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Carbohydrate Research 338 (2003) 251–256
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Heparin antiproliferative activity on bovine pulmonary artery smooth muscle cells requires both N-acetylation and N-sulfonation

María O. Longas,[a,*] Hari G. Garg,[b] Jennifer M. Trinkle-Pereira,[a] Charles A. Hales[b]

[a] *Purdue University Calumet, Department of Chemistry and Physics, Hammond, IN 46323, USA*
[b] *Department of Medicine, Pulmonary/Critical Care Unit, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114, USA*

Received 24 July 2002; accepted 15 October 2002

Abstract

The antiproliferative activity of Heparin (HP) on bovine pulmonary artery smooth muscle cells (BPASMC) *in vitro* requires both N-acetylation and N-sulfonation. This was demonstrated by quantifying the relative N-acetylation of three commercial heparins of known antiproliferative activities, using their Fourier-transform infrared (FTIR) band areas at 1381–1378 and 1320–1317 cm^{-1}, which combined resulted in 1.0, 1.0 and 1.3 cm^2 for Choay, Elkins–Sinn and Upjohn HP, respectively. These results show that Upjohn HP, which is at least 44% more antiproliferative than the other two, is 30% more N-acetylated. Upjohn HP was also N-desulfonated chemically, and its antiproliferative activity was determined. Its total sulfonate ($-SO_3^-$) content (*O*- and *N*-sulfonate) was quantified using the FTIR band area at 1260–1200 cm^{-1} for the S=O stretching; a drop in sulfonate content from 21.87% (w/w) before N-desulfonation to 16.51% (w/w) after N-desulfonation, resulted in a 67% decrease in its inhibitory potency. In addition to the requirement that ~24% of the sulfonate content be bonded to N, the data show a direct correlation between the extent of Upjohn HP N-acetylation and its antiproliferative activity on BPASMC.

1. Introduction

Heparin (HP), an acidic glycosaminoglycan (GAG) and potent anticoagulant with numerous biologic functions, contains D-GlcNAc, D-GlcA and L-IdoA that may be O- or N-sulfonated ($O–SO_3^-$ or $N–SO_3^-$). The glycosidic linkages are: α-(1→4) from D-GlcNAc to D-GlcA or to L-IdoA; α-(1→4) from L-IdoA to D-GlcNAc and β-(1→4) from D-GlcA to D-GlcNAc.[1,2] The amino group of the GlcN residue may be substituted with an acetyl or sulfonate group or unsubstituted. The hydroxyl groups at the 3- and 6-positions of GlcN residues and the 2-position of hexuronic acid residues can either be substituted with sulfonate groups or unsubstituted. This microheterogeniety of heparin appears to be responsible for the variance in the antiproliferative activity of different commercially available heparin preparations.[3] The antiproliferative activity of HP on arterial smooth muscle cells has been demonstrated *in vivo* and *in vitro*.[4–6] Both *N*- and *O*-sulfonates are believed to play important roles on the inhibitory potency HP has on these cells.[4–6]

Using synthetic HP oligosaccharides as well as polymeric HP, Castellot and co-workers demonstrated that although *N*-sulfonates were essential for antiproliferative activity on smooth muscle cells, their replacement with *N*-acetyl moieties did not render HP less active.[4–6] Specifically, N-desulfonation resulted in loss of anticoagulant and cell-growth inhibition activities, but the latter function was regained upon N-reacetylation.[4–6] Obviously, N-acetylation appears to play a more effective role on antiproliferative activity, as it could replace *N*-sulfonates. These reports indicate that the functional groups responsible for the anticoagulant and antiproliferative functions of HP are different.[4–6]

In this paper we report that the extent of HP N-acetylation has a direct correlation with its inhibitory potency on bovine pulmonary artery smooth muscle cells (BPASMC), and that around 24% of the total HP-sulfonate compostion must be *N*-sulfonate.

Abbreviations: BPASMC, bovine pulmonary artery smooth muscle cells; FBS, fetal bovine serum; GAG, glycosaminoglycan; HP, heparin.

* Corresponding author. Tel.: +1-219-9892286; fax: +1-219-9892130
E-mail address: mol@calumet.purdue.edu (M.O. Longas).

