Quality Check of Heparin Injections by ^{1}H-Nuclear Magnetic Resonance Spectroscopy

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The quality of commercial heparin injections was examined by 400-MHz proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy using several measuring modes. The signals of the *N*-acetyl protons, as well as the sugar-ring protons, attached to the sulfamino and sulfato group-bearing carbons could be easily distinguished from other proton signals and quantified. Measuring at a high temperature (60 °C) enabled clear isolation of the H-5 proton signal in the sulphated iduronic acid residue (Is-5) from other proton signals including that of water. The heparin contents of various heparin injections were estimated by using this signal as an index. However, the signal intensity was not parallel with anticoagulant activity. On the other hand, the *N*-acetyl proton signal was highly correlated to anticoagulant activity. The present method was also useful for concurrent identification of additives in heparin injections.

Keywords heparin; ^{1}H-NMR; quality check; iduronic acid residue; methyl *p*-hydroxybenzoate; benzylalcohol; sorbitol

Heparin has versatile physiological effects, such as, anticoagulant, antilipemic, anticomplementary, and anti-inflammatory activities. For this reason heparin preparations obtained from various biological sources, including bovine lung and liver, as well as porcine intestines, are commercially distributed. Among these physiological activities anticoagulant activity is the most important and there are a number of biological assay methods using fresh bovine blood. However, since these methods are cumbersome and require proficiency, an alternative chemical or physicochemical method that can correlate obtained values to anticoagulant activity has been desired.

The structure of heparin is complex and still retains an ambiguous part to be elucidated, but it is basically a polysaccharide composed of glucosamine and iduronic acid (partly glucuronic acid) residues linked alternatively through the β-1,4-linkage. The amino group in the glucosamine residue is either *N*-sulfated or *N*-acetylated, and some of the hydroxyl groups in both monosaccharide residues are sulfated. Chart 1 shows a typical expression of heparin molecule.

Mulloy and Johnson extensively studied the structure of some heparin samples by the proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy using a two-dimensional ^{1}H–^{1}H chemical shift correlation spectroscopy (COSY) technique.[1] Ayotte and Perlin[2] also studied the structure of heparin and its derivatives by ^{1}H-NMR. The latter group also described some comments with regard to activity change in modification of the heparin molecule.

We have examined various preparations of heparin injection by ^{1}H-NMR operating at 400 MHz, placing emphasis on a variation of relative intensities of several characteristic signals. In this report, we demonstrate the usefulness of this method for the estimation of heparin as well as additives in heparin preparations, and discuss the correlation of some particular signals to anticoagulant activity.

Experimental

A standard sample of heparin was obtained from Sigma (St. Louis, MO, U.S.A.). All other heparin injections were obtained from pharmaceutical companies (Novo, Shimizu, Sankyo, Mitsui and Dainippon, from intestinal mucosa; Upjohn, from bovine lung; Eizai, from bovine liver). Each injection sample (5000 units) was dialyzed against water and lyophilized, and the residue was dissolved in 99.85% deuterium oxide (0.5 ml). The anticoagulant activity of each sample was measured according to the method described in the XIth Japanese pharmacopeia (p. 857). ^{1}H-NMR spectra were recorded on a JEOL spectrometer at a probe temperature of 25 °C or 60 °C. Spectra were taken in the homogated decoupling mode to suppress the solvent peak of water. Thirty-two transients of 32 kilo data points were accumulated over a spectral width of 6000 Hz. Chemical shifts were referred to an internal reference of 1.0% potassium methansulfonate (2.797 ppm). Measurement of two-dimensional correlation spectroscopy (COSY)[3] was carried out for a spectral width of 2000 Hz in each domain by using the standard JEOL program. A 256 × 512 data matrix with a final resolution of 0.24 Hz, after zero filling in the F2 dimension, was used with 16 scans in each measurement.

The signals of additives in injections were observed for intact (nondialyzed) samples of heparin injection.

