

Chemical Microdetermination of Heparin in Plasma

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A new, simple, and highly sensitive method for the determination of heparin has been established. Heparin was first converted into unsaturated disaccharides through the action of heparin lyases I, II, and III. A major trisulfated unsaturated disaccharide product results, consistent with structural analysis of a number of pharmaceutical heparins using one- and two-dimensional ¹H NMR spectroscopy. This disaccharide was analyzed by HPLC using fluorometric postcolumn derivatization. The correlation between the amount of this trisulfated unsaturated disaccharide and anticoagulant activity of heparin as measured by anti-IIa was determined. The analysis of these pharmaceutical heparins showed a linear correlation between both HPLC and bioassay. This HPLC method was then applied to a pharmacokinetic study of heparin intravenously administered to rabbits. © 1997 Academic Press

Heparin is a polydisperse, highly sulfated, linear polysaccharide composed of repeating 1 → 4 linked uronic acid and glucosamine residues (1). When pharmaceutical heparin is subjected to consecutive fractionation by chromatography or electrophoresis, it is found to consist of many different, closely related, sulfated polysaccharides of which only a fraction have anticoagulant activity (2). Heparin also has another level of structural complexity, associated with its primary structure or sequence (3). Thus, the failure to completely understand heparin's structure is not the result of a lack of effort but rather is due to its extremely complex structure (3).

The presence of heparin in biological fluids and drugs is usually estimated by bioassays such as coagulation tests (4). When these coagulation tests were originally

introduced, it was noted that an individual calibration curve was required for each heparin preparation (4). This point has often been ignored resulting in considerable variation in coagulation response to different heparin preparations. Differences are often observed between the amount of heparin measured by coagulation tests and the actual *in vivo* drug concentration (5). While chemical measurements of heparin in plasma have been proposed, they generally lack sufficient sensitivity to be clinically useful (6, 7). A sensitive method that measures the amount of chemical heparin in biological samples would be of great importance in regulating its dose and preventing side effects. This paper describes such a simple and sensitive procedure for the determination of heparin in plasma. Heparin is enzymatically depolymerized and its major trisulfated unsaturated disaccharide is analyzed by HPLC using postcolumn derivatization. This method has been applied to the pharmacokinetic study of heparin intravenously administered to rabbits.

MATERIALS AND METHODS

Six different heparin samples, sodium salts, from porcine and bovine intestinal mucosal and bovine lung heparin were obtained from Sigma Chemical Co. (St. Louis, MO), Nacalai Chemical Co. (Tokyo, Japan), Fluka Chemika-Biochemika Japan Co. (Tokyo, Japan), and in large quantities from Celsus Laboratories (Cincinnati, OH). Low-molecular-weight heparin (average molecular weight, 6000) prepared from bovine intestinal mucosa was purchased from Sigma. Heparin disaccharide standards were from Seikagaku Co. (Tokyo, Japan), Sigma, Dextra Laboratories (Reading, UK), or Grampian Enzymes (Aberdeen, Scotland). Heparin lyase I (EC 4.2.2.7), heparin lyase II (No EC assigned), and heparin lyase III (EC 4.2.2.8) from *Flavobacterium heparinum* were from Seikagaku, Grampian Enzymes, or Sigma. Actinase was from Kaken Pharmaceutical Co. (Tokyo, Japan).

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¹H NMR

¹H NMR spectroscopy was performed using conditions described previously (8). Briefly, a heparin sample (approximately 2 mg) was dissolved in 0.5 mL of D₂O (99.9%) and freeze-dried repeatedly to remove exchangeable protons. The sample was kept in a desiccator over phosphorus pentoxide *in vacuo* overnight at room temperature. The thoroughly dried sample was then dissolved in 0.5 mL of D₂O (99.96%), passed through a 0.45- μ m syringe filter, and transferred to a NMR tube (5.0-mm-o.d. \times 25-cm; Wilmad Glass Co., Buena, NJ). 1D and 2D NMR experiments were performed on a JEOL GSX500A spectrometer equipped with a 5-mm field gradient tunable probe with standard JEOL software at 303 K for NOE spectra or 333 K for other experiments on 500- μ L samples. The HOD signal was suppressed by presaturation for 3 or 1.5 s for 1D or 2D spectra, respectively. To obtain 2D spectra, 512 experiments resulting 1024 data points for a spectral width of 2000 Hz were measured, and the time domain data were multiplied after zerofilling (data matrix size, 1K \times 1K) with a shifted sine-bell window functions for 2D double quantum filtered (DQF)²-COSY, NOESY, or TOCSY experiments. An MLEV-17 mixing sequence of 100 ms was used for 2D TOCSY and NOESY experiments by using 150, 250, and 500 ms as the mixing time were performed.

