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Comparison of ovine, bovine and porcine mucosal heparins and low molecular weight heparins by disaccharide analyses and ^{13}C NMR

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Ovine, porcine and bovine heparin were compared by NMR, disaccharide composition, optical rotation, elemental analyses, HPSEC and USP analyses. There were some differences found in the NMR and disaccharide composition of the heparins. Ovine heparin contained less monosulphated and disulphated disaccharides than porcine or bovine mucosal heparins. The heparins were approximately equivalent in USP activity.

Low molecular weight ovine, porcine and bovine heparin were prepared by both nitrous acid and copper/peroxide depolymerisation. Products were compared to each other and to Fragmin® by disaccharide composition, optical rotation, HPSEC, NMR, elemental analyses, and the USP assay for anticoagulant activity.

The low molecular weight products showed a greater similarity to each other than to the parent heparins and it was concluded that the species of origin of a low molecular weight heparin would be difficult to determine by these methods alone.

INTRODUCTION

A small amount of ovine mucosal heparin is sold as an anticoagulant for people whose religious beliefs discourage them from using porcine or bovine derived heparin. Despite the present use of ovine heparin, to date, few structural studies of it have been reported. We hope that further analyses of ovine heparin and comparisons of ovine heparin with porcine and bovine heparins may allow the species of origin of a heparin or low molecular weight heparin to be verified by analytical methods. In addition to the importance of such verification to people with particular religious beliefs, such verification may become increasingly important if fears of diseases linked to certain animals, such as bovine spongiform encephalopathy, may be allayed.

In recent years the use of low molecular weight (LMW) heparin has become an increasingly popular alternative to heparin as an antithrombotic agent. LMW heparins are considered to be superior to heparin for the prevention of thrombosis. Reports on the pharmacological activity of LMW heparins indicate they have a higher *in vivo* bioavailability, a longer physiological half life and a reduced prohemorrhagic action when compared to native heparin (Coccheri, 1990; Desai and Linhardt, 1994). Several methods are used to prepare commercial LMW heparins and the structure and biological activity of the products are related to the method of depolymerisation, additional processing, the species and the tissue source. The majority of commercial LMW heparins are prepared from porcine mucosal heparin (e.g. Logiparin® and Fragmin®) although at least one product is prepared from bovine

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Abbreviations—ATIII, antithrombin III; LMW, low molecular weight; HPSAX, high performance-strong ion exchange chromatography; USP, United States Pharmacopoeia; ΔDiH-0S, 4-*O*-(4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid)-2-deoxy-2-acetamidoglycopyranose; ΔDiH-NS, 4-*O*-(4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid)-2-deoxy-2-sulphamidoglycopyranose; ΔDiH-6S, 4-*O*-(4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid)-2-deoxy-2-acetamidoglycopyranose-6-sulphate; ΔDiH-(6,N)S, 4-*O*-(4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid)-2-deoxy-2-sulphamidoglycopyranose-6-sulphate; ΔDiH-(U2,N)S, 4-*O*-(4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid-2-sulphate)-2-deoxy-2-sulphamidoglycopyranose; ΔDiH-(U2,N,6)S, 4-*O*-(4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid-2-sulphate)-2-deoxy-2-sulphamidoglycopyranose-6-sulphate; ΔDiH-(3,N,6)S, 4-*O*-(4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid)-2-deoxy-2-sulphamidoglycopyranose-3,6-disulphate; ΔDiH-(3,N,)S, 4-*O*-(4-deoxy-α-L-threo-hex-4-enopyranosyluronic acid)-2-deoxy-2-sulphamidoglycopyranose-3-sulphate.

mucosal heparin (Fluxum®) (Linhardt, 1992; Desai and Linhardt, 1994). No LMW heparins are produced from ovine mucosal heparin. In the present study we prepared two distinct LMW ovine heparins for comparison with porcine and bovine LMW heparins. This allows us to extend our comparison of ovine, bovine and porcine native mucosal heparins to their LMW products to determine whether species differences are retained by the respective LMW heparin.

