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Covalent Heparin Cofactor II-Heparin and Heparin Cofactor II-Dermatan Sulfate Complexes

CHARACTERIZATION OF NOVEL ANTICOAGULANTS*

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Heparin cofactor II is a naturally occurring anticoagulant that acts by specifically inhibiting thrombin and is facilitated by the binding of glycosaminoglycans such as heparin and dermatan sulfate. *In vivo*, heparin cofactor II-glycosaminoglycan complexes dissociate, leaving the inhibitor less active in its ability to function as a component of the anticoagulation pathway. We have produced permanently activated heparin cofactor II molecules by covalent linkage to either heparin or dermatan sulfate. Covalent heparin cofactor II-heparin and heparin cofactor II-dermatan sulfate complexes had catalytic antithrombin activities similar to those of the corresponding starting heparin and dermatan sulfate (86% and 110% of standard heparin and dermatan sulfate activity, respectively). Both heparin cofactor II-heparin and heparin cofactor II-dermatan sulfate had fast bimolecular rate constants of 1.4×10^7 M^{-1} s^{-1} and 1.3×10^7 M^{-1} s^{-1}, respectively, for reaction with thrombin. The intravenous half-life of the covalent complexes in rabbits was significantly longer than that of free heparin or dermatan sulfate (4.4, 3.4, 0.33, and 0.50 h for heparin cofactor II-heparin, heparin cofactor II-dermatan sulfate, heparin, and dermatan sulfate, respectively). Given their unique properties, these conjugates may have a clinical application for long term, selective inhibition of thrombin.

Coagulation occurs by the activation of proenzymes in a cascade leading to generation of thrombin, which in turn converts fibrinogen to fibrin. Thrombin is also responsible for feedback activation of other coagulation factors (1) and is considered the pivotal enzyme in the coagulation pathway (2). Control of thrombin generation regulates plasma coagulant activity.

Heparin cofactor II (HC)[1] is an important plasma protein that selectively inhibits thrombin (3). A number of glycosaminoglycans (GAGs) catalyze HC to markedly accelerate the reaction of HC with thrombin (4). While dermatan sulfate (DS) has been shown to exclusively facilitate thrombin inhibition by HC (3), other GAGs, such as heparin and heparan sulfate, can catalyze inactivation of thrombin by either HC or plasma antithrombin (AT) (4–6). Fluid phase thrombin is readily inhibited by heparin activated AT; however, clot-bound thrombin is resistant to inactivation by either AT+ heparin or AT + low molecular weight heparin (7–9) due to the formation of thrombin-heparin-fibrin ternary complexes (10). In contrast, noncovalent HC·DS has been shown to be superior to AT·heparin for inhibition of thrombin in the presence of fibrin (9).

There are a number of limitations associated with clinical use of GAGs, due in part to the undesired effects of GAGs upon dissociation from the thrombin inhibitor. First, with regard to the undesired effects of heparin, anticoagulation with standard or low molecular weight heparins can lead to bleeding (11, 12) as well as heparin-induced thrombocytopenia (13). Heparin activity may be reduced due to cell surface and plasma protein binding (14). Application of heparin in other spaces, such as the lung for treatment of fibrin deposition during neonatal respiratory distress syndrome (15, 16), is hampered by the rapid loss of the GAG intravascularly (17). Long term heparin use can result in osteoporosis (18, 19). DS also has a number of undesirable effects *in vivo*. While DS has decreased cell surface binding (20) and causes reduced endothelial intracellular PAI-1 levels (21), compared with heparin derivatives, hemorrhagic effects (22) and loss of activity from plasma protein binding (23) are still significant. Both GAGs (heparin and DS) have short intravenous half-lives (24, 25) and, by acting as a large negative surface, can assist in activation of factor XI (26).

We hypothesized that covalent linkage of HC to either DS or heparin may reduce the problems involved in therapeutic use of GAGs. HC-GAG conjugates would always be in an active form. Since covalent HC-GAG complexes would be large and would not dissociate, clearance due to glomerular filtration or protein-mediated uptake by organs might be reduced, resulting in longer intravenous half-lives. Similarly, since part of the GAG in the covalent complex would be covered by HC, hemorrhagic effects, heparin induced thrombocytopenia, and osteoporosis may be decreased due to steric hindrance. Conjugates of HC and heparin (HCH) may, in addition to direct inactivation of thrombin, also catalyze thrombin inhibition by plasma AT. Covalent HC-DS (HCD) should selectively inhibit thrombin

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[1] The abbreviations used are: HC, heparin cofactor II; GAG, glycosaminoglycan; DS, dermatan sulfate; AT, antithrombin; HCH, covalent HC-heparin; HCD, covalent HC-DS; ATH, covalent AT-heparin; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; TSP, Tris/saline/polyethylene glycol.

and may give better long term inhibition of clot-bound thrombin compared with non-covalent HC + DS mixtures.

Recently, we have prepared covalent complexes of AT and heparin (ATH) (27). ATH was made without modification of either inhibitor protein or GAG, and gave a product with high anticoagulant activities and long half-life *in vivo*. We decided to use the same techniques employed for ATH synthesis in order to prepare HCH and HCD covalent complexes. Both HC-GAG conjugates were produced, and their properties and intravenous clearance investigated.