Enzymatic Digestion of Heparin

Complete depolymerization of heparin and heparan sulfate uses a mixture of heparin lyases I, II, and III (9, 10). Briefly, sample (\leq 1 mg in 100 μ L of water) is placed in a 500- μ L polypropylene centrifuge tube. Sodium phosphate buffer (10 μ L, 500 mM, pH 7.5) is added to the sample, after which 5–10 mU each of heparin lyase I, II, and III is added. The sample is digested for 8–12 h at 35°C and analyzed immediately by the HPLC or stored frozen at –60°C until analysis.

HPLC Condition

The established conditions for the determination of the trisulfated unsaturated disaccharide from heparin were as follows: The HPLC system was assembled with gradient pumps (Jusco 880-PU, intelligent HPLC pump; Jusco, Tokyo, Japan), a variable sample

injector (VMD-350; Shimamura Instrument Co., Tokyo, Japan), a double-plunger pump (PSU-2.5W, Shimamura Instrument Co.) for delivery of the post-column reagents, a dry reaction bath (Type DB-3, Shimamura Instrument Co.), a fluorescence spectrophotometer (Hitachi Model F-1050; Hitachi Seisakusho, Tokyo, Japan), and a chromatointegrator (D-2500, Hitachi Seisakusho). The separation column, Asahipak NH₂ P-50 (250-mm \times 4.6-mm-i.d.), was from Shodex Co. (Tokyo, Japan). A linear gradient elution program was started at 100% of eluent A (0.1 M sodium phosphate buffer, pH 10.0, containing 0.1 M sodium NaCl) to 100% of eluent B (0.1 M phosphate buffer, pH 10, containing 0.5 M NaCl) for 10 min, maintained at 100% of eluent B for 10 min, and then returned to 100% of the initial buffer. Flow rate was constant at 0.5 mL/min sodium hydroxide (0.5 M) and 50 mM guanidine was added, to the eluate of the separation column, at a flow rate of 0.25 mL/min using a double-plunger pump. The mixture passed through a polytetrafluoroethylene (PTFE) reaction coil (10-m \times 0.5-mm-i.d.) set in a dry reaction bath thermostated at 110°C and then through a PTFE cooling coil (2-m \times 0.25-mm-i.d.). The effluent was monitored by fluorescence with excitation at 320 nm and emission at 420 nm.

Anti-IIa Activity Measurement

Anti-IIa activity (11) was determined by incubating 50 mL of heparin in diluted normal human plasma with 50 mL of human thrombin (12 NIH units/mL) at 37°C for 30 s. Then, 50 mL (2.5 mmol/mL) of Chromogenic TH (ethylmalonyl-Pro-Arg-*p*-nitroanilide hydrochloride) was added, and the amidolytic thrombin activity was measured at 405 nm. Measurements were performed on an ACL 300 plus from Instrumentation (Lexington, MA) and calculated in comparison with USP Heparin Reference Standard (K-3) supplied by U.S. Pharmacopeial Convention (Rockville, MD).

Isolation of Heparin from Blood Plasma

The pretreatment procedure, described for the determination of dermatan sulfate (12), was modified for preparation of heparin from blood plasma. Briefly, to a 100- μ L portion of plasma and 100 μ L of 0.05 M Tris-HCl buffer (pH 8.0) containing 1% actinase were added and plasma proteins were digested at 45°C for 3 h. Acetic acid (1.5 mL of 15 mM) containing 10% sodium chloride was then added, and the mixture was boiled for 5 min in a water bath. After cooling in an ice bath, the solution was centrifuged at 2300g for 15 min. The supernatant was transferred to a new tube, 5 mL of cold ethanol saturated with sodium acetate was added, and the mixture was kept at 0°C for 1 h. After centrifugation at 2300g for 15 min at 4°C, the pellet was recov-

² Abbreviations used: DQF, double quantum filtered; PTFE, polytetrafluoroethylene; GlcNpS6S, 2-deoxy-2-sulfamino- α -D glucopyranose 6-O-sulfate; IdoAp25, α -L-idopyranosyluronic acid; GlcNpAc, 2-deoxy-2-sulfamino- α -D-glucopyranose; GlcNp5356S, 2-deoxy-2-sulfamino- α -D-glucopyranose 3,6-O-disulfate; GlcAp, β -D-glucopyranosyluronic acid; IdoAp, α -L-idopyranosyluronic acid; ATIII, antithrombin III; HCII, heparin cofactor II; GAGs, glycosaminoglycans; COSY, correlation spectroscopy; NOESY, nuclear Overhauser effect spectroscopy; TOCSY, totally correlated spectroscopy.