The most detailed structural studies of ovine heparin have been reported by Casu *et al.* (1983 and Loganathan *et al.* (1990). Ovine mucosal heparin was found by Casu *et al.* (1983) to be of the highly sulphated 'beef lung' type. Loganathan *et al.* (1990) examined ovine heparin by treatment with heparinase and subsequent HPSAX of the resulting oligosaccharides. This method of analysis quantitatied the major ATIII binding sites in heparin, but did not yield information on the proportions of several disaccharide components present in heparin. These include the disaccharide components that result in the unsaturated disaccharides ΔDiH-0S, ΔDiH-6S, ΔDiH-(6,N)S and ΔDiH-(U2,N)S (for an explanation of these abbreviations, see Fig. 2) when cleaved from the polymer with heparinases. A method of disaccharide analysis reported by Volpi *et al.* (1992) yields information on the amounts of these components, and therefore this is the method we have chosen for the analyses of ovine heparin, and its low molecular weight products.

MATERIALS AND METHODS

Materials

The porcine and ovine mucosal heparins were products of New Zealand Pharmaceuticals Ltd (Palmerston North, New Zealand). Bovine mucosal heparin was obtained from Sigma Chemical Company. Fragmin® (Kabi Pharmacia AB, Sweden) was obtained from a local hospital and freeze dried prior to use. Heparinase I (heparin lyase I, EC 4.2.2.7), heparinase II (heparin lyase II, no assigned EC number), and heparinase III (heparin lyase III, EC 4.2.2.8) were obtained from Sigma Chemical Company.

Nitrous acid depolymerisation

Heparin was depolymerised using a modified version of the method of Shively and Conrad (1976). Heparin (3.00 g) was dissolved in 1% acetic acid (150 ml), 24 ml of 0.4% $NaNO_2$ was added and the solution stirred at $20 \pm 1°C$. The reactions were monitored by HPSEC. When the peak molecular weights of the products approached 5600 Da the pH of the solutions was adjusted to 6.5, and NaCl (10% w/v) was added followed by 3 volumes of methanol. The precipitates were collected by filtration using Whatman No. 50 paper and dried overnight *in vacuo* at 45°C.

H_2O_2/Cu^{2+} depolymerisation of heparin

Ovine, porcine and bovine heparins were depolymerised using a modified version of the method of Volpi *et al.* (1992). The reactions were monitored by HPSEC until the peak molecular weight approached 5000 Da. Thus heparin (1.5 g) and anhydrous sodium acetate (1.5 g) were dissolved in water (15 ml). Copper acetate monohydrate (0.10 g) was added and the reaction mixture warmed to 50°C. The pH was constantly adjusted to 7.5 with 2M NaOH, and hydrogen peroxide (50% w/w, 0.2 ml) was added every 30 min. After the desired molecular weight was achieved (6–9 h), 0.1M EDTA (pH 7, 15 ml) was added to quench the reaction, and NaCl (10% w/v) followed by 3 volumes of methanol were added to precipitate the product. The precipitate was dissolved in a small volume of water, eluted through a 20 ml column of Amberjet 1200 (H^+) ion exchange resin and neutralised with 2M NaOH. The product was then dialysed overnight in benzoylated dialysis tubing (9 mm, Sigma Cat. D-2272, LMW cutoff) against 0.05M EDTA and 0.05M NaCl (pH 7). The products were then dialysed for 24 h versus frequently changed Milli Q water and finally freeze dried to give white amorphous solids.

Disaccharide compositional analyses

Constitutive disaccharide quantitation was carried out using heparinases I, II, and III, as described by Volpi *et al.* (1992). HPSAX was conducted using a Spectraphysics Model SP8700 HPLC pump, a Spectraphysics SP8750 solvent delivery system, a Spectraphysics SP8440 UV/VIS detector (232 nm), a Waters Millennium 2010 chromatography manager, and a Spherisorb 5, SAX, 250 × 4.6 mm column. The flow rate was 1 ml min^{-1}. From 0 to 8 min, 0.15M NaCl was eluted isocratically in a pH 6.00, 0.001M NaH_2PO_4 buffer. From 8 to 30 min the NaCl concentration was linearly increased to 0.47M (keeping the pH and phosphate concentration constant), and then from 30 to 35 min the NaCl concentration was linearly increased to 2.0M. The column was then eluted isocratically for 5 min with 2.0M NaCl in a pH 6.00, 0.001M NaH_2PO_4 buffer.

