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The Anticoagulant, Antiprotease and Antithrombotic Actions of Various Heparin Fractions

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THE ANTICOAGULANT, ANTIPROTEASE AND ANTITHROMBOTIC ACTIONS OF VARIOUS HEPARIN FRACTIONS

by

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VITA

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His elementary education was obtained at St. Mary's school, Riverside, Illinois. The author attended Marmion Military Academy, Aurora, Illinois, obtaining his high school diploma in 1973.

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LIST OF ABBREVIATIONS

1.	APTT	Activated Partial Thromboplastin Time
2.	AT - III	Antithrombin III
3.	AUC	Area Under Concentration Time Curve
4.	CBC	Complete Blood Count
5.	Cl_p	Plasma Clearance
6.	CPDA	Citrate, Phosphate, Dextrose, Adenine
7.	CY 216	Low Molecular Weight Heparin Code Name
8.	FPA	Fibrinopeptide - A
9.	FPAGT	Fibrinopeptide - A Generation Test
10.	GPC	Gel-Permeation Chromatography
11.	HPLC	High Performance Liquid Chromatography
12.	IV	Intravenous
13.	IU	International Unit
14.	K_e	Rate constant of Elimination
15.	M.W.	Molecular Weight
16.	NHP	Normal Human Plasma
17.	PCC	Prothrombin Complex Concentrate
18.	pNA	para Nitroaniline
19.	PT	Prothrombin Time
20.	RVV	Russell's Viper Venom
21.	SMAC	Sequential Multiple Analyte Computer
22.	t 1/2	Half-life
23.	t-PA	Tissue Plasminogen Activator
24.	U	Unit
25.	USP	United States Pharmacopeia
26.	Vd_a	Apparent Volume of Distribution

CHAPTER I

INTRODUCTION AND REVIEW OF THE LITERATURE

I. INTRODUCTION TO HEPARIN

A. History

Heparin is a commonly used anticoagulant drug indicated for the prophylaxis and treatment of thromboembolic disorders. Although it has been investigated for almost 70 years and been in clinical use for over thirty years, many questions remain relative to its pharmacodynamic actions (Jaques, 1979).

The discovery of heparin is attributed to the serendipitous observations of Jay McClean, a medical student at Johns Hopkins working under the direction of professor W.H. Howell. Howell's interests were in the investigation of natural thromboplastins. He assigned McClean the task of extracting and observing the activity of various phosphatides such as cephalin and a hepatic extract termed heparophosphatide. During these studies McClean noticed that the thromboplastic activity of the phosphatide was not only lost after prolonged storage, but actually prolonged coagulation times in his assay system. Further investigations by Howell and Holt, (1918) demonstrated that these substances were also active after intravenous administration in dogs. Since these

substances were especially abundant in the liver, Howell named them heparins. Further characterization revealed that these substances contained no phosphorus and were not actually phosphatides, but rather sulfur containing carbohydrates (Howell & Holt, 1928). These heparins demonstrated weak anticoagulant activity and were to toxic for therapeutic use.

Using a different extraction method, Scott and Charles, (1933) reported that a relatively pure high yield of heparin could be obtained from beef lung. In this system, purification by tryptic digestion and alcohol precipitation removed protein and fatty contaminants. Additional work by Scott and Charles, (1936) resulted in the preparation of a crystalline form of heparin as either a barium or sodium salt. The enhanced efficiency of these extraction and purification processes resulted in sufficient quantities of suitable material for clinical evaluation.

B. The Chemistry of Heparin

Chemically heparin is best characterized as a family of linear anionic polysaccharide chains with a highly heterogeneous distribution of molecular weights. The molecular weights of the individual components generally range from 1,000 through 50,000 daltons. The mean molecular weight is usually between 9,000 and 15,000

daltons, however these figures vary with both the source and method of extraction. It has been shown that beef lung heparin has different molecular weight characteristics compared to porcine mucosal heparin (Cifonelli & King, 1970 ; Bianchini et al., 1976).

Reports from various investigators have shown that commercial heparin preparations contain at least 21 discrete molecular species identifiable by electrofocusing (Mc Duffie et al., 1975 ; Dietrich et al., 1975). Most likely twenty-one is the minimal number since the separation loses resolution at the longest chains. For this reason the number may be as high as 23 or 24. These molecular species vary in molecular weight, net charge, charge density and ratio of hexose residues. The heterogeneity within the components of heparin has been shown to be a consequence of the natural synthetic process rather that an artifact of preparation (Cifonelli, 1974). A conceptual representation of the diversity of heparin is seen in appendix I. Recently, heparin preparations have also been shown to vary in terms of the proportion of individual molecular weight components (Barlow, 1983, 1985). Not only are heparins heterogeneous in terms of their composition of molecular weight components, but also in the distribution of these components. Thus, it is possible that heparins with similar mean molecular weights may differ in the relative proportion of individual

components.

The polysaccharide chains of heparin are constructed with alternating residues of uronic acid (either L iduronic or D-glucuronic acid) and D-glucosamine monosaccharide units joined via alpha 1-4 glycosidic linkages. These residues are sulfated and acetylated to a varying degree, however most glucosamine units possess an O-sulfate at carbon six, while most iduronic acid residues are sulfated at carbon two. An O-sulfate group at carbon three is thought to be critical to the antithrombin III binding sequence (Lindahl et al., 1980). A drawing representing the chemical structure of heparin is seen in appendix II.

Commercial heparin is generally extracted from either bovine lung or porcine intestinal mucosa and prepared as either a sodium or calcium salt. However, barium and potassium salts are also prepared. These metals are usually present at concentrations of about 12 %. No difference has been observed in the pharmacologic properties between any of the different salt preparations and it is generally assumed that they are equivalent (Cocchetto and Bjornsson, 1984).

C. Standardization of Heparin

Due to the inherent variability within the sources and methods of manufacturing, it is necessary to standard-

ize commercial heparin preparations in terms of biological actions. These actions are quantified in units of anti-coagulant activity.

The first unit was defined by Howell as the amount of heparin that would prevent the clotting of one ml. of freshly drawn cat blood overnight in the cold (Howell, 1928). The Connaught unit was essentially similar to the Howell unit, however the potency was determined by comparison to a defined reference heparin preparation (Scott & Charles, 1933). The need for international standardization of heparin resulted in the establishment of the first international standard and unit of heparin in 1942. The unit was defined as 1/130 mg of the international standard heparin preparation (prepared for and held by the World Health Organization Division of Biological Standards Geneva, Switzerland). Since its origin, the international standard has been successively modified such that currently the fourth international standard is utilized. Unfortunately, no specific assay was described for use with the international standards. Consequently these standards have been used in a variety of assays for heparin's potency evaluation. Since the values of the international standards vary in different assay systems, the international standards have proven an unreliable method for the standardization of heparin (Brozovic & Bangham, 1974).

The U.S. Pharmacopeial Unit (USP) was originally defined as the amount of activity present in 1/130 mg of the second international standard heparin. Unlike the international unit, the USP unit utilizes a specific coagulant method to evaluate the potency of heparin. The assay measures heparin's inhibition upon recalcified activated sheep plasma (U.S. Pharmacopeia XX, 20th revision). Although this method relies on a semi-quantitative visual evaluation of the clot endpoint, it has proven to be very reproducible among individual heparin preparations.

The British Pharmacopeial (BP) assay has also been widely used for heparin potency evaluation and standardization. This assay is based upon the inhibition of thromboplastin activated oxalated ox blood by heparin. When compared to USP standardized heparins, this assay gives higher results for lung heparins but lower values for mucosal heparins (Brozovic & Bangham, 1974).

Recently, the USP has established an assay for standardizing heparin in terms of anti Xa units. This assay measures the inhibition of standardized bovine factor Xa in the presence of heparin using a Xa specific chromogenic substrate. The amount of anti factor Xa activity per mg in USP units must not be less than 80 % nor more than 120 % of the potency of heparin in USP heparin units per mg as determined by the assay

(Pharmacopeial Forum, 1984).

D. Overview of Coagulation

In order to understand the mechanisms and sites of action of heparin, a basic comprehension of the coagulation network is helpful.

Blood coagulation occurs through a series of complex interactions between at least 15 distinct plasma glycoproteins. A description of the names, functions, approximate molecular weights and normal plasma concentrations is listed in appendix III. With the exception of fibrinogen these glycoproteins are classified as either protein cofactors or enzyme precursors. The precursors circulate as non-activated zymogens and are converted to active enzymes upon proteolytic activation. Most of these enzymes are serine proteases which produce limited proteolytic cleavage at peptide bonds on the carboxyl side of arginine.

In its most simplified form, the coagulation network can be represented as two distinct, but interacting pathways which result in the generation of thrombin. These pathways, the intrinsic and extrinsic are so designated based upon their mechanism of activation and are conceptually illustrated in appendix IV.

Intrinsic activation is a surface mediated reaction which results when the contact factors (prekallikrein and

factor XII) are adsorbed to a negatively charged surface. Such a negatively charged surface is provided in vitro by glass or kaolin and in vivo by collagen. The interactions between the contact factors and a negatively charged surface result in a conformational change which exposes the active serine site of the molecule. During this process, the contact factors are activated to the proteases kallikrein and factor XIIa. In the presence of the protein cofactor high molecular weight kininogen, factor XIIa activates factor XI to XIa. A intermediary stage of intrinsic activation is then initiated when factor XIa activates factor IX. This is the first calcium and phospholipid dependent phase of intrinsic activation. Calcium and phospholipid are also necessary for the next step where factor IXa converts factor X to Xa. The protein cofactor factor VIII is also required for this process. Factor X activation is also the first step of the common pathway so designated because it is at this point that both intrinsic and extrinsic pathways converge. After the generation of factor Xa, factor II (prothrombin) is converted by Xa in the presence of the protein co-factor, factor V, to IIa (thrombin). Finally, factor IIa converts fibrinogen into soluble fibrin monomers and fibrinopeptides A and B. At this point the fibrin monomers are able to spontaneously polymerize, however they remain soluble (in 5 M urea) until acted upon by the

transaminase, factor XIIIa.

Extrinsic activation results when factor VII comes into contact with the intracellular lipoprotein, tissue thromboplastin. Since this lipoprotein normally resides within cells, it is extrinsic to plasma. For this reason, initiation of coagulation by this mechanism has been termed extrinsic activation.

Unlike other precursors of the coagulation enzymes, factor VII has been demonstrated to possess proteolytic activity (Zur, 1978). When tissue thromboplastin enters the blood, the proteolytic activity of factor VII is enhanced sufficiently to activate factor X (Nemerson, 1983). The activated factor X then catalyzes the conversion of VII to VIIa, thus creating an amplification loop. Factor VII-thromboplastin has also been shown to activate factor IX, and by this mechanism may further amplify the initiation of coagulation by contributing to factor X activation (Osterud & Rapaport, 1977). Because of the powerful amplification mechanisms surrounding factor VII activation, it has been suggested that factor VII may be the key enzyme for the initiation of coagulation (Nemerson, 1983).

Although most of the proteins of the coagulation network are procoagulant, a variety of enzymes and protease inhibitors display some anticoagulant actions. Alpha-1 antitrypsin, C-1 esterase inhibitor and alpha-2

antiplasmin inhibit serine proteases by forming a 1:1 stoichiometric complex which inhibits serine proteases upon association. Antithrombin III (discussed later in detail) also functions in this manner. When compared for their ability to inhibit thrombin, antithrombin III is the most potent of the aforementioned inhibitors. Alpha-2 macroglobulin has also been shown to inhibit thrombin (Thompson & Harker 1983).

Recently protein C, a vitamin K dependent protein, has been shown to be an important regulator of coagulation (Comp, 1984). Upon activation by thrombomodulin bound thrombin, protein C exerts it's anticoagulant actions by digesting the active forms of factors V and VIII.

A regulatory mechanism which functions after the formation of a clot, involves the enzyme plasmin. This protein circulates as the zymogen plasminogen and can be activated to plasmin during contact activation or by the activity of various tissue plasminogen activators. After activation, plasmin can dissolve insoluble fibrin monomers and produce in clot lysis. The activity of plasmin is regulated by the inhibitors $alpha_2$ antiplasmin and $alpha_2$ macroglobulin.

E. Mechanism of Heparin's Action (Antithrombin III Dependent)

It has been recognized for many years that heparin

requires the plasma protein antithrombin III for the expression of most of its anticoagulant effects. The initial observation of this cofactor was made by Howell, (1928) and Quick, (1938). It was observed that heparin tested against pure thrombin and fibrinogen had little effect. However when heparin was tested against thrombin in plasma, it was an extremely potent anti-thrombin agent. Since this antithrombin activity of heparin in plasma could be destroyed by heating, it was hypothesized that the components necessary for the action of heparin were probably proteins (Howell, 1928 ; Quick, 1938). By separating the albumin and globulin fractions of plasma, the heparin cofactor activity was primarily found in the albumin fraction (Quick, 1938). These observations led to the hypothesis that components of the albumin fraction contained antithrombin activity and that the addition of heparin resulted in the intensification of these actions.

