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Mapping and quantification of the major oligosaccharide components of heparin

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A new method of determining the oligosaccharide composition of commercial glycosaminoglycan heparin is described in which heparin was first depolymerized using heparin lyase (EC 4.2.2.7), and then analysed by a single h.p.l.c. step. All 20 of the porcine and bovine heparins examined were found to contain a small number of major oligosaccharide components, which on average comprised 86% of their mass. The five most abundant oligosaccharides have defined chemical structures. Although the relative abundance of oligosaccharides varied, the heparins examined were surprisingly similar. Porcine, bovine, low-M_r, and high and low antithrombin III (ATIII)-affinity heparins, however, each had distinctly different proportions of these major oligosaccharide components. The concentration of one of these five oligosaccharides, containing a portion of the ATIII binding site, correlated with the anticoagulant activity of the ATIII-affinity-fractionated porcine-mucosal heparins from which it was derived. An additional oligosaccharide of undetermined structure was found in significant quantities in both bovine heparin and high ATIII-affinity porcine-mucosal heparin. The correlation between oligosaccharide concentration and anticoagulant activity suggests that the oligosaccharide is derived from a structural variant of the ATIII-binding site. Finally, for the heparins examined chondroitin/dermatan sulphate formed 0.6–7.4% of their mass.

INTRODUCTION

Heparin is a polydisperse, highly sulphated, alternating copolymer of 1→4-linked glucosamine and hexuronic acid (Casu, 1985). Although it has been closely studied for a half-century, its chemical structure still has not been established. Heparin is biosynthesized as a proteoglycan consisting of a core protein to which polysaccharide or glycosaminoglycan chains are attached (Jacobsson *et al.*, 1984). Although synthesized as a proteoglycan, most scientific studies on heparin's structure and biological activities are performed on commercially-prepared glycosaminoglycan heparin.

Heparin's biological activities have been classified into its effects on cells and on proteins (Jacques, 1979). Of all these activities the most clinically important is heparin's primary use as an anticoagulant and antithrombotic agent. This activity is primarily mediated through the presence of an unique antithrombin (ATIII) binding site in the heparin polymer (Lindahl *et al.*, 1983; Atha *et al.*, 1984). The presence and the number of these binding sites within a particular commercial heparin preparation can be estimated using ATIII-affinity chromatography (Hovingh *et al.*, 1986), a variety of chromatogenic assays based on the ATIII-mediated inhibition of coagulation factor IIa or Xa (FIIa, FXa), or coagulation-based assays.

The structure of heparin has recently been under intensive investigation by our group (Linhardt *et al.*, 1985; Merchant *et al.*, 1985; Linhardt *et al.*, 1986; Rice *et al.*, 1987) and others (Bienkowski & Conrad, 1985; Reynertson *et al.*, 1983; Linker & Hovingh, 1984). Generally, heparin is first depolymerized either chemically or enzymically into a mixture of oligosaccharides which is then fractionated and structurally characterized. The picture which has emerged is a heparin that is complex, comprised of a large number of oligosaccharide components with subtle differences in sulphation, acetylation and stereochemistry. In many of these previous studies the recovery (as a wt.% of heparin) of these oligosaccharides was not accurately established, making it difficult to determine whether a given oligosaccharide represents a major or minor component of heparin's structure.

This paper reports a method for the quantitative determination of heparin's composition, including the molar concentration of its ATIII-binding site. Heparin was first enzymically depolymerized and then analysed by a single h.p.l.c. step. This method was applied to over 20 different commercial heparins in an effort to assess their structural similarities and differences.

EXPERIMENTAL

Materials

Heparin samples were obtained from: (A) Dr. Whyte Owen of the Mayo Clinic (unbleached porcine mucosal heparin); (B) Fluka, Buchs, Switzerland (lot no. 255763-286, > 140 units/mg); (C) Daiichi Pure Chemicals, Tokyo, Japan (lot no. 201DVI, 160 units/mg); (D) Abbott Laboratories, N. Chicago, IL, U.S.A.

Abbrevations used: aPTT, activated partial thromboplastin time; FIIa, coagulation factor IIa; FXa, coagulation factor Xa; ATIII, antithrombin III; HAH, high ATIII-affinity heparin; LAH, low ATIII-affinity heparin; UH, unfractionated heparin; CS, chondroitin sulphate; DS, dermatan sulphate; AUFS, absorbance units full scale; s.a.x., strong-anion-exchange.