ered and dried *in vacuo* for 6 h. The residue was redissolved in 50 μ L of 50 mM sodium acetate buffer (pH 7.0) containing 10 mM of calcium acetate and incubated with 50 mU of chondroitinase ABC at 37°C for 3 h to remove the chondroitin sulfate linked to inter- α -trypsin inhibitor normally found in plasma (13). The heparin in the sample was next digested using 4 U of heparin lyase I or a mixture of heparin lyase I (2 U), heparin lyase II (0.5 U), and heparin lyase III (2 U) at 35°C for 15 h. After centrifugation at 2300g for 10 min, the resulting supernatant was analyzed by HPLC with postcolumn derivatization.

Pharmacokinetic Study

Male albino rabbits (3–3.5 kg body wt) were used for the pharmacokinetic study of intravenously administered heparin. Heparin dissolved in saline (2 mg/mL) was injected through the marginal ear vein at a dose of 1 mg/kg body wt. Blood samples were collected from the marginal ear vein on the opposite side from the injection at from 0 to 360 min after the administration. Blood was collected in 0.2% disodium EDTA and immediately centrifuged at 1500g for 10 min to obtain plasma, which was transferred to a polypropylene plastic vials and stored briefly in a refrigerator or for longer time in a deep freezer.

RESULTS AND DISCUSSION

Compositional Analysis of Heparin

The 1D proton magnetic resonance spectra in Fig. 1 are representative of bovine lung, bovine intestinal mucosal, and porcine intestinal mucosal heparins, respectively. The major signals, labeled in Fig. 1A, correspond to the main constituent monosaccharide residues, i.e., 2-deoxy-2-sulfamino- α -D-glucopyranose 6-*O*-sulfate (GlcNpS6S) and α -L-idopyranosyluronic acid 2-*O*-sulfate (IdoAp2S) (14). Furthermore, Figs. 1A–1F also show a group of important minor signals corresponding to protons in 2-acetamido-2-deoxy- α -D-glucopyranose (GlcNpAc), 2-deoxy-2-sulfamino- α -D-glucopyranose 3,6-*O*-disulfate (GlcNpS3S6S) (14), β -D-glucopyranosyluronic acid (GlcAp) (15), and α -L-idopyranosyluronic acid (IdoAp) (14, 15). The presence of the H-2 and H-3 signals of GlcNpS3S6S, contained in the antithrombin III (ATIII)-binding pentasaccharide sequence, was confirmed by a two dimensional double quantum filtered-chemical shifts correlation (DQF-COSY) experiment (Fig. 2). Cross-peaks between H-1/H-2 and H-2/H-3 of GlcNpS3S6S residue in the DQF-COSY spectrum clearly shows the down-field shift of each signal resulting from the presence of the 3-*O*-sulfate group in this residue (Fig. 2). The integrations of the signals of the *N*-acetylmethyl group at 2.05 ppm and H-2 of GlcNpS3S6S at 3.35 ppm vary significantly

in each heparin sample. As previously pointed out, heparin obtained from bovine lung contains a far lower amount of GlcNpAc than that obtained from porcine and bovine intestinal mucosa (16, 17). In the current study this is again observed as demonstrated by the reduced intensity of the methyl signal at 2.05 ppm in Fig. 1A when compared with those in Fig. 1B–F. Table 1 shows the monosaccharide composition of each heparin sample as determined with by 1 H NMR spectroscopy (18). The integration of *N*-acetylmethyl protons at 2.05 ppm was used to determine the amount of GlcNpAc(6S or 6OH) and the integration of each anomeric proton signal at 4.5 to 5.5 ppm (see Fig. 1) was used to determine mol% of each monosaccharide residue in heparin sample. While the sensitivity of 1 H NMR spectroscopy is lower than that of other analytical methods, such as HPLC and CE, this method is useful in providing important qualitative and quantitative information on heparin.

The presence of dermatan sulfate residue in some pharmaceutical heparin samples has been demonstrated by Holme and Perlin (19) using 1 H NMR. However, no signals corresponding to dermatan sulfate were detected in any of the spectra of the heparins analyzed in this study. Apparently, the procedure used for the manufacture of these pharmaceutical heparins was effective in removing contaminants such as dermatan sulfate.