Further biochemical characterization finally revealed that antithrombin III is an $alpha_2$ globulin with a molecular weight of about 64,000 (Abligaard et al., 1967).

The observation that factor Xa as well as thrombin could be inhibited by a plasma protein and that this process was accelerated by heparin was reported by Biggs et al., (1970) and Yin et al., (1971). They concluded that the heparin accelerated factor inhibiting factor Xa

and thrombin were the same protein. These observations were confirmed by the studies of Rosenberg & Damus, (1973) and Damus et al., (1973) who offered the first mechanistic analysis of the actions of antithrombin. Using an extract of antithrombin judged homogeneous by disc gel electrophoresis, SDS gel electrophoresis and immunoelectrophoresis they were able to demonstrate that anti factor Xa and antithrombin activities reside on the same molecule. The proposed mechanism for the inhibition of thrombin involves binding between arginine residues on antithrombin and the active serine sites of thrombin. The binding occurs in a stable 1 : 1 stoichiometric complex which is not dissociable by denaturing or reducing agents. Heparin accelerates this process by binding allosteric lysyl residues on antithrombin which result in a conformational change providing a more favorable orientation of one or more arginines for interaction at the active site of thrombin (Rosenberg & Damus, 1973 ; Rosenberg, 1975 ; Damus et al., 1973). A critical tryptophan residue has also been proposed to reside within the heparin binding site (Blackburn & Sibley, 1980). This proposal is based upon the observation that modification of a single tryptophan residue blocks not only the binding of heparin to antithrombin, but also the heparin induced enhancement of thrombin inactivation. Since these actions can be accounted for by allosteric effects, additional

experimentation is necessary to prove the critical role of tryptophan (Blackburn & Sibley, 1980).

The reactivity between arginine and active serine sites led to the hypothesis that the heparin-antithrombin complex should inhibit all serine proteases within the coagulation network (Rosenberg, 1975). Consistent with this view, it has been demonstrated that in vitro, the heparin-antithrombin complex exerts it's anticoagulant effects by inhibiting factors XIIa, XIa, Xa, IXa and thrombin (Damus et al., 1973 ; Rosenberg et al., 1975 ; Snead et al., 1976 and Yin et al., 1971). The possible exception to this theory is the serine protease factor VIIa (Rosenberg et al., 1977 & Jesty, 1978). Although some investigators have reported inhibition of factors VII or VIIa by heparin-antithrombin (Godal et al., 1974 ; Broze & Majerus, 1980), these observations are controversial and currently the subject of investigation.

The heparin-antithrombin complex has also been shown to inhibit the serine protease plasmin (Highsmith et al., 1974).

F. Mechanisms of Heparin's Action (Antithrombin III Independent)

Many investigators studying the antithrombotic actions of heparin have reported that the in vitro anticoagulant actions of heparin are not always

quantitatively related to the in vivo antithrombotic actions. Furthermore, antithrombotic activity often shows poor relationship to antithrombin III affinity (Merton et al., 1984 ; Barrowcliffe et al., 1984). Consistent with these observations, Ofosu et al., (1982) have shown anticoagulant actions by low antithrombin affinity fractions. Using fractions prepared by antithrombin affinity chromatography methods, low affinity heparin inhibited activation of prothrombin, but did not inhibit factor Xa or IIa. Unlike the anticoagulant actions of high affinity heparin, this inhibition was not dependent upon the presence of antithrombin. The mechanism of the low affinity component in this action was attributed to disruption of the binding of factor Xa to phospholipid, Ca^{++} and factor II by low affinity heparin. It was concluded that the contribution by the low affinity component in terms of the overall antithrombotic effect of heparin was probably insignificant (Ofosu et al., 1982).

Using similar affinity chromatographic methods it has been shown that about one third of commercial heparin binds with high affinity to antithrombin III (Holmer et al., 1981). If antithrombin III dependent mechanisms are solely responsible for heparins anticoagulant actions, then it would appear that almost two-thirds of the heparin mixture is inactive. In support of this view, removal of the high antithrombin III affinity fraction from heparin

results in almost complete loss of the in vitro anti-coagulant activity. Based on these observations, it was predicted that high antithrombin III affinity fraction of heparin would be a more effective in vivo antithrombotic agent than conventional heparin. Recently, several investigators have shown this not to be true. Merton et al., (1984) compared high affinity with unfractionated heparin for their ability to inhibit stasis induced thrombosis in a rabbit model. Although similar circulating anti Xa and clotting activity was observed, the unfractionated heparin provided greater protection from thrombosis that the high affinity fraction. The greater antithrombotic response of the unfractionated heparin was attributed to its content of low antithrombin affinity components. Further proof of the antithrombotic effects of low affinity heparin was demonstrated by mixing low affinity with high affinity heparin. The addition of low affinity components restored antithrombotic potency to the high affinity fraction equivalent to unfractionated heparin (Merton et al., 1984 ; Barrowcliffe et al., 1984). These observations clearly indicate that at least a part of heparins antithrombotic effects are mediated through non antithrombin III dependent mechanisms. Speculation about the nature of these mechanisms has included effects on endothelial cells, pro-fibrinolytic actions, anti-coagulant actions mediated through heparin cofactor II,

effects upon blood viscosity and interactions with cellular components of the blood.

1. Heparin's Action on Endothelial Cells

It has been demonstrated that heparin binds to endothelial cells both in vitro and in vivo (Mahadoo et al., 1977 ; Barzu et al., 1984). Because of their critical role in hemostasis, binding of heparin to endothelial cells is thought to play an important antithrombotic role which is indirectly independent of the antithrombin III mechanism. Recently, several investigators have shown that heparin like molecules exist on the surface of endothelial cells and are important determinants of the anticoagulant nature of these cells (Rosenberg, 1985). It is thought that anti heparin proteins such as platelet factor - 4 and histidine rich glycoprotein, constantly compete with anticoagulant proteins for the limited number of binding sites on these heparin like molecules. The binding of exogenous heparin to endothelial cells would alter this competition simply by creating additional binding sites (Rosenberg, 1985). These additional binding sites could contribute to heparin's overall antithrombotic actions by increasing the difficulty for neutralizing the natural antithrombotic characteristics of endothelial cells by anti heparin proteins.

2. Heparins Effect on Fibrinolysis

A profibrinolytic action, resulting from heparin-endothelial interactions may also contribute to overall antithrombotic effects. Heparin administration has been associated with an increase in fibrinolytic activity as demonstrated by decreased euglobulin lysis times, increased fibrin plate activity, increased circulating B_B 15-42 related peptides and increased circulating immunoreactive tissue plasminogen activator (t-PA) (Gaffney et al., 1982 ; Variel et al., 1983 ; Vinazzer et al., 1982 ; Fareed, 1985). Although the mechanisms resulting in the increased fibrinolytic activity are not well understood, it is likely that several mechanisms are involved. Among the possible profibrinolytic mechanisms of heparin are direct release of t-PA from endothelial cells, an increased synthesis of t-PA and inhibition of plasmin or plasminogen activator inhibitors (Fareed, 1985).

3. Heparin Cofactor II

An additional heparin cofactor, similar to antithrombin III in composition and molecular weight has been recently identified (Griffith et al., 1983 ; Tollefsen et al., 1982). This cofactor has been termed heparin cofactor II. Unlike antithrombin III, it is thought to inhibit only thrombin and not the other

proteases of the coagulation network inhibited by antithrombin III (Tollefsen et al., 1984). Like antithrombin III, heparin cofactor II binds heparin and other sulfated polysaccharides in a 1 : 1 stoichiometric manner. The binding of heparin, chondroitin sulfate, dermatan sulfate, pentosan polysulfate or dextran sulfate results in a dramatic increase in the rate at which thrombin is neutralized (Yamagishi et al., 1986)..

Although in vitro, the heparin-heparin cofactor II complex inhibits thrombin, it's role as a physiological mediator of heparins anticoagulant actions is questionable. Heparin cofactor II's concentration in the plasma is only about 1.0 micro molar, approximately half that of antithrombin III. Also, heparin cofactor II requires a two fold greater concentration of heparin to neutralize an equivalent amount of thrombin when compared to antithrombin III. Consistent with these observations, animal studies indicate that after intravenous injection, thrombin is exclusively neutralized by antithrombin III (Tollefson, 1984). Thus it seems unlikely that under normal physiologic conditions, heparin cofactor II is a significant contributor to heparins anticoagulant actions.

4. Heparin's Effect on Blood Rheology

It is thought that antithrombotic actions can be produced by agents which affect the rheology of blood

(Angelkort et al., 1979). Consistent with this, it has been suggested that a lowering in blood viscosity by heparin may contribute to it's overall antithrombotic effects (Kitsos et al., 1986 ; Reggiero et al., 1983 ; Copley and King, 1984). Preliminary reports by Kitsos et al., (1986) have demonstrated some reduction in whole blood viscosity by heparin and heparin fractions. These effects were only detectable using an extremely sensitive controlled stress rheometer.

Currently, the mechanism involved in heparin's effect on viscosity is not well understood, however it seems likely that cell membrane characteristics and plasma protein levels are altered. It has been hypothesized that both a reduction in plasma fibrinogen levels, as well as a direct effect on fibrinogen may be responsible for heparin's decrease in plasma viscosity (Ruggiero et al., 1983 ; Copley and King, 1984).

5. Cellular Interactions

Several investigators have suggested that actions by heparin on cellular components of the blood may contribute to it's overall antithrombotic effects. Most reports have identified platelets and leukocytes as the components involved in these actions (Zucker, 1977 ; Czarnetzki et al., 1980).

a. Platelets

Platelets plays an important role in the development of both arterial and venous thrombosis. The former being more dependent on the activity of platelets than the latter. It seems likely that heparin affects this role since it is known to produce effects upon platelets both in vitro and in vivo. The exact nature of heparins influence on platelets and the resulting contribution to hemostasis is unclear at this time. Both stimulation and inhibition of in vitro platelet aggregation have been reported by heparin (Salzman et al., 1980 ; Gillett & Besterman, 1973). These effects appear to be dependent on the source of heparin, the individual platelets and the method of aggregation.

Heparin has been suggested to exert antithrombotic actions by inhibiting the adherence of platelets to subendothelial connective tissue. Evidence for this comes from experiments using heparin coated arterio-venous shunts in dogs. In these studies, heparin coated shunts remained patent longer and displayed a reduced accumulation of platelets (Zucker, 1977).

b. Leukocytes

Leukocytes have been suggested to play a role in blood coagulation. Although both anticoagulant and procoagulant activities have been reported, it seems

likely that leukocytes act in a procoagulant manner by stimulating thromboplastin production (Niemetz, 1972 ; Lerner et al., 1977). Heparin has been shown to inhibit chemotactic factors involved in the migration of leukocytes (Czarnetzki et al., 1980). This inhibition may result in reduced thromboplastin production and by this action contribute to the overall anticoagulant action.

G. The Pharmacokinetics of Heparin

Heparin has been used clinically for almost 30 years, yet its pharmacokinetics remain poorly understood. The difficulty in studying this complex problem originates from heparin's polycomponent nature (Estes, 1980). Because heparin is not one molecule but rather a mixture of diverse molecules, a suitable method for measuring the kinetics of all components does not exist.

In an attempt to study the pharmacokinetics of heparin several investigators have utilized radio-labelled heparin. The use of this method is questionable since it appears that the radioactive label may not distribute identically to the individual components of heparin. A greater proportion of label may bind to low molecular weight heparin species. This is suggested by results from radio-labelled studies which demonstrate a slightly larger volume of distribution (Estes, 1980). The larger distribution volume may also be explained by free

label resulting from migration off the heparin or metabolic degradation.

In the absence of accurate direct kinetic measurements, a variety of bio-assays have been used to estimate the pharmacokinetics of heparin. These bio-assay methods utilize calibration curves to determine the plasma concentration of heparin. Most commonly, various activated clotting time assays are used in these studies. A brief review of the various bio-assays for heparin has been provided by Wessler and Gitel, (1979).

Comparison of pharmacokinetic studies using different bio-assays has demonstrated great variation. Results about absorption, volume of distribution (Vd) and clearance (Cl) vary significantly and are dependent upon the method of assay.

In reviewing the pharmacokinetics of heparin it is important to mention some of the components with which it is likely to react in vivo. In the blood, heparin not only binds to antithrombin III, but also to many constituents of the plasma including fibronectin, albumin, fibrinogen, platelet factor-4 and histidine rich glycoprotein (Dawes et al., 1985 ; Estes, 1980). The effect of binding by heparin to these plasma components upon the pharmacokinetics and pharmacodynamics is largely unknown. Heparin has also been shown to bind and be taken up by endothelial cells (Barzu et al., 1984 ; van Rijn et al.,

1987).