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(Panheprin, lot no. 40-095-AF); (E) Sigma Chemical Co., St. Louis, MO, U.S.A. (lot no. 12F-6821); (F) Diosynth, Holland (170 units/mg); (G) Sigma (Porcine Intestinal, Grade I; lot no. 25F-0683, 177 units/mg); (H) Sigma (Porcine Intestinal, Grade II, lot no. 95F-0294, 143 units/mg); (I) Sigma (Bovine Lung, lot no. 16F-0188, 149 units/mg); (J) Evans, (BP-Mucous, lot no. 2LQ450); (K) Elkins-Sinn, Cherry Hill, NJ, U.S.A. (Porcine Intestinal, lot no. 016057); (L) U.S. Biochemical Corporation, Cleveland, OH, U.S.A. (lot no. 16921, 154 units/mg); (M) Lancaster Synthesis, Windham, NH, U.S.A. (CAT no. 4467, > 115 units/mg); (N) Cal-Biochem, La Jolla, CA, U.S.A. (Bovine Lung, lot no. 505602, 145 units/mg); (O) Hepar, Franklin, OH, U.S.A. (Porcine Mucosal, lot no. PM152284, 167 units/mg); (P) Hepar (Porcine Mucosal, lot no. PM17385, 158 units/mg); (Q) Hepar (Porcine Mucosal, lot no. ST 82261, 145 units/mg); (R) Hepar (Porcine Mucosal, lot no. 16585, 164 units/mg); (S) Hepar (RD Heparin, lot no. 10183, 98 units/mg); and (T) Calbiochem (RD Heparin 3000, lot no. 507438, 76 units/mg). USP reference standard (Rockville, MD, U.S.A.), heparin sodium, K1 with 355 units/ml was used as a standard in the amidolytic assays.

Heparan monosulphate from bovine kidney, and dermatan sulphate (DS) from porcine skin, were obtained from Sigma. Heparinase (EC 4.2.2.7) was purified from *Flavobacterium heparinum* [5 units (μmol/min)/mg] (Yang *et al.*, 1985), and was also obtained from Sigma (1.5 units/mg). Heparitinase from *F. heparinum* (EC 4.2.2.8; 1 unit/mg) and chondroitinase ABC from *P. vulgaris* (EC 4.2.2.4; 1 unit/mg) were obtained from ICN Immunobiologicals, Lisle, IL, U.S.A.

Carbazole, concanavalin A–Sepharose, thrombin (human, 1000 units/mg of protein) and thrombin-assay substrate Chromozym TH (tosyl-Gly-Pro-Arg-*p*-nitroanilide acetate) were obtained from Sigma. Activated thrombofax reagent optimized [for activated partial thromboplastin time (aPTT)] was obtained from Ortho Diagnostics Systems Inc. Factor Xa-amidolytic kit, including S-2222 (benzoyl-Ile-Glu,Gly-Arg-*p*-nitroanilide) was from Hellena Laboratories, TX, U.S.A. Dialysis tubing Spectropore 3500 was from Spectrum Medical Industries, Los Angeles, CA, U.S.A. Polyethylene glycol, M_r 6000, was from E.M. Merck, Darmstadt, West Germany. H.p.l.c. was performed on dual Constametric II pumps connected through a gradient mixer from LDC, Milton Roy, Riviera Beach, FL, U.S.A. A fixed-loop injector #7125 from Rheodyne, Cotati, CA, U.S.A. and a variable wavelength UV-5 detector from ISCO, Lincoln, NE, U.S.A. were used. Gradient control and data collection used an Apple IIe microcomputer running Chromatochart software from Interactive Microware, State College, PA. Strong-anion-exchange (s.a.x.) h.p.l.c. was performed on a Spherisorb (5 μm particle size) column of dimensions 4.6 mm × 25 cm from Phase Separations, Norwalk, CT, U.S.A. U.v. spectroscopy used a Shimadzu model UV-160 spectrophotometer equipped with a thermostated cell.

Preparation of commercial heparins

All heparin samples were prepared by dissolving approx. 20 mg into 1 ml of water and dialysing (in 3500-M_r cut-off bags at 4 °C) first against 10 vol. of 1 M-NaCl followed by 3 × 1000 vol. of deionized water. After dialysis the heparins were removed from their dialysis bags and syringe filtered into pre-weighed vials, freeze-dried, and stored together dessicated over anhydrous $CaCl_2$ for 2 days. Each of the heparin samples was then carefully weighed and, using a density of 2 g/ml for heparin, the appropriate value of distilled water was added to obtain a 20 mg/ml stock of solution. For example, if a heparin sample weighed exactly 20 mg, 990 μl of water was added to obtain a 20 mg/ml solution. These stock solutions were stored at −70 °C and dilutions were made for uronic acid assay, aPTT, antithrombin-mediated anti-FXa and anti-FIIa assays, DS assay, and heparinase-catalysed depolymerization.