Table 2 shows the total disaccharide composition of the heparin samples determined using a previously described HPLC with the postcolumn derivatization method (20). An estimate of the trisulfated disaccharide content of the heparin samples was obtained from the integrated area/peak height of the peak in the chromatogram. This HPLC method required 40 min to elute the trisulfated unsaturated disaccharide of interest using conditions required for the simultaneous determination of the minor undersulfated unsaturated disaccharides produced from heparin (or heparan sulfate) (20). Thus, it was necessary to reexamine the optimum conditions for the determination of the trisulfated unsaturated disaccharide. Based on this study, a retention time of 12 min was found to be optimum. High sensitivity fluorescence detection of the trisulfated unsaturated disaccharide was achieved by its postcolumn derivatization with guanidine. Figure 3 shows a typical chromatogram of the trisulfated unsaturated disaccharide obtained when analyzing heparin in a plasma sample according to this optimized method (described in detail under Materials and Methods).

To establish a method for the chemical microdetermination of heparin, two strategies were first examined: (i) The intact heparin polysaccharide was directly determined after its separation from other biological contaminants by electrophoresis or chromatography (21–24). (ii) The heparin polysaccharide was first frag-

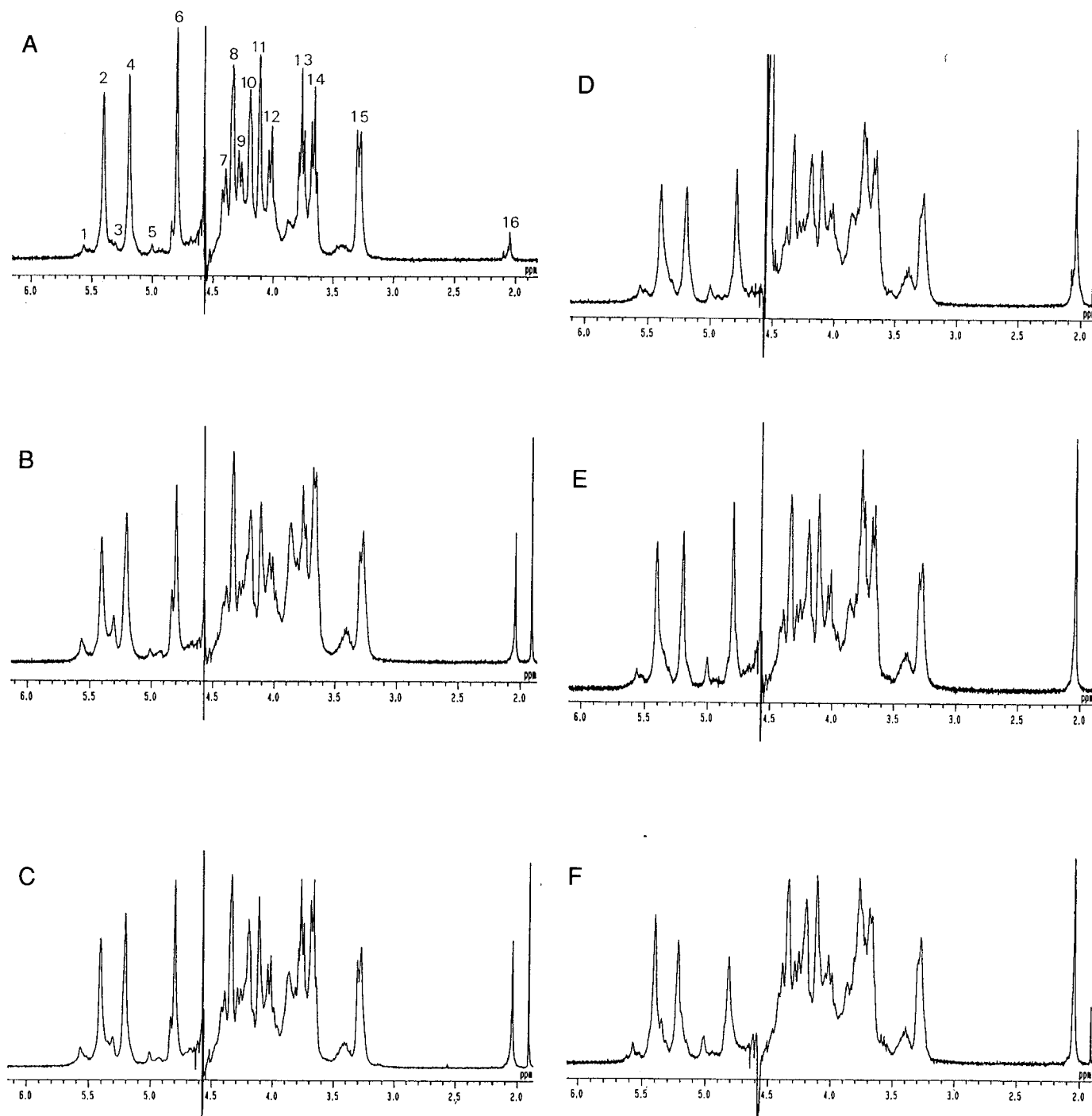


FIG. 1. One-dimensional ^1H NMR spectra of heparin samples. (A) Bovine lung heparin from Fluka, (B) bovine intestinal heparin from Fluka, (C) bovine intestinal mucosal heparin from Sigma, (D) porcine intestinal mucosal heparin from Nacalai, (E) porcine intestinal mucosal heparin from Celsus, (F) low-molecular-weight heparin from porcine intestinal mucosal from Celsus. Signals: 1, H-1 of GlcNpS6S linked to GlcAp; 2, H-1 of GlcNpS6S linked to IdoAp2S; 3, H-1 of GlcNpS linked to IdoAp2S; 4, H-1 of IdoAp2S; 5, H-1 of IdoAp; 6, H-5 of IdoAp2S linked to GlcNpS6S; 7, H-6a of GlcNpS6S; 8, H-2 of IdoAp2S; 9, H-6b of GlcNpS6S; 10, H-3 of IdoAp2S; 11, H-4 of IdoAp2S; 12, H-5 of GlcNpS6S; 13, H-4 of GlcNpS6S; 14, H-3 of GlcNpS6S; 15, H-2 of GlcNpS6S; 16, N-acetylmethyl protons of GlcNAc. Multiplets resonating around 3.4 ppm are assigned as H-2 protons of GlcAp, GlcNpS6S linked to GlcAp, and GlcNpS3S6S.