EXPERIMENTAL PROCEDURES

Chemicals—All chemicals were of analytical grade. The HC (66 kDa), used in all studies, was from Affinity Biologicals (Hamilton, Ontario (ON), Canada) and gave a single band on sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE). Heparin (sodium salt, from porcine intestinal mucosa) and dermatan sulfate (sodium salt, from bovine mucosa) were both from Sigma (Mississauga, ON, Canada). Heparin was 179.2 units/mg (grade I-A), and the dermatan sulfate was 85% chondroitin sulfate B with any remainder being chondroitin sulfates A and C. Heparinase (heparin lyase, EC 4.2.2.7 (hepase)) and chondroitinase ABC (chondroitin ABC lyase, EC 4.2.2.4 (ABCase)) were from ICN (Costa Mesa, CA). Tosyl-glycyl-prolyl-arginyl-4-nitroanalide acetate and general protease P-5147 (which was devoid of GAG degrading activities) were from Sigma. Thrombin (IIa) was obtained from Enzyme Research Laboratories (South Bend, IN), while $Na^{125}I$ was from NEN Life Science Products (Mississauga, ON, Canada). Chloramine T was from BDH (Toronto, ON, Canada).

Complex Preparation and Purification—The average molecular weights of the heparin and DS were 15,000 and 29,200 (determined by gel filtration), respectively. Heparin and DS were analyzed for chains that contained a free aldose aldehyde end group by reaction with 3,4-dinitrobenzoic acid (7.5 mM) in Na_2CO_3 (0.24 M) at ≈95 °C for 1 min, followed by measurement of absorbance at 548 nm to determine the amount of 4-amino-3-nitrobenzoate produced (28). The number of aldose aldehyde termini per GAG molecule was calculated by using a xylose standard curve and the average molecular weight for heparin or DS. Synthesis of HCH and HCD followed a procedure similar to that reported previously for ATH, based on the principle of covalent linkage through Schiff base -Amadori rearrangement at 40 °C (27). HC (0.5 mg in 52.5 μl of 0.02 M phosphate, 0.15 M NaCl, pH 7.3 (PBS)) was mixed with either heparin (27.8 mg in 0.5 ml of PBS) or DS (74.0 mg in 1 ml of PBS) in capped plastic tubes, followed by incubation at 40 °C for 10 days. Subsequently, the reaction mixtures were made 0.05 M in $NaBH_3CN$ (by addition of 0.5 M $NaBH_3CN$ in PBS), and incubation continued at 37 °C for 5 h, in order to reduce any remaining Schiff base.

Purification of the complexes formed was achieved by slight modification of the method used previously for production of ATH (27). Each reaction mixture was made 2.5 M in $(NH_4)_2SO_4$ by addition of 3.0 M $(NH_4)_2SO_4$, 0.02 M phosphate, pH 7.0. In each case, the resultant mixture was immediately combined with 1.5 ml of pre-equilibrated, wet, packed butyl-agarose beads (agarose with ether-linked butyl groups, from Sigma)/ml of solution added. After gentle mixing for 30 min, the supernatant was discarded and, after washing the beads with 2.2 M $(NH_4)_2SO_4$ in buffer, the bound complex + free HC were eluted with 0.02 M phosphate, pH 7.0. Eluted material was dialyzed *versus* 0.01 M Tris·HCl, pH 8.0, followed by gentle mixing for 30 min with 0.5–1.0 ml of pre-equilibrated, wet, packed DEAE-Sepharose Fast Flow beads (Pharmacia, Uppsala, Sweden). The supernatant was discarded and, after washing the beads with 0.3 M NaCl, 0.01 M Tris·HCl, pH 8.0, purified complex was eluted with 2.0 M NaCl, 0.01 M Tris·HCl, pH 8.0. Purified HCH and HCD were concentrated by dialysis (12,000–14,000 molecular weight cut-off), *versus* PBS at 4 °C, under 1 atm N_2 pressure.