0008-6215/03/$ - see front matter
PII: S0008-6215(02)00441-X

2. Materials and methods

2.1. Materials

Cation-exchange resin (AG-50 W-X8, 200–400 mesh), was purchased from Bio-Rad, Hercules, CA. Chromatography columns (0.9 by 20.5 cm) were prepared using 10-mL Pasture pipettes. Filtered water was purified by distillation after passing it through organic removal and mix-bed ion-exchanger cartridges (Millipore Corp., Bedford, MA). Purified water was collected in glass and stored under nitrogen until used. It was devoid of sugars, as demonstrated by the results of gas chromatography–mass spectrometry analysis of the silylated sugars in 1 L of water concentrated to 100 μL, and subjected to the silylation reaction described by Pierce (P.O. Box 117, Rockford, IL). Under the conditions employed, no silylated sugars were detected, even though the sensitivity of the method was 1 μg/μL. Commercial HP's were from Choay, Elkins–Sinn and Upjohn. Cellulose polyacetate membranes were a product of Schleicher & Schuell, Keene, NH. BPASMC were derived from explants of the central pulmonary artery as described by us earlier.[3] All other reagents were of the best quality commercially available. Unless otherwise indicated, purified H_2O was used in all the experiments.

2.2. Heparin electrophoresis

The purity of the commercial HP's was tested, using 2-D electrophoresis on cellulose polyacetate membranes.[7] Every preparation revealed only one GAG spot at the position of standard HP (not shown).

2.3. N-desulfonation

The cation-exchange resin described above was washed three times using 500 mL of H_2O per 39 mL of packed exchanger. Each wash involved placing the ion exchanger in H_2O at 4 °C overnight. The column was packed under gravity and eluted with H_2O until the pH of the effluent was pH 5.0, the pH of the H_2O used.

Heparin (14.0 mg) was dissolved in 0.5 mL of H_2O, loaded on the column, and eluted with water in seven fractions, 1.0 mL each. The number of fractions collected was determined by the pH of the effluent, which changed from pH 5.0 (the pH of the water used) to pH 1.0 and back to pH 5.0. Fractions were pooled, and the pH was determined to be pH 2.63. This pool was flushed with N_2 for 1 min and placed in an oven at 60 °C for 24 h. The control contained all the reagents except HP.

The course of the N-desulfonation reaction was followed by reaction of the sulfonate removed with aq $KMnO_4$. Thus, 250 μL of the HP-containing solution was mixed with 10–20 μL of 0.1 M $KMnO_4$; this reaction mixture turned orange-brownish only after desulfonation had been allowed for 24 h at 60 °C. The control that contained all other reagents, except HP, displayed no color change even after 24 h desulfonation time.[8]

The reaction was terminated at 24 h, because the results of preliminary N-desulfonations conducted for 24, 30 and 36 h indicated no further chemical changes after a 24-h reaction time. At the end of the incubation period, the reaction mixture was adjusted to 0.33 M sodium acetate and the volume was determined. N-desulfonated HP was precipitated from this solution with five solution-volumes of absolute ethanol at 4 °C overnight and removed by centrifugation at $3{,}000 \times g$ and 5 °C for 20 min. The ethanol supernatant was concentrated to 1.0 mL and used to confirm N-desulfonation by precipitating the sulfate in it with 0.14 mL of 1 M $BaCl_2$.[8] The HP precipitate was washed three times with 10 mL of chilled 80% (v/v) aq ethanol, dissolved in a minimum volume of H_2O, lyophilized, and the yield (9.79 mg) was determined. The percentage of *N*-desulfonation was established using Fourier-transform infrared (FTIR) spectroscopy.[9]

2.4. Spectroscopic analysis

In these experiments we used the diffusion reflectance technique in a Perkin–Elmer Spectrum 1000 FTIR spectrophotometer. HP (900 μg) was dissolved in H_2O, combined with 109 mg of KBr, allowed to form a homogeneous solution and then lyophilized. The dry material was stored desiccated over P_2O_5 for at least 1 week. The band at 1260–1200 cm^{-1} was used to quantify sulfur as sulfonate ($-SO_3^-$).[10] Band areas from six different FTIR spectra were cut and weighed; the average was used to read the corresponding sulfonate concentration on a standard curve of pentosan polysulfate generated under the same conditions.[9]

