CHARACTERIZATION OF THE PLASMA AND BLOOD ANTICOAGULANT POTENTIAL OF STRUCTURALLY AND MECHANISTICALLY NOVEL OLIGOMERS OF 4-HYDROXYCINNAMIC ACIDS[@]

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Running Title: Anticoagulant Activity of Sulfated DHPs

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Abstract

Recently, we designed sulfated dehydropolymers (DHPs) of 4-hydroxycinnamic acids that displayed interesting anticoagulant properties (Henry et al. *J. Biol. Chem.* 2007; **282**: 31891-9). Structurally and mechanistically, sulfated DHPs are radically different from all the anticoagulants studied to-date. To assess whether their unique mechanism and structure is worth exploiting for further rational design of homogeneous DHP-based molecules, we investigated their anticoagulant potential in human plasma and blood using a range of clotting assays. Sulfated DHPs prolong plasma clotting times, prothrombin and activated partial thromboplastin times, at concentrations comparable to the clinically used low molecular weight heparin, enoxaparin. Fibrin formation studies in human plasma show that there is a structural dependence of anticoagulant action. Human whole blood studies using thromboelastography and Hemostasis Analysis System indicate that they are 17–140-fold less potent than enoxaparin. The results demonstrate that sulfated DHPs possess good in vitro and ex vivo activity, which will likely be improved through rational design.

Keywords

Anticoagulant agents, dehydropolymers, thrombin inhibition; clotting times; thromboelastography; Hemostasis analysis system

Abbreviations

APTT, activated partial thromboplastin time

CEM, clot elastic modulus

DHP, dehydropolymer

DTI, direct thrombin inhibitor

FXa, factor Xa

HAS, hemostasis analysis system

LMWH, low molecular weight heparin

MA, maximum amplitude of a TEG signal

M_R, average molecular weight

PCF, platelet contractile force

PEG, polyethyleneglycol

PT, prothrombin time

T_{50}, time to reduce fibrin formation by 50%

TC, time to clot

TEG, thromboelastography

TGT, thrombin generation time

UFH, unfractionated heparin

Introduction

Thrombin and factor Xa (FXa), two key serine proteases of the coagulation cascade, have been the prime target of rational drug design for the past decade (1). Both proteases can be targeted through either the antithrombin -dependent (indirect) or -independent (direct) inhibition pathways. The direct inhibitors include peptides or peptidomimetics, e.g., hirudin, argatroban, and ximelagatran, while indirect inhibitors include heparin, low-molecular weight heparins and fondaparinux. Heparins work through antithrombin, a plasma serine proteinase inhibitor (serpin) and a major natural regulator of clotting. Full-length heparin, or unfractionated heparin (UFH) (Fig. 1A), greatly enhances the rate of antithrombin inhibition of thrombin, FXa and factor IXa under physiological conditions, which is the major mechanism involved in its anticoagulant action (2). Yet, UFH suffers from several limitations including bleeding risk, variable patient response, heparin-induced thrombocytopenia and the inability to inhibit clot-bound thrombin (3, 4). Low molecular weight heparins (LMWHs), derivatives of UFH with reduced polymeric length, and fondaparinux, a specific sequence of five saccharide residues (Fig. 1A), have been introduced in the past two decades as agents with higher specificity for FXa. Another heparin pentasaccharide, idraparinux, is likely to be introduced shortly [5,6]. Yet, each newer agent retains bleeding risk and is unable to inhibit clot-bound thrombin (7,8).

The problems of heparin-based therapy arguably arise from the structure of UFH. The large number of sulfate groups introduces phenomenal anionic character in the polysaccharide. The average molecular weight (M_R) of UFH is ~15,000 implying the presence of ~65–85 negative charges on average on a single chain (9). In addition to this polyanionic character, heparin biosynthesis results in millions of sequences that differ from each other in the placement of sulfate groups, thereby generating considerable microheterogeneity and polydispersity. Both

these structural features introduce a large number of interactions with plasma proteins and cells (10), which are potentially the cause of heparin's adverse effects.

With respect to the other pathway of regulating thrombin and fXa, the direct inhibition pathway, several molecules have been put forward such as argatroban, ximelagatran and dabigatran for thrombin and, rivaroxaban, DX9065a and razaxaban for factor Xa [11-13]. Direct thrombin inhibitors (DTIs) and factor Xa inhibitors form a major class of clotting regulators that are considered to be superior to heparins primarily because of the expectation that they can inhibit both circulating and clot-bound enzymes. Yet, challenges exist in the development of these inhibitors including establishing enzyme-binding affinity that is not associated with excessive bleeding and avoiding liver toxicity (14).