Affinity fractionation of heparin

Heparin was fractionated based on its affinity to ATIII (Denton *et al.*, 1981). A concanavalin A–Sepharose column (1 cm × 10 cm) at 4 °C was equilibrated with 0.02 M-Tris/HCl containing 50 mM-NaCl, 1 mM-$MgCl_2$, 1 mM-manganese chloride and 1 mM-$CaCl_2$ at pH 7.4. Excess ATIII (Griffith *et al.*, 1985), in the same buffer, was applied and 20 mg was bound to the column. Heparin [5 mg of unfractionated porcine heparin (UH) from Hepar] in 1 ml of the same buffer was loaded onto the column and the column was washed with 15 ml of starting buffer. The heparin which failed to bind (2.6 mg) was recovered in this fraction. The NaCl concentration in the buffer was increased to 0.25 M and 15 ml was used to release the heparin (1.4 mg) that was non-specifically bound to the column. Heparin bound (1 mg) to the immobilized ATIII [high ATIII-affinity heparin (HAH)] was eluted using 15 ml of the same buffer containing 1 M-NaCl. Each fraction was dialysed, freeze-dried, redissolved in a small volume of water and its heparin concentration measured by uronic acid assay. The 2.6 mg of non-binding heparin was fractionated a second time on the same column and again the non-binding fraction was recovered, dialysed, freeze-dried, redissolved and assayed, resulting in 1.3 mg of low ATIII-affinity heparin (LAH).

Coagulation and amidolytic assays

The HAH, UH and LAH heparin samples were assayed by aPTT by first constructing a standard curve using porcine mucosal heparin (R) from 0 to 0.5 units/ml of human plasma vs. log aPTT (in s). The aPTT was determined using a manual method previously described in detail (Linhardt *et al.*, 1982). Each heparin was prepared in fresh citrated human plasma at several concentrations ranging from 1 to 4 μg/ml.

The heparin samples were assayed for anti-FXa activity using a Kabi Vitrum Coatest Heparin Kit by the 'End-Point' method. A standard curve was constructed using USP heparin from 0.1 to 0.7 units/ml of test plasma vs. absorbance at 405 nm. Test plasma containing each heparin was prepared at concentrations of 0.5–10 μg/ml.

The heparin samples were prepared (0.05–1.5 μg/340 μl) in buffer containing 0.05 M-Tris/HCl, 7.5 mM-disodium ethylenediamine tetra-acetic acid and 0.15 M-NaCl, pH 8.4. A standard curve was constructed using USP heparin between 0 and 0.02 units/340 μl of buffer. Heparin in buffer (340 μl), ATIII (10 μl of 5 mM-ATIII in 0.02 M-Tris/HCl, 0.25 M-NaCl, pH 7.4) and FIIa (50 μl of 6.7 NIH units/ml) were combined and incubated at 37 °C for 1 min, when 200 μl of chromozym TH (0.75 mM in distilled water) was added. After exactly 1 min of further incubation at 37 °C, 200 μl of 50 % (v/v) aqueous

acetic acid was added to terminate the reaction. The log absorbance at 405 mm vs. units/ml was plotted.

Uronic acid determination of heparin

The uronic acid content of each of the heparin samples was compared by the carbazole method (Bitter & Muir, 1962). A standard curve of absorbance at 530 nm vs. μg (1–30 μg) of heparin was prepared with one of the standard heparin solutions (O).

Determination of chondroitin (dermatan) sulphate content of heparin

A standard curve was prepared using 0–10% (w/v) DS solution [chondroitin sulphate (CS)B] in 100–90 wt. % of standard heparin. To 125 μl of this solution (containing a total of 1.5 mg of heparin plus DS), 225 μl of 0.25 M-sodium acetate and 2.5 mM-calcium acetate solution, adjusted to pH 7.0, was added along with 50 μl of chondroitinase ABC (2.5 units). The reaction mixture was sealed and incubated for 24 h at 37 °C at which time the reaction was cooled and its absorbance at 232 nm was measured directly in a quartz microcuvette having a 1 mm pathlength. Controls with either substrate or enzyme absent were prepared. Each heparin solution (without added DS) was analysed in a similar fashion.