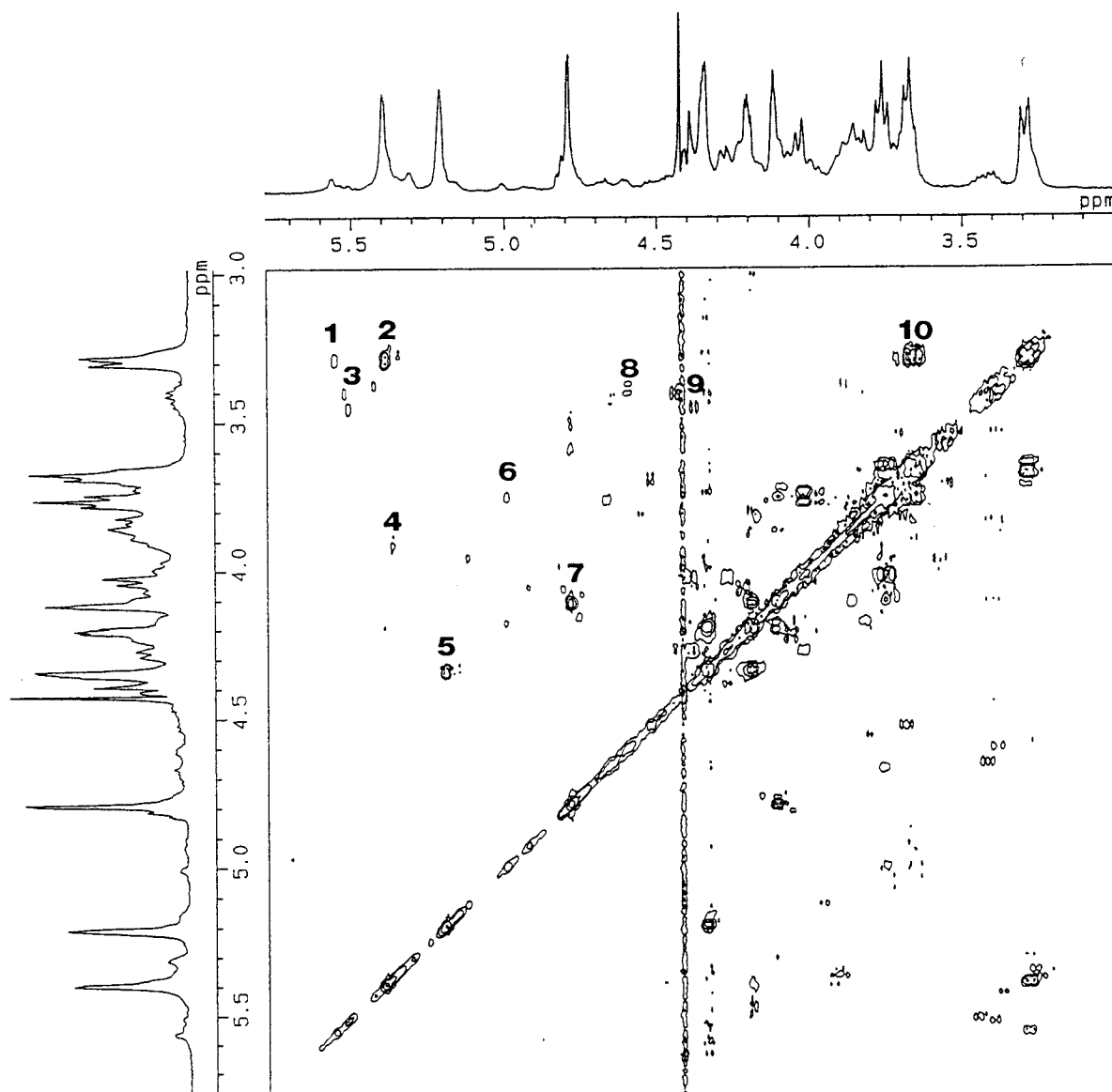


FIG. 2. Two-dimensional DQF-COSY spectrum of bovine intestinal mucosal heparin. Cross peaks: 1, H-1/H-2 of GlcNpS linked to GlcAp; 2, H-1/H-2 of GlcNpS6S linked to idols; 3, H-1/H-2 of GlcNpS3S6S; 4, H-1/H-2 of GlcNpAc(6S or 6OH); 5, H-1/H-2 of IdoAp2S; 6, H-1/H-2 of IdoAp; 7, H-4/H-5 of IdoAp2S; 8, H-1/H-2 of GlcAp; 9, H-2/H-3 of GlcNpS3S6S; 10, H-2/H-3 of GlcNpS6S. All other cross peaks are observed between the neighboring coupling protons indicated in Fig. 1.

mented using heparin lyase, and the unsaturated disaccharide products were determined using electrophoresis or chromatography relying on UV detector at 232 nm (25, 26). The former strategy has been successfully used in the determination of dermatan sulfate from porcine skin (27) and fucosylated chondroitin sulfate from sea cucumber (28). The fluorometric reaction used for the direct detection of dermatan sulfate or fucosylated chondroitin sulfate, however, requires the presence of a 4-*O*-sulfated GalNAc to produce the fluorophor(s). Heparin (and heparan sulfate) can not be detected by this reaction (28) as they contain GlcNp

residues and are only 6- and/or 3-*O*-sulfated. Heparin (or heparan sulfate) can be indirectly determined by the second strategy, which relies on disaccharide compositional analysis (25, 26). Based on the results obtained from a disaccharide analysis of heparin using this approach, approximately 70–90% of disaccharide units are trisulfated unsaturated disaccharide, Δ UAp2S1 \rightarrow 4GlcNpS6S. This observation, suggests that by determining the concentration of Δ UAp2S1 \rightarrow 4GlcNpS6S, the total, absolute amount of heparin can be rapidly and accurately measured. Although there are a number of reports for the separation and determi-