Analysis of Complexes—The physicochemical properties and some activities of the HCH and HCD preparations were determined. Conjugates were analyzed by SDS-PAGE (29). Prior to electrophoresis, complexes were incubated for 24 h at 37 °C with PBS; 0.01 unit of hepase/ml of 0.001 M $CaCl_2$, 0.001 M sodium acetate, 0.15 M NaCl; 0.5 unit of ABCase/ml of 0.001 M sodium acetate, 0.15 M NaCl; or 1 μg of P-5147/ml of 0.05 M Tris·HCl, pH 8.0. Gels were stained with Coomassie Blue (for protein) and with alcian blue followed by silver (for GAG) (30). Lanes containing either hepase/ABCase or protease-treated conjugate were scanned for either protein- or GAG-stained material, respectively, by laser densitometry (LKB Ultroscan XL). Comparison of the scans to standard curves, produced by scanning lanes containing varying amounts of either protein (HC) or GAG (heparin or DS), run on the same gel, allowed the mass of HC or GAG (heparin or DS) in each complex to be determined. Given the molecular weights of HC, heparin, and DS, the HC:GAG molar ratio for each conjugate could be calculated. Anticoagulant activity of HCH and HCD was determined by anti-factor Xa and anti-IIa assays in which catalysis of the inhibition of enzyme by exogenous plasma AT and HC were measured (reactions in which the increase in amount of Xa or thrombin inhibited by plasma AT and plasma HC was in vast excess to the conjugate being assayed). Anti-factor Xa activity was measured on an ACL300 using a kit (Stachrom anti-Xa, Diagnostica Stago, Asnières, France), which involved incubation of the sample in plasma with excess factor Xa for 30 s, followed by addition of a chromogenic substrate to measure residual activity. The amount of enzyme activity inhibited during the reaction was compared with a standard curve, constructed from reactions with plasma samples containing standard heparin (from Diagnostica Stago) of known concentrations (units/ml). The amount (units/ml) of the sample was divided by the amount (mg/ml) of heparin or DS present to obtain specific activities for the compound. The antithrombin activity in HCH preparations was determined using an assay similar to that for anti-factor Xa activity except that an anti-IIa kit was used (Instrumentation Laboratory, Lexington, MA). DS could not be measured by the anti-IIa kit due to the reduced sensitivity of the kit for DS. Therefore, DS and HCD antithrombin activity was quantitated by a modified activated partial thromboplastin time assay (using an ST4 machine (Diagnostica Stago, Asnières, France)); where 50 μl of 0.02 M $CaCl_2$ was added to a mixture of 50 μl of Thrombosil I (Hemoliance, Raritan, NJ) + 50 μl of complex diluted in plasma (pre-incubated for 5 min at 37 °C), and the resultant clot time compared with a standard curve of dermatan sulfate in plasma. Addition of anti-human HC antibody neutralized all inhibitory activity, thus verifying that the DS antithrombin assay only detected activity against thrombin (HC only reacts with thrombin; Ref. 3). Results for anti-factor Xa assays of HCH and HCD were expressed as units per mg of GAG in the conjugate. Anti-IIa results for HCH and HCD were calculated as the activity per mg of GAG assayed, and expressed as a percentage of the activity attained by the same amount (by mass) of standard heparin or DS. In order to determine that direct antithrombin activity was due to covalent binding of thrombin, HC (1 μM) or complexes (0.25 μM) were reacted with 0.18 μM ^{125}I-thrombin (labeled using chloramine T; Ref. 31) at pH 7.4 for 2 h at 37 °C and the resultant products electrophoresed on SDS-PAGE, followed by autoradiography.

Kinetics of Thrombin Inhibition—Rate of reaction with thrombin was investigated using pseudo first order kinetics. Ten μl of 2 nM thrombin in 0.02 M Tris·HCl, 0.15 M NaCl, 0.6% polyethylene glycol 8000, pH 7.4 (TSP) was added rapidly to a well (Nunc 96-well plate; Becton Dickinson, Lincoln Park, NJ) containing 10 μl of inhibitor in TSP at 23 °C. At various times, the inhibition mixture was neutralized by rapid addition of 200 μl of 222 μM tosyl-glycyl-prolyl-arginyl-4-nitroanalide acetate in 0.01 mg of Polybrene/ml of TSP and the remaining thrombin activity against the substrate measured at 405 nm over 10 min. Biomolecular rate constants for inhibition of thrombin were calculated by a method similar to that used previously for ATH (27), which was derived from the work of Kitz and Wilson (32). The following model (Reaction 1) was used as a kinetic mechanism for thrombin inhibition.

$$\mathrm{IIa} + \mathrm{A} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \underset{\text{(non-covalent complex)}}{\mathrm{IIa \cdot A}} \xrightarrow{k_2} \underset{\text{(covalent complex)}}{\mathrm{IIa\text{-}A}}$$

REACTION 1

The inhibitor A = HCH, HCD, non-covalent HC·H, or non-covalent HC·DS complexes.

Apparent rate constants (k_{app}) were calculated from plots (semi-natural log) of the proportion of thrombin (IIa) activity remaining *versus* time (k_{app} = −[ln(remaining thrombin activity/initial thrombin activity)]/time). Plots of $1/k_{app}$ *versus* 1/A allowed for determination of bimolecular rate constants, given as the ratio k_2/K_i = 1/slope (where k_2 = 1/ordinate intercept and K_i (the equilibrium constant for disappearance of IIa·A) = −1/abscissa intercept = $(k_{-1} + k_2)/k_1$).