1. Absorption

Because the molecules of heparin are so highly charged, it is not well absorbed from all routes of administration. Generally heparin is administered by either subcutaneous or intravenous routes. Intrapulmonary administration has also been shown to be a useful method (Bick and Ross, 1985). More recently, absorption of orally administered heparin has been reported (Ueno et al., 1982 ; Lasker, 1985 ; Larsen et al., 1986). Although there is wide variation in both the rate and extent of absorption after various routes of administration, the total amount of heparin required to achieve the same anticoagulant effect over equivalent time periods has been reported to be similar for intravenous, subcutaneous and intrapulmonary routes (Cocchetto and Bjornsson, 1984). Similar data following oral administration of heparin is not available, however preliminary data indicates that only low molecular weight molecules displaying anti Xa activity are recovered from the plasma (Larsen et al., 1986).

2. Distribution

Most studies have reported the Vd of heparin to be about 0.07 L/Kg (Estes, 1980). This volume approximates distribution to the plasma compartment, however slightly

larger distribution volumes have been reported depending on the method of assay. Bjornsson et al., (1982) demonstrated the differences in Vd calculated from three assay methods. Significant differences were obtained, with the values obtained from chemical methods being slightly greater than those based upon coagulant assays. It should be mentioned that when ^{35}S labelled heparin has been used to estimate Vd, volumes between those of extracellular and total body water are reflected (Estes, 1980). This is not an accurate reflection of heparins distribution volume and illustrates the inadequacy of radiolabelled heparin for such studies. The large Vd is explained by the loss of the radioactive label from the heparin molecule.

Cellular uptake of heparin has been reported by several investigators (Mahadoo and Jaques, 1979 ; Barzu et al., 1984). If these observations are true, they may explain reports indicating a volume of distribution for heparin which slightly exceeds the plasma compartment. The major cell populations responsible for heparin uptake were reported to be macrophages and endothelial cells.

3. Half-life and Clearance

Unlike many drugs, heparin exhibits dose dependent pharmacokinetics (Estes, 1980 ; Bjornsson et al., 1982 ; Raasch, 1980 ; Cocchetto and Bjornsson, 1984). Half-life increases with increasing dose in humans and animals with

no Michaelis-Menton type kinetics being apparent (Cocchetto and Bjornsson, 1984). After administration to normal volunteers, the half-life of heparin is about 1.5 hours, however reports have ranged from 23 minutes to 2.48 hours depending on the dose and method of assay (McAvoy, 1978). Clearance rates have been reported at about 0.5 to 0.6 ml/min/Kg (Estes, 1980). Most investigators have emphasized the many variables affecting heparins half-life and clearance, thus estimates of its true values are difficult.

Recently it has been shown that different delivery systems may affect the half-life of heparin. Using heparin encapsulated in liposomes, a half-life up to three times that of non-encapsulated heparin was observed after intravenous administration (Kim et al., 1986). The prolonged half-life was attributed to gradual release of the heparin from liposomes trapped within the reticulo-endothelial system.

4. Metabolism and Elimination

The metabolism and elimination of heparin are rather poorly understood due to heparins polycomponent nature. Unlike more conventional drugs, metabolites of heparin are not readily identifiable. Heparin metabolism involves depolymerization and desulfation by a variety of heparin-ases, desulfatases and endoglycosidases. Thus during

metabolism, high molecular weight components may be metabolized to molecules resembling non-metabolized low molecular weight species.

Heparin is primarily eliminated by the reticulo-endothelial system, liver and kidneys. Evidence for this is found in the prolonged anticoagulant effects observed in nephrectomized and hepatoectomized animals (Cocchetto and Bjornsson, 1984). The reticuloendothelial system has been associated with this process largely because it is the only system which can account for the reports of variation and dose dependent nature of heparins half-life (Estes, 1980).

II. THE MOLECULAR WEIGHT DEPENDENT EFFECTS OF HEPARIN

A. Fractionation

As previously discussed, there are a variety of oligosaccharide chains of differing molecular weight within any given heparin preparation. Assuming that individual molecular weight components differ in their biological function, many investigators have studied the biologic actions of heparin as a function of molecular weight. To accomplish this task, various fractionation techniques have been employed to create molecular weight fractions. The most commonly employed fractionation techniques have been gel-filtration chromatography or depolymerization with either nitrous acid or heparinase.

The depolymerization methods typically result in end residues which are characteristic of the process (Casu, 1984). Heparinase an enzyme from Flavobacterium heparinum, splits glycosidic bonds between N - sulfated glucosamine and iduronic acid 2 - sulfate. This process results in fragments which terminate with 4,5 - unsaturated iduronic acid at the non reducing end and N-sulfated glucosamine at the reducing end. Using heparinase, more that 90 % of a commercial heparin has been shown to be digested to a hexasaccharide (Jaques, 1978, 1978).

Nitrous acid is less specific than heparinase digestion and splits glycosidic bonds between N - sulfated glucosamine and any kind of uronic acid. This results in

fractions with 2,5 - anhydromannose at the reducing end (Casu, 1984).

Under carefully controlled conditions, the use of both heparinase and nitrous acid results in the production of low molecular weight compounds without major deviation from the original glycosaminoglycan structure (Coyne, 1985). Because of these characteristics, both depolymerization techniques have been utilized in studies on the molecular weight dependent effects of heparin.

Unlike heparinase and nitrous acid digestions, gel filtration chromatography does not in any way alter the native chemical composition or structure of heparin. For the separation of various molecular weight components, gel filtration depends on a size exclusion principal. During the gel filtration process, the individual heparin molecules elute at a rate dependent upon their size. High molecular weight species elute at a faster rate than those of low molecular weight. Thus depending upon the size exclusion properties of the gel, an effective separation based upon molecular weight can be achieved. Using this technique, only natural fractions are separated from the original preparation. These characteristics contribute to the effectiveness of gel filtration a fractionation technique for studying the molecular weight dependent effects of heparin.

B. In Vitro Studies

1. Molecular Weight Dependent Inhibition of Thrombin

Initial studies largely utilized gel filtration techniques to fractionate a single source of heparin into molecular weight fractions (Scott et al., 1957 ; Laurent, 1961 and Lasker, 1966).

Using gel filtered molecular weight fractions in whole blood or plasma clotting assays, some correlation was observed between molecular weight and heparin's anticoagulant activity (Laurent, 1961). Generally, higher molecular weight heparins were more potent than those of lower molecular weight, however variation was observed with both the source of heparin and the assay utilized. Correlations between molecular weight and anticoagulant activity were consistent only between molecular weights of 11,000 - 4,000 daltons (Scott et al., 1957 ; Laurent, 1961 ; Lasker and Stivala, 1961).

Additional studies by Laurent et al., (1978) and Shen et al., (1978) utilized gel matrices with differing size exclusion properties to study molecular weight dependent effects over a greater range of molecular weights. Fractionation of single sources of porcine mucosal and beef lung heparin yielded fractions ranging from 36,000 - 5,600 daltons. Using these gel filtered fractions, anticoagulant activity determined by both a whole blood clotting and a amidolytic antithrombin assay

demonstrated a molecular weight dependence in ranges from 7,800 through about 16,500 daltons. Molecular weights above 16,500 did not exhibit an increase in anticoagulant activity with increasing molecular weight. Both investigators reported that the lowest molecular weight fractions (< 5,600 & < 6,200) did not show any significant anticoagulant activity. It is interesting to note that a molecular weight of about 5,600 correlates well with the minimum size of heparin necessary to accelerate thrombin inhibition in the presence of antithrombin (Oosta et al., 1981). This observation, along with reports of anti Xa activity at even lower molecular weights, suggest that the molecular weight dependent effects reported by Laurent et al., (1978) and Shen et al., (1978) largely reflected the molecular weight dependent effects of heparin upon thrombin.

The relationship of molecular weight to thrombin inhibition was thought to relate to the necessity of heparin binding simultaneously to both antithrombin III and thrombin for maximal inhibition (Nordenman et al., 1978 ; Porter et al., 1976 ; Wilson-Gentry and Alexander, 1973). Since heparin is a linear polysaccharide, higher molecular weights are equated with longer chains. With increasing chain length, there is also increased probability that the chain will be of sufficient length to bind both antithrombin III and thrombin. More recently

Oosta et al., (1981) have shown that heparin fragments of about 14 monosaccharide residues are capable of catalyzing thrombin inactivation. Using heparin fragments of various length, the kinetic rate constants for the inactivation of thrombin by antithrombin - heparin were studied as a function of the length of the heparin fragment. Hexasaccharide, octasaccharide and decasaccharide sequences displayed no effect on the rate of thrombin inactivation although they were found to bind tightly to antithrombin III. Heparin fragments of longer length (14 residues) bound to antithrombin with equal affinity, but were capable of catalyzing thrombin inactivation. Using a slightly longer heparin fragment, additional rate enhancement of thrombin inactivation was observed. These findings suggested that both fragments possessed similar ability to activate antithrombin relative to thrombin inactivation, however the larger fragment possessed an additional structural element which may have contributed in approximating free enzyme with protease inhibitor (Oosta et al., 1981).

The direct binding of heparin to thrombin provided a partial explanation of the molecular weight dependent effects of heparin. Additional explanations of the molecular weight dependent actions of heparin upon thrombin were thought to involve binding to antithrombin III. It has been proposed that molecular weight dependent

effects may relate to the probability of finding the antithrombin III binding site (Laurent et al., 1978). If a specific sequence of heparin is required to bind to antithrombin III, then the anticoagulant activity should be related to the probability of finding that sequence on the heparin molecules. This theory assumed equal probability of these sequences occurring along the heparin chains. Although these assumptions provided an explanation of the molecular weight dependent effects of heparin, additional factors were thought to be involved based on studies using high antithrombin III affinity heparin. Since these fractions were already selected for their ability to bind antithrombin III, it was proposed that they should no longer display molecular weight dependent effects. However, a high correlation between molecular weight and anticoagulant activity was still observed in these fractions.

2. Molecular Weight Dependent Inhibition of Xa

A molecular weight dependence to the inhibition of factor Xa has also been reported (Lane et al., 1978 ; Andersson et al., 1978 ; Thunberg et al., 1979). As with investigations involving thrombin, gel filtered fractions of heparin were utilized in these studies along with specific anti Xa assays. Unlike the antithrombin effects of heparin, the specific activity per unit weight of

heparin increased with decreasing molecular size in both amidolytic and clot based assays (Andersson et al., 1979 ; Lane et al., 1978 ; Graham and Pomeroy, 1979). Interestingly, this negative correlation between molecular weight and anti Xa activity was observed in plasma but not in purified systems (Andersson et al., 1979). In the purified system, the molecular weight dependence resembled that of thrombin where higher molecular weights resulted in greater inhibition (Andersson et al., 1979 and Ellis et al., 1987). Investigation of this discrepancy between plasma and purified systems revealed that the high molecular weight anti Xa components were preferentially neutralized in plasma (Anderssom et al., 1979). Ellis et al., (1987) speculated that this effect may be due to interactions with lipoproteins. This neutralization sharply decreases at low molecular weights, thus the increased anti Xa potency of low molecular weight heparin is primarily due to a decreased neutralization of this component in plasma. These observations are also consistent with reports of differential neutralization of various molecular weight heparins by protamine (Hubbard and Jennings, 1985 ; Holmer and Soderstrom, 1983 ; Racanelli et al., 1985). All investigators observed resistance by the anti Xa component of heparin to neutralization by protamine. Although the addition of protamine to heparin did result in a measurable reduction

of the anti Xa activity, complete neutralization was never observed (Holmer and Soderstrom, 1983). Similar observations were reported for the ability of platelet factor - 4 to neutralize the anti Xa activity of heparin (Lane et al., 1984).

The observation that low molecular weight heparin displays such high anti factor Xa activity also suggests that unlike thrombin, direct binding of heparin to factor Xa is not necessary for maximal inhibition (Andersson et al., 1979 ; Thunberg et al., 1979). This speculation has been confirmed by kinetic studies on the rate of enhancement of factor Xa inhibition in the presence of antithrombin and low molecular weight heparin (Jordan et al., 1980). These studies demonstrated that the heparin induced acceleration of Xa inhibition by antithrombin III is solely dependent upon the binding of heparin to this inhibitor.

The effects of specific heparin fragments from the same source of unfractionated heparin on factor Xa inhibition were reported by Oosta et al., (1981). Hexa-saccharide, octasaccharide and decasaccharide displayed a significant capacity to accelerate factor Xa inhibition in the presence of antithrombin. A distinct relationship between the molecular size of these fragments and their biologic activity was observed. Increasing chain length resulted in increasing activity. This relationship was

consistent from the hexasaccharide to a sequence containing about 16 monosaccharide residues. Interestingly, the affinity of these fragments to antithrombin as determined by a competitive binding assay also increased as a function of molecular size. (Oosta et al., 1981).