We reasoned that reducing UFH's high negative charge density would reduce its adverse effects. At the same time, enhancing its hydrophobic character would possibly induce greater specificity of action. Thus, we designed sulfated dehydropolymers (DHPs) of 4-hydroxycinammic acids as advanced mimics of UFH and LMWH (Fig. 1B). Sulfated DHPs are typically prepared in high yields in two simple steps, an enzymatic coupling of 4-hydroxycinnamic acid monomers followed by a chemical sulfation step (15). Initial studies suggested that the designed sulfated DHPs can reduce clotting (15). Recently, we discovered that although designed as mimics of heparin, sulfated DHPs do not prefer to utilize the indirect pathway of thrombin and factor Xa inhibition. In fact, the dominant mechanism of inhibition is direct allosteric inhibition of thrombin and factor Xa (16). Allosteric inactivation of the two key proteinases arises from binding in anion-binding exosite II. This is the first observation of thrombin inhibition arising from exclusive exosite II interaction. The dominant mechanism of factor Xa inhibition remains unstudied, but is likely to involve its anion-binding exosite II.

In addition to this unique mechanistic feature, sulfated DHPs are also structurally distinct. Except for the presence of sulfate groups, DHPs possess a scaffold unlike any other anticoagulant investigated to-date. The unique mechanism of action and novel structure of DHPs may lead to a homogeneous molecule that exhibits more specificity and reduced side-effects in comparison to current agents. However, before a homogeneous molecule can be designed, it is important to answer the question whether DHPs possess sufficient anticoagulant activity in plasma and blood for further rational design. To answer this question, we studied the anticoagulant potential of DHPs in several *in vitro* and *ex vivo* systems including activated partial thromboplastin time (APTT), prothrombin time (PT), thromboelastography (TEG®) and Hemostasis Analysis System (HAS™) and compared it to a clinically used anticoagulant, enoxaparin. Our studies show that sulfated DHPs are fairly potent anticoagulants in human plasma and blood. These results support the need to explore the DHP scaffold in the design of novel anticoagulants.

Methods

Proteins, Chemicals and Coagulation Assay Conditions

Sulfated DHPs, CDSO3, FDSO3 and SDSO3 (Fig. 1B) were prepared in two steps from 4-hydroxycinnamic acid monomers, caffeic acid, ferulic acid and sinapic acid, as described previously (15). Stock solutions of sulfated DHPs were prepared in deionized water and stored at –80^{O}C. Pooled normal human plasma for coagulation time assays was purchased from Valley Biomedical (Winchester, VA). Activated partial thromboplastin time reagent containing ellagic acid (APTT-LS), thromboplastin-D and 25 mM $CaCl_2$ were obtained from Fisher Diagnostics (Middletown, VA). Thromboelastograph® Coagulation Analyzer 5000 (TEG®), disposable cups

and pins, and 200 mM stock $CaCl_2$ were obtained from Haemoscope Corporation (Niles, IL). LMWH (M_R 5,060) was purchased from Sigma (St. Louis, MO), while enoxaparin (M_R 4,500) was from Aventis Pharmaceuticals. All other chemicals were analytical reagent grade from either Sigma Chemicals (St. Louis, MO) or Fisher (Pittsburgh, PA) and used as obtained.

` **Inhibition of $CaCl_2$-initiated Fibrin Formation in Plasma by Sulfated DHPs**

A 650 µL aliquot of freshly thawed pooled human plasma was co-incubated with 10 µL sulfated DHP (either 3 or 5 mg/ml in H_2O) and 200 µL APTT-LS reagent at 37 ^{O}C for 5 minutes. Following incubation, 850 µL of this sample was transferred to a PEG 20000-coated polystyrene cuvette. Fibrin formation was initiated by rapidly adding 50 µl of 20 mM Tris-HCl buffer, pH 7.4, containing 100 mM NaCl, 25 mM $CaCl_2$, and 0.1% PEG8000, and 200 µl of 25 mM $CaCl_2$. Following initiation, the transmittance at 600 nm was continuously monitored until a plateau was reached corresponding to the formation of a solid fibrin polymer.

Inhibition of Thromboplastin-D-initiated Fibrin Formation in Plasma by Sulfated DHPs

A 200 µL aliquot of freshly thawed citrated human plasma was co-incubated with 10 µL sulfated DHP (or the reference molecule) and 390 µL of water at 37 ^{O}C for 5 minutes. Following incubation, 600 µL of the sample was transferred to a PEG 20000-coated polystyrene cuvette. Clotting was initiated by rapidly adding 400 µL of pre-warmed thromboplastin-D reagent and the decrease in transmittance at 600 nm was monitored continuously until a plateau was reached. The time to clot, or the lag time, was calculated as the time necessary for the transmittance to decrease by 1% from the initial value. Likewise, the time necessary for a 50% decrease in transmittance from the initial value was also obtained.