The disaccharides were identified by their retention times using standards from Seikagaku (H-mix Cat. No. 400576), and Sigma Chemical Company [α-αUA2S(1→4)GlcNAc, α-ΔUA2S(1→4)GlcNS and α-→UA2S(1→4)GlcNAc6S]. Quantitation was achieved by comparison of the response of the unknowns with known amounts of the Seikagaku heparin disaccharides.

NMR

^{13}C and ^{1}H NMR spectroscopy was performed on a Varian Unity 500 spectrometer operating at 125.7 MHz for the collection of ^{13}C spectra. The carbon spectra

were acquired at 80°C, an acquisition time of 1.3 s, and 10 000–20 000 transients were acquired until a satisfactory signal to noise ratio was achieved. The spectra were referenced to internal methanol (50.232 ppm).

HPSEC

HPSEC was performed with a TSKgel-G2000SW HPLC column and a TSKgel-G4000SW HPLC column in series (both 7.5 mm × 30 cm). A Waters 410 Differential Refractometer detector was used, and a 0.15M phosphate buffer (pH 6.4) was run at a flow rate of 1.0 ml/min. The samples were dissolved in the running buffer at a concentration of 10 mg/ml, and the injection volume was 20 μl. Dermatan sulphate standards of known molecular weight were used for calibration. The GPC data was collected and processed using a Waters Millennium 2010 chromatography manager.

USP assay

The USP assay for anticoagulant activity was carried out as described in the United States Pharmacopoeia (USP, 1989).

Optical rotations

Optical rotations of 1% concentration aqueous solutions in a 20 cm cell were measured at 25°C at the sodium D-line on a Bellingham and Stanley P10 polarimeter.

RESULTS AND DISCUSSION

Comparison of porcine, ovine and bovine heparins

The method of disaccharide analysis used in this work was based on the method reported by Dietrich *et al.* (1980). This method was also used by Volpi for the comparison of natural, LMW (enrichment process) and free-radical depolymerised heparin (Volpi *et al.* 1992). Six disaccharides (Figs 1 and 2) were identified by coelution with disaccharide standards. Several components were not identified; these may include disaccharides containing a 3-*O*-sulphated glucosamine residue, or (ΔDiH-(3,6,N)S and ΔDiH-(3,N)S), which are also present in the AT III binding site of heparin (Loganathan *et al.*, 1990). Rice and Linhardt (1989) showed that the combined action of heparin lyase I and heparin lyase III can produce ΔDiH-(3,6,N)S from heparin. The findings of Desai *et al.* (1993)