TABLE 1
Monosaccharide Composition of Various Heparins by ¹H NMR Analysis

Sample ^a	Hexosamine residue, mol%				Uronic acid residue, mol%			
	GlcNpS6S	GlcNpS	GlcNpS3S6S	GlcNpAc	IdoAp2S	IdoAp	GlcAp	Others ^b
A	91.4	3.0	2.7	2.9	90.3	3.0	6.2	0.5
B	77.4	13.3	2.4	6.9	88.5	2.8	7.8	0.9
C	74.9	10.9	3.8	10.4	87.1	4.1	8.0	0.8
D	62.6	10.8	2.2	24.4	86.8	6.0	6.8	0.4
E	66.3	6.8	5.1	21.8	75.4	8.8	13.9	1.9
F	65.5	11.0	4.9	18.6	80.6	9.8	8.8	0.8

^a The samples are designated as in Fig. 1.

^b Not identified.

nation of Δ UAp2S1 \rightarrow 4GlcNpS6S prepared from heparin by HPLC and CE methods with the UV detection at 232 nm, these methods are not sufficiently sensitive for the analysis of therapeutic doses of heparin in biological samples. Recently, we developed a highly sensitive HPLC method for the determination of the unsaturated disaccharides from chondroitin sulfate/dermatan sulfate with 2-cyanoacetamide reagent as a postcolumn derivatization reagent (29). Since heparin (and heparan sulfate) disaccharides did not react with 2-cyanoacetamide, the reactivity of other derivatization reagents useful in fluorometric detection were reexamined for the fluorometric detection of Δ UAp2S1 \rightarrow 4GlcNpS6S from heparin. The heparin-derived disaccharide, Δ UAp2S1 \rightarrow 4GlcNpS6S, produced a strong fluorescence after its reaction with guanidine in alkaline medium. The detection limit of Δ UAp2S1 \rightarrow 4GlcNpS6S was 1 pmol (signal to noise = 3) using the HPLC conditions and postcolumn derivatization method described in this paper. This amount of disaccharide corresponds to 1 ng of intact heparin sample.