Prothrombin Time and Activated Partial Thromboplastin Time

Clotting time was determined in a standard 1-stage recalcification assay with a BBL Fibrosystem fibrometer (Becton-Dickinson, Sparles, MD). For PT and APTT assays, the reagents were pre-warmed to 37°C. For PT assays, 10 µL sulfated DHP (or the reference molecule) was mixed with 90 µL of citrated human plasma, incubated for 30 s at 37 ^{O}C followed by addition of 200 µL pre-warmed thromboplastin. For APTT assays, 10 µL sulfated DHP was mixed with 90 µL citrated human plasma and 100 µL 0.2% ellagic acid. After incubation for 220 s, clotting was initiated by adding 100 µL of 25 mM $CaCl_2$. Each experiment was performed at least twice. The averaged data was fitted by a quadratic equation to calculate the concentration of the anticoagulant necessary to double the clotting time (2×APTT or 2×PT).

Thromboelastograph (TEG®) Analysis of Clot Formation in the Presence of Sulfated DHPs

The TEG® assays were performed essentially as reported earlier (17). Briefly, the assays were initiated by transferring 20 µL of 200 mM $CaCl_2$ into the Haemoscope™ disposable cup, oscillating through 4^0 45' angle at 0.1 Hz, followed by the addition of a mixture of 340 µL of sodium citrated whole blood containing 10 µL sulfated DHP or dH_2O (control) at 37 ^{O}C. This recalcification initiates clot formation in the TEG® coagulation analyzer, which operates until all necessary data collection (R, K, α and MA) is completed in an automated manner.

Hemostasis Analysis System (HAS™) Analysis of Clot Formation in the Presence of Sulfated DHPs

Analysis of platelet function and clot structure was performed using the HAS™ (Hemodyne, Inc., Richmond, VA). A mixture of 700 µl of citrated whole blood and 10 µl sulfated DHP or ddH_2O (control) was co-incubated at room temperature for 5 minutes and then

700 µl was placed in a disposable cup. To initiate clotting, 50 µl of 150 mM $CaCl_2$ was added to 700 µl of the blood – DHP mixture to give a final $CaCl_2$ concentration of 10 mM, while the cone was simultaneously lowered into the recalcified blood sample. As the clotting proceeds, platelets attach to both surfaces generating tension within the fibrin meshwork. This tension is measured with a displacement transducer in terms of platelet contractile force (PCF). The onset of PCF is a measure of thrombin generation time (TGT), while clot elastic modulus (CEM) is the ratio of the applied force (stress) by the transducer to the measured displacement (strain). The HAS^{TM} system operates in an automated manner until all data is collected.

Results and Discussion

Structure of Sulfated Dehydropolymers (DHPs) of 4-Hydroxycinnamic Acids

Three synthetic sulfated DHPs – CDSO3, FDSO3 and SDSO3 (Fig. 1B) – were studied. The molecules were prepared in two steps from caffeic acid, ferulic acid and sinapic acid, each of which contains a common scaffold, the 4-hydroxycinnamic acid monomer (Fig. 1B) (15). Briefly, the sulfated DHPs (M_R ~2,500 – 4,000) are a mixture of oligomeric chains that contain 4 – 15 monomers suggesting that the molecules are comparable in size to enoxaparin (M_R ~5,000) (15). In addition, the DHPs contain several types of inter-monomeric linkages (Fig. 1B), thereby generating polydispersity and heterogeneity, a property they share with LMWHs. Yet, sulfated DHPs are significantly less sulfated than heparins. Whereas sulfated DHPs contain an average of 0.33 sulfate group per monomer, LMWHs possess an average of 1 – 1.3 sulfate groups for every saccharide residue. More importantly, sulfated DHPs possess a large number of aromatic rings in the backbone, while heparins have none. Thus, sulfated DHPs are significantly more hydrophobic than the LMWHs.

Effect of Sulfated DHPs on Fibrin Formation in Normal Human Plasma

To determine whether our sulfated DHPs prolong fibrin formation in plasma, we utilized *in vitro* transmittance assays. Addition of $CaCl_2$ to normal pooled human plasma under APTT-like conditions triggers 'coagulation' resulting in the synthesis of fibrin, which blocks the passage of light through the sample. A characteristic decrease in transmittance at 600 nm as a function of time is observed from which the time to clot (TC) and the time it takes to reduce the transmittance, i.e., clotting, by 50% (T_{50}) can be measured. The presence of all three sulfated DHPs prolonged fibrin synthesis, as shown by the delayed decrease in transmittance at 600 nm (Fig. 2A). For SDSO3, the T_{50} value changed from 79 s to 161 s as the concentration was increased from 27 to 45 μg/ml (Fig. 2A, Table 1). Similarly, the T_{50} values were 272 and 593 s, and 93 s and 247 s at 27 and 45 μg/ml for CDSO3 and FDSO3, respectively. These initial results demonstrated that sulfated DHPs prolong fibrin formation in a dose-dependent manner. The results also suggest that the anticoagulation potency varied with the structure of the sulfated DHP (see Fig. 1).