3. Molecular Weight Dependent Effects on Other Coagulation Factors

The majority of studies investigating the molecular weight dependence of heparin utilized global clotting assays such as the APTT or the British Pharmacopoeia assay. These tests reflected multiple effects upon the coagulation system and did not demonstrate actions upon individual proteases. When factor specific amidolytic assays were used to investigate the molecular weight dependent effects of heparin, similar effects as observed in the global assays were reported for the inhibition of thrombin. A different pattern of molecular weight dependence was observed for the inhibition of factor Xa. These observations led to speculation about the molecular weight dependent effects on the other coagulation factors known to be inhibited by heparin.

Holmer et al., (1981) reported the effects of gel filtered molecular fractions of heparin on coagulation factors XIIa, XIa, Xa, IIa, and Kallikrein in amidolytic and clotting assays specific for these factors. As with

previous reports, anti IIa potency increased with increasing molecular weight. An inverse relationship was observed for anti Xa activity. Potency appeared to increase with decreasing molecular weight. The relationships between molecular weight and potency of the other coagulation factors could be classified as resembling those of either factor Xa or thrombin (Holmer et al., 1981).

Factors IXa and XIa resembled thrombin in that the specific activity of the heparin increased with increasing molecular weight. These reports were consistent with observations indicating that the inhibition of factor IXa is dependent upon binding of heparin both to antithrombin III and directly to the free enzyme (Jordan et al., 1980).

Kallikrein and factor XIIa resembled factor Xa in that the inhibition of these factors was less dependent upon the length of the polysaccharide chain.

Based upon these observations, it was hypothesized that the molecular weight dependent effects of heparin upon other coagulation factors could be characterized as resembling either thrombin or factor Xa. In addition, it was suggested that the mechanisms relevant to the molecular weight dependent effects of heparin on coagulation factors in these categories would be similar (Holmer et al., 1981).

C. Molecular Weight and Antithrombin III Affinity

It is now well known that the anticoagulant activity of heparin is largely mediated by the plasma cofactor antithrombin III (Rosenberg et al., 1973 ; Damus et al., 1973). Purification of antithrombin III by Miller-Andersson in 1974 resulted in the development of several affinity chromatography techniques utilizing antithrombin immobilized to sepharose. The use of these techniques greatly facilitated the understanding of the requirements for the binding of heparin to antithrombin III.

Hook et al., (1976) demonstrated that heparin could be separated into components of high and low anticoagulant activity based upon affinity to the immobilized antithrombin. Interestingly, these affinity fractions appeared to have similar structures and molecular weight distributions. Some differences were observed in the net charge with the high affinity fractions tending to have a greater negative charge.

Andersson et al., (1976) performed a similar affinity fractionation with additional gel filtration to achieve molecular weight subfractions of the high affinity component. These subfractions demonstrated that the high affinity fraction of heparin was not homogeneous. Within the high affinity fraction there was still a variety of components differing in molecular weight. As previously indicated, the high molecular weight high affinity

fractions showed greater IIa inhibition, while low molecular weight high affinity fractions showed greater anti Xa activity. Although no molecular weight sub-fractionation was performed on the low affinity fraction, in vitro screening in various anticoagulant assays revealed them to be virtually inactive (Andersson et al., 1976).

These observations by Andersson and Hook resulted in speculation about the relationship between heparins antithrombin affinity and molecular weight. It was theorized that high affinity heparin should contain a greater degree of binding sequences for antithrombin than low affinity heparin. Thus, the total number of these units in a heparin molecule would be proportional to antithrombin affinity (Laurent et al., 1978).

Theories about the relationship between molecular weight and antithrombin affinity were obscured by an imprecise knowledge of the antithrombin binding sequence of heparin. Laurent et al., (1978) proposed that a segment of ten disaccharides was needed for the full activity of heparin. Although the term specific dodeca-saccharide was used, it was emphasized that this should not be interpreted as only one possible sequence but rather several sequences which may have in common the location of only one sugar unit.

Using affinity and size fraction techniques,

Rosenberg et al., (1978) reported characteristics essential for antithrombin affinity. Fractions of heparin with high affinity had a significantly higher content of glucuronic acid and a lower amount of N - sulfated glucosamine per molecule compared to the low affinity fraction. Within both fractions, a tetrasaccharide sequence responsible for antithrombin affinity was identified. Differences in affinity were suggested to be due to the relative abundance of this sequence. Within the high affinity fraction, sufficient amounts of this sequence are present such that each molecule may contain the this structure. However, the low affinity fraction contains only enough tetrasaccharide for one fifth of the molecules to contain the sequence. Although about 20 % of the molecules in the low affinity fraction contain the tetrasaccharide sequence, only about 1 % of the anti-coagulant activity of the high affinity fraction is observed. This suggests that an additional structural feature may either be included in or missing from the low affinity molecules containing the tetrasaccharide sequence (Rosenberg et al., 1978).

Using nitrous acid digestion followed by anti-thrombin affinity fractionation, Lindahl et al., (1979) observed that the smallest fraction binding to anti-thrombin was between six and eight disaccharide units. Based on this observation, it was reported that the

antithrombin binding site was more a tetradecassacharide. The binding site was thought to contain both variable and invariable regions, with the non variable regions being of critical importance to antithrombin binding. The tetrasaccharide sequence reported by Rosenberg et al., (1978) was thought to be essential only in that it contributed to a portion of the non variable region.

Recently, strong evidence for the minimal binding sequence of heparin to antithrombin III has been reported (Choay et al., 1983). Unlike previous studies, extensive heparinase digestions, combined with a unique chemical synthesis were utilized to define the binding requirements of heparin to antithrombin III. Chemical analysis of a hexasaccharide obtained by heparinase digestion revealed a 4,5 unsaturated uronic acid at the non reducing end of the molecule. Since this residue was not associated with anticoagulant activity it was speculated that the minimal binding sequence of heparin was a pentasaccharide. To prove this theory, a specifically substituted pentasaccharide (molecular weight 1,585) was synthesized along with two of the possible tetrasaccharide combinations contained within the pentasaccharide sequence. Subsequent in vitro analysis in anti Xa and specific antithrombin binding assays revealed that neither tetrasaccharide bound to antithrombin III nor did they exhibit any anti Xa activity (Choay et al., 1983). However, the penta-

saccharide bound to antithrombin with an affinity constant of the same order of magnitude as that reported for high antithrombin affinity heparin (Choay et al., 1983). Similarly, the pentasaccharide exhibited high in vitro anticoagulant activity in both anti Xa amidolytic and clotting assays however no antithrombin activity was observed.

Using a similarly synthesized tetrasaccharide (molecular weight 1,268), Petitou (1984) reported in vitro anticoagulant activity. These results, and those of Rosenberg et al., (1978) suggest that the minimal sequence of heparin capable of binding to antithrombin, is a tetrasaccharide. This minimal tetrasaccharide sequence while capable of binding to antithrombin does not exhibit significant anticoagulant activity (Petitou, 1984). Thus, it is likely that the minimal sequence of heparin capable of binding to antithrombin III is a tetrasaccharide. However, the pentasaccharide appears to be the minimal sequence capable of antithrombin III binding and eliciting significant anticoagulant activity. By substituting the molecular weights of the penta and tetrasaccharides it is possible to determine the molecular weight dependency for these functions as 1,268 and 1,585 respectively.

The structural characteristics necessary for antithrombin III binding were reported by Thunberg et al., (1982). Enzymatic and chemical modifications revealed

that three sulfate groups played key roles in antithrombin affinity. These sulfates were identified as the N-acetyl - 6 - O - sulfate on D - glucosamine, the N-sulfate in the three position on 3,6 - di - O - sulfate-D - glucosamine and the N - sulfate in the three position on N - sulfate - 6 -O - sulfate - D - glucosamine. The essential role of the 6 - O - sulfate and 3 - O-sulfate groups for antithrombin III binding were reported by Lindahl et al., (1983) (1980). Choay et al., (1983), provided strong additional evidence for the essential role of the 6 - O - sulfate group. Using the synthetic penta and tetrasaccharide fragments, interactions with anti-thrombin III were only observed with the pentasaccharide. The only difference between the penta and tetrasaccharides was the 6 - O - sulfate group on the monosaccharide at the non reducing end of the pentasaccharide. The absence of interactions with antithrombin without this group provides strong evidence for it's functional role (Choay et al., 1983). Interestingly, definitive proof for the essential role of the 3 - O - sulfate group reported first by Lindahl et al., (1980), could not be demonstrated in the pentasaccharide studies (Choay et al., 1983). Additional studies by Petitou, (1984) did confirm this observation. A pentasaccharide deficient in the 3 - O - sulfate group, as determined by NMR spectroscopy did not bind to anti-thrombin III nor exhibit any anticoagulant activity

suggesting the critical role of this group. In these same studies, a tetrasaccharide deficient in both the O-sulfate and N - sulfate groups on the residue at the non reducing terminal demonstrated minimal anticoagulant activity suggesting that their importance must be reconsidered.

D. Molecular Weight Dependent Events Not Mediated By Antithrombin III

Most reports investigating the molecular weight dependent actions of heparin have focused upon anti-thrombin mediated anticoagulant actions. However, some studies have indicated molecular weight dependence to functions not involving antithrombin III.

1. Lipoprotein Lipase Activation

It is well known that administration of heparin results in increased lipoprotein lipase activity in the blood. This increase has been attributed not to effects upon the kinetic properties of the enzyme but rather to an increased bioavailability resulting from displacement from capillary endothelium by heparin (Olivecrona et al., 1977). Harenberg et al., (1985) reported a molecular weight dependence to these effects. Plasma lipoprotein lipase activity was twice as high following intravenous administration of low molecular weight heparin when

compared to unfractionated heparin in humans. The molecular weight dependent effects were even more dramatic after subcutaneous administration. These observations suggest that low molecular weight heparin is more effective in displacing lipoprotein lipase from its endothelial binding sites than heparin of higher molecular weight.

2. Endothelial Cell Binding

Choay et al., (1986) reported the molecular weight dependent properties of heparin binding to endothelial cells. Affinity constants were determined using a competitive binding assay between radioiodinated heparin and fractions of various molecular weight. Using this technique, it was shown that heparin of higher molecular weight possessed greater affinity for cultured endothelial cells than lower molecular weight fractions. Similar observations were reported by Barzu et al., (1984). By increasing the sulfate content of the various fractions it was observed that a higher charge density could compensate for decreased molecular size.

3. Heparin Cofactor II Activation

A molecular weight dependent activation of heparin cofactor II has also been reported (Choay et al., 1986). Yamagishi et al., 1986). Using fragments of heparin

obtained by nitrous acid depolymerization and sized by gel filtration, heparin cofactor II mediated thrombin inhibition decreased steadily with decreasing molecular weight (Choay et al., 1986). This observation was consistent between molecular weights of 9,700 through 6,200 daltons. When low molecular weight fragments were chemically over sulfated, it appeared to increase the affinity constants for these fragments. This observation suggested that the molecular weight dependent effects were likely due to an increased charge associated with the high molecular weight molecules (Choay et al., 1986). Yamagishi et al., (1986) observed similar results relating charge and molecular weight to dextran sulfate enhanced heparin cofactor II activity.

4. Pro-fibrinolytic Actions

Another affect of heparin which has shown some dependence upon molecular weight and or sulfate content, are it's pro-fibrinolytic actions. Vinazzer et al., (1982) compared high (18,000) and low (3,000) molecular weight fractions from the same source for their effect on euglobulin lysis time. A significant difference was observed between the two fractions. The low molecular weight fraction had no effect, while the high molecular weight fraction showed a dose dependent enhancement of the euglobulin lysis time. It was thought that this effect

was mediated through endogenous activation of factor XII which paralleled the effect on euglobulin lysis time. Although the use of the molecular weight fractions demonstrated the effect to be a function of molecular weight, a similar effect involving sulfate content was observed. Using a highly sulfated synthetic heparin analog (3,000 molecular weight) a comparable effect to that of the high molecular weight heparin was observed (Vinazzer et al., 1982). These results suggested that the molecular weight dependent effect of heparin on euglobulin lysis time are at least in part due to charge characteristics. Similar pro-fibrinolytic effects involving charge mediated binding of heparin to plasminogen activators has been reported by Paques et al., (1986).

5. Heparin - Platelet Interactions

Many studies have demonstrated effects by heparin on platelet function, the most notable being platelet aggregation. A molecular weight dependence to heparin induced platelet aggregation has been reported by several investigators. Salzman et al., (1982) reported that gel filtered fractions of the same source of heparin differed in their ability to both induce and enhance aggregation induced by other agonists. The high molecular weight fraction (22,000 daltons) was more active in these actions than the low molecular weight fraction (7,000 daltons).

similar results comparing unfractionated heparin with various depolymerized fractions were observed by Brace and Fareed, (1985) and Blockmans et al., (1986). Further studies by Brace and Fareed, (1986) expanded the range of molecular weight dependence to heparin induced platelet aggregation. Using molecular weight fractions of depolymerized heparin ranging from 6,200 - 1,800 daltons platelet aggregation was studied in individual human platelet rich plasmas. A direct correlation between aggregation and molecular weight was established. In all cases platelets which aggregated to heparin showed a decreased response with fractions of decreasing molecular weight.