2.5. Cultured pulmonary artery smooth cell proliferation assay

Smooth muscle cell proliferation assays were preformed as previously described, but without platelet derived growth factors.[3] Briefly, isolated BPASMC were seeded at 1.5×10^4 cells/well into six-well tissue culture plates, grown for two days, then growth was arrested for 48 h by reducing the serum concentration of the medium from 10% (v/v) to 0.1% (v/v). The medium was then changed for experimental samples, to contain either standard medium [RPMI-1640 with 10% (v/v) fetal bovine serum (FBS)], growth arrested media (0.1%, v/v, FBS) or standard media containing Upjohn HP or its N-desulfonated derivative at the concentrations described below. All media contained streptomycin (10

μg/mL), penicillin (100 U/mL) and amphotericin B (1.25 μg/mL). After 4–5 days, the BPASMC present in the cell culture wells were rinsed with Hank's balanced salt solution to remove the remaining cell culture medium and unattached dead cells. No dead cells (Trypan Blue exclusion) were obtained either in the control or in the cultures treated with the most potent Upjohn HP preparation or its N-desulfonated derivative. After detachment from the culture wells with trypsin/EDTA, cell numbers were determined using a Coulter counter.

The Upjohn HP preparation or its N-desulfonated derivative was dissolved in distilled, sterile water to a final concentration of 1 mg/mL; varying aliquots of this solution were combined separately with 13.50 mL of culture medium, and 2 mL of the resulting media were added to each culture well. Upjohn HP and its N-desulfonated derivative were completely soluble, as the culture medium was clear (no turbidity) after addition of the HP samples.

In the present study we used 1 μg/mL, 10 μg/mL, and 100 μg/mL of the Upjohn HP preparation and its N-desulfonated derivative.

The percent growth was calculated as:

$$\%\ \text{growth} = \frac{\text{net cell growth in treated medium}}{\text{net cell growth in standard medium}} \times 100$$

where, net cell growth = cell growth in standard or treated medium minus cell growth in growth arrested media.

2.6. Cell-growth statistics

Results are presented as mean ± standard error of the mean. Comparisons among groups were made with a factorial analysis of variance (ANOVA), using the Stateview software package (SAS Institute Inc., Cary, NC) for Macintosh computers. If ANOVA were significant, multiple comparisons were made using Fisher protected least significant difference (PSLD) test. In all cases, the significance was set as $p < 0.001$.

3. Results and discussion

The relative values of the FTIR bands areas arising from the C–H (1381–1378 cm^{-1}) and the C–C–H (1320–1317 cm^{-1}) stretching vibrations of acetamido ($-NHCOCH_3$) groups,[10] and the corresponding percent