To assess whether anticoagulant potency of sulfated DHPs is retained if coagulation is initiated through the extrinsic pathway, thromboplastin-D was used as an initiator of clotting. Once again, the presence of all three sulfated DHPs significantly slowed down the formation of fibrin in normal plasma suggesting that three molecules inhibit clotting (not shown). Figure 2B and 2C show the change in TC and T_{50} as a function of the concentration of each sulfated DHP (and reference molecule, enoxaparin). Both TC and T_{50} increase as the concentration of sulfated DHP increases. The increase is not linear and is accelerated at higher concentrations of the anticoagulant. More interestingly, the three sulfated DHPs display an anticoagulation profile similar to enoxaparin. While, CDSO3 is more potent than enoxaparin, FDSO3 and SDSO3 are

less potent. For example, the concentration of anticoagulant needed to double the time for 50% fibrin formation was found to be ~3.5 μM for CDSO3 and 6, 11 and >25 μM for enoxaparin, FDSO3 and SDSO3, respectively.

Effect of Sulfated DHPs on Clotting Times

PT and APTT are commonly used to assess the coagulation status of human plasma (18). All three sulfated DHPs exhibited a significant concentration-dependent prolongation of PT and APTT (not shown). A typical parameter for describing anticoagulant activity in these assays is the concentration of the anticoagulant needed for doubling the normal plasma clotting time (2×PT or 2×APTT). The 2×PT value for sulfated DHPs ranged from 13.1–33.3 μM, while that for enoxaparin was 75.3 μM suggesting the new molecules are 2.3–5.7-fold more potent in the PT assay (Table 1). The doubling of APTT required 2.9–6.4 μM concentration of the three sulfated DHPs, while enoxaparin required 1.2 μM. This indicates that the sulfated DHPs are ~2.4–5.3-fold weaker anticoagulants in the APTT assay as compared to enoxaparin and the order of activity is CDSO3 > FDSO3 > SDSO3 (Table 1).

Thromboelastographic Measurement of Effect of Sulfated DHPs on Whole Blood Clotting

Whole blood clotting is a dynamic process that involves many components including cells, which may alter anticoagulant potency. To compare sulfated DHPs and enoxaparin in a whole blood system, we employed thromboelastography (TEG®), a technique used in clinical settings for following anticoagulation with LMWHs (19-21). TEG® measures various responses of a formed clot to shearing force. In this technique, a pin is inserted into an oscillating cup containing whole blood. As fibrin polymerizes, the pin starts to move with the oscillating cup and the movement of the pin is recorded as amplitude, which in time reaches maximum

amplitude (MA) (Fig. 3A). The stronger the clot, the more the pin moves with the cup and the higher the MA. Shear elastic modulus strength (G), a measure of clot stiffness, is calculated from MA. Additionally reaction time R and angle α (Fig. 3A) are also obtained in a TEG® experiment. R is the time required for the initial fibrin formation, while α is the acute angle in degrees between an extension of the R tracing and the tangent of the maximum slope produced by the TEG® tracing during clot stiffening. Angle α is a measure of the rate of formation of three-dimensional fibrin network. Parameters that affect MA include fibrin concentration and structure, concentration and functional state of platelets, deficiency of coagulation factors and presence of clotting inhibitors (22).

All three sulfated DHPs affect R, α, MA and G parameters in a dose-dependent manner (see Table S1 in Supplementary Material). Briefly, as the concentration of CDSO3 increases from 0 to 24.3 μM, R increases from 7.0 to 21.5 min. This effect parallels the time to clot results obtained in the plasma assay. Likewise, sulfated DHPs lower the value of angle α from 59° for normal blood to 13.5–17° at the highest concentrations studied. This indicates that the kinetics of fibrin polymerization and networking is significantly retarded by the presence of sulfated DHPs. Enoxaparin exhibits similar characteristics, except that it is 23–51-fold more potent than sulfated DHPs when comparisons are made at doubling the R value from its value in the absence of any anticoagulants (not shown). Likewise, enoxaparin is 17–32-fold and 18–37-fold more potent when comparisons are made for a 50% reduction in the angle α and shear elastic modulus G, respectively (Fig. 3B).

Effect of Sulfated DHPs on Whole Blood Coagulation as Evaluated by Hemostasis Analysis System

To further compare the whole blood anticoagulant potential of the sulfated DHPs with enoxaparin, we performed an *ex-vivo* study using HAS™, which measures the forces generated by platelets within a clot (23). In this technique, the clot is allowed to form between a temperature-controlled lower surface (cup) and a parallel upper surface (cone). As the clot grows, it attaches to both the surfaces pulling the fibrin strands inward. This pull is measured by a displacement transducer, which produces an electrical signal on the cone proportional to the amount of force generated by the platelets. HAS™ also provides detailed information on clot structure through the measurement of clot elastic modulus (CEM), which is the ratio of stress induced by platelets to strain arising from the change in clot thickness (24). PCF is observed to increase as soon as thrombin is formed suggesting that appearance of PCF can be used as surrogate marker for TGT (thrombin generation time), the minimal time required for production of thrombin following initiation of clotting (23).