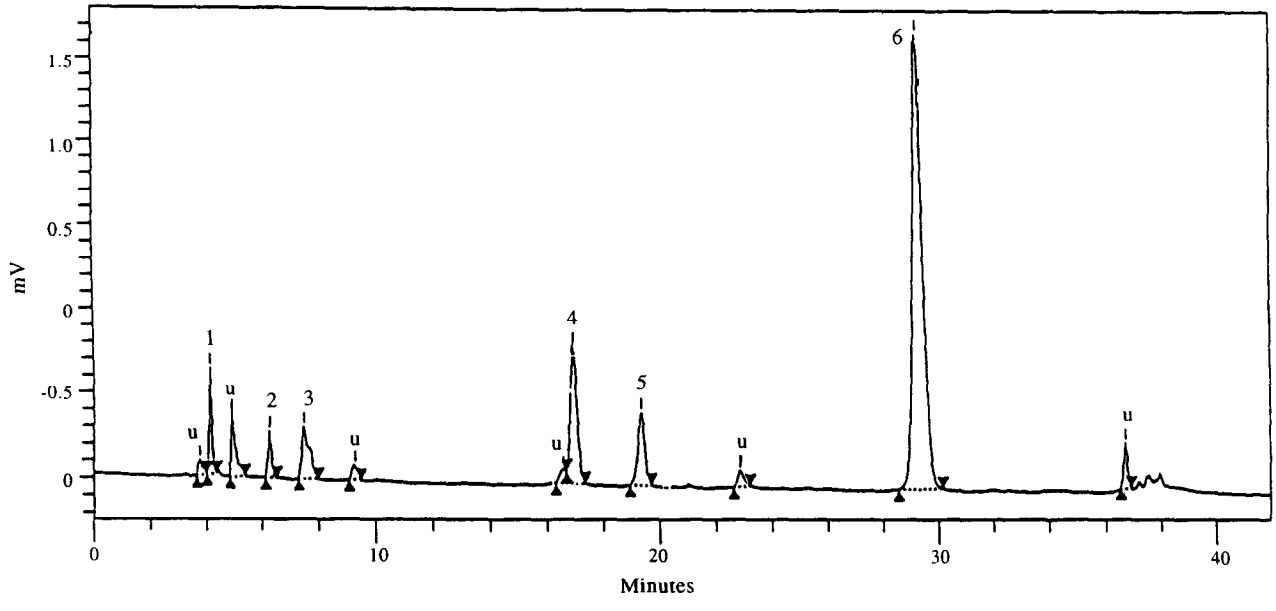

Fig. 1. HPSAX chromatogram of disaccharides from native porcine heparin.

Number	Name	Ret time(min)	% Area
	U	3.76	0.87
1	ΔDiH-0S	4.15	4.04
	U	4.94	3.94
2	ΔDiH-NS	6.29	2.12
3	ΔDiH-6S	7.51	4.94
	U	9.29	0.95
	U	16.57	0.97
4	ΔDiH-(6,N)S	16.98	12.13
5	ΔDiH-(U2,N)S	19.38	6.40
	U	22.87	1.21
6	ΔDiH-(U2,N,6)S	29.28	60.05
	U	36.74	2.38

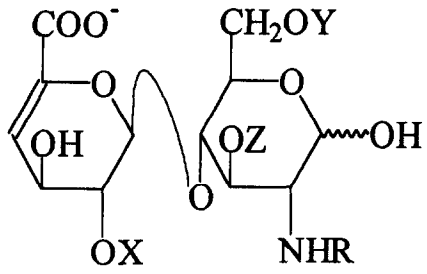

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Fig. 2. Structure of disaccharides accounted for by enzymic hydrolyses and HPSAX.

Component	Notation	R	X	Y	Z
1	ΔDiH-0S	Ac	H	H	H
2	ΔDiH-NS	SO_3-	H	H	H
3	ΔDiH6S	Ac	H	SO_3-	H
4	ΔDiH-(6,N)S	SO_3-	H	SO_3-	H
5	ΔDiH-(U2,N)S	SO_3-	SO_3-	H	H
6	ΔDiH(U2,N,6)S	SO_3-	SO_3-	SO_3-	H
	ΔDiH-(3,N,6)S	SO_3-	SO_3-	H	SO_3-
	ΔDiH-(3,N)S	SO_3-	SO_3-	H	SO_3-

seem to contradict this earlier work, and therefore little can be concluded about the production of such a disaccharide from heparin. The expected disaccharides containing 3-*O*-sulphated glucosamine residues were not formally identified in the HPSAX as these compounds were not available.

The disaccharide analyses gave similar amounts of the trisulphated disaccharide ΔDiH-(U2,N,6)S for the three different heparins (Table 1). Ovine heparin contained significantly less *N*-sulphated disaccharides than did porcine and bovine heparins (9.6% compared with 17.6% and 21.5% for porcine and bovine, respectively). Bovine heparin contained significantly more ΔDiH-(U2,N)S than ovine or porcine (12.5% compared with 2.0% and 5.5% for ovine and porcine, respectively).

