaminoglycans in human heparin. PAGE analysis showed both human and porcine mucosal heparins were identical. Bovine lung heparin gives a distinctly different oligosaccharide map (not shown) and is devoid of the band corresponding to hexasaccharide **8** (Loganathan et al., 1990).

Quantitative oligosaccharide mapping (Linhardt et al., 1990a,b, Linhardt et al., 1988; Rice et al., 1987) was done by SAX-HPLC on the heparinase-depolymerized heparins (Figure 3). The results of these analyses were used to calculate disaccharide composition (Table I). Mass balance closure of 90% was excellent for both human and porcine heparins, confirming their purity. Again, the similarity in the disaccharide composition of the human and porcine mucosal heparin was striking. One very important difference between human and porcine heparin was the higher content of the →4)-β-D-GlcAp(1→4)-α-D-GlcpN2S3S6S(1→ sequence in the human heparin (observed in oligosaccharide **8**, Figure 3). This unusual sequence contains sulfation at position 3 of the glucosamine residue, characteristically found in porcine mucosal heparin's ATIII binding site (Figure 4) (Atha et al., 1984; Lindahl et al., 1983). The ATIII binding site in heparin can tolerate some structural variability without losing its high affinity for ATIII (Pejler et al., 1987; Atha et al., 1985). Recently, we reported structural variability in heparins from

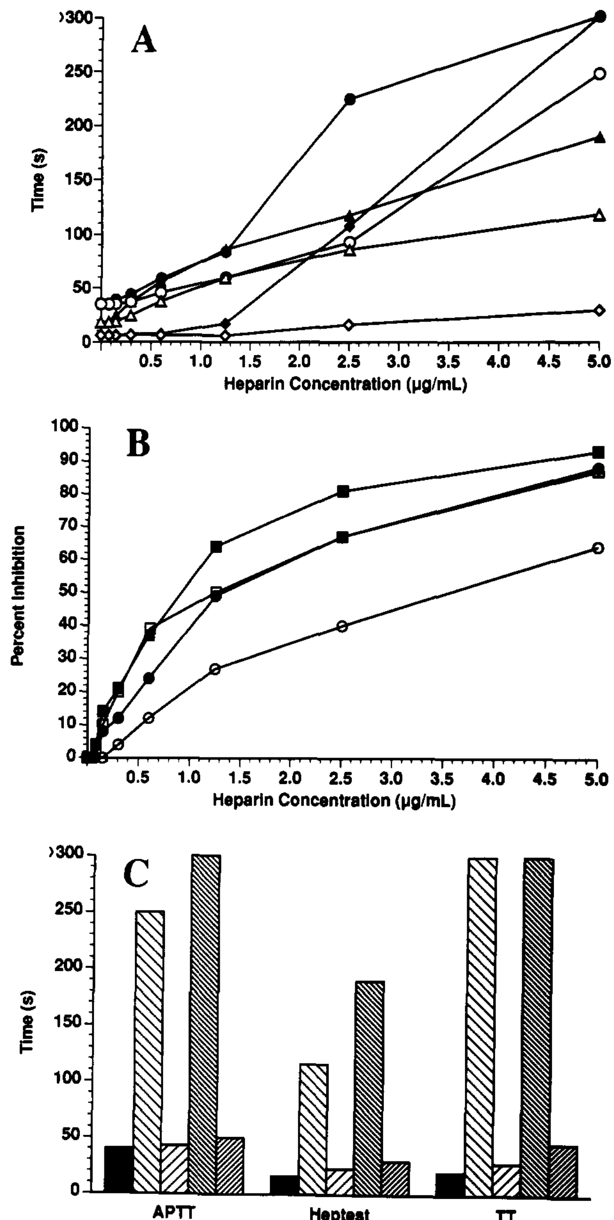


FIGURE 5: Comparison of the activity of human and porcine heparin. A solution of 184 μg/mL of each heparin was prepared in normal saline for analysis (Walenga et al., 1986). The filled symbols correspond to the human heparin and the open symbols to the porcine heparin. (A) Anticoagulant activity is measured by clotting time as a function of heparin concentration using APTT (○), CaTT (◇), and Heptest (△) assays. (B) Amidolytic activity is percent (%) inhibition (change in absorbance at 405 nm compared to control) as a function of heparin concentration using ATIII-mediated anti-Xa (○) and anti-IIa (□) assays. (C) Protamine neutralization of APTT, Heptest, and TT activities. In each assay, the first bar (solid) is the control, followed by porcine heparin and porcine heparin plus protamine (wide diagonals), and then human heparin and human heparin plus protamine (narrow diagonals). The PT remained unchanged at 10 s for both heparins at concentrations from 0 to 5 μg/mL (not shown).

different species, within and immediately adjacent to the ATIII binding site (Loganathan et al., 1990). The ATIII binding site in human heparin is identical to that found in porcine intestinal mucosal heparin (Figure 4) but markedly different from that found in beef lung heparin (Loganathan et al., 1990). It is unclear what physiological role this variability in ATIII binding site structure might play.

Not only does human heparin have the same ATIII binding site as porcine mucosal heparin, it is enriched in this ATIII binding site. Quantitative oligosaccharide analysis showed that human heparin contained 23% more ATIII binding sites (calculated from the mole percent of oligosaccharide **8**) than porcine mucosal heparin. $^{1}$H-NMR confirms this difference, indicating the presence of 12% more *N*-acetylglucosamine (found in oligosaccharide **8**) in the human heparin (Figure 1a,b). This increased number of ATIII binding sites in human heparin is accompanied by increased anticoagulant activity. The anticoagulant, anti-serine protease amidolytic and protamine neutralization activities (Walenga et al., 1986) of human and porcine heparins were profiled (Figure 5). By anti-Xa activity, human heparin (255 units/mg) had 43% greater potency than did porcine heparin (145 units/mg). The concentration of heparin used in these bioassays was first estimated by carbazole assay and then confirmed by quantitative $^{1}$H-NMR.

These results also provide direct chemical evidence for the existence of a human mast cell heparin. The predominant ATIII binding site in human heparin is identical to that found in porcine intestinal mucosal heparin and different from that found in bovine lung heparin. Although human heparin is similar in structure to porcine mucosal heparin, it is more active as an anticoagulant. The presence of a highly anticoagulant heparin within a hemangioma provides the basis for better understanding of bleeding complications associated with these vascular tumors as well as insight into heparin's possible role, as an angiogenesis regulator, in the growth and regression of these common vascular tumors.

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