Intravenous Clearance—Samples of HC, HCH, and HCD were labeled with ^{125}I using brief (30 s) reaction with chloramine T (31) and recovery of the product in rabbit albumin (CooperBiomedial Inc., Malvern, PA) as a carrier. Under these conditions, labeled preparations were obtained that retained their integrity, as determined by PAGE. Rabbits (New Zealand White) were then injected intravenously with either H, DS, ^{125}I-HC, ^{125}I-HCH, or ^{125}I-HCD. At predetermined time points, 0.45-ml samples of blood were taken into 50 μl of 3.8% (m/v) Na_3 citrate. The citrated blood was centrifuged at 3000 × *g* for 15 min and

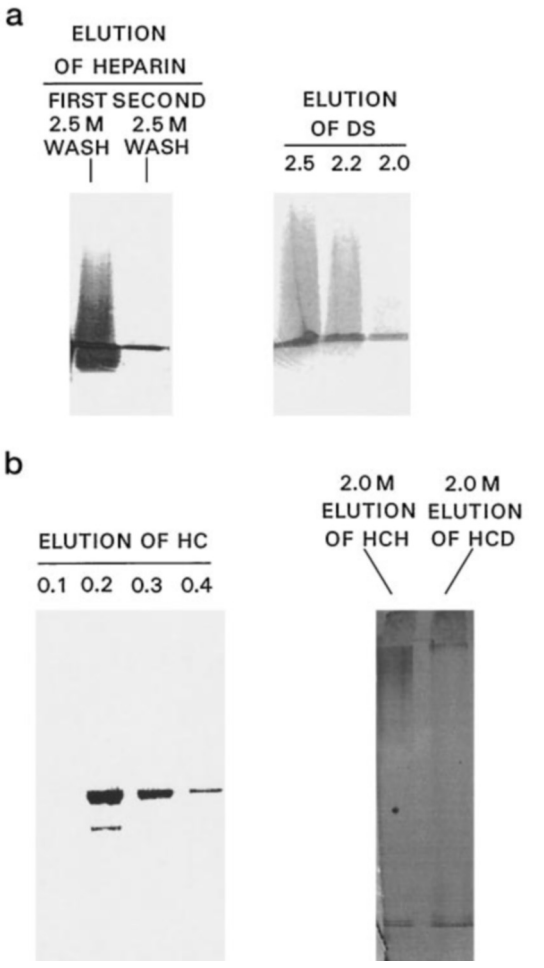

FIG. 1. **Purification of covalent heparin cofactor II-heparin (HCH) or heparin cofactor II-dermatan sulfate (HCD) complexes.** HCH and HCD were purified from unreacted heparin and DS, respectively, by chromatography on butyl-agarose followed by purification from unreacted HC on DEAE-Sepharose. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5% separating gel, reducing conditions) of fractions from butyl-agarose and DEAE-Sepharose chromatographies are shown in *panels a* and *b*, respectively. *Numbers* above the lanes represent molar concentrations of $(NH_4)_2SO_4$ (*a*) and NaCl (*b*).

aliquots of the supernatant plasmas taken for anti-factor Xa heparin assay, DS analysis (modified activated partial thromboplastin time assay, as described above), or counting in a LKB 1272 Clinigamma γ-counter.

RESULTS

Purification of Products—Synthesis of HCH and HCD relied on Schiff base formation of HC lysyl amino groups with aldose aldehyde groups, followed by Amadori rearrangement. Analysis of free aldose aldehydes from aldose hemiacetal termini in heparin and DS resulted in calculated values of 0.18 and 0.56 per molecule, respectively. Thus, 0.82 and 0.44 of the molecules of heparin and DS, respectively, contained terminal monosaccharide residues which did not have a free aldehyde (likely glycosidically linked to serine; Ref. 33). Isolation of HCH and HCD from starting materials was achieved by chromatography of the reaction mixtures on butyl-agarose followed by DEAE-Sepharose. SDS-PAGE of the elution of H, DS, HC, HCH, and HCD from butyl-agarose and DEAE-Sepharose is shown in Fig. 1. H and DS were displaced from butyl-agarose beads at high $(NH_4)_2SO_4$ concentrations (2.5 and 2.2 M, respectively) while HC, HCH and HCD remained bound (Fig. 1*a*). After elution of HC + HCH or HC + HCD (devoid of free H or DS, respectively)

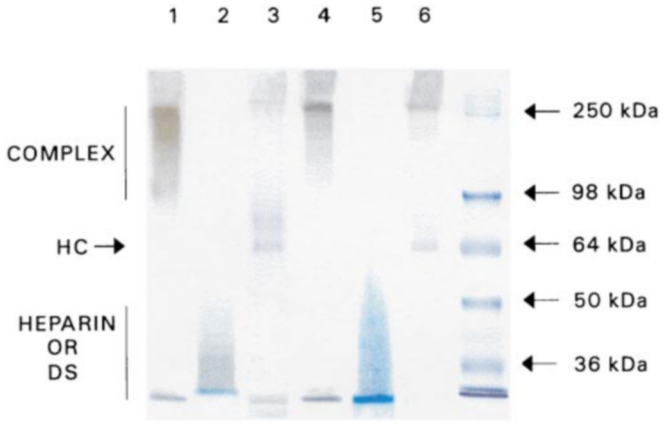

FIG. 2. **Qualitative analysis of covalent heparin cofactor II-glycosaminoglycan complexes.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5% separating gel, reducing conditions) of covalent heparin cofactor II-heparin complex (*lane 1*) treated with protease (*lane 2*) or heparinase (*lane 3*) and covalent heparin cofactor II-dermatan sulfate complex (*lane 4*) treated with protease (*lane 5*) or chondroitinase ABC (*lane 6*) are shown. Molecular size markers are indicated on the *right*, and the positions of HC, heparin, or DS are indicated on the *left*.