Kinetics of heparinase depolymerization of heparin

Heparin (20 μl of 20 mg/ml) was added to 300 μl of 0.25 M-sodium acetate and 2.5 mM-calcium acetate solution adjusted to pH 7.0, at 30 °C in a microcuvette. Commercial heparinase (0.016 units in 80 μl) was added to substrate and the change in absorbance was measured continuously at 232 nm in a thermostated u.v. spectrophotometer. The initial rate [dA_{232} (1 cm path length)/dt (min)] was measured over the first 9 min of the reaction and the final absorbance at 232 nm was measured directly after 100 min.

Preparation of heparin-derived oligosaccharides using heparinase

Heparin (50 μl of 20 mg/ml) was added to 425 μl of 0.2 M-NaCl and 5 mM-sodium phosphate, pH 7.0. Purified or commercial heparinase (25 μl, 0.015 units) was added to make the total volume of the solution 500 μl. The reactions were run at 30 °C to completion in 8 h. The heparin depolymerization reactions were terminated by heating at 100 °C for 1 min (Rice *et al.*, 1987). Aliquots (4, 40 and 180 μl) were removed, added to 1 ml of 0.03 M-HCl to obtain a total volume of 1 ml and absorbance at 232 nm was determined.

Analytical s.a.x.h.p.l.c. separation of heparin-derived oligosaccharides

Depolymerized heparin was injected at two concentrations, 40 μg/40 μl and 4 μg/40 μl, onto an analytical Spherisorb, 5 μm particle size (0.46 cm × 25 cm) s.a.x.h.p.l.c. column equilibrated with 0.2 M-NaCl, pH 3.5. The sample was eluted from the column with a linear gradient [concentration (y, in M) at any time (x in s) = $0.0002\,x + 0.2$] of NaCl at pH 3.5 and a flow rate of 1.5 ml/min. The elution profile was monitored by absorbance at 232 nm at 0.02 absorbance units full scale (AUFS) and a chart speed of 15 cm/h. The amount of oligosaccharides F1–F5 was assessed by computer integration of peak area using a standard curve. Peaks were tentatively identified by either co-elution with an authentic sample or retention time. The identity of the peaks assigned to F1–F5, F2′ and F5′ in the depolymerized heparins was confirmed by comparing each with purified oligosaccharide standards analysed on a single-gradient polyacrylamide gel (Rice *et al.*, 1987).

Molar absorptivities, standard curves and error analysis. The five major oligosaccharides obtained from depolymerization of heparin by heparinase were prepared at > 95% purity (Rice, 1987) and characterized (Merchant *et al.*, 1985; Linhardt *et al.*, 1986; Rice *et al.*, 1987). These compounds were desalted twice by Sephadex G-10 column chromatography, frozen, freeze-dried, and stored desiccated over anhydrous $CaCl_2$ for several days. Three 1 mg samples of each of the purified, dried oligosaccharides were accurately weighed, dissolved in 1 ml of 0.03 M-HCl, and three dilutions of each (in 0.03 M-HCl) were prepared. Absorbance at 232 nm was determined after blanking against 0.03 M-HCl. The molar absorption coefficient (ϵ) was calculated from the plot of absorbance at 232 nm vs. molarity determined from the M_r of the sodium salt of each compound.

Aliquots of the samples having approx. absorbance at 232 nm of 0.9 (0.173 nM), were used to construct calibration curves. These compounds were injected, using a 100 μl-calibrated syringe, into a 200 μl loop connected to the analytical s.a.x.h.p.l.c. column and elution was monitored by absorbance at 232 nm. Each sample was applied in triplicate, at each of three sample volumes, 15 μl, 30 μl and 45 μl. The average elution peak area vs. μg injected, was plotted for each of the oligosaccharides F1–F5.

Error analysis was performed on the compositional analysis by depolymerizing heparin (R) in triplicate and analysing each of the resulting oligosaccharide mixtures by s.a.x.h.p.l.c.

RESULTS

The analysis of the heparin samples at concentrations of 5, 10 and 15 μg/ml by carbazole assay gave a collection of lines with an average equation:

$$A_{530} = 0.0183\ A_{530}/\mu\text{g of heparin} - 0.0006\ A_{530}$$

with the slope having an S.D. of 0.0034 ($n = 20$).

Each heparin was depolymerized under identical conditions using heparinase to afford a mixture of oligosaccharides. The rate of depolymerization, under conditions of substrate saturation, was nearly identical for all the heparins examined. An initial rate of 91.9 μmol/min was determined with an S.D. of 8.3 μmol/min ($n = 20$). The final yield of depolymerization product is shown in Table 1. Fractionation of the depolymerization mixture by direct injection onto s.a.x.h.p.l.c. at two concentrations resulted in all peaks being on-scale and allowed the accurate determination of integration area. Five major oligosaccharide fragments (F1–F5), tentatively assigned by elution position, were observed in all heparins and their concentration is given in Table 1.