Anti-IIa Activity

The total anticoagulant activity of the heparin samples were analyzed using Chromozyme TH kit from

Boehringer-Mannheim with whole human plasma as a source of inhibitory substances activated by heparin, such as ATIII and heparin cofactor II (HCII). Heparin binds to thrombin and ATIII in a ternary complex, accelerating the rate of thrombin inhibition by ATIII by 2000-fold (30). HCII is structurally similar to ATIII with a molecular weight of 65,000 (31) and a *pI* of 4.9–5.3 (32), having a similar carboxyl terminal sequence but a distinctly different amino terminal sequence (33). The physiological role of HCII might be as a reserve of thrombin inhibitor when the plasma concentration of ATIII becomes abnormally low (34). Unlike ATIII, HCII can inhibit thrombin but no other coagulation proteases (30, 35). In addition to this unusual specificity, HCII also can be potentiated by GAGs, other than heparin, including dermatan sulfate (36) and heparan sulfate, both of which are found lining the luminal surface of the endothelium (37). The mechanism by which HCII inhibits thrombin is similar to that proposed for ATIII (29). While ATIII interacts with heparin through a specific and unique pentasaccharide binding site, HCII primarily binds heparin in its highly sulfated regions, such as those rich in trisulfated disaccharide (38).

The correlation between the content of trisulfated unsaturated disaccharide residue and the anti-IIa ac-

TABLE 2
Disaccharide Composition of Various Heparins

Heparin ^a	Disaccharide, mol% ^b			
	Δ UAp2S-GlcNpS6S	Δ UAp-GlcNpS6S	Δ UAp2S-GlcNpS	Other
A	86.2	3.5	Trace	10.3
B	54.6	9.7	8.3	27.4
C	74.4	6.6	7.8	11.2
D	62.1	8.4	9.9	19.6
E	57.6	9.7	4.8	27.9
F	44.1	18.9	8.3	28.7

^a The samples are designated as in Fig. 1.

^b Samples were digested by heparin lyases I, II, and III and determined by HPLC.

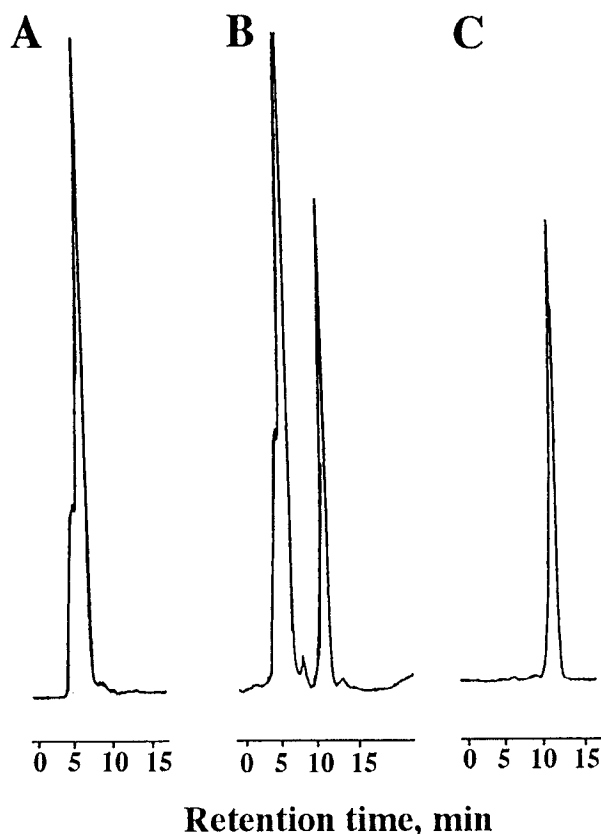


FIG. 3. Typical chromatograms of a trisulfated unsaturated disaccharide purified from plasma. (A) Plasma collected at 0 h, (B) plasma collected at 0.5 h after heparin injection, (C) standard $\Delta\text{UAp}2\text{S} \rightarrow \text{GlcNpS}6\text{S}$.

tivity is shown in Fig. 4. The correlation between the anti-IIa activity and the amount of $\Delta\text{UAp}2\text{S}1 \rightarrow 4\text{GlcNpS}6\text{S}$ in each heparin ($r = 0.849$ obtained by heparin lyase I and $r = 0.686$ obtained by the mixture of heparin lyases I, II, and III) suggested that this method would be useful for determining the chemical concentration of heparin with nanogram sensitivity. This method not only identifies heparin but also provides information on the nature of its anticoagulant activity. Although it is well known that intact heparin contains the significant amounts of the sequence which resists against heparin lyase digestion, the amount of trisulfated disaccharide unit obtained by heparin lyase treatment is not only abundant in heparin's HCII but is also present in heparin's ATIII binding site as well and its concentration correlates with heparin's anti-IIa activity.