TABLE I
Physical properties of covalent heparin cofactor II-glycosaminoglycan complexes

HCH and HCD preparations were analyzed for relative protein and GAG content by SDS-PAGE of complexes and standards, followed by differential staining and densitometry. Given the average molecular weights of the component species, the HC:GAG molar ratios could be determined. Molecular mass values of the conjugates were estimated by gel filtration on Sephadex G-200 under dissociative conditions (2 M NaCl), in comparison with purified reference proteins.

Complex	HC:GAG molar ratio	Molecular mass range
		kDa
HCH	0.93	84–126
HCD	1.09	90–135

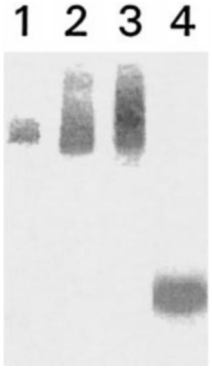

FIG. 3. **Reaction of complexes with thrombin.** Heparin cofactor II (HC; 1 μM), covalent heparin cofactor II-heparin (HCH; 0.25 μM) and covalent heparin cofactor II-dermatan sulfate (HCD; 0.25 μM) were each reacted with ^{125}I-thrombin (0.18 μM) in pH 7.4 buffer at 37 °C for 2 h. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5% separating gel, reducing conditions) of HC + ^{125}I-thrombin, HCH + ^{125}I-thrombin, HCD + ^{125}I-thrombin, and buffer + ^{125}I-thrombin were run in *lanes 1*, *2*, *3*, and *4*, respectively, followed by autoradiography.

from butyl-agarose in buffer (no $(NH_4)_2SO_4$), the material was applied to DEAE-Sepharose (0.01 M Tris·HCl, pH 8.0). HC was removed from DEAE beads by 0.3 M NaCl, followed by elution of either HCH or HCD in buffered 2 M NaCl (Fig. 1*b*). Analysis of the final concentrated preparations showed that, in the separate syntheses, 6.4% of starting HC (by mass) was recovered as HCH and 3.4% was recovered as HCD. Low yields of the conjugates were not due to protein loss during purification

since most of the HC could be recovered after DEAE-Sepharose chromatography. Analysis of the products by SDS-PAGE, followed by staining for protein or GAG and laser densitometry, showed that HCH was >99% and >96% free of unreacted HC and H, respectively, while HCD was >92% and >90% free of uncomplexed HC and DS, respectively (Fig. 2). Purification of HCH or HCD by gel filtration of the reaction mixtures on Sephadex G-200 did not improve yield or purity.

Physicochemical Properties—SDS-PAGE of either HCH or HCD resulted in bands of polydisperse, high molecular weight species, which did not dissociate after heating at 100 °C in 2-mercaptoethanol + SDS, pH 8.8 (Fig. 2). Stability of the products was indicative of covalently bonded protein and GAG. Treatment of the compounds with protease or GAG degrading enzymes caused release of the constituent GAGs and HC, respectively (Fig. 2). Quantitative analyses of conjugate composition is given in Table I. Both HCH and HCD preparations had protein:GAG molar ratios of close to 1:1. Gel filtration of HCH and HCD on Sephadex G-200 in 2 M NaCl (to prevent multimerization) gave molecular mass ranges of 84–126 kDa and 90–135 kDa, respectively (using protein molecular weight standards as references).

TABLE II
Anticoagulant activities of covalent heparin cofactor II-glycosaminoglycan complexes

HCH and HCD were tested using a commercially available kit to measure inhibition of factor Xa activity (chromagenic end point assay). Inhibition of thrombin (IIa) was determined using either an anti-IIa kit, which analyzed reaction with exogenous IIa by measuring residual chromagenic activity (heparin and HCH), or a clot time based activated partial thromboplastin time assay (DS and HCD). Heparin standards were used in the anti-factor Xa and anti-IIa assays while a DS standard curve was used in the clot-based assay. All assays were in the presence of normal plasma and results represent catalysis of reaction of factor Xa or IIa with exogenous inhibitors (antithrombin or heparin cofactor II) by the test compounds. Anti-factor Xa activity is given as units/mg of glycosaminoglycan assayed. Anti-IIa activity is reported as percentage of activity observed for the same mass concentration of standard heparin or DS.

Compound	Anti-factor Xa	Anti-IIa
	units/mg	%
Standard heparin	184	100
Dermatan sulfate	1.7	100
HCH	180	86
HCD	3.1	110

Anticoagulant Activity—Reaction of each conjugate with ^{125}I-thrombin verified that 1:1 complexes are produced between inhibitor and enzyme (Fig. 3). Purified preparations were titrated with human thrombin in order to measure inhibitory capacity. For every mole of HCH and HCD used, 0.943 and 0.756 mol, respectively, of thrombin were consumed. Therefore, >94% of HCH and >75% of HCD molecules were active in forming inhibitor complexes with thrombin. Although stable at 4 °C, freezing of concentrated HCH and HCD solutions at −60 °C, followed by a single thawing at 37 °C, reduced thrombin reactive complexes to <10%. Specific activities against factor Xa and thrombin, in plasma, are shown in Table II. In the presence of exogenous plasma HC and antithrombin, both HCH and HCD displayed catalytic activity similar to that of starting H and DS (Table II). Interestingly, HCH was able to catalyze reaction of factor Xa with antithrombin (mechanism of anti-factor Xa assay); however, anti-factor Xa activity of HCD was minimal. Antithrombin (anti-IIa) activities of both conjugates were similar to those of the GAG starting materials (Table II). Calculation of the rate of reaction with thrombin was carried out on measurements of remaining thrombin activity