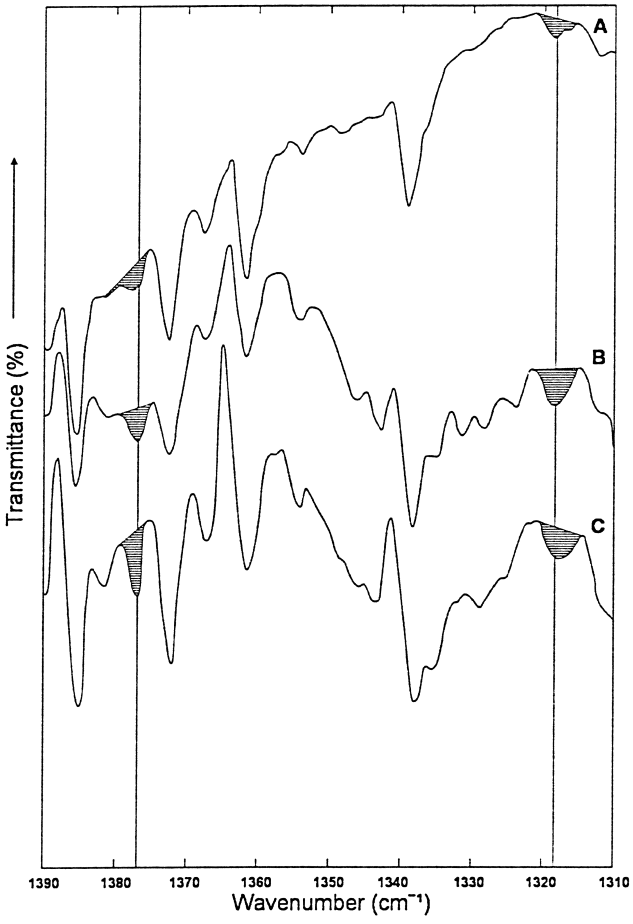

Fig. 1. Expanded FTIR spectra of: A, Choay; B, Elkins–Sinn; C, Upjohn heparin. The whole spectrum for Upjohn HP appears in Fig. 3. Whole spectra for Choay and Elkins–Sinn HP's have been reported previously.[9] Hatched band areas were determined in cm^2. Their relative values appear in Table 1.

Table 1
Quantitation of infrared bands of N-acetyl groups in commercial heparins

Heparin	Bonds Stretching	Area [a] (Σ) (cm^2)	S.D.	Area ratio (cm^2)	Inhibition [b] (%)
Choay	C–H, C–C–H	0.895	0.083	1.0	4.0
Elkins–Sinn	C–H, C–C–H	1.050	0.084	1.0	1.4
Upjohn	C–H, C–C–H	1.305	0.072	1.3	48.0

S.D., Standard deviation of the mean. Values represent the averages from 6 different determinations. Refer to the text for additional experimental details.

[a] Combined FTIR band areas at 1381–1378 and 1320–1317 cm^{-1} of the C–H and C–C–H stretching vibrations of monosubstitued amides. Calculated using the FTIR spectra of unmodified HP's enlarged between 1390 and 1310 cm^{-1} (Fig. 1).

[b] Percent growth inhibition of BPASMC.[3]

cell-growth inhibition effected by the various HP's investigated appear on Table 1. Fig. 1 shows the FTIR bands used to calculate the relative, HP N-acetylation. Upjohn HP, with ~30% more N-acetylation, displays an antiproliferative potency greater than that of Choay HP by 44% and than that of Elkins–Sinn HP by 47% (Table 1).

Fig. 2 shows the effects of Upjohn HP and its N-desulfonated derivative on the growth of BPASMC at three concentrations (1 μg/mL, 10 μg/mL and 100 μg/mL). Cell growth in GAG-free medium was defined as 100% growth. Treatment of BPASMC cultures with 1 μg/mL, 10 μg/mL and 100 μg/mL of Upjohn HP produced the following decreases in cell growth: 79.13 ± 3.38, 49.84 ± 2.91, 20.24 ± 1.76, respectively. When 1 μg/mL, 10 μg/mL and 100 μg/mL of N-desulfonated Upjohn HP derivative was added to the BPASMC cultures, the respective percentages of cell growth were 92.55 ± 3.97, 86.66 ± 4.23, 74.79 ± 4.38 (Fig. 2).

The sulfonate content dropped from $21.87\% \pm 3.36$ (w/w) to $16.51\% \pm 2.05$ (w/w) during chemical desulfonation. This represents a 24.49% decrease in sulfonate composition, which produced a reduction in HP inhibitory potency of ~67% of the original activity (Fig. 2). Although chemical sulfonation of native heparin did not affect its antiproliferative activity on BPASMC,[11] the data reported here demonstrate that HP N-sulfonation is essential for its cell-growth inhibition of BPASMC, and confirm previous findings by Castellot et al.[4,5] and Tiozzo et al.[6] These workers showed that N-desulfonated HP lost most of its capacity to inhibit the growth of rat aortic smooth muscle cells *in vitro*.[4] The minimum N-sulfonation for best antiproliferative activity of HP on smooth muscle cells remains to be determined.

Heparin N-desulfonation was complete in 24 h, as demonstrated by the yield of $BaSO_4$ precipitated from the aq ethanol supernatant containing the sulfonate that was cleaved off N-sulfonated HP during the N-desulfonation reaction. About the same amount of $BaSO_4$ was obtained in preliminary experiments conducted under the same conditions, but at reaction times varying from 24 to 36 h.