Pharmacokinetic Study

Plasma concentrations of heparin measured by the present HPLC method were detectable up to 6 h after intravenous drug injection (1 mg/kg body wt). No circu-

lating endogenous heparin was detected in rabbit blood plasma by the present method, even when analyzing 10 times more plasma sample than the procedure calls for. The concentration of heparin measured by this method decayed biexponentially in all animals and is consistent with a two-compartmental model. The half-life of the initial elimination phase was 0.30 ± 0.05 h, while that of the terminal elimination phase was 6.0 ± 3.0 h (Fig. 5).

Heparin's anticoagulant activity was measurable only during the initial elimination phase (up to 4 h), and the half-life was strongly dependent on the amounts of dosed heparin using the method of Boneu *et al.* (39). It is, however, difficult to compare the results of the HPLC assay to those obtained using catalyzed thrombin inhibition tests and radioactive measurements because the amounts of administered heparin were completely different.

In summary, this work demonstrates that the sensitive determination of heparin using HPLC with post-column derivatization gave heparin concentrations that correlated with its anti-IIa activity. The high sensitivity determination of chemical heparin by this method has also revealed that the pharmacokinetic be-

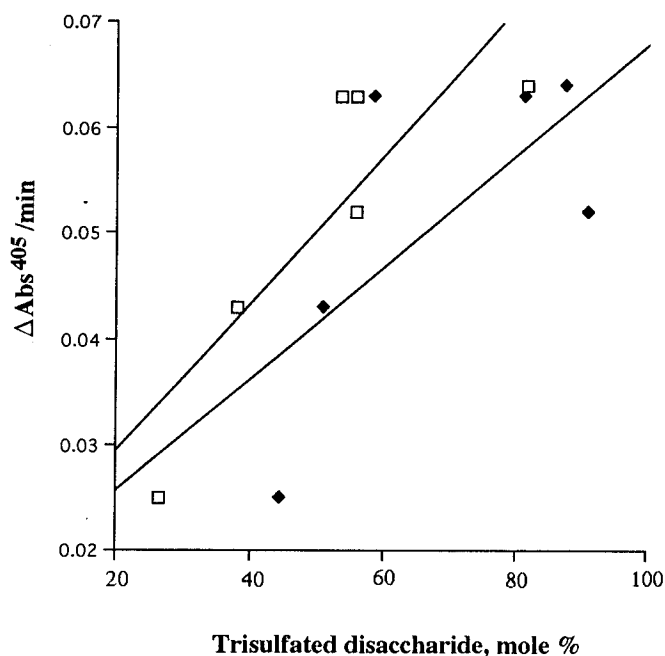


FIG. 4. Correlation between the content of trisulfated disaccharide and the anti-IIa activity. Each point was obtained by heparin lyase I digestion (\square), $y = -0.0007x + 0.015$, $r = 0.849$. Each point was obtained by heparin lyases I, II and III digestion (\blacklozenge), $y = 0.0005x + 0.015$, $r = 0.686$. The $\Delta\text{Abs}^{405}/\text{min}$ is the difference absorbance per minute between control plasma and plasma to which heparin was added. Normal human plasma was a source of the coagulation factors and the anticoagulant activity was measured using Chromozyme TH.

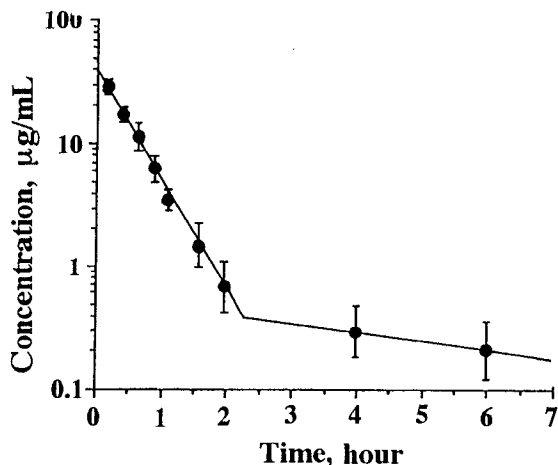


FIG. 5. Plasma levels of heparin following the intravenous injection of 1 mg/kg to rabbits. Experiments were performed by using three animals and data were designated as means \pm SD.

havior of heparin intravenously administered to rabbits biexponential decay consistent with a two-compartmental model.

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