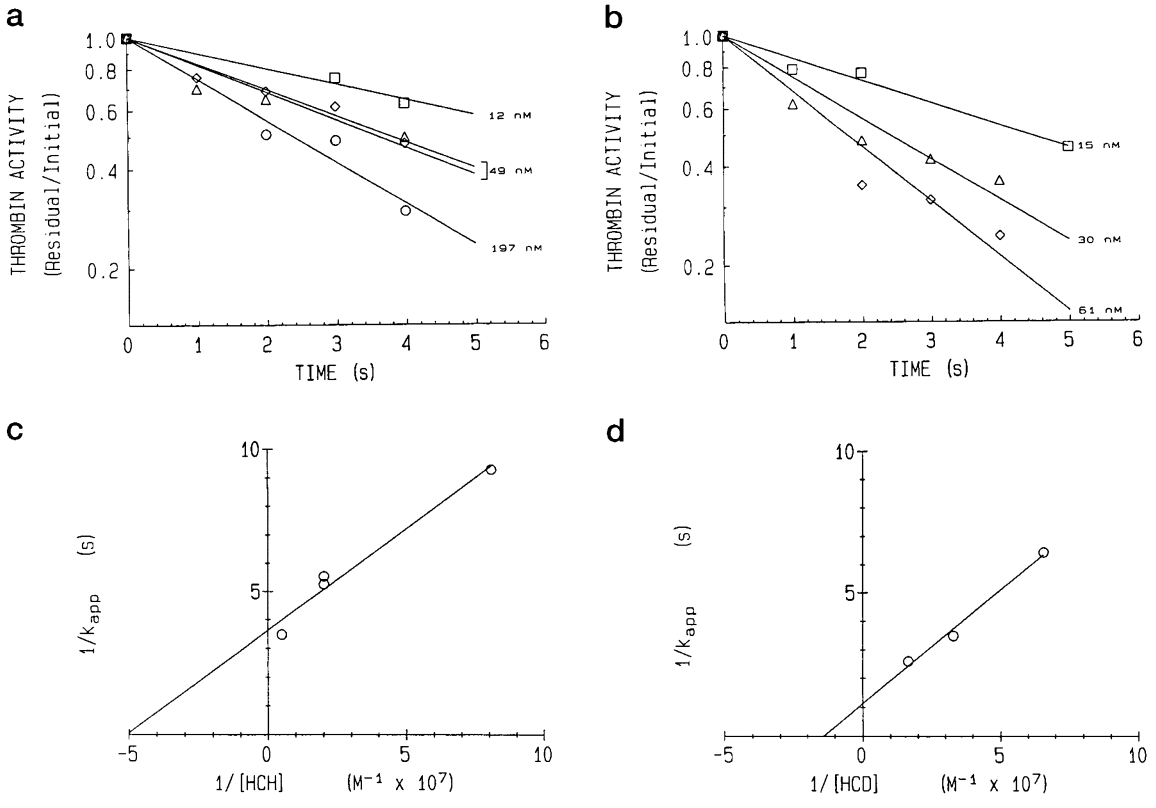

FIG. 4. **Kinetics of thrombin inhibition by covalent complexes.** Thrombin was reacted with heparin cofactor II-heparin (HCH) or heparin cofactor II-dermatan sulfate (HCD) in pH 7.4 buffer. At various times, reaction was terminated and residual thrombin activity measured by addition of tosyl-glycyl-prolyl-arginyl-4-nitroanalide substrate in Polybrene. Thrombin activity (residual activity (ΔA_{405}/10 min)/initial activity (ΔA_{405}/10 min at time = 0)) *versus* time for HCH + thrombin (*a*) and HCD + thrombin (*b*) are shown at different HCH and HCD concentrations. Plots of $1/k_{app}$ (k_{app} = −slope for curves in *panels a* and *b*) *versus* 1/[HCH] or 1/[HCD] are shown in *panels c* and *d*, respectively.

over time at different inhibitor concentrations (Fig. 4). The bimolecular rate constants of HCH and HCD were determined to be 1.4×10^7 M^{-1} s^{-1} and 1.3×10^7 M^{-1} s^{-1}, respectively. These values compared with rate constants of 8.0×10^6 M^{-1} s^{-1} for heparin and 6.8×10^6 M^{-1} s^{-1} for DS; both measured in the presence of saturating amounts (>10-fold molar excess) of HC. Interestingly, k_2 (rate for conversion of non-covalent thrombin-inhibitor complex to covalent thrombin-inhibitor complex) was ≈3 times slower for HCH than HCD (resulting in a ≈3 times lower value for K_i (the equilibrium constant for disappearance of the non-covalent thrombin-inhibitor complex)).