Results and Discussion

Assignment of Signals Figure 1 shows the COSY spectrum of a sample of lung heparin at 60 °C. On the basis of a perpendicular survey, major signals could be unambiguously assigned to the corresponding sugar-ring protons. The results are summarized in Table I. The sugar-ring proton at C_n of the glucosamine or iduronic acid residue is designated as As-*n* or Is-*n*, respectively. The reported assignment by Mulloy and Johnson,[1] as well as Ayottes and Perlin,[2] based on decoupling experiments, also supported the present results. Figure 2 compares the spectra of heparin samples from two different sources measured at 25 and 60 °C. The spectra obtained at 25 °C showed rather broadened peaks in the range of 3—5 ppm. The water signal still remained at *ca.* 4.7 ppm, even after homogate-decoupled,

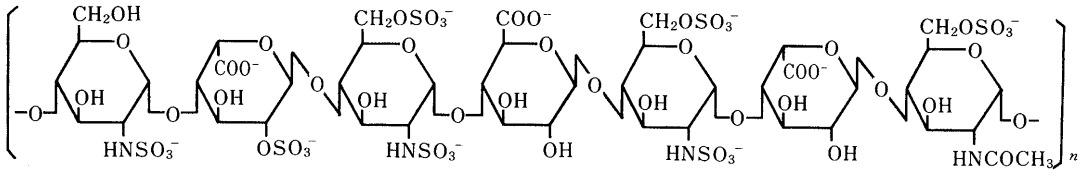

Chart 1

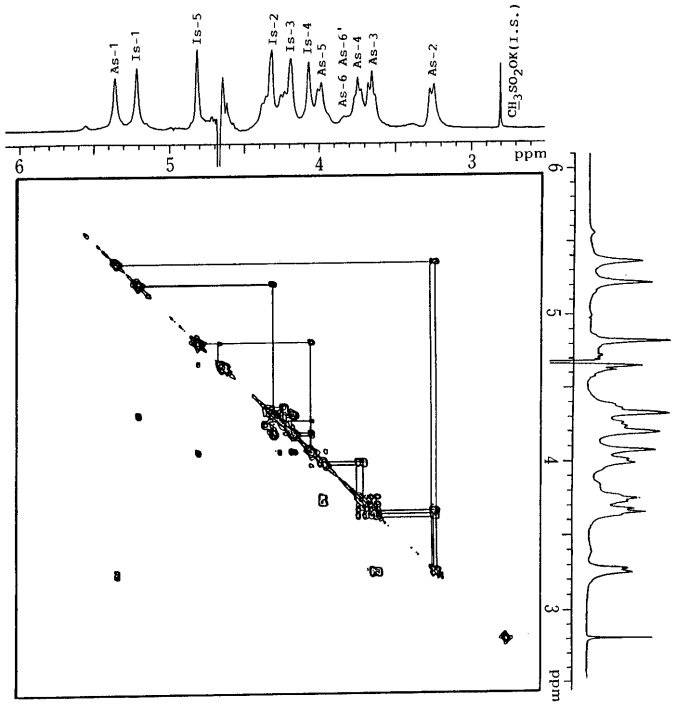

Fig. 1. ^{1}H–^{1}H COSY Spectrum of a Heparin Sample Obtained from Bovine Lung

As: *N*-sulfated glucosamine residue. Is: 2-sulfated iduronic acid residue.