Integration of ^{13}C NMR is not generally applicable to determine amounts of material present in a sample because the relaxation time of the nucleus as well as its abundance has an effect on peak area. However, for carbons in a similar environment the correlation of peak

Table 1. Disaccharide analysis of porcine, ovine and bovine heparins and their low molecular weight products(%w/w)

	ΔDiH-OS	ΔDiH-NS	ΔDiH-6S	ΔDiH-(6,N)S	ΔDiH-(U2,N)S	ΔDiH-(U2,N,6)S	Unknown (R. time 37.6 min)
Porcine							
Native	2.4	1.4	3.2	10.7	5.5	63.0	
LMW (HNO_2)	2.5	0.8	2.8	6.1	3.2	42.5	8.6
Fragmin® (HNO_2)	1.0	0.6	2.6	6.8	2.7	47.0	16.6
LMW (Cu^{2+})	1.2	0.7	2.3	8.0	3.2	49.4	
Ovine							
Native	1.1	0.3	1.0	7.5	2.0	60.0	
LMW (HNO_2)	1.5	0.5	1.0	4.2	1.2	39.4	7.2
LMW (Cu^{2+})	0.7	0.2	1.0	6.6	1.7	54.9	
Bovine							
Native	2.1	1.3	0.9	7.7	12.5	64.2	
LMW (HNO_2)	2.9	0.5	0.8	2.7	5.5	31.3	11.1
LMW (Cu^{2+})	1.1	0.6	0.8	5.3	7.1	46.5	

Table 2. Relative intensities for selected signals in the ^{13}C NMR of heparins and LMW heparins

Chemical shift	23.2	54.9	57.9	96.8	97.4	98.1	98.8	100.5	103.2
Assignments[a]	$NHCOCH_3$	C-2	C-2	C-1	C-1	C-1	C-1	C-1	C-1
	7,8	7,8	9	8	9	10	11	3	12
Native Heparin									
Porcine	0.34	0.20	0.14	0.24	0.11	1.0	0.18	1.10	0.40
Ovine	0.14	0.08	0.13	0.15	0.11	1.0	0.22	0.86	0.26
Bovine	0.23	0.10	0.08	0.12	0.10	1.0	0.20	0.99	0.24
LMW HNO_2									
Porcine	0.33	0.25	0.14	0.27	nr[b]	1.0	nr[b]	1.12	0.45
Ovine	0.17	0.12	0.15	0.20	nr[b]	1.0	nr[b]	1.17	0.32
Bovine	0.18	0.08	nr[b]	0.22	nr[b]	1.0	nr[b]	1.22	0.34
LMW Cu^{2+}/H_2O_2									
Porcine	0.33	0.22	0.15	0.23	nr[b]	1.0	0.18	1.13	0.35
Ovine	0.11	0.15	0.11	0.14	nr[b]	1.0	0.21	1.16	0.33
Bovine	0.13	0.07	0.08	nr[b]	nr[b]	1.0	0.24	0.98	0.15

[a]Positions of carbons shown in Fig. 5.
[b]Not resolved.