Gradient-polyacrylamide-gel electrophoresis analysis was performed to check the tentative assignments made in each chromatogram (Rice *et al.*, 1987). All the heparins clearly contained F1–F5 and F2′. The porcine heparins contained primarily F5, while the bovine heparins contained primarily F5′ as well as a higher level of F4. HAH and LAH were, respectively, enriched and

Table 1. Compositional analysis of heparins

Heparin	Oligosaccharide (μg/100 μg of heparin) F1	F2	F3	F4	F5	Mass balance wt. % (μg of F1–F5/100 μg)	μmol of F1–F5 100 μg	A_{232}/ϵ (μmol of product mixture/100 μg)	Mass balance (mol %) (μmol of F1–F5/μmol of product mixture)	CS or DS (μg/100 μg of heparin)
A	41.3	4.04	11.1	14.5	8.71	79.7	0.090	0.110	81.3	5.7
B	50.0	4.37	12.8	10.9	9.66	87.7	0.102	0.126	102.4	5.6
C	43.1	5.72	12.1	11.6	6.75	79.3	0.091	0.107	84.8	7.2
D	52.6	4.79	14.4	17.8	9.98	99.5	0.113	0.131	86.3	1.4
E	51.9	5.66	13.3	13.0	7.04	90.9	0.107	0.126	85.3	–
F	49.7	6.55	14.0	13.8	7.28	91.4	0.106	0.121	87.3	0.7
G	48.6	6.37	14.7	16.4	7.92	94.0	0.107	0.142	75.4	2.8
H	38.3	5.53	12.6	12.9	6.50	75.9	0.086	0.106	80.9	4.8
I	67.0	4.32	14.2	22.9	2.31	110.8	0.134	0.120	111.6	1.4
J	45.5	6.27	13.3	13.2	6.34	84.6	0.098	0.119	82.1	0.9
K	43.7	2.95	12.7	10.0	5.79	75.1	0.089	0.118	75.7	3.5
L	39.1	3.91	10.1	12.5	6.98	72.6	0.083	0.107	77.9	6.0
M	44.5	5.49	11.7	9.9	6.12	77.7	0.092	0.110	82.8	7.5
N	66.2	6.41	9.9	27.8	3.02	113.4	0.135	0.140	96.8	1.0
O	53.1	5.65	14.9	16.4	8.35	98.4	0.113	0.124	91.4	0.7
P	49.0	5.83	12.9	13.6	5.34	86.6	0.102	0.129	79.0	0.7
Q	47.9	8.80	13.0	12.2	5.45	87.4	0.102	0.127	80.2	–
R	49.0	5.10	13.9	15.8	6.51	90.9	0.105	0.127	82.5	0.6
S	55.9	6.90	14.3	16.2	6.19	99.5	0.117	0.119	98.2	0.9
T	32.4	4.72	7.8	11.0	2.21	58.1	0.068	0.104	65.6	0.7
LAH	37.1	4.86	10.3	10.1	2.16	64.5	0.076	0.094	66.4	–
HAH	35.8	2.52	9.0	5.0	11.10	63.5	0.072	0.109	80.7	–

disenriched in both F5 and F5′. Depolymerized heparins (A–T) were also combined in equal parts and analysed by s.a.x.h.p.l.c. The resulting chromatograms were no more complex than those obtained on analysing any of the individual heparins, indicating that the major peaks in each depolymerized heparin have identical retention times.

The molar absorption coefficients (M^{-1} cm^{-1}) for the major oligosaccharides were: F1, 5063±8% (cf. Linker & Hovingh, 1972, 5200) for M_r 665; F2, 5331±2% for M_r 1228; F3, 5066±4% for M_r 1228; F4, 5657±3% for M_r 1330; and F5, 5275±9% for M_r 1833. Calibration curves constructed for F1–F5 by plotting peak area vs. μg of oligosaccharide each showed a correlation $r^2 > 0.999$ on linear regression analysis. Three samples of heparin (R) were depolymerized and analysed by s.a.x.h.p.l.c. The ratio of S.D. to μg determined (multiplied by 100) for each of the oligosaccharides were: F1, 3.6%; F2, 5.7%; F3, 5.1%; F4, 1.7%, and F5, 4.7%. The wt.% mass balances (μg of F1–F5/100 μg) shown in Table 1 were measured to within 5% based on these standard deviations.