Intravenous Clearance—Elimination of standard heparin, DS, HCH, HCD, or HC, injected intravenously into rabbits, was followed by analysis of plasma samples taken over time. Testing of time samples by SDS-PAGE, followed by autoradiography, showed that detectable plasma radioactivity was bound to macromolecules similar in molecular weight profile to those injected. Results for plasma clearance are given in Fig. 5. As expected, heparin and DS rapidly disappeared from the circulation with single-phase half-lives of 0.33 and 0.50 h, respectively (Fig. 5*a*). HC-containing species were retained in the plasma phase for much longer times and their loss followed exponential decay patterns (Fig. 5, *a* and *b*). Using a two-compartment model ($Y = A_1e^{-B1X} + A_2e^{-B2X} + C$; where Y = plasma activity, X = time, A_1 and A_2 are fractional constants for the two compartments, B_1 and B_2 are rate constants for exchange between compartments, and C is the background baseline), the half-lives for the β-phase (B_1) for plasma clearance of HCH, HCD, and HC were calculated to be 4.40, 3.36, and 13.9 h, respectively. In a three-compartment model, the γ-phase half-lives of HCH, HCD, and HC were significantly longer and similar to each other (50, 40, and 48 h, respectively). Thus, the intravenous pharmacokinetics of HCH and HCD were more comparable to that of HC than clearance of the free GAGs.

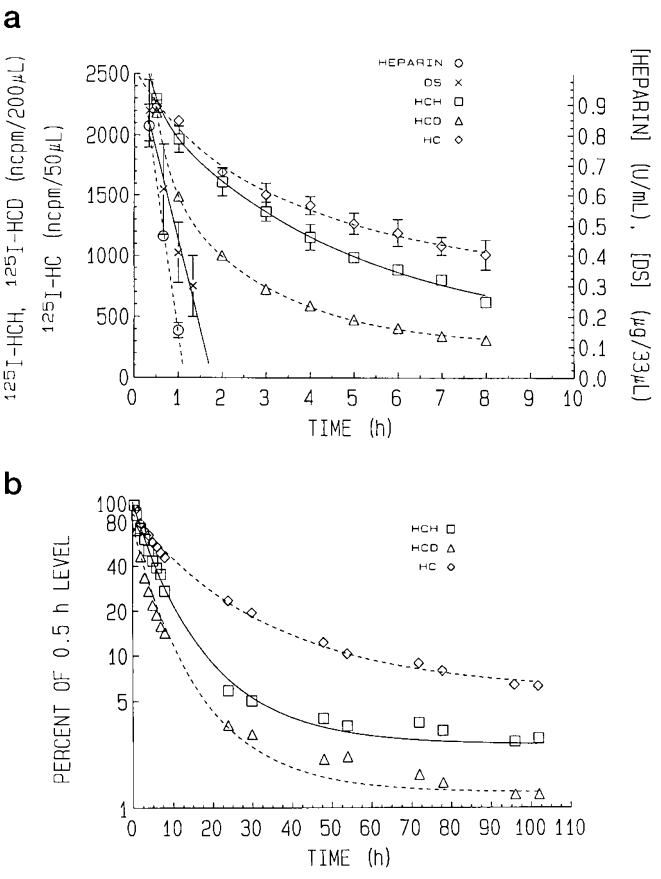

FIG. 5. **Plasma clearance of covalent heparin cofactor II-glycosaminoglycan complexes in rabbits.** Heparin (H), dermatan sulfate (DS), ^{125}I-heparin cofactor II-heparin (HCH), ^{125}I-heparin cofactor II-dermatan sulfate (HCD), and ^{125}I-heparin cofactor II (HC) were each intravenously injected into rabbits (n = minimum of 2 for each compound). Plasma samples, taken over time, were analyzed for anti-factor Xa activity (H), antithrombin DS activity, or γ-radioactivity (HCH, HCD, or HC). Plasma levels from 0–8 h and 0–102 h are shown in *panels a* and *b*, respectively. Error = ±1 S.E. Where not indicated, error bars are smaller than the symbol.

DISCUSSION

The ideal clinical anticoagulant would reliably and predictably inhibit thrombin, in particular clot-bound thrombin, without substantially increasing risk of bleeding. GAGs, commonly heparin but also DS, are used clinically for both prophylaxis and treatment of thrombosis (34, 35). The disadvantages of these GAGs, to differing degrees, include risk of bleeding (35–38), resistance of clot bound thrombin, short intravenous half-life compared with the plasma protein inhibitors (antithrombin and HC), whose action they catalyze (24, 25, 39), and decreased activity due to binding to proteins *in vivo* (14, 20, 23). We have produced and investigated the properties of two HC-GAG covalent complexes (HCH and HCD), which have a number of desirable properties that suggest they may have clinical applications related to selective thrombin inhibition.