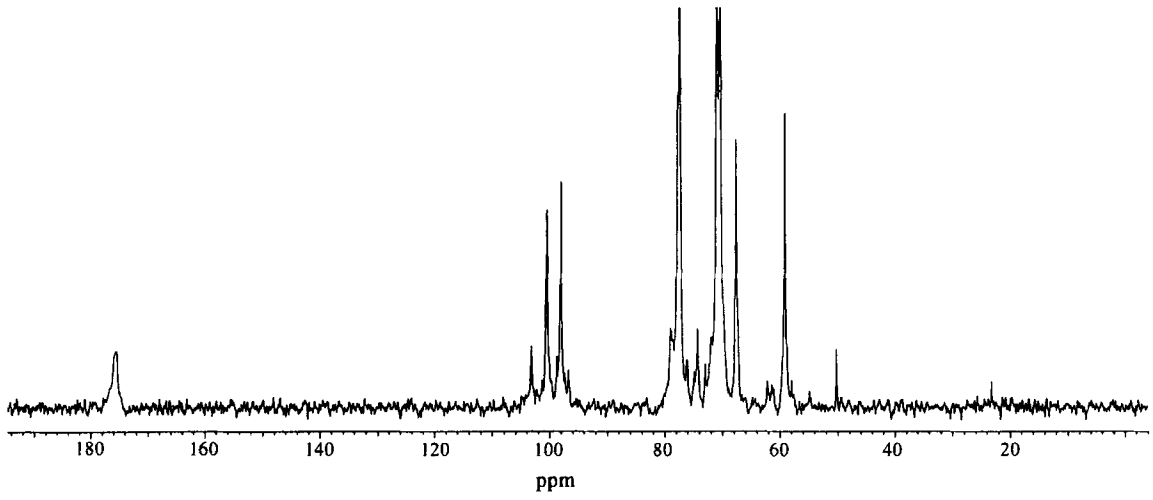

Fig. 3. ^{13}C NMR of native porcine heparin.

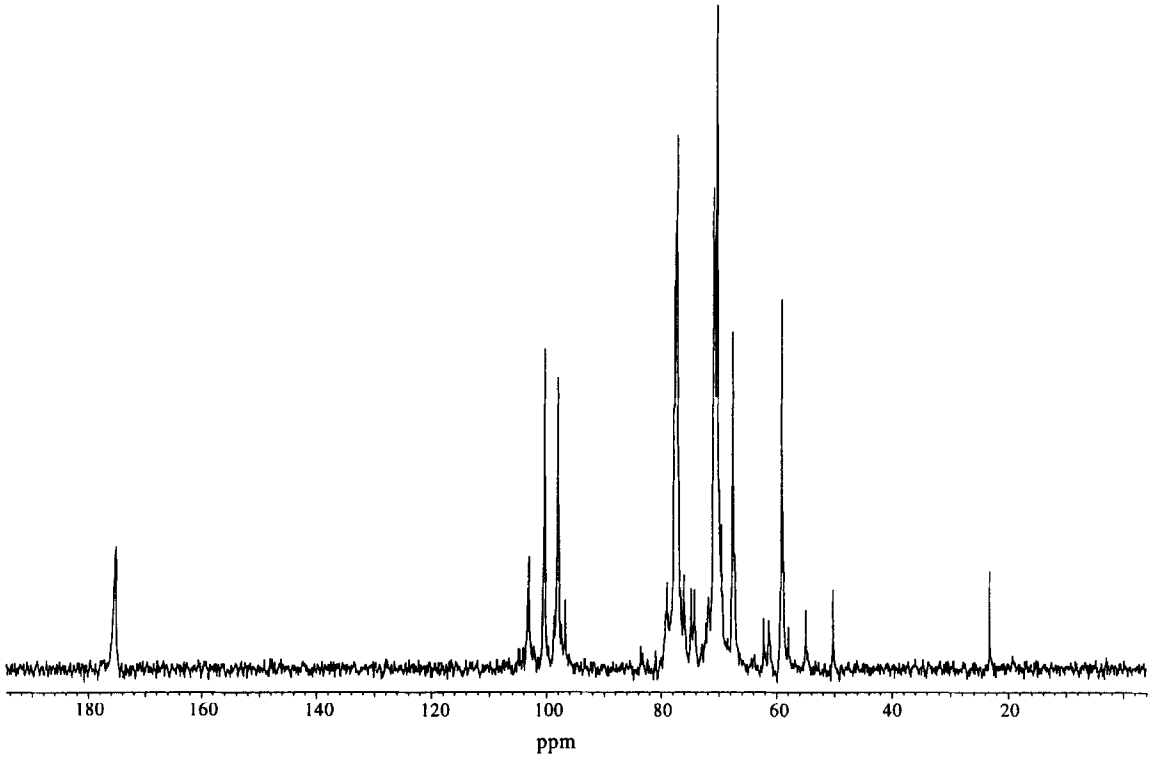

Fig. 4. ^{13}C NMR of native ovine heparin.

area with relative concentration is a reasonable assumption (Slim *et al.*, 1994).