E. In Vivo Studies

1. The Effect of Molecular Weight on the Pharmacokinetics of Heparin

The pharmacokinetics of heparin fractions of different molecular weight have not been directly investigated, however several investigators have compared the pharmacokinetics of native and various commercial low molecular weight heparins. Like pharmacokinetic studies on unfractionated heparin, these studies have primarily calculated kinetic parameters from estimates of plasma heparin concentrations determined in anticoagulant assays. Unfortunately, many of these assays were developed for use

with unfractionated heparin and are variable in their response to low molecular weight heparin. Consequently, reports comparing the pharmacokinetics of unfractionated and low molecular weight heparin have yielded contrasting values for the various kinetic parameters.

An additional problem in comparing the pharmacokinetics or other biological actions of low molecular weight heparins was the lack of a international reference preparation (recently a low molecular weight reference has been proposed Thomas, 1987). In the absence of such a reference, the various low molecular weight heparins were standardized for potency in a variety of assays which differed with the manufacturer. This resulted in low molecular weight heparins which although standardized in anticoagulant units, differed significantly in potency and gravimetric amounts. Furthermore the standardized units of low molecular weight heparins did not correlate well with units of unfractionated heparin (Barrowcliffe et al. unpublished report). Most pharmacokinetic studies comparing unfractionated and low molecular weight heparins have used these unit dosages. Comparisons were inaccurate since it was likely that the various heparins were not compared at equigravimetric concentrations. The pharmacokinetics of heparin are dose dependent and vary with the method of assay. For these reasons, many reports comparing the pharmacokinetics of native and low molecular

weight heparin are of little value.

a. Absorption and Distribution

Bergqvist et al., (1983) compared the absorption of two low molecular weight fractions (obtained by gel-filtration and depolymerization) with unfractionated heparin after subcutaneous administration. In this study, plasma anti Xa activity was observed over a twenty four hour period after a single injection of a 5,000 International Unit (IU) dose. In contrast to the unfractionated heparin, it was reported that there was significantly greater absorption of both low molecular weight fractions. Peak absorption for both low molecular weight and unfractionated heparin appeared at about four hours post administration (Bergqvist et al., 1983). The activities of both low molecular weight heparins remained greater at 11 hours post injection than the peak activity of the unfractionated heparin. These differences were attributed to molecular weight dependent differences in both the rate and amount of absorption (Bergqvist et al., 1983). It is interesting to note that a molecular weight dependent difference in the rate and degree of absorption was also observed between the two low molecular weight fractions.

Bratt et al., (1985, 1986) reported a small dose dependent change in the volume of distribution after

intravenous administration of a low molecular weight fraction of heparin (Kabi 2165) as determined by plasma anti Xa activity. At a dose of 60 IU/Kg the apparent volume of distribution was reported to be 2.9 L. Increasing the dose to 120 IU/Kg resulted in an approximate 15 % increase in the volume of distribution to 3.4 L. This apparent dose related increase in distribution volume was thought to reflect a larger fraction of drug not bound to antithrombin III at the higher dose. The unbound drug would be available for binding outside the plasma perhaps to endothelial cells (Bratt et al., 1986).

b. Half-life

The half life of low molecular weight heparin fractions has been reported to be significantly longer than unfractionated heparin. In most studies, the half-life of low molecular weight heparin is two to three times longer than that of unfractionated heparin (Fareed et al., 1985 ; Lockner et al., 1985 ; Boneu et al., 1985 ; Harenberg et al., 1986). Other investigators have reported the half-life of low molecular weight heparin to be up to 4 times that of unfractionated heparin (Bara et al., 1985). As with unfractionated heparin, the half-life of low molecular weight heparin appears to be dose dependent. Half-life increases with increasing dose but does not display typical Michaelis-Menten (capacity-limited) type

kinetics (Bara et al., 1985 ; Harenberg et al., 1986 Bergqvist et al., 1983).

c. Clearance and Elimination

The clearance of low molecular weight heparin is also dependent on dose (Bratt et al., 1985 1986). After a 120 IU/Kg dose, Cl was reported to be 25 ml/min., compared to 15 ml/min. after a 60 IU/Kg dose.

Recently it has been reported that the clearance of native and low molecular weight heparin may be different (Goudable et al., 1986). Native and low molecular weight heparin were compared for differences in half-life in healthy human patients and those with impaired renal function. No difference was observed in the half-life of native heparin between the healthy and renal impaired individuals. However, a dramatic difference was observed with the low molecular weight fraction. These results suggest that renal mechanisms may play a greater role in the clearance of low molecular weight compared to native heparin.

d. Bioavailability

Several reports have demonstrated that low molecular weight heparins are more efficiently absorbed after sub-cutaneous administration than unfractionated heparin (Fareed et al., 1985 ; Bergqvist et al., 1983 ; Harenberg

et al., 1986). In an attempt to quantify the extent of these differences in absorption, investigators have evaluated the relative bioavailability after subcutaneous and intravenous administration. It must be kept in mind that such a comparison is only acceptable when the rate constant of elimination (K_e) is constant with respect to drug plasma concentration. Since the K_e of unfractionated and low molecular weight heparin has been reported to be dependent upon concentration, the validity of such studies is questionable.

By comparing the area under the plasma concentration time curve (AUC) for both intravenous and subcutaneous routes of administration, several investigators have reported higher bioavailability with low molecular weight compared to unfractionated heparin. Bara et al., (1985) reported 91 % bioavailability in terms of anti Xa activity, for low molecular weight heparin in contrast to 28 % for unfractionated heparin. The greater bioavailability seen with the low molecular weight heparin was dependent upon the method of assay. When the same agents were compared in an anti IIa assay, no significant difference between the low molecular weight or unfractionated heparin was observed.

Increased relative bioavailability of low molecular weight heparin, as measured by anti Xa methods have been reported by Bratt et al., (1986) and Harenberg et al.,

(1986).

3. The Effect of Molecular Weight on Antithrombotic Actions

The antithrombotic actions of unfractionated and low molecular weight heparins in both clinical and animal studies have been reviewed (Thomas et al., 1981 ; Fareed et al., 1985 ; Kakkar, 1984). In these studies, low molecular weight heparins have exhibited effective antithrombotic actions. When compared to unfractionated heparin, they require greater gravimetric concentrations to achieve similar antithrombotic actions after intravenous administration. However in subcutaneous regimens, equal gravimetric dosages of unfractionated and low molecular weight heparin have produced similar antithrombotic actions.

The antithrombotic activities of unfractionated and low molecular weight heparin correlate reasonably well with their in vitro anticoagulant actions. However, the significance of the correlation varies between different assay methods (anti Xa, anti IIa, APTT). For relating in vitro anticoagulant to in vivo antithrombotic actions, no one assay appears clearly superior.

A study directly examining the effect of molecular weight on antithrombotic actions was reported by Bergqvist et al., (1985). In this study, various molecular weight

fractions (molecular weights ranging from 22,000 - 4,900 daltons) and unfractionated heparin were compared for their ability to inhibit in vivo thrombosis in animal models. These studies utilized a high molecular weight fraction obtained by gel filtration as well as heparinase digested low molecular weight fractions. All except the lowest molecular weight fraction decreased the frequency of venous thrombosis and at equigravimetric dosages were not less effective that unfractionated heparin.

In a dose response study, the unfractionated heparin was determined to be more efficacious than low molecular weight heparin. This observation was especially interesting since the low molecular weight heparin was determined to have greater circulating anti Xa activity (Bergqvist et al., 1985). This observation was consistent with those of Carter et al., (1981). These observations suggest that the anti IIa activity of heparin is an important determinant of antithrombotic actions.

Recently, Walenga et al., (1986) reported antithrombotic properties for a synthetic pentasaccharide. Although this agent displayed no thrombin inhibition, antithrombotic actions were observed. When compared to unfractionated heparin an approximate 4 fold greater concentration was required for similar antithrombotic activity. This observation supports earlier observations that an anti IIa component of heparin is important for

antithrombotic actions, but not necessary. These observations suggest that the relationship between molecular weight and antithrombotic actions is similar to the molecular weight dependent relationships of antithrombin III affinity and thrombin inhibition.

CHAPTER II

STATEMENT OF PURPOSE

Molecular weight is an important factor in understanding the pharmacological actions of heparin. Heparin's antiprotease actions, pharmacokinetic behavior and antithrombotic effects have been shown to be dependent upon this parameter (Laurent et al., 1961 ; Harenberg et al., 1986 ; Bergqvist et al., 1983). A greater understanding of the pharmacological actions of heparin as they relate to molecular weight may contribute to greater safety and efficacy of this agent.

It was the purpose of this dissertation to investigate the molecular weight dependence of the pharmacological effects of heparin in terms of its anticoagulant, antithrombotic and pharmacodynamic behavior.

In vitro anticoagulant studies were conducted for the purpose of identifying molecular weight dependent effects associated with specific coagulation pathways. These studies utilized conventional clotting and amidolytic assays as well as novel protease generation tests. These tests were specifically designed to investigate possible actions of heparin not previously reported. Of particular importance were the protease generation systems using factor VII-thromboplastin and those based upon the release of fibrinopeptide-A. These assays were used to

investigate the molecular weight dependent effects of heparin on the extrinsic pathway of coagulation.

The molecular weight fractions were studied for their antithrombotic effects in a rabbit thrombosis model. These studies were conducted for the purpose of relating molecular weight to antithrombotic activity. The fractions were administered by both subcutaneous and intravenous routes of administration. Comparison of the antithrombotic efficacy in both routes provided information about the effect of molecular weight on the absorption of active components after subcutaneous administration.

The molecular weight fractions were also studied to determine the plasma concentration time course. These studies were carried out to investigate any molecular weight dependence in heparin's apparent volume of distribution, biologic half-life or clearance after both subcutaneous and intravenous administration. Comparison of the area under the plasma concentration time curve after both routes of administration provided information about the effect of molecular weight on absolute absorption.

The information obtained from this dissertation may provide a clearer pharmacological profile of the effects of heparin relative to molecular weight. This information may have practical value relative to therapeutic indi-

cations, routes of administration and toxic effects.

Several low molecular weight heparins are currently in pre-clinical and clinical trials. Knowledge about the molecular weight dependent effects of heparin may be especially important for predicting the pharmacological actions of these new agents. Furthermore, the test systems developed for this dissertation may be of value for potency determination, standardization and clinical monitoring of both heparin and its low molecular weight fractions.

CHAPTER III

MATERIALS AND METHODS

A. Heparin and its Fractions

Unfractionated porcine sodium mucosal heparin (lot # H 410) and CY 216 (lot # P157 XH) a low molecular weight heparin fraction, were obtained from Choay Institute (Paris, France). The CY 216 was prepared by ethanol extraction of porcine mucosal heparin, followed by gel filtration on a Ultrogel ACA 44 column (Choay et al., 1980). The analytical profile of the porcine mucosal heparin is seen in appendix V.

B. Reagents

1. Clotting Assays

Thromboplastin-C reagent (lot #'s TPCD - 318, 348 and 358) a rabbit brain thromboplastin containing .0116 M $CaCl_2$, was obtained from American Dade (Miami, FL) and used in the prothrombin time assay (PT). A description of this assay is in appendix VI. APTT reagent (lot # 4W6-34) was obtained from General Diagnostics, (Morris Plains, NJ) and used in the activated partial thromboplastin assay. The APTT reagent was a rabbit brain cephalin with a micronized silica activator. The assay is described in appendix VII. Heptest heparin assay (lot #'s A 85, B-86-2) were obtained from Haemachem (St. MO). A description of the assay is in appendix VII.

Human thrombin (Fibrindex brand lot # 3B340 50 U/vial) was purchased from Ortho Diagnostics (Raritan, NJ) in powder form. The thrombin was reconstituted in distilled water, diluted with saline, standardized to 10 NIH U/ml using a plasma clotting assay and stored at -70° C until used. Bovine factor Xa (lot B44) was purchased from RQP Laboratories (South Bend, IN). The Xa was reconstituted in 1.0 ml tris buffer containing 1.0 mg/ml bovine serum albumin, diluted 1:4 in saline and stored at -70° C until use. Human factors VII, X and Xa were purchased from Diagnostica Stago (Asniers, France). Human factor VII (lot # 67 100 ug/vial) was reconstituted with distilled water, diluted to 5 ug/ml with saline and stored at-70° C until use. Human factor X (lot # H 11 F U/vial) was reconstituted in 0.5 ml of amidolytic assay buffer prior to each use. Human factor Xa (lot # H 11 25 nKat/vial) was reconstituted with distilled water prior to each use. Human antithrombin III (lot # 85 07 01 86576 500 U/vial) obtained from Kabi Vitrum (Stockholm, Sweden), was reconstituted in 1.0 ml of distilled water, diluted to 5 U/ml with saline and stored in aliquots at -70° C until use. The chromogenic substrates used in the amidolytic assays, spectrozyme TH (lot # 1051 /81) and spectrozyme Xa (lot # 1213 /85) were purchased from American Diagnostica (Greenwich, CT). These substrates were

reconstituted with distilled water to the desired molarity prior to use.