Previous work has shown that both heparin and DS catalyze the inhibition of thrombin by HC (4). In contrast to heparin, DS causes selective inhibition of thrombin by solely activating HC which, in turn, only reacts with thrombin (3). If a permanently activated HC derivative could be constructed that possessed the prolonged intravenous half-life of the native plasma protein, the resultant product would potentially be an improved anticoagulant for thrombin regulation *in vivo*. Recently, we prepared a permanently activated inhibitor of thrombin and factor Xa by covalent linkage of heparin to antithrombin (ATH) (27). Formation of ATH conjugate relied on the existence of a subpopulation of aldose-terminating molecules in commercial heparin (33), which could form a Schiff base with antithrombin lysyl groups, followed by Amadori rearrangement (27). Since both heparin and DS have aldose termini, we subsequently applied similar methodology used for ATH synthesis in the conjugation of GAGs and HC. Covalent HC-heparin (HCH) and HC-DS (HCD) were prepared and their physical properties, *in vitro* activities, and intravenous clearance investigated.

The conditions that resulted in good yields of ATH were employed in HCH and HCD formation (pH 7.3 buffer, 40 °C). Although ATH was prepared with a conversion of ≥50% of the antithrombin into product at this concentration, pH, and temperature (data not shown), HCH and HCD were obtained at yields that were 10-fold lower. There are a number of possible reasons for the synthetic inefficiency. Lysyl groups on HC, close to where ionic GAG binding occurs (⊕ charged region of N-terminal loop; Ref. 4), may have higher pk_a values so that a higher pH is required for nucleophilic attack on the aldose carbonyl (Schiff base formation). Alternatively, temperature may not have been optimal for thermal activation. Additionally, if the number of heparin or DS molecules with high affinity HC binding sites close to their aldose termini was much less than the number of HC molecules present, only a small proportion of the non-covalent HC·GAG complexes could form covalent linkages. Possibly, critical lysyl groups required for covalent linkage of the ionically bound HC·GAG complexes are sterically hindered. Nevertheless, significant amounts of HCH and HCD conjugates were isolated. HC:GAG molar ratios were approximately 1:1 (Table I), which is in agreement with the non-covalent binding stoichiometry (4). The difference in molecular mass of HCD (90–135 kDa) *versus* HCH (84–126 kDa)

was likely a reflection of the longer average chain length of DS compared with H, as well as possible increased hydration spheres around HCD.

Both HCH and HCD directly inhibited thrombin by forming a 1:1 complex with the enzyme. The thrombin reactivity recovered in the final products was somewhat different, with HCH having a higher proportion of active molecules than HCD (94% compared with 76%). Both complexes were sensitive to denaturation, since freeze-thawing drastically reduced activity. The bimolecular rate constants of reaction of HCH or HCD with thrombin were slightly faster than similar reactions with non-covalent HC + heparin or HC + DS mixtures. This result was expected since one reaction step was eliminated in the case of the conjugates (binding of the GAGs to HC). As was observed previously with ATH (27), HCH and HCD were able to catalyze the reaction of exogenous inhibitors with thrombin (Table II). Catalysis of the inhibition of factor Xa by HCH was similar to that of starting heparin, indicating the presence of antithrombin binding sites on the heparin moiety. As expected, HCD had the same reduced factor Xa inhibition capability as starting DS. Although catalytic activities were observed for both conjugates, the specific activities for HCH's catalysis of either factor Xa or thrombin inhibition by plasma antithrombin were severalfold less than that reported for ATH (27). ATH complex formation by simple incubation involves initial non-covalent interaction between the AT and high affinity binding sites (pentasaccharide sequences) on heparin. Therefore, we proposed for ATH formation that AT selected for heparin chains which were enriched in pentasaccharide sequences (27, 40). Initial non-covalent binding of HC to heparin does not involve pentasaccharide sequences. Thus, HCH retained the same proportion of binding sites for antithrombin, in its covalently linked heparin chains, as the starting heparin.

Study of the clearance of the complexes in rabbits (Fig. 5) revealed that HCH and HCD had 7–10-fold longer intravenous half-lives than the corresponding free GAGs. By comparison, the slow rates of disappearance of the conjugates approached that of HC (HC being only ≈3 times longer (two-phase model)). HCH was lost from the circulation ≈30% more slowly than HCD. This slight difference in clearance between HCH and HCD may be simply due to the fact that, as the DS chains in HCD are longer than the heparin chains in HCH, the proportion of the DS moiety occupied by HC in HCD would be less than for the heparin component in HCH. Thus, a larger portion of the DS chain in HCD is exposed to cell surface (endothelium, hepatocyte) interactions *in vivo*, which could cause more rapid removal than HCH. Clearly, more investigation of plasma and cell surface binding of HCH and HCD will be required to determine the mechanism(s) involved in their *in vivo* pharmacokinetics.

To our knowledge, this is the first report of covalent conjugation of HC and GAGs. The coupling technique involves very mild conditions that allow for spontaneous complex formation. The HCH and HCD products react rapidly with thrombin to produce inhibitor-thrombin complexes and can also catalyze reaction of exogenous plasma inhibitors (antithrombin or HC) with coagulant enzymes. Both HCH and HCD have long intravenous half-lives, compared with heparin and DS, and clearance profiles similar to that of HC. These conjugates (particularly HCD) may have applications in the prophylaxis or treatment of clot bound thrombin, since DS-activated HC has been shown to be superior for the inhibition of clot-associated coagulant activity (9). In conclusion, the covalent HC-GAG complexes offer exciting potential alternatives for anticoagulation therapy.

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