The relative heights of signals in the ^{13}C NMR spectra can be used to compare the various heparins (Table 2). The ^{13}C NMR was assigned according to Braud *et al.* (1985); a small fixed adjustment in ppm was used to account for differences in the referencing of the spectra. The ^{13}C NMR spectra of porcine and ovine heparins are shown in Fig. 3 and Fig. 4. The intensities reported in Table 2 are relative to the intensity of the C-1 signal from the *N*,6-disulphated glucosamine residues, the major glucosamine constituent of the heparins. The acetate signal at 23.2 ppm and the signal from the C-2 of the *N*-acetylated glucosamine at 54.9 ppm are both less intense in spectra of the ovine and bovine heparins than those in spectra of the porcine heparin. This result is confirmed by examination of the disaccharide composition, where the sum of the *N*-acetylated disaccharides is 5.6% for porcine heparin, and 1.1% and 3.0% for the ovine and bovine heparins, respectively.

The ^{13}C NMR signal at 96.8 ppm is assigned to C-1 of the *N*-acetyl-6-*O*-sulpho-glucosamine residues (Braud *et al.*, 1985); the intensity of this signal relative to the signal for the C-1 of the *N*,6-disulphated glucosamine

Table 3. USP anticoagulant activities for heparins and low molecular weight heparins (IU/mg)

	Ovine	Bovine	Porcine
Native	165	157	167
HNO_2	89	114	92
Cu^{2+}/H_2O_2	109	87	106
Fragmin®			115

was 0.24, 0.15, and 0.12 for the porcine, ovine and bovine heparins, respectively. Owing to a relatively high level of noise, the ratios obtained from NMR can only be regarded as approximate. They can be compared with the disaccharide analyses which gave a value of the percent composition of the ΔDiH-6S disaccharide of 3.2%, 1.0%, and 0.9% (w/w) for porcine, ovine, and bovine heparins, respectively.

The ^{13}C NMR signals at 57.9 ppm and 97.4 ppm are attributed to the C-2 and C-1, respectively, of both the *N*,3-disulphated glucosamine residues and the *N*,3,6-trisulphated glucosamine residues. These 3-*O*-sulphated glucosamine residues are important components of the ATIII binding sites (Loganathan *et al.*, 1990; Taylor *et al.*, 1995) of heparin. By the relative intensity of these signals in ^{13}C NMR it can be seen there are similar amounts of these residues in the three heparins relative to the amount of *N*,6-disulphated glucosamine (Table 2). The biological activity is similar across the species according to the USP coagulation assay (Table 3), yet real differences exist between the NMR spectra of the heparins.

The ratio of the intensity of the C-1 of glucuronate (103.2 ppm) to the intensity of the 2-*O*-sulpho-iduronate C-1 signal (100.5 ppm) was 0.36, 0.30, and 0.24 for porcine, ovine, and bovine heparins, respectively. Owing to differences in the relaxation times of the nuclei it cannot be stated that porcine heparin contains glucuronate acid at a level of 36% of the 2-*O*-sulpho-iduronate. However, it can be stated that the level of glucuronate relative to 2-*O*-sulpho-iduronate decreases by a factor of 0.30/0.36 from porcine to ovine heparin, and by a factor of 0.24/0.36 from porcine to bovine heparin.

Using the above data one can readily differentiate between the three different types of mucosal heparins, and these heparins can be easily differentiated from bovine lung heparin by ^{13}C NMR. The ^{13}C NMR of bovine lung heparin shows that it contains very little glucuronate (Gatti *et al.*, 1979).

Preparation and analyses of LMW heparins

To the authors' knowledge this is the first time that preparation of low molecular weight ovine heparin has been reported. Porcine, ovine, and bovine heparins were depolymerised by both nitrous acid (Shively and Conrad, 1976) and free-radical mediated depolymerisation (Volpi *et al.*, 1992). The reactions were monitored by HPSEC and when the peak molecular weight of the heparins reached 5600 Da the reactions were quenched. The molecular weight parameters of the starting materials and the low molecular weight products are presented in Table 4.