A chromatogram of oligosaccharide products of the porcine mucosal heparin (P) at 4 μg/40 μl injection concentration is shown in Fig. 1. Chromatograms (40 μg/40 μl injections) of the oligosaccharide products of two different lots of porcine heparin from the same manufacturer (P and R), porcine heparin (Panheprin) from a second manufacturer (D), unbleached porcine heparin (A), low-M_r heparin chemically prepared from porcine mucosal heparin (S), and bovine lung heparin (I) are shown in Fig. 2. ATIII-affinity fractionated heparins, LAH and HAH are shown in Fig. 3.

Porcine heparin was fractionated on an ATIII-affinity column to prepare high- and low-affinity heparin. Together with unfractionated heparin (an equal weight mixture of heparin O, P, Q and R), these heparins were

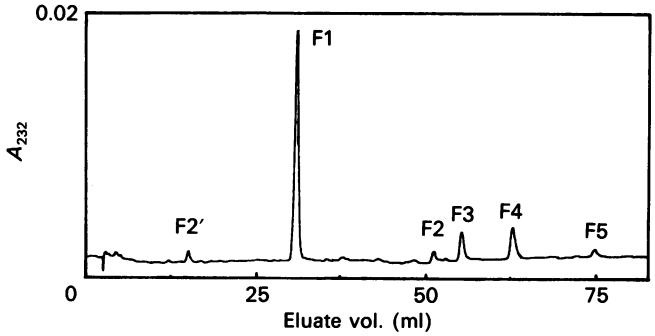

Fig. 1. Porcine mucosal heparins analysed by analytical s.a.x.-h.p.l.c.

The oligosaccharide product mixture (4 μg in 40 μl) obtained from each heparin was fractionated and is shown as a plot of A_{232} (0.02 AUFS) vs. elution volume in ml. Final oligosaccharides F1 through to F5 and disulphated disaccharide F2′ are: F1, ΔUAp2S(1→4)-α-D-GlcNp2S6S; F2, ΔUAp2S(1→4)-α-D-GlcNp2S(1→4)-α-L-IdoAp2S(1→4)-α-D-GlcNp2S6S; F3, ΔUAp2S(1→4)-α-D-GlcNp2S6S(1→4)-β-D-GlcAp(1→4)-α-D-GlcNp-2S6S; F4, ΔUAp2S(1→4)-α-D-GlcNp2S6S(1→4)-α-L-Id-oAp2S(1→4)-α-D-GlcNp2S6S; F5, ΔUAp2S(1→4)-α-D-GlcNp2S6S(1→4)-α-L-IdoAp(1→4)-α-D-GlcNAcp6S-(1→4)-β-D-GlcAp (1→4) -α-D-GlcNp2S3S6S; F2′, ΔUAp-2S(1→4)-α-D-GlcNp2S; (ΔUA, 4-deoxy-α-L-*threo*-hex-4-enopyronosyluronic acid; GlcA, glucuronic acid, and IdoA, iduronic acid).

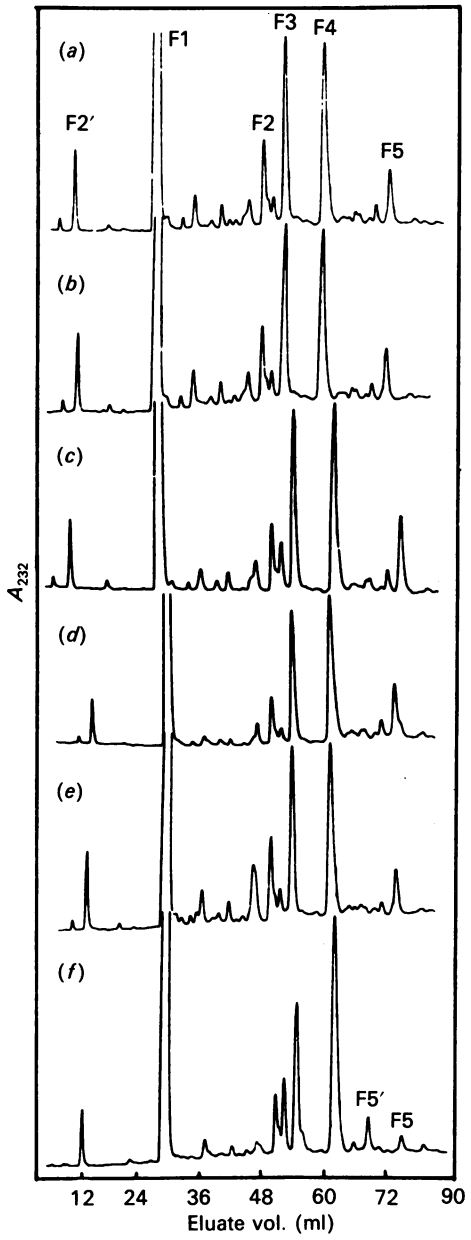

Fig. 2. Analysis of heparins by analytical s.a.x.h.p.l.c.