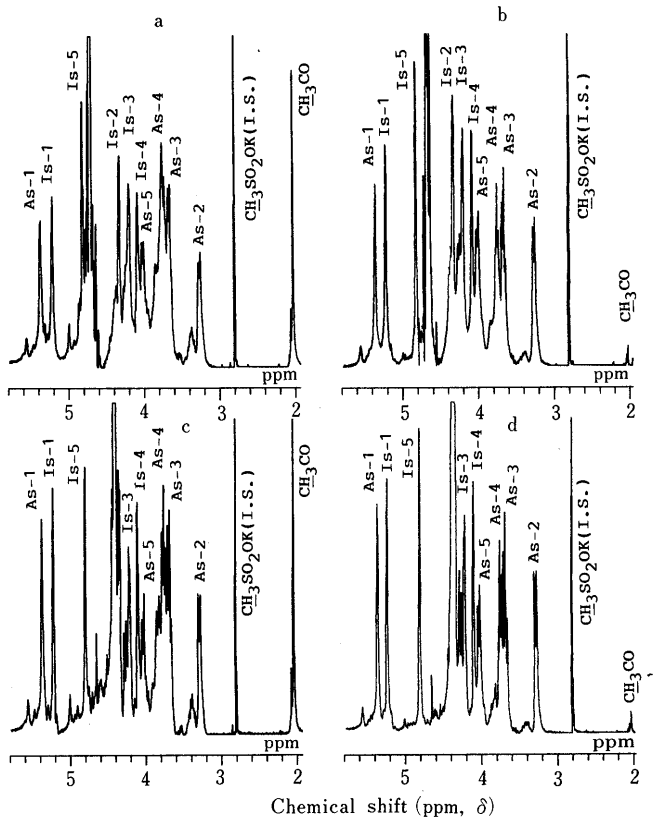

Fig. 2. ^{1}H-NMR Spectra of Samples of Heparin from Porcine Intestinal Mucosa (a, c; Novo) and Bovine Lung (b, d; Upjon), Measured at 25 °C (a, b) and 60 °C (c, d)

TABLE I. Assignment of Major Signals, Measured at 25 °C and 60 °C

Chemical shift (ppm, δ)	25 °C	60 °C
As-1	5.35	5.34
As-2	3.26	3.27
As-3	3.65	3.67
As-4	3.74	3.74
As-5	3.99	4.01
As-6	4.24	4.25
As-6′	4.36	—
Is-1	5.21	5.22
Is-2	4.32	—
Is-3	4.19	4.20
Is-4	4.07	4.09
Is-5	4.82	4.79

As: *N*-sulphated glucosamine residue. Is: 2-*O*-sulphated iduronic acid residue.

and the Is-5 signal which appeared at 4.81 ppm. The spectra at 60 °C showed better resolution due to effective relaxation, giving well split signals. The water signal was shifted, 0.3 ppm upward to 4.4 ppm. The well isolated, intense signal of Is-5 could be selected as the representative of the heparin molecule.

Estimation of Heparin Contents of Heparin Injections Using the Is-5 signal, the heparin contents of heparin injections were estimated. Potassium methansulfonate was used as an internal standard (I.S.), which gave the signal of the methyl proton at 2.797 ppm, completely separated from the signals of heparin. The selective irradiation of the water peak at 60 °C in the homogate-decoupling mode eliminated the problem of interference. The plot of relative intensity of the Is-5 signal to that of I.S. showed linearity at least in the range of 20—80 mg/ml of heparin as shown in Fig. 3, which covers the range of heparin concentration in commercial heparin injections.

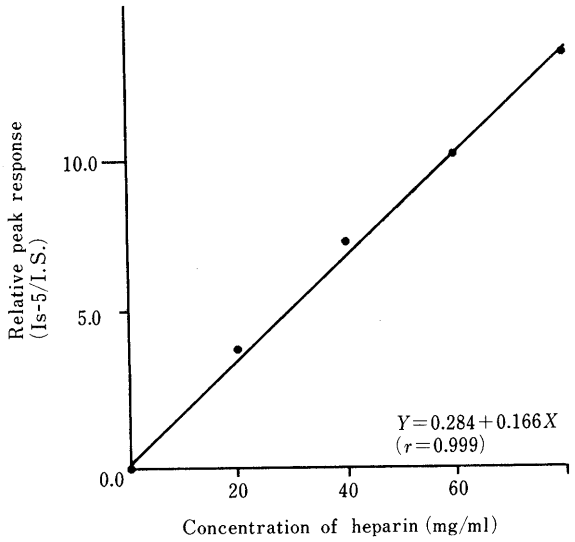

Fig. 3. Calibration Curve of Heparin, as Observed from the Response of the Is-5 Signal

Sample: sodium salt of heparin from porcine intestinal mucosa. Temperature, 60 °C. Measuring mode: homogate decoupling.

Identification of Additives in Heparin Injections Some compounds such as methyl *p*-hyroxybenzoate, benzylalcohol and sorbitol are often added to heparin injections as preservatives or an agent to keep isotonicity. The spectra of heparin injections containing these additives gave characteristic signals due to these compounds, as shown in Fig. 4. Figure 4b shows an example of the spectra of

benzylalcohol-containing samples. It gave proton signals of 4-substituted benzene at 7.38—7.47 and those of the hydroxymethyl protons at 4.64 ppm. Figure 4c shows the addition of methyl *p*-hydroxybenzoate as evidenced by the presence of a pair of two-proton doublets at 7.94 and 6.98 ppm assignable to 1,4-substituted benzene ring protons, together with the methyl ester protons at 3.89 ppm. In Fig. 4d, large multiple peaks are observed at around 3.6 ppm, partly overlapped with those of heparin, indicative of the addition of sorbitol. Thus, the present method enabled direct observation of the protons in both heparin and additives in intact injection samples, and was proved to be a powerful tool for the quality check of heparin injections in manufacturing and circulating processes, as well as in hospital management.