Table 4. Yields of low molecular weight heparins by nitrous acid and free radical process and physicochemical characteristics of the native heparins and their low molecular weight products

	Yield (%)	S (%)	a_D	M_w	M_p	M_n
Porcine						
Native		9.4	+46.8	14300	14800	10600
H_2O_2	54	9.9	+46.6	7100	5900	5600
HNO_2	82	9.8	+44.0	7500	5800	5700
Ovine						
Native		10.8	+45.1	12200	12500	8700
H_2O_2	57	10.4	+42.0	7600	6300	6000
HNO_2	86	10.4	+43.4	6900	5100	5300
Bovine						
Native		10.4	+48.2	14900	15000	10700
H_2O_2	53	10.2	+48.0	7100	5800	5700
HNO_2	82	10.7	+42.6	6000	4500	4800

The most significant difference in the oligosaccharide composition of the nitrous acid depolymerised products compared with the native heparins is the appearance of an unknown oligosaccharide which is eluted at high salt concentration. Nitrous acid depolymerises heparin via deamination of the *N*-sulphated glucosamine residues (Shively and Conrad, 1976). Owing to the unknown oligosaccharide's high affinity for the anion exchange medium, it is likely the unknown oligosaccharide contains more acidic groups than ΔDiH-(U2,N,6)S, and therefore it is thought to be a mixture of tetrasaccharides with 2,5-anhydromannose residues at the reducing ends. For the purposes of quantitation, the unknown oligosaccharide was assumed to be a tetrasaccharide derived from the prevalent repeating units of heparin; 2-*O*-sulpho-iduronate, and *N*-sulpho-6-*O*-sulpho glucosamine. In the case of Fragmin®, the reducing end of the polymer has been converted to the alditol during manufacture (Desai and Linhardt, 1994), and therefore the oligosaccharides derived from the reducing end would be oligosaccharide-alditols. However under the HPLC conditions used here these tetrasaccharide-alditols were not distinguished from the non-reduced forms. These tetrasaccharides are presumably resistant to hydrolysis by the heparinases due to the presence of the anhydromannose residue. The ^{13}C NMR spectra of the nitrous acid depolymerised products contained signals at 81.4, 83.5, 86.6, and 90.6 ppm which were not in the parent heparins, and are attributed to the anhydromannose residues by comparison to data for the corresponding anhydromannitol alditols (Uchiyama and Nagasawa, 1991).

All of the low molecular weight heparin preparations in this study gave lower yields of disaccharides by disaccharide analyses than did the parent heparins. This is thought to be due at least partially to the effect of the increased number of non-reducing end groups that would

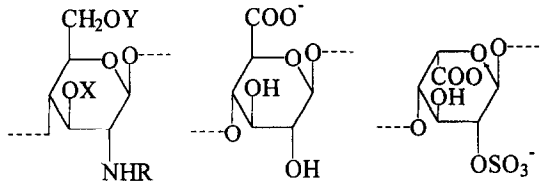

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Fig. 5. Structure of monosaccharides accounted for by ^{13}C NMR.

Component	R	X	Y
7	Ac	H	H
8	Ac	H	SO_3-
9	SO_3-	SO_3-	H,SO_3-
10	SO_3-	H	SO_3-

be released by the heparinases. These end groups would not have any double bonds to act as chromophores. In addition it has previously been demonstrated that Cu^{2+}/H_2O_2 depolymerisation reduces the yield of disaccharides that undergo enzymic cleavage (Volpi *et al.*, 1992).

Concluding remarks

We have demonstrated that porcine, ovine, and bovine heparins can be distinguished by a combination of ^{13}C NMR and disaccharide analyses. We have analysed several samples, but, for analytical laboratories to be confident of these conclusions made on the species of origin of a particular heparin by disaccharide and NMR analyses, further studies using a broad range of heparins from different suppliers need to be carried out.

Unlike Casu *et al.* (1983) we have determined that ovine mucosal heparin is not like the highly sulphated beef-lung type, but of a highly sulphated type more closely related to porcine and bovine mucosal heparins.

We have prepared low molecular weight ovine heparin and compared it with low molecular weight ovine and bovine heparins by several methods including ^{13}C NMR and disaccharide analyses. This is the first reported synthesis of LMW ovine heparin. The analytical results of the LMW ovine heparin show a greater similarity to the LMW porcine and bovine heparins than was seen for the full-length natural materials. The results indicate that the species of origin will be more difficult to identify for LMW heparins.

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