$BaSO_4$ resulted from reaction of Ba^{2+} ions formed in aq $BaCl_2$, and the sulfate (SO_4^{2-}) ions in the aq ethanol supernatant that was obtained after removal of N-desulfonated HP from the N-desulfonation reaction mixture. Sulfite ions (SO_3^{2-}) removed from N of HP were converted to sulfate ions (SO_4^{2-}) in the aq reaction medium. Sulfate ions are known to form in aq solutions due to oxidation of sulfite ions (SO_3^{2-}) by the oxygen in air.[12]

The FTIR spectrum of N-desulfonated HP was basically the same as that of the unmodified one (Fig. 3). It indicates that as expected, N-desulfonation was specific for the N-sulfonates of HP.[8] Namely, HP did not suffer any structural alterations detectable by this technique.

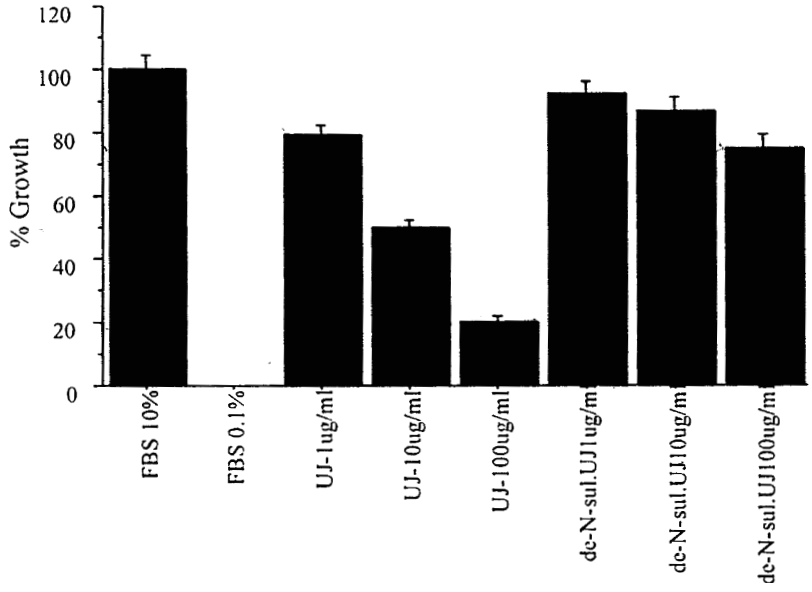

Fig. 2. Percent growth of bovine pulmonary artery smooth muscle cells cultured in fetal bovine serum plus the Upjohn heparin preparation and its N-desulfonated derivative. Standard: Without any HP derivative (Column 1); BPASMC grown in 0.1% (v/v) FBS (Column 2); Upjohn HP (1 μg/mL) (Column 3); Upjohn HP (10 μg/mL) (Column 4); Upjohn HP (100 μg/mL) (Column 5); N-desulfonated HP derivative (1 μg/mL) (Column 6); N-desulfonated HP derivative (10 μg/mL) (Column 7); N-desulfonated HP derivative (100 μg/mL) (Column 8). In each experiment, cells were seeded at 1.5×10^4 cells/well. See the text for additional experimental conditions.