3. Plasma Preparation

Normal human pooled plasma was prepared from at least 10 healthy male and female volunteers under the guidelines established by the Institutional Review Board for the protection of human subjects (permit # 5F appendix IX). Blood was collected by venipuncture using double syringe technique through a 21 gauge butterfly needle. The initial 2 - 3 ml's of blood were discarded and the subsequent blood was immediately added to 3.8 % citrate (1 : 10) in plastic tubes. The tubes were then centrifuged for 20 minutes at 2,500 RPM to obtain platelet poor plasma. Additional human plasma was purchased from the Loyola University Medical Center Blood Bank. This plasma was made by collecting blood from healthy donors into CPDA1 anticoagulant. The blood was centrifuged and the resulting plasma was freshly frozen in 250 ml packs. Prior to use, the individual plasma packs were thawed in a water bath at 37^0 C and pooled. A minimum of at least 5 individual plasma preparations were used to prepare a pool. Aliquots of all plasmas were stored at -70^0 C prior to use.

Factor I deficient plasma (made deficient by plasma phoresis of normal donors) was obtained from George King

Biomedical (Overland Park, KS) This plasma was stored at -70° C until use. Plasma deficient in antithrombin III was prepared by heparin affinity chromatography using a method described by Ofusu et al., (1981). Heparin-sepharose CL 6B (lot # FE 15465) was obtained from Pharmacia (Piscataway, NJ). A column (Pharmacia K 15/90, total volume 156 ml) was packed with the heparin-sepharose, and equilibrated with 0,06 M $NaHPO_4$ and 0.5 M NaCl pH 7.5. To obtain the antithrombin deficient plasma, 200 ml of citrated normal human, platelet poor plasma was applied to the column. The plasma was eluted at a flow rate of about 2.5 ml/ minute with equilibration buffer. The eluted plasma was dialyzed against 0.4% sodium citrate containing 0.15 M NaCl, aliquoted and frozen at -70° C prior to use. Antithrombin III levels were determined using a synthetic substrate based automated method (aca automated chemistry analyzer E.I. Du Pont Co). Reference antithrombin III deficient plasma was provided dy Dr. F. Ofusu (McMaster Univ. Hamilton Ontario Canada).

Platelet rich plasma was prepared in the following manner. Blood was drawn from individual donors into 3.8 % citrate (1:10 ratio) and centrifuged at 225 x g for 20 minutes to obtain plasma rich in platelets. The platelet count was then adjusted (using autologous platelets) to about 250,000 using a light microscope and a bright line hemocytometer.

4. Thrombogenic Reagents

Konyne brand of prothrombin complex concentrate (PCC) lot # NC 912, obtained from Cutter Laboratories (Berkley, CA) was reconstituted in 20 ml of sterile water to obtain a working solution of 25 U /ml. This solution was kept frozen at -70° C until use. Russell's viper venom (RVV) in cephalin lot # 20F 39581, obtained from Sigma Chemical Co. (St. Louis, MO) was reconstituted with sterile water to 0.1 U/ml prior to each use.

5. Anesthetics

The anaesthetic agent utilized in both animal models was ketamine hydrochloride (Ketalar, Parke Davis Morris Plaines, NJ). In the rabbit stasis thrombosis model, an additional anaesthetic xylazine (Rompum Bayvet division, Miles Labs Shawnee, KS) was used. Both anaesthetics have been shown to have no effect on the normal coagulation profile or heparinizability of either primates or rabbits (Fareed et al., 1985). Pentobarbital sodium (Nembutal Abbott Chicago, IL) was used in rabbit euthanasia.

6. Animals

A mature primate colony (Macaca mulatta) consisting of 17 male and female animals (weight range 7-12 Kg) was housed in the AAA LAC approved animal research facility of Loyola University Medical Center. The health of all

primates was routinely evaluated by a licenced doctor of vetrinary medicine. Abnormal primates were excluded from experimentation. All primates were maintained on a standard diet of Purina monkey chow, had free access to water and kept on a regular 12 hour light /dark cycle during all experiments.

New Zealand white rabbits (Oryctolagus cuniculus) weight range 0.5 - 2.0 Kg, were obtained from Langshaw farms (Augusta, MI). These rabbits ranged in age from 9-18 mos. Rabbits were also housed in the animal research facility and exposed to a regular 12 hour light / dark cycle. The rabbits were fed a standard diet of Wayne Rabbit Ration and allowed free exposure to water.

For all animal studies, ethical guidelines established by the committee for animal welfare were strictly adhered to and all protocols were approved by the aforementioned committee.

7. Analytical Instruments

Most of the instruments used in this study were available through the departments of pharmacology and pathology at Loyola University Medical Center. These included a Biogamma gamma counter (Beckman Instruments Fullerton, CA) used in radioimmunoassays, a DU-7 spectrophotometer (Beckman Instruments Fullerton, CA) used in chromogenic substrate assays and an Multistat centrifugal

analyzer (Instrumentation Labs Lexington, MA) used in the factor VII-thromboplastin assays. Several fibrometers, (BBL Cockeysville, MD) used in clotting assays as well as two cone plate viscometers (Wells-Brookfield Stoughton, MA) were available in the hemostasis research laboratory. An personal computer XT (IBM Personal Computers Boca Raton, FL) and support software was utilized for statistical and word processing applications. The software used in these applications was SYSTAT version 2.0 (Systat Inc. Evanston, IL) and WORDPERFECT 4.1 (SSI Oren, UT) respectively.

A K 100/100 chromatography column and Pf-30 fraction collector (Pharmacia Laboratory Separation Division Piscataway, NJ) was used in the molecular weight fractionation. A Waters (Milford, MA) HPLC-GPC chromatography system, equipped with a model 710 WISP sample processor, a model 490 Multiwavelength Detector, a model 510 HPLC pump, and a Digital 350 computer running Waters 840 software was utilized for molecular weight determinations. These instruments were made available at Choay Chemie (Roen, France). The columns used in the molecular weight determinations were LKB Ultropac tsk 2,000 and 3,000 (Bromma, Sweden). The porosity of these columns was 125 and 250 angstroms respectively.

C. Molecular Weight Fractionation

Sodium porcine mucosal heparin (lot # H 410) was fractionated into molecular weight subfractions utilizing a method similar to that of Johnson and Malloy (1976). The fractionation was performed at Choay Chemie (Roen, France) under the following conditions. A Pharmacia K 100/100 column (dimensions 10 X 100 cm., total bed volume 1 L) was packed with Ultrogel ACA 44 agarose acrylamide matrix, size exclusion range 10,000-140,000 (Reactifs Clichy, France). The column was connected to a mariott flask and a sample reservoir through a three way valve to facilitate sample application and maintain a consistent flow rate. Prior to the fractionation process, the column was equilibrated with 0.5 M NaCl and the flow rate was adjusted to 400 ml / hour.

Five grams of the unfractionated heparin was dissolved in 70 ml's of distilled water and applied to the column through the sample reservoir. Heparin elution was monitored by manually recording absorbance of the eluent at 205 nM using a Beckman model 35 spectrophotometer. At the first absorbance peak, 15 fractions were collected with the aid of a Pharmacia Pf-30 fraction collector. The interval between fractions was 35 minutes. Since the absorbance peaks indicated low heparin concentrations in the initial and latter fractions, these fractions were pooled such that a total of nine fractions were obtained. The fractions were recovered using twice ethanol precip-

itation. The ethanol precipitation was performed using 1 volume of the fraction with 1.5 volume of 100 % ethanol while stirring. This mixture was centrifuged at 5,000 rpm and the supernatant discarded. The precipitate was then homogenized and vacuum dried in glass filter funnels at 40^0 C. The heparin content of the fractions was determined using a toluidine blue assay described in (NCCLS 5: (13) 373-376). The calibration curve for this assay is seen in appendix X.

D. Molecular Weight Determination

In order to determine the molecular weights of the gel filtered fractions, gel permeation chromatography was performed on a Waters liquid chromatography system. The system was equipped with a Digital 300 series minicomputer running Waters 840 software to facilitate data reduction. The Waters 840 system was specifically designed for applications in molecular weight determinations of polymers. The system calculated not only mean molecular weight, but also values indicating the molecular weight distribution. These values were calculated from the chromatographic characteristics of standards with similar molecular composition on columns of specific porosity. Since the molecular weight range of heparin was large, two columns of different porosity were used. Thus prior to use, the columns were calibrated with anionic

polymers consisting of sulfated glucuronic acid, uronic acid and glucosamine. A description of these standards is given in appendix XI. Appendix XII and XIII show the calibration curves obtained by running the standards on the LKB tsk 2000 and 3000 columns respectively. Values for retention time (RT) and polymer dispersity (D) were determined, and used along with the molecular weights of the standards to calculate a calibration curve. This curve was calculated by third polynomial regression and yielded polynomial coefficients (D_0, D_1, D_2, D_3) which characterized the average standard curve. The following equation was used:

$$MW = D_0 + D_1 (RT) + D_2 (RT)2 + D_3 (RT)^3$$

The molecular weight of the samples were determined from their chromatographic characteristics under the following conditions:

Columns: LKB tsk 2,000 and 3,000

Mobile Phase: $NaSO_4$ 0.5 M

Detector: UV 205 nm

Flow Rate: 1 ml/min.

Sample: 20 ul of 10mg/ml

Following chromatography, the total area under the elution curve for each sample was determined by integration. The computer divided the total area into about 50 equal time slices. The retention time value of each slice was used in the previous equation to calculate the molec-

ular weight of the individual slices. This provided approximately 50 (MW) and (RT) values for the following calculations where (S_x = the area of the x slice), (M_x = the molecular weight of the x slice), (M_w = the weight average molecular weight), (M_n = the number average molecular weight), (M_z = the z number molecular weight) and (D = the dispersity of the polymer).

$$M_w = \frac{(S_x M_x)}{(S_x)} \qquad M_n = \frac{(S_x)}{(S_x/M_x)}$$

$$M_z = \frac{(S_x M_x)^2}{(S_x M_x)} \qquad D = \frac{M_w}{M_n}$$

Thus, M_w indicated the value for the mean molecular weight of the polymer. M_n and M_z were molecular weight values which characterized the high and low molecular weight areas of the elution curve. These values were of particular importance for evaluating the relative distribution of differing molecular weight components within a given fraction. For example, if $M_w = M_n = M_z$, then this indicated that the polymer was monodisperse. Inequality in these values indicated a polydisperse mixture (Waters, 1985).

Verification of the molecular weights determined by the HPLC-GPC method were achieved by measuring the viscosity of the fractions (Johnson and Malloy, 1976 and Laurent, 1961). For this purpose a Wells Brookfield cone plate viscometer was utilized. Prior to viscosity measurements, the instrument was calibrated with silicone

fluids of known viscosity (5 and 10 centipoise) standardized by methods traceable to the U.S. National Bureau of Standards. Measurements were made by adding 1 ml of the individual fractions (conc. 10 mg/ml) to the viscometer. The solution was allowed to equilibrate to 25^0 C at which time the viscosity measurements were recorded at shear rates of 90, 225 and 450^{sec-1}. To obtain viscosity values in centipoise (mPa), the values read from the viscometer were multiplied by a constant based on the geometry of the cone and the shear rate. These values were .0514 for shear rate 450, .102 for shear rate 225 and .257 for shear rate 90 (Wells - Brookfield Technical Product Information).

E. In Vitro Anticoagulant Studies

From the original nine, five fractions yielding the widest range of molecular weights were selected for the experimental studies. These fractions were supplemented to pooled normal human platelet poor plasma at concentrations ranging from 0.625 - 20 ug/ml and profiled in clotting assays routinely used for coagulation profiling. These tests included: prothrombin time (PT), activated partial thromboplastin time (APTT) and Heptest. All assays were performed exactly as previously described.

The test fractions were also supplemented to antithrombin III deficient plasma (the antithrombin III

deficient plasma was prepared as previously described) at a concentration of 20 ug/ml and screened in an identical pT assay.