Chromatograms (*a* and *b*) are porcine heparins (P) and (R) respectively, prepared by Hepar, (*c*) porcine heparin (D) from Abbott, (*d*) unbleached porcine heparin (A), (*e*) low-M_r porcine heparin (S), and (*f*) bovine heparin (I). Each heparin was depolymerized and injected at 40 μg/40 μl.

analysed for F5 content. HAH, UH and LAH had respective activities of: 77, 172 and 237 units/mg by aPTT; 110, 218 and 700 units/mg by ATIII-mediated anti-FIIa assay; and 56, 181 and 555 units/mg by ATIII-mediated anti-FXa assay. A plot of these activities vs. μg of F5 gives a good correlation ($r^2 = 0.81$) which improved when the F5′ found in HAH (3.95 μg/100 μg of heparin) was added ($r^2 = 0.84$). Bovine lung heparin also contains an elevated level of oligosaccharide F5′ (Fig. 2). The concentration of contaminating DS was also determined for each heparin using chondroitinase ABC (Perlin & Folkman, 1987) (Table 1).

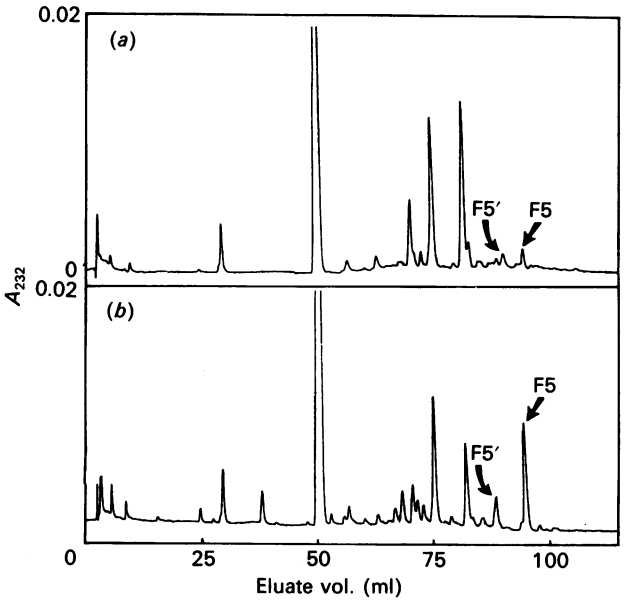

Fig. 3. Analysis of ATIII-affinity-fractionated heparin by analytical s.a.x.h.p.l.c.

LAH (*a*) and HAH (*b*) were depolymerized and injected at 40 μg/40 μl. The elution volume for F1–F5 is different from that given in Fig. 1 and 2 due to the use of a second s.a.x.h.p.l.c. column and a slightly different gradient.

DISCUSSION

Heparin, from a variety of sources, was carefully prepared by predialysis, freeze-drying to a uniform dryness, and dissolving to a concentration of 20 mg/ml. Analysis of these stock solutions by carbazole assay confirmed that each contained a comparable mass of heparin. On treatment with heparinase all heparins gave nearly identical initial rates and similar final molar concentrations of oligosaccharide products (Table 1).

Analysis of proteins for the presence of conserved sequences has relied heavily on peptide mapping. In an effort to develop a similar approach for heparin, the oligosaccharide maps for 15 commercial porcine mucosal heparins were prepared. At 4 μg/40 μl injections all the heparins looked remarkably similar, with each showing five major oligosaccharides (Fig. 1). These major oligosaccharides (F1–F5) are each released from the polymeric substrate at similar rates by heparinase (Rice, 1987). In addition, each heparin shows an early peak at 8 min corresponding to a disulphated disaccharide (F2′) which directly arises from the polymeric substrate, but can also be produced as the result of prolonged action of heparinase at a secondary site contained in tetrasaccharide F2 (Rice, 1987). Oligosaccharide F4 also contains a secondary site for heparinase. Although this site is over 100-fold less sensitive to heparinase than is the polymeric substrate, prolonged enzymatic treatment results in its breakdown to 2 mol of F1 (Rice, 1987). All of the major oligosaccharides are stable under the reaction conditions used in this study, thus the amounts of these products (Table 1) correspond to their content in the heparin polymer.