In addition to its dependence on thrombin, PCF is sensitive to platelet number, platelet metabolic status, presence of thrombin inhibitors and degree of GPIIb/IIIa exposure (20,25-27). Likewise, CEM is a complex parameter that is sensitive to changes in clot structure, fibrinogen concentration, the rate of thrombin generation and red blood cell flexibility, while TGT is sensitive to clotting factor deficiencies, antithrombin concentration and presence of anticoagulants. Low PCF and low CEM coupled with a prolonged TGT are associated with increased bleeding risk, while elevated PCF and CEM paired with a decreased TGT are associated with thrombotic disease states.

All three DHPs affect TGT, PCF and CEM parameters in a dose-dependent manner (see Table S2 in Supplementary Material). For example, as the concentration of FDSO3 increases from 0 to 23.8 μM, the TGT value increases from 235 seconds to 465 seconds (Fig. 4A). This effect parallels the results obtained in the plasma thrombogenesis assay and TEG®. More importantly, the presence of sulfated DHPs in blood decreases PCF from 7.6 Kdynes to 2.4–1.2 Kdynes at 14–37 μM (Fig. 4B), while enoxaparin induces a PCF of 0.9 Kdynes at 0.44 μM. When comparisons are made for a 50% reduction in PCF, enoxaparin is 63–140-fold more potent. Likewise, sulfated DHPs decrease CEM from 21.6 Kdynes/cm^2 for normal blood to 4.5–1.3 Kdynes/cm^2 at the highest concentrations studied. Comparison of CEM values indicates that enoxaparin is 43–90-fold more potent than sulfated DHPs (Fig. 4C). These results confirm that sulfated DHPs behave in a manner similar to enoxaparin, except for the concentration at which these are effective.

Conclusions and Significance

The major conclusion of this work is that sulfated DHPs display whole blood anticoagulation properties similar to a clinically used anticoagulant, enoxaparin, although the effective concentration range is different. In TEG® and HAS™ assays, sulfated DHPs are 17–51-fold and 43–140-fold less active than enoxaparin, respectively. Considering that the structure and mechanism of action of sulfated DHPs is radically different from all known anticoagulants (15,16), this is a significant observation. It implies that a new class of more potent molecules, most likely homogeneous and synthetically accessible, may be possible to design from the DHP scaffold. We expect that this class of molecules will utilize allosteric modulation of thrombin and factor Xa activity through binding in exosite II, the first molecules to display this unique mechanism (16). In addition, our previous results using enzyme inhibition assays show that

unsulfated DHPs also possess significant anticoagulation potential (15). This suggests that it may be possible to design un-sulfated, synthetic molecules with a unique mechanism of action. A specific advantage expected of these un-sulfated homogeneous DHP-based structures is that absence of sulfate group would make the molecules orally bioavailable.

The work presented here shows that in the PT assay, the three sulfated DHPs are effective at concentrations in the range of enoxaparin, while in the APTT assay they are only 2–6-fold weaker. Despite major mechanistic differences, both sulfated DHPs and enoxaparin prolong APTT better than PT. Inhibition of fibrin formation in plasma shows that CDSO3 was comparable to enoxaparin. Yet, sulfated DHPs are much weaker in whole blood than enoxaparin. It is possible that the significant hydrophobic character of sulfated DHPs induces binding to cells resulting in significant sequestering of active agent. It is likely that this non-specific binding will be reduced with homogeneous, synthetic small molecules.

Overall, the results demonstrate that sulfated DHPs possess good plasma and whole blood anticoagulation activity. This does not imply that our novel molecules will be clinically effective. Toxicity studies will have to be performed to ascertain that these novel structures do not induce abnormal effects. An important point to note in this regard is that in vivo enoxaparin does not prolong PT and APPT at concentrations sufficient to anticoagulate suggesting that in vitro or ex vivo potency does not translate directly into in vivo effectiveness. Yet, the results described here suggest that the novel structure and mechanism of sulfated DHPs may lead to a new class of potent anticoagulants.

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Figure Legends

Figure 1. **Structures of heparins (A) and sulfated DHPs (B).** A) Fondaparinux is based on heparin pentasaccharide, while heparin and LMWHs are polydisperse, heterogeneous mixture of polysaccharide chains (M_R ~15,000 and ~5,000 Da, respectively) arising due to variations in X, Y, Z and R groups. B) Sulfated DHPs possess a radically different structure from the heparins (and other anticoagulants) and are synthesized in two steps from the corresponding 4-hydroxycinnamic acid monomers, caffeic acid (CA), ferulic acid (FA) or sinapic acid (SA). The M_R of the sulfated DHPs is in the range of 2,500–4,000. Linkages, β-*O*-4 and β-5, are commonly present in sulfated DHPs (shown as shaded ovals).