Amidolytic antifactor Xa and IIa assays were performed to assess the relative inhibition of these two enzymes by the various fractions. The test fractions were supplemented to pooled normal human platelet poor plasma and the assays were performed as follows. For the amidolytic anti Xa method: 375 ul of 0.05 M Tris and 0.175 M NaCl buffer pH 8.4 was equilibrated with 25 ul of plasma sample to 37° C. 50 ul of 0.5 nkat/ml human or bovine factor Xa was added and allowed to react for exactly 2 minutes. After the 2 minute incubation, 50 ul of substrate (spectrozyme-Xa 2.5 mM) was added and the change in absorbance was recorded at 405 nm over a one minute period. To determine the effect of the individual fractions, the heparinized samples were compared to a saline control, and a value of relative % inhibition was calculated.

The anti IIa method utilized the same buffer with a volume of 400 ul, to which 25 ul of plasma sample was added. The mixture was equilibrated as before and 25 ul of 10.0 NIH unit thrombin was added and incubated for exactly for 1.0 minute. At the end of one minute, 50 ul of substrate (spectrozyme-TH 2.5 mM) was added and the change in absorbance per unit time is recorded. Percent

inhibition was calculated in an identical manner as described for the anti Xa assay.

Fibrinopeptide-A generation tests (FPAGT) were also used to examine the test fractions. This test was used essentially as previously described (Emanuele et al., 1984, 1985) however, it was slightly modified to yield more reproducible results. The test was performed as follows. 0.625 - 5.0 ug/ml concentrations of the test fractions were supplemented to pooled normal human platelet poor plasma. Thromboplastin C (Dade PT reagent) was standardized in a prothrombin time assay to achieve consistent and measurable amounts of (FPA). The standardization was accomplished by diluting the thromboplastin C in 0.025 M $CaCl_2$ to obtain a PT value of about 35 seconds for citrated human plasma. FPA generation was initiated by adding 100 ul of the standardized thromboplastin to 400 ul of test plasma. A control FPA generation was also performed by adding 100 ul of saline to 400 ul of test plasma. FPA generation proceeded for exactly 2.0 minutes, at which time 100 ul of inhibitor cocktail containing 10 mg/ml EDTA, 500 KIU/ml aprotinin, 1 ug/ml indomethecin (Indocin Merk Sharp & Dohme, Philadelphia, PA) and a thrombin inhibitor 5 antithrombin U/ml was added. The plasma was then treated with bentonite (2:1 bentonite to plasma) mixed well and centrifuged. The supernatant was then assayed for FPA using a Mallinckrodt

radioimmunoassay (RIA) kit (St. Louis, MO). This kit utilized competitive binding between I^{125} labeled and unlabeled fibrinopeptide - A for a limited number of antibody binding sites. Quantitation of the generated FPA was achieved by comparing the assay results to a FPA standard curve.

The FPAGT was performed identically in platelet rich, and antithrombin III deficient plasma.

A whole blood system for the FPAGT was also utilized. The test was performed as follows: 12 X 75 non siliconized glass tubes were washed with saline, marked at a 2.0 ml level and supplemented with 100 ul of an appropriate concentration of heparin or heparin fraction. Blood was drawn from normal human volunteers using a double syringe technique. After discarding the first 2-3 ml's, the whole blood was immediately added to the tubes and filled up to the 2.0 ml mark. FPA generation was allowed to proceed for exactly 2.0 minutes. At the end of 2.0 minutes, 200 ul of the inhibitor cocktail previously described was added to prevent further FPA generation. Plasma was obtained by centrifugation, treated with bentonite, re-centrifuged and assayed as before.

The molecular weight dependent effects of heparin upon the extrinsic network were studied by developing an assay using thromboplastin and factor VII to activate factor X to Xa. This amidolytic Xa generation assay was

performed on a centrifugal analyzer (Mulitstat III, IL Lexington KY) as follows: 25 ul of 80 ug/ml factor X, 25 ul of 2.5 ug/ml factor VII and 25 ul of the individual heparin fractions (25 ug/ml heparin diluted in 1.25 U/ml AT-III) were added to well # 1 of the centrifugal rotor. 140 ul of 0.06 mM Tris and .1 mM $CaCl_2$ buffer pH 8.1, 25 ul of 2.5 mM spectrozyme Xa and human thromboplastin diluted 1:20 in 0.025 M $CaCl_2$ were added to well # 2 of the rotor. After incubation to 37° C, the contents of the two wells were mixed by accelerating the rotor and the change in absorbance at 405 nM was recorded at 30 second intervals over a 5 minute time period, after a 5 second delay.

This assay was slightly modified to allow for the use of a plasma matrix. In this modification, the plasma provided factors VII and X, thus additional buffer was added to achieve a similar volume. Dilute (1 : 2 in saline) fibrinogen deficient plasma was used in these studies to avoid clotting.

The results from assays run on the multistat kinetic analyzer were expressed in terms of uM of substrate cleaved per unit time. The multistat provides a recording of the absorbance change over time. Using the molar absorbance value for para-nitroanaline (9.2×10^3 L mol^{-1} cm), molar values were calculated per unit of time. Since the multistat has a .5 cm path length, absorbance values

were first divided by two. Values were calculated at three different time periods in the linear portion of the assay and averaged to obtain the rate of substrate cleavage in terms of uM/time.

For all of the previously described in vitro assays, an appropriate dose response was determined by studying the test fractions in at least four concentrations. Since the pooled plasma was considered a reagent for the in vitro test systems, all fractions were assayed on five separate occasions to achieve statistical significance.

F. In Vivo Studies

1. Pharmacodynamic time course

The pharmacodynamic time course of three test fractions (molecular weights 23,000; 13,300 and 5,100 daltons) along with the unfractionated heparin and a commercial low molecular weight heparin (Fraxiparine) were investigated in a primate model (Macaca mulatta) using both intravenous and subcutaneous routes of administration. The model was similar to that described by Fareed et al., (1985). Before initiating the study, all primates were profiled using clinical laboratory methods including SMAC, CBC and coagulation studies for the purpose of excluding abnormal animals. Before all blood sampling or drug injections, the animals were anaesthetized/immobilized by injection of 10 mg/kg

ketamine HCl. It has been shown that repeated administration of this anaesthetic does not significantly alter the normal coagulation or heparinization profile (Fareed et al., 1985). At all times when test drug or anaesthesia was administered the safety and comfort of each primate was continually monitored.

For subcutaneous administration, all fractions were compared at a dose of 1.0 mg/kg (stock solution conc. 10.0 mg/ml). For intravenous administration, a dose of 250 ug/kg was used (stock solution 1.0 mg/ml). Prior to injection of the test fraction, a baseline blood sample was drawn from the saphenous vein of the individual monkeys. For the intravenous route, the fractions were administered by a single bolus injection to the same vein. For the subcutaneous route, a single bolus was injected at a site in the lower abdomen. For both intravenous and subcutaneous routes, the test agents were injected through syringes incorporating sterile, pyrogen free 0.2 um Nalgene filters (Nalge Co. Rochester, NY). After injections and blood sampling, the animals were returned to their respective cages until the next sampling time. At the prescribed time intervals, the primates were anaesthetized and a blood sample was taken as before. For the subcutaneous route blood samples were taken at 0, 2, 4, 6, 8 , 10, 12 and 24 hours post injection. For the intravenous route blood samples were taken at 0, 5, 10,

15, 30, 60, 180, and 360 minutes post injection. At each sampling time 3.0 ml of blood was collected into siliconized glass tubes containing 0.3 ml of 3.8 % sodium citrate and centrifuged to obtain plasma. Clotting times were performed on the fresh plasma samples in the intravenous study. The protease and fibrinopeptide - A assays were performed using the same plasma which had been stored at- 70^0 C for not more than two months. In the subcutaneous study, all assays were performed in plasma which had been stored at - 70^0 C.

In the intravenous study, each fraction was tested in the same five animals. Since all animals received all test agents, a dosing schedule was utilized to account for effects possibly due to the order in which the agents were administered. The schedule was as follows:

Animal	Week 1	Week 2	Week 3	Week 4	Week 5
1	I	IV	IX	heparin	CY 216
2	IV	I	CY 216	IX	heparin
3	IX	heparin	IV	CY 216	I
4	heparin	CY 216	I	IV	IX
5	CY 216	IX	heparin	I	IV

During the study, the following protocol for blood sampling was strictly adhered to.

9:15 A.M. injection of anaesthesia.

9:30 A.M. 0 hour sample followed by test fraction injection.

9:35 A.M. 5 min. sample

9:40 A.M. 10 min. sample

9:45 A.M. 15 min. sample

10:00 A.M. 30 min. sample

10:45 A.M. animals were examined for determination if additional anaesthesia was required and injected if necessary.

10:30 A.M. 1 hour sample

12:15 P.M. additional anaesthesia administered

12:30 P.M. 3 hour sample

3:15 P.M. additional anaesthesia administered

3:30 P.M. 6 hour sample

For the subcutaneous study, the different molecular weight fractions were examined in 15 healthy primates. These animals were randomly assigned to three groups of 5 for the purpose of testing all 5 fractions on each experimental day. Each fraction was tested in 5 different primates. The limited number of primates necessitated that individual monkeys be used more that once. At least 10 washout days were allowed before a any animal was utilized for the second time. The following blood sampling time schedule was utilized.

8:00 A.M. injection of anaesthesia

8:15 A.M. 0 hour blood sample followed by test fraction injection

10:00 A.M. injection of anaesthesia

10:15 A.M. 2 hour blood sample

12:00 P.M. anaesthesia

12:15 P.M. 4 hour blood sample

2:00 P.M. anaesthesia

2:15 P.M. 6 hour blood sample

4:00 P.M. anaesthesia

4:15 P.M. 8 hour blood sample

6:00 P.M. anaesthesia

6:15 P.M. 10 hour blood sample

8:00 P.M. anaesthesia

8:15 P.M. 12 hour blood sample

8:00 A.M anaesthesia

8:15 A.M. 24 hour blood sample

For both intravenous and subcutaneous studies, the pharmacodynamic time course was characterized in terms of the biological half-life ($t_{1/2}$), apparent volume of distribution (V_d), plasma clearance (Cl_p) and area under the plasma - drug time curve (AUC). Individual parameters were calculated from heparin concentrations determined using each of the previously described in vitro assays (Heptest, FPAGT, Anti Xa, Anti IIa). A dilute thromboplastin clotting time (PT) was also performed by using the standardized thromboplastin from the FPAGT in a PT assay. To convert the bio-assay parameter into relative heparin concentrations, calibration curves were used. To ensure the most accurate calibration, the curves were constructed

from test fraction supplementation of the individual zero hour plasma samples.

To determine Vd, the following formula was used.

$$V_d = \frac{x}{C_p} \quad \text{where}$$

C_p = maximal plasma concentration after distribution determined by back extrapolation of the beta slope to time zero and x = dose of drug given. $t_{1/2}$ was determined by observing the time required for the plasma concentration to decrease by 1/2 during the decay phase obtained from a semi-log plot of concentration / time. Cl_p was calculated using the following equation: $Cl_p = Vd \times Ke$ where,

$$K_e = \frac{.693}{t_{1/2}}$$

AUC was calculated using the trapezoidal rule. To determine the area between sampling times the following equation was used.

$$[AUC]_{t_{n-1}}^{t_n} = \frac{C_{n-1} + C_n}{2} (t_n - t_{n-1})$$

The total area was calculated by summing the individual areas between consecutive time intervals. Absorption of the anticoagulant components after subcutaneous administration was determined relative to the intravenous route using the following formula.

$$\text{Absorption} = \frac{\text{AUC SC / dose}}{\text{AUC IV / dose}}$$

2. Antithrombotic actions

The antithrombotic effects of the test fractions were studied in a modified stasis thrombosis model (Fareed et al., 1985) using both subcutaneous and intravenous routes of administration. In the subcutaneous route, all fractions were compared at 1.0 mg/kg. For the intravenous route all fractions were compared at 25, 50 and 100 ug/kg.

White New Zealand male rabbits were anaesthetized with intramuscular injections of Xylazine (20 mg/kg) and ketamine (80 mg/kg). After induction of anaesthesia, the rabbits were weighed and prepared for surgery. Baseline blood samples were also taken at this time. The surgical procedure entailed the isolation of both right and left jugular vein segments. To minimize trauma and to ensure hemostasis, battery operated cauteries were used in the surgical procedures. After isolating the jugular vein segments, the test fractions were injected by intravenous route through a marginal ear vein and allowed to circulate for five minutes. For the subcutaneous route of administration, the surgical procedure was initiated 3.5 hours post subcutaneous injection in the lower abdomen.

At the appropriate time, thrombogenic challenge of Konyne brand of PCC (25 U/kg) followed by RVV (0.01 U/kg) was administered through the marginal ear vein and allowed to circulate for exactly 20 seconds. At this time, the isolated jugular vein segments were ligated and stasis

produced. Blood samples, drawn by cardiac puncture for ex vivo analysis, were taken immediately prior to and after injection of the thrombogenic challenge. After exactly 10 minutes of stasis time, the isolated segments were removed and examined for blood clots in a saline filled petri dish. Clot formation was visually graded using a + - system. In this system, - represented blood only with no evidence of clotting, + indicated some small clots but mostly blood, ++ indicated mostly small but some medium clots, +++ indicated a large clot with some blood while ++++ indicated a fully formed, casted clot with no blood. In order to analyze the data, the + - grades were transformed into numerical values using the following scale.