The depolymerized heparins were also injected at 40 μg/40 μl (resulting in an off-scale F1 peak) to amplify

the intensity of the minor oligosaccharide components (Fig. 2). Two lots of porcine heparin from the same manufacturer resulted in remarkably similar maps (Fig. 2*a–d*). The lot-to-lot similarity, however, was no more remarkable than the similarity between heparins from different manufacturers or between commercial and unbleached porcine heparins (Fig. 2*a–d*). As little as 1 μg of heparin (at 0.005 AUFS) could be used to prepare an oligosaccharide map.

The picture of porcine heparin which emerges from these chromatograms is of a simple polymer which is primarily poly-F1 (Fig. 1), but which also contains a small number of additional characterized and uncharacterized (Fig. 2*a–d*) oligosaccharides. Integration of the signals corresponding to oligosaccharides F1–F5 results in an average mass balance recovery of 86 wt. % ($n = 16$, S.D. 8) for porcine mucosal heparins. The addition of DS measured as a contaminant of each heparin preparation (average concentration 3.4 wt. %) and F2′ which is found at an average concentration of 1.6 wt. % (as judged from the F1 calibration curve and an M_r of 563) in heparin brings the average mass balance to 91 wt. %. The recovery of uncharacterized minor oligosaccharides from all commercial porcine heparins, as well as unbleached porcine heparin, suggests that these are more than just artifacts arising from the commercial processing of heparin. Although it is possible that some of these products might result from minor contaminants (sulphoesterase, sulphamidase, heparitinase) in our enzyme preparation (Yang *et al.*, 1985), nearly identical fragmentation patterns were observed when a commercially prepared heparinase was used. It would be unexpected for both enzyme preparations to contain identical amounts of the same minor contaminating activities. The addition of up to 10 wt. % of heparan monosulphate to a standard heparin, before depolymerization, did not appreciably alter the oligosaccharide map. Further studies using a homogeneous enzyme preparation (Yang *et al.*, 1985) (currently available only in very small amounts) are required to resolve the question of the source of the minor oligosaccharides representing the remaining 9 % required to close mass balance. We previously published the structures of two *N*-acetylated tetrasaccharides derived from heparinase treatment of heparin (Merchant *et al.*, 1985). Although we suggested that these oligosaccharides represented major structural components of the heparin polymer, we now believe that the gel-permeation chromatography step which preceded s.a.x.h.p.l.c. enriched these two minor tetrasaccharides (corresponding to the two unassigned minor peaks at 42–54 ml in Fig. 2*a–d*).

The mass balance of over 100 wt. % for bovine heparins (I and N) can be rationalized by their high F1 content. The 8 % error associated with the measurement of molar absorption coefficient for F1 (this error was particularly difficult to reduce due to residual salt in the F1 sample), when combined with a 5 % error in analysis, probably results in the high-mass recovery.

Low-M_r heparins and bovine lung heparins show similar but distinctly different oligosaccharide maps from each other and porcine heparin (Fig. 2*e* and *f*). Low-molecular-mass heparin contains higher concentrations of minor tetrasaccharide components eluting between 42 and 54 ml. Bovine heparin shows another major oligosaccharide eluting near F5, labelled F5′ in Fig. 2.

LAH and HAH were depolymerized, and their content of oligosaccharides F1–F5 was determined (Table 1 and Fig. 3*a* and *b*). As expected HAH resulted in a large amount of F5 on heparinase depolymerization and exhibited a high level of ATIII-mediated activities. On the other hand, the oligosaccharide mixture formed by the depolymerization of LAH was depleted in F5 and this heparin exhibited low ATIII-mediated activities. A plot of activity vs. μg of F5 for affinity-fractionated heparins clearly demonstrated the relationship of F5 to ATIII-mediated activity.

Like porcine heparin, bovine heparin contains the five major oligosaccharides. Unlike porcine mucosal heparin, bovine lung heparin has a considerably higher degree of *N*-sulphation (Linker & Hovingh, 1979) affecting the concentration of F1, F4 and F5. Oligosaccharide F5 is greatly diminished in bovine heparin due to the fact that its major ATIII-binding site is a structural variant (Pejler *et al.*, 1987). A second peak appeared near F5 in the chromatogram (Fig. 2*f*), labelled F5′, may correspond to the structural variant of the ATIII-binding site which predominates in bovine heparin. The additional observation that this oligosaccharide is greatly enriched by ATIII-affinity chromatography (Fig. 3*b*) supports this premise. Further research is required to establish this point and define the precise chemical structure of F5′.

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