Figure 2 **Inhibition of fibrin formation in pooled normal human plasma in the presence of sulfated DHPs.** Fibrin formation as a function of time was monitored using the decrease in transmittance of light at 600 nm following initiation of 'clotting' under APTT conditions (A) and PT conditions (B). A) CDSO3 (bold black lines), FDSO3 (bold grey lines) and SDSO3 (thin black lines). Concentrations of sulfated DHPs used were either 27 μg/ml (denoted as 'L') or 45 μg/ml ('H'). B) Time to clot (TC) and C) time needed for 50% formation of fibrin (T_{50}) values are plotted as a function of the concentration of sulfated DHP and enoxaparin. Solid lines in both B) and C) are trendlines, not non-linear regressions. Δ = CDSO3; ♦ = enoxaparin; ● = FDSO3; □ = SDSO3. See text for details.

Figure 3 **Comparison of the effect of sulfated DHPs and enoxaparin on clot formation in whole blood using TEG®.** Inset in (A) shows a typical thromboelastogram

expected of any anticoagulant. MA, R, α and G are parameters obtained from TEG® analysis. See Methods for details. (B) shows the variation in G as a function of concentration of the sulfated DHPs and enoxaparin. Solid lines are trendlines (not regression fits) from which concentration of anticoagulant needed to reduce shear elastic modulus G by 50% (shown as shaded line) of the starting value was derived.

Figure 4 **Comparison of the effect of sulfated DHPs and enoxaparin on platelet function in whole blood using HAS™.** A) shows selected HAS™ profiles obtained with FDSO3, B) and C) show the variation in PCF and CEM, respectively, as a function of concentration of the sulfated DHPs and enoxaparin. Solid lines are trendlines from which the concentration of anticoagulant needed to reduce PCF or CEM by 50% (shaded line) of the starting value was derived.

Table 1. Effect of sulfated DHPs and enoxaparin on human plasma clotting using fibrin formation and clotting time assays.

	Fibrin Formation Assay		*Clotting Time Assay*	
	27 μg/ml	***45 μg/ml***	***2 × PT[a]***	***2 × APTT[b]***
	(s)	*(s)*	(*μM*)	(*μM*)
CDSO3	272	593	13.1 ± 1.3	2.9 ± 0.8
FDSO3	93	247	16.6 ± 1.7	4.1 ± 0.4
SDSO3	79	161	33.3 ± 3.0	6.4 ± 1.2
Enoxaparin	--[c]	--	75.3 ± 2.0	1.2 ± 1.1

[a]The normal human plasma PT was found to be 12.2 sec. [b] The uninhibited human plasma APTT was 29.5 sec. [c] not performed.

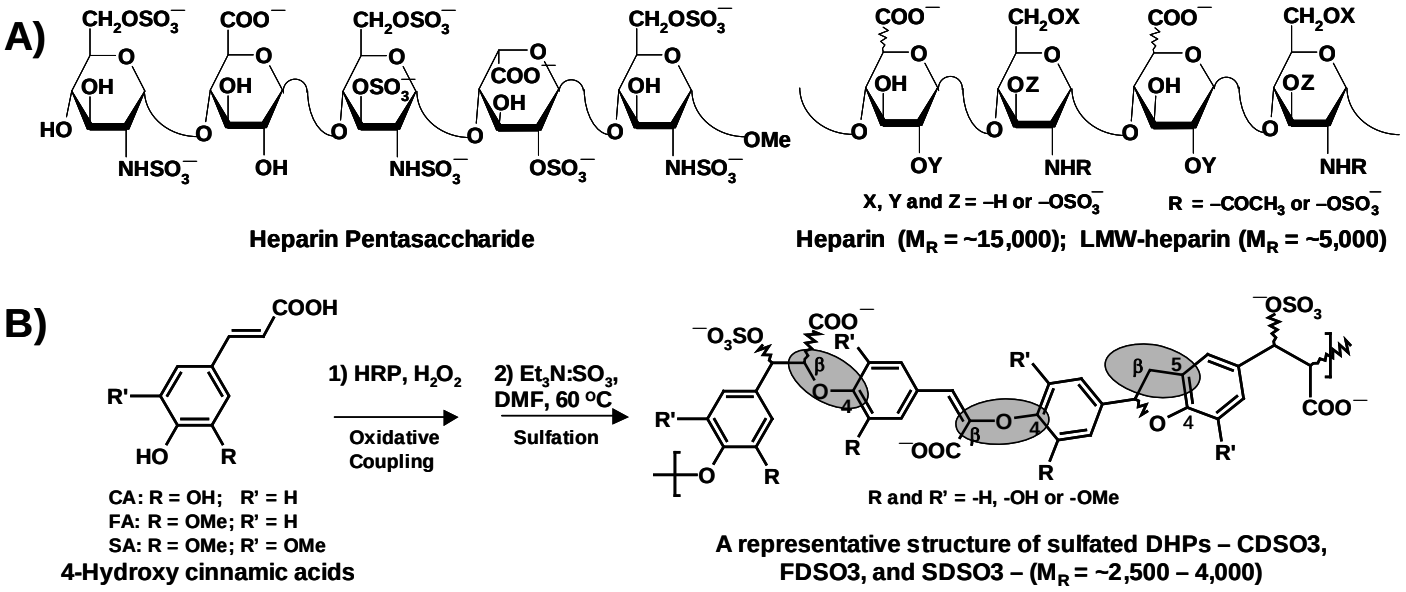
A)
Heparin Pentasaccharide
X, Y and Z = –H or –OSO3−
R = –COCH3 or –OSO3−
Heparin (MR = ~15,000); LMW-heparin (MR = ~5,000)
B)
1) HRP, H2O2
Oxidative Coupling
2) Et3N:SO3, DMF, 60 °C
Sulfation
CA: R = OH; R' = H
FA: R = OMe; R' = H
SA: R = OMe; R' = OMe
4-Hydroxy cinnamic acids
R and R' = -H, -OH or -OMe
A representative structure of sulfated DHPs – CDSO3, FDSO3, and SDSO3 – (MR = ~2,500 – 4,000)