Differentiation of the Calcium Salt from the Sodium Salt Heparin injections are usually available as either calcium or sodium salts. The NMR spectra of the calcium salt showed a significant difference from that of the sodium salt in that the Is-1 and Is-5 signals in the former are remarkably shifted toward lower field (Fig. 5). The magnitude of the Is-1 shift was so great that the Is-1 signal passed over the As-1 signal. Thus, the chemical shifts of Is-1 and Is-5, as well as the magnitude of separation of the As-1/Is-1 pair in the lowest field, readily discriminated the counter ion. In association with these shifts, the As-4 signal was also notably moved downfield. The downfield shift of the Is-5 signal is ascribable to binding of the carboxyl group to a calcium ion, and the shifts of the As-1 and As-4 signals are possibly due to interaction of the interglycosidic oxygen atom with another calcium ion.

Correlation of ^{1}H-NMR Spectrum to Anticoagulant Activity The signals of As-2 and Is-5, as well as the *N*-acetyl protons are easily distinguishable from other proton signals. With regard to the functions of *N*-sulfate and *O*-sulfate groups there have been extensive studies,[2] but the results were perplexing; entire removal of the *N*-sulfate and *O*-sulfate groups from the heparin molecule

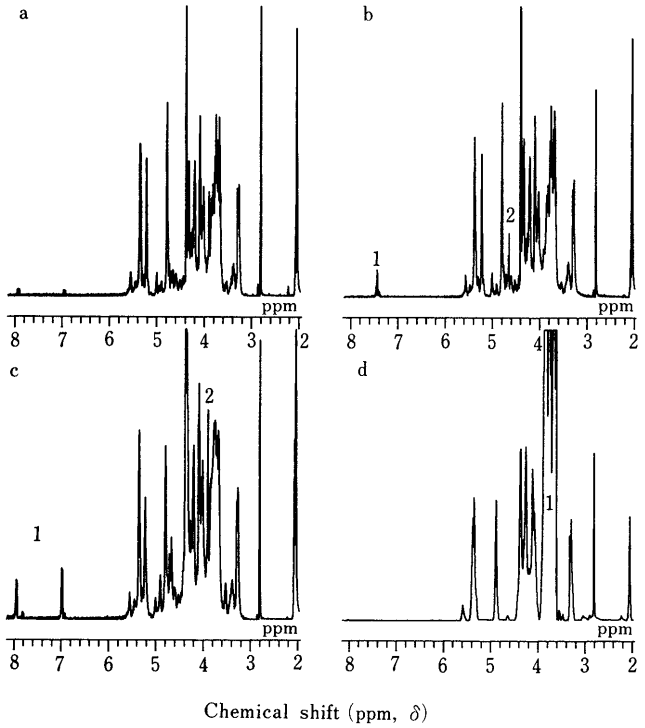

Fig. 4. Examination of Additives in Heparin Preparations

a, no additives; b, benzylalcohol (1, benzene ring protons; 2, hydroxymethyl protons); c, methyl *p*-hydroxybenzoate (1, benzene ring protons; 2, methyl protons); d, sorbitol. The analytical conditions were the same as those described for Fig. 3.