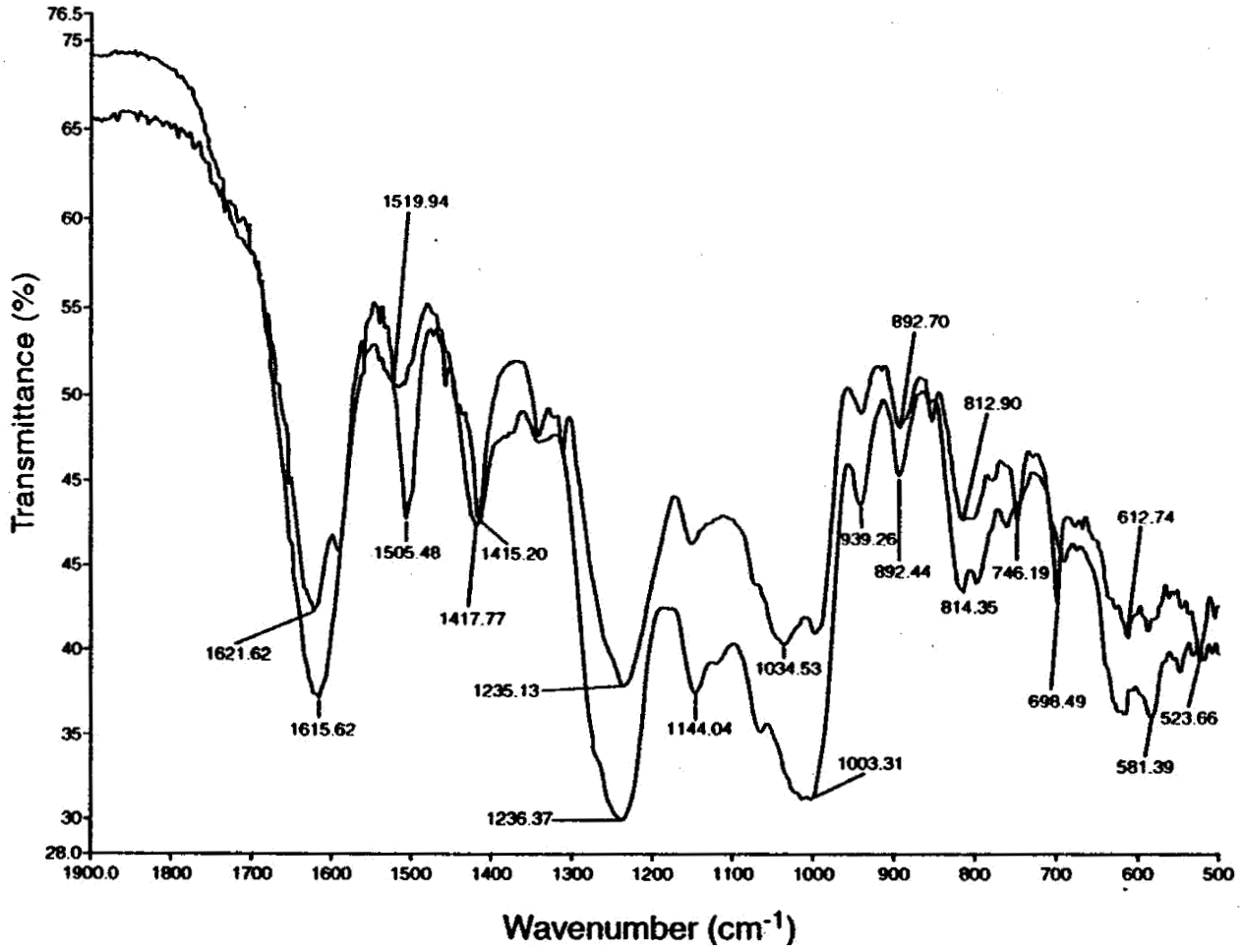

Fig. 3. FTIR spectra of Upjohn Heparin (HP) obtained using the conditions described in the text. The bottom spectrum is from unmodified Upjohn HP. Upper spectrum is from N-desulfonated Upjohn HP.

Furthermore, the N-acetylation of Upjohn HP, expressed as combined FTIR band areas at 1381–1378 and 1320–11 317 cm^{-1} (Fig. 1), was about the same before (1.30 cm^2, Table 1) and after (1.28 cm^2) N-desulfonation.

These data show that Upjohn HP is $\sim$30% more N-acetylated than the Choay and Elkins–Sinn HP's and also has at least 44% more antiproliferative activity on BPASMC. The 24.49% (w/w) drop in sulfonate content during chemical N-desulfonation decreased the original biological activity by $\sim$67% when the HP concentration used in the cell-growth assay was 100 μg/mL (Fig. 2). These results suggest that at least 24% (w/w) of the sulfonate content must be bonded to N. Although Castellot and co-workers showed that HP-inhibitory activity on smooth muscle cells was recovered after replacement of its N-sulfonates with N-acetyl moieties,[4–6] our results demonstrate that both HP N-acetylation and N-sulfonation are essential for its inhibitory potency on BPASMC *in vitro*.

Acknowledgements

This work was supported by a Scholarly Research Release Award from Purdue University Calumet and by an NS-FILIP DUE 9650826 to María O. Longas and by the National Institutes of Health Grant HL 39150 to Charles A. Hales.

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