Figure 1

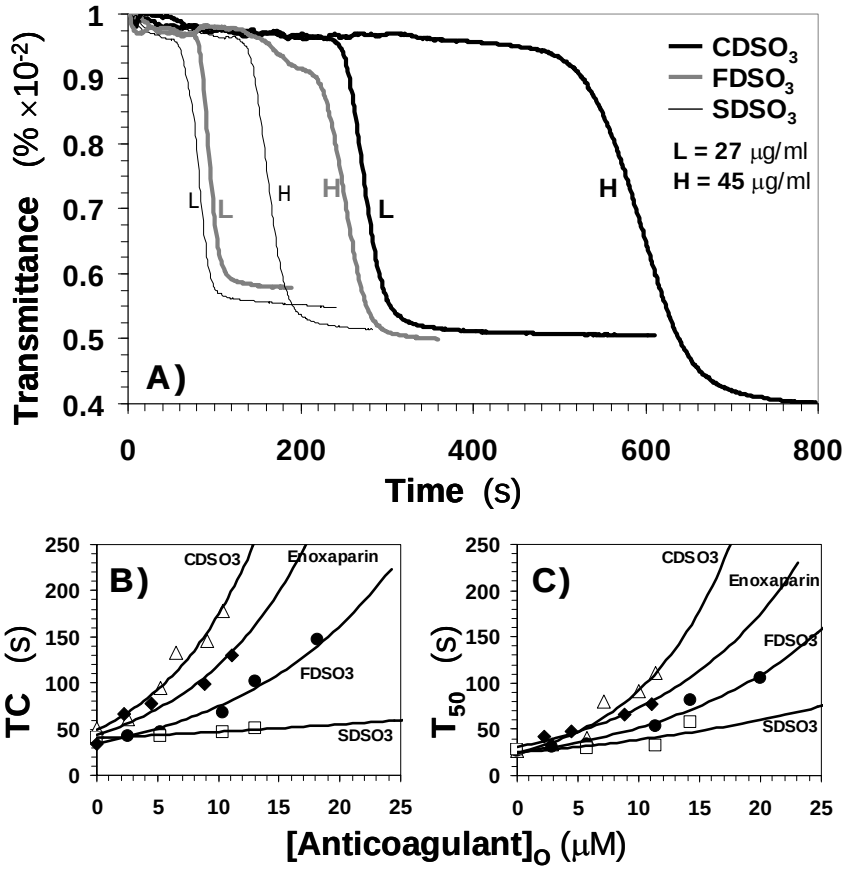
A)
Transmittance (% ×10-2)
Time (s)
CDSO3
FDSO3
SDSO3
L = 27 μg/ml
H = 45 μg/ml
L
L
H
H
L
H
B)
TC (s)
CDSO3
Enoxaparin
FDSO3
SDSO3
C)
T50 (s)
CDSO3
Enoxaparin
FDSO3
SDSO3
[Anticoagulant]O (μM)