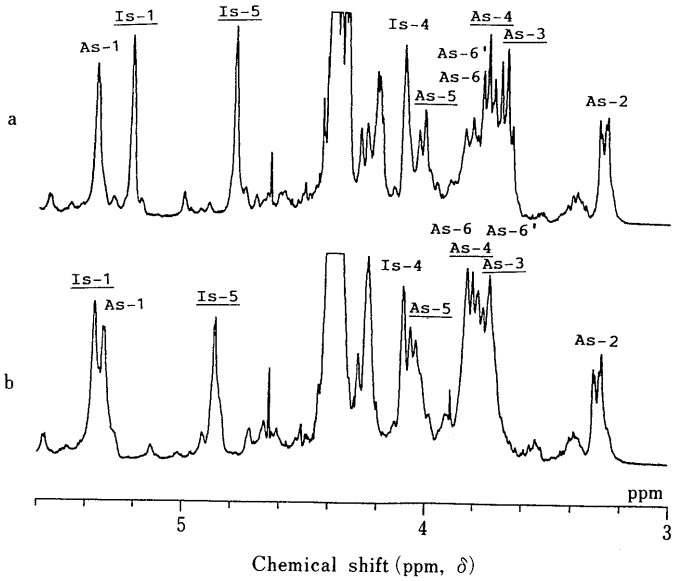

Fig. 5. Comparison of the ^{1}H-NMR Spectra between the Sodium and Calcium Salts of Heparin Samples

a, sodium salt; b, calcium salt.

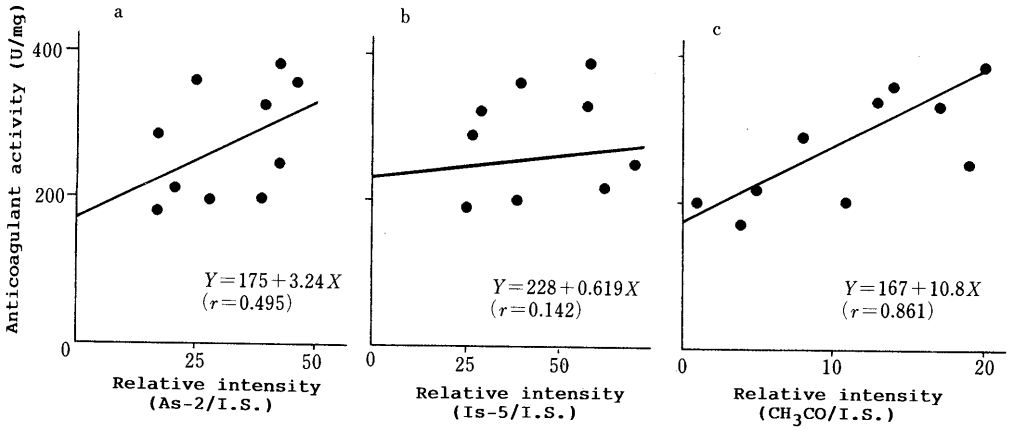

Fig. 6. Correlation of the Relative Intensities of the As-2(a), Is-5(b) and *N*-Acetyl(c) Proton Signals to I.S., to Anticoagulant Activity

resulted in complete loss of activity, but partial substitution of the *N*-sulfate group by the *N*-acetyl group enhanced activity.[2] Therefore, we reexamined the correlation of the intensities of the As-2 (as doublet) and Is-5 signals, as well as the *N*-acetyl signal, to anticoagulant activity. Figure 6 indicates the correlation curves. It is observed that the relative intensities of the As-2 (as doublet) and Is-5 signals to that of I.S. are not well correlated to anticoagulant activity, giving low values (0.495 and 0.142, respectively) of coefficient of correlation (r), whereas the relative intensity of the *N*-acetyl proton signal showed relatively high correlation ($r=0.861$). Since the As-2 and Is-5 signals are the most outstanding signals for the *N*-sulfated glucosamine and 2-*O*-sulfated iduronic acid residues, respectively, the foregoing results imply that a higher degree of sulfation of both monosaccharide residues had little effect on anticoagulant activity (Fig. 6). The high correlation of the relative intensity the *N*-acetyl proton signal to anticoagulant activity is consistent with the results reported by Ayotte and Perlin.[2] This finding suggests the possibility of production of heparin preparations having higher anticoagulant activity, by chemical modification of the *N*-sulfate group to the *N*-acetyl group. A study of this modification is now in progress in order to verify this suggestion.

References

1) B. Mulloy and E. A. Johnson, *Carbohydr. Res.*, **170**, 151 (1987).
2) L. Ayotte and A. S. Perlin, *Carbohydr. Res.*, **145**, 267 (1986).
3) C. A. G. Haasnoot, F. J. M. van de Ven, and C. W. Hilbers, *J. Mag. Resonance*, **56**, 343 (1984).