Figure 2

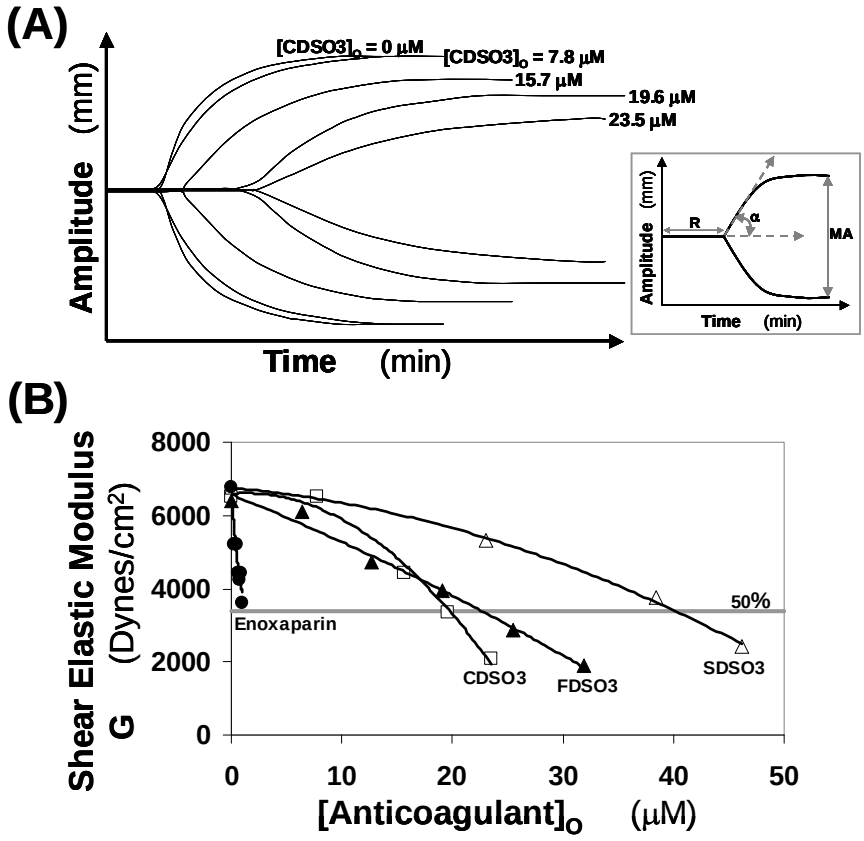
(A)
[CDSO3]o = 0 µM
[CDSO3]o = 7.8 µM
15.7 µM
19.6 µM
23.5 µM
Amplitude (mm)
Time (min)
R
α
MA
Amplitude (mm)
Time (min)
(B)
Shear Elastic Modulus G (Dynes/cm2)
8000
6000
4000
2000
0
Enoxaparin
50%
CDSO3
FDSO3
SDSO3
0
10
20
30
40
50
[Anticoagulant]o (µM)

Figure 3

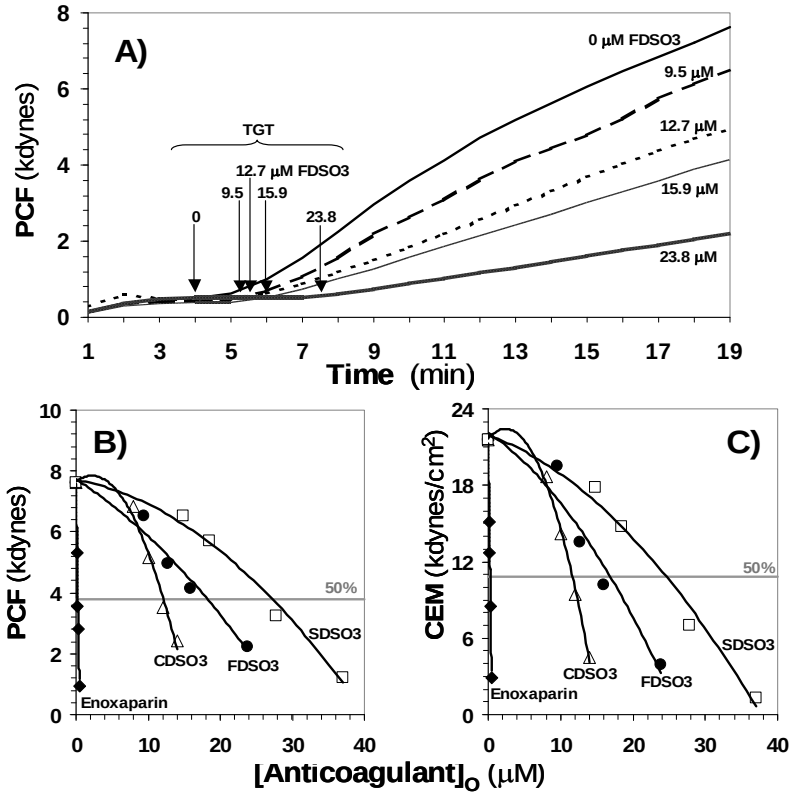
A)
PCF (kdynes)
Time (min)
TGT
0
12.7 µM FDSO3
9.5
15.9
23.8
0 µM FDSO3
9.5 µM
12.7 µM
15.9 µM
23.8 µM
B)
PCF (kdynes)
50%
SDSO3
CDSO3
FDSO3
Enoxaparin
C)
CEM (kdynes/cm²)
50%
SDSO3
CDSO3
FDSO3
Enoxaparin
[Anticoagulant]o (µM)

Figure 4