- = 0
+ = 1.25
++ = 2.5
+++ = 5.0
++++ = 10.0

After transformation, mean values were determined from the average of the left and right stasis scores.

For statistical significance, all drugs were compared in 5 animals for each route of injection and concentration.

G. Statistical analysis

Various statistical tests were performed on the experimental data. For this purpose an IBM PC XT

utilizing SYSTAT and an Hewlett - Packard 32 E were used to facilitate data reduction and test statistic calculation.

SYSTAT is a commercial software program for statistical applications. It has the ability to perform most commonly used statistical tests, and was utilized in the calculation of means, standard deviations, correlation coefficients, t-tests, one way analysis of variance (AVOVA) and Student-Newman-Keuls test statistics. All test statistics were calculated at a significance level of 0.05. Critical values for t, F and H distributions were obtained from Zar, (1974).

To establish relationships between molecular weight and effects in the various in vitro assays, regression analysis was used to calculate correlation coefficients.

To test for a difference in effect of the fractions in the various in vitro tests, one way ANOVA was utilized. If ANOVA revealed a significant difference, the Student-Newman-Keuls test was used to more precisely determine which fractions differed. This procedure was selected in preference to both the Tukey and Duncans tests since it tends to be more powerful and more widely accepted (Zar, 1984). When using SYSTAT to perform the Student-Newman-Keuls test, only critical values for differences in ordered means were provided. These values were utilized by calculating the differences in ranked mean values for

the comparison groups and comparing this value at the appropriate gap order to determine significance.

To test for a difference in the pharmacodynamic time course of the test fractions, ANOVA followed by Student-Newman-Keuls testing was again utilized.

To test for difference between the antithrombotic effects of the test fractions, the Kruskal - Wallis nonparametric ANOVA was used. Multiple comparison testing of nonparametric ANOVA was done similar to the Newman-Keuls test, however the ranked sums were used in the calculation of the test statistic.

To represent the variation in mean values, standard deviation calculated using SYSTAT. Thus in all figures, error bars represent the standard deviation.

CHAPTER IV

RESULTS

A. Fractionation of Heparin

From the initial five grams of starting material, 4.425 grams were recovered from the gel-filtration procedure in the following proportions. Fraction I 250 mg, II 725 mg, III 575 mg, IV 650 mg, V 626 mg, VI 550 mg, VII 450 mg, VIII 500 mg and IX 100 mg. The elution profile of heparin lot # 410 is seen in figure 1. The elution pattern reflected the composition of molecular weight components in the unfractionated preparation. A normal distribution of these components was observed.

To test whether the heparin content was similar for each of the gel-filtered fractions, a toluidine blue heparin assay was used. In this assay, the toluidine blue dye reacted with the sulfate groups on the heparin molecules to produce a color change. This color change increased with increasing heparin content and was measurable by absorbance at 606 nm. Appendix X shows the direct relationship between heparin content and absorbance at 606 nm. Figure 2 illustrates the results of the toluidine blue assay on the gel-filtered fractions. All fractions displayed similar absorbance characteristics suggesting similar glycosaminoglycan content.

B. Molecular Weight Determination

The results of the HPLC-GPC chromatography of the fractions, the starting heparin and CY 216 using two columns of different porosity (LKB tsk 2000 and 3000) are represented in appendix XIV and XV respectively. A detailed description identifying various regions of the elution curves is shown in appendix XX. Due to the differences in porosity, slightly different molecular weights were obtained from the two columns. To achieve accurate values, the results from both columns were averaged used in the calculation of apparent mean and peak molecular weights. These values along with the approximate molecular weight ranges are seen in table 1. Although significant overlap existed between the fractions, they were different when analyzied using HPLC in terms of mean molecular weight, peak molecular weight and molecular weight distribution.

Table 2 shows the results of the viscosity measurements on the same heparin preparations. The viscosity of the individual fractions ranged from 0.76 mPa for the 5,100 M.W. fraction to 1.28 mPa for the 23,000 M.W. fraction. For all test heparins, the viscosity increased with increasing molecular weight. When the viscosity values were plotted as a function of molecular weight, a linear relationship was observed (figure 3).

c. In Vitro Screening (Clotting and Amidolytic Assays)

Five fractions with the broadest difference in molecular weight, the native heparin from which these fractions were obtained and CY 216 were supplemented to normal human pooled plasma. These agents were tested at 5 concentrations for in vitro anticoagulant activity using assays described in the methods section. All assays were performed in duplicate on 5 separate days to achieve statistical validation.

1. APTT Assay

The results for the APTT, expressed in terms of clotting time in seconds, are given in table 3. For this and all other clotting assays, the prolongation of this parameter was directly related to the anticoagulant action of the respective fraction. For all fractions a concentration dependent response was observed within the 0.6-10 ug/ml range. The lowest concentrations resulted in slight increases from the baseline clotting time of 27 seconds. Higher concentrations, displayed greatly prolonged times for all fractions. At the higher concentrations, the 23,000 ; 17,450 ; 13,300 and 9,000 M.W. fractions, along with the native heparin, inhibited the assay beyond its linear range of 200 seconds.

When compared for potency at a concentration of 2.5

ug/ml, large differences between the individual molecular weight fractions were observed. The 13,300 M.W. fraction was the most potent with a clotting time of 82 seconds. At the same concentration, the 5,100 M.W. fraction displayed the least potent actions, with a time of 36 seconds. The apparent potency rank order of the different heparins in this assay was 13,300 M.W. > Native Heparin > 17,450 M.W. > 9,000 M.W. > 23,000 M.W. > CY 216 > 5,100 M.W.. ANOVA revealed that these potency differences were significant ($p < .0001$). Newman-Keuls (.05) comparison suggested significant differences between the following: 13,300 $\neq$ heparin $\neq$ 17,450 $\neq$ 9,000 = 23,000 = CY 216 = 5,100.

Testing the 5 gel-filtered fractions for correlation between potency and M.W. indicated a poor relationship ($r = .187$). Although a good correlation existed from 5,100 through 13,300, beyond this point, further increases in molecular weight did not result in greater potency.

2. PT Assay

The results of the PT assay are shown in table 4. Since this assay was not sensitive to the actions of heparin below 1.25 ug/ml, relatively higher concentrations were used. Between 1.25 - 20 ug/ml, concentration dependent effects were observed for all fractions. These concentration dependent effects varied in potency response

between the different molecular weight fractions. At 20 ug/ml, the 23,000 M.W. fraction prolonged the clotting time to 55 seconds while a identical concentration of the 5,100 M.W. only elevated the time to 15 seconds. When ranked in terms of apparent potency at the 20 ug/ml concentration, the 23,000 M.W. > 17,450 M.W. > 13,300 M.W. > Native Heparin > 9,000 M.W. > CY 216 > 5,100 M.W.. ANOVA suggested that these potency differences were significant ($p < .0001$). The overall conclusion determined by Newman-Keuls at .05 suggested significant differences between the following: 23,000 $\neq$ 17,450 $\neq$ 13,300 $\neq$ heparin $\neq$ 9,000 = CY 216 $\neq$ 5,100.

Regression analysis demonstrated a strong relationship between clotting time in the PT assay and molecular weight ($r = .97$). From 5,100 through 23,000 M.W., potency directly increased with increasing M.W. It was interesting to note that the potency of both the native heparin and CY 216 both correlated well to their respective molecular weights.

An identical PT assay was run in plasma deficient in antithrombin III (antithrombin III levels were determined to be 6.2 % of normal using a synthetic substrate based functional assay [aca method] (table 5). In this plasma, 20 ug/ml concentrations of all test agents produced a slight prolongation from the baseline clotting time. No difference in potency was observed between any of the test

fractions ($p = .83$).

3. Heptest Assay

The results for the heptest assay are presented in table 6. The baseline value of the assay in normal human pooled plasma was 16 seconds. Supplementation of the test fractions at concentrations between 0.6 and 10 ug/ml to the same plasma resulted in concentration dependent increases in clotting time. Increasing concentration resulted in increased clotting time for all fractions. These concentration dependent actions were different in terms of potency response between the various test agents. When compared at a concentration of 2.5 ug/ml, the apparent rank order of the test heparins was as follows: Native heparin > 13,300 > 9,000 > 23,000 > 17,450 > CY 216 > 5,100. ANOVA indicated significant differences between these potency responsesn ($p = .000$). The overall conclusion determined by Newman-Keuls analysis at .05 suggested the following differences in potency were significant: Heparin $\neq$ 13,300 $\neq$ 9,000 = 23,000 $\neq$ 17,450 $\neq$ CY 216 $\neq$ 5,100.

The correlation observed between potency and molecular weight in this assay was poor ($r = .08$). Potency increased with increasing molecular weight from 5,100 to 13,300. Higher M.W.'s (17,450 and 23,000) did not demonstrate greater potency but rather were less potent when

compared to the 13,300 M.W. fraction.

4. Anti Xa Assay

The results of the amidolytic anti Xa assay are shown in table 7. The results of this assay are expressed in terms of % inhibition and were calculated as previously described in methods. These values represent the inhibition of factor Xa by the different heparin preparations. For all test fractions, this inhibition increased with increasing concentration, however large differences in potency were observed. At 10 ug/ml, the most potent fractions resulted in inhibition ranging from 87 - 88 %. The least potent fraction displayed 69 % inhibition at an identical concentration. When ranked in order of apparent potency determined at a concentration of 2.5 ug/ml, 13,300 > Heparin > 23,000 > 17,450 > 9,000 > CY 216 > 5,100. ANOVA suggested that the apparent potency differences were significant ($p = .031$). Newman-Keuls multiple comparison test (.05) suggested actual differences between the following fractions: 13,300 = heparin =23,000 = 17,450 $\neq$ 9,000 $\neq$ 5,100 = CY 216.

Potency correlated well with M.W. from 5,100 through 13,300. However, above 13,300 M.W. all fractions displayed similar potency, thus the overall correlation between potency and M.W. was poor ($r = .67$).

5. Anti IIa Assay

The results for the anti IIa amidolytic assay are seen in table 8. These results are expressed in terms of % inhibition and reflect the actions of the fractions on factor IIa. As with all previous assays, concentration dependent effects were observed for all test agents between 0.626 and 10 ug/ml concentrations. Between the individual fractions, large differences in potency were observed. At 10 ug/ml, the most potent fractions resulted in inhibition ranging from 90 - 93 %. Less potent fractions produced about 60 % inhibition at identical concentrations. The apparent potency rank order observed at a concentration of 2.5 ug/ml was 23,000 > 13,300 > 17,450 > Heparin > 9,000 > 5,100 > CY 216. ANOVA suggested the potency differences were significant ($p = .000$). Newman-Keuls (.05) demonstrated significant differences between the following fractions: 23,000 = 17,450 = 13,300 = heparin $\neq$ 9,000 $\neq$ 5,100 $\neq$ CY 216.

The correlation between potency and M.W. was $r = .60$. These results were similar to those achieved in the anti Xa assay. Potency increased with increasing molecular weight from 5,100 through 13,300. Molecular weights above 13,300 showed no additional increases in potency.

It is interesting that in most conventional clotting and amidolytic assays, similar patterns of molecular

weight dependent potency were observed. The noteworthy exception was the PT assay. This assay was also the only method which utilized an extrinsic activation system.

For the purpose of further examining the molecular weight dependent effects of heparin on extrinsic activation, a assay based upon the release of fibrinopeptide-A was utilized. The assay was used as described in methods.

6. FPA Generation Assays

The test agents were examined in four FPA generation matrices (normal platelet poor plasma, antithrombin III deficient plasma, normal platelet rich plasma and whole blood). For all plasma systems, thromboplastin was used as an activator (extrinsic activation). The whole blood system was activated by contact with glass (intrinsic activation). The results of all FPA generation assays were expressed in terms ng/ml of FPA generated over a two minute period. This value was inversely related to the potency of the test fractions in the assay.

a. FPA Generation In Normal Human Plasma

The results of the test fractions in normal platelet poor plasma are shown in table 9. Between the ranges of 0.6 - 5 ug/ml, concentration dependent effects were observed for all test fractions. Differences in potency between the fractions were observed and appeared to be

related to molecular weight. The 23,000 M.W. fraction displayed the most potent inhibition of FPA. At 5 ug/ml 42 ng/ml were generated. In contrast, the 5,100 M.W. fraction displayed the least inhibition. At an identical concentration, 763 ng/ml of FPA were generated over the two minute time period. When the test fractions were compared for their ability to inhibit FPA generation at a concentration of 2.5 ug/ml, the following potency rank order was observed 23,000 > Heparin > 17,450 > 13,300 > 9,000 > CY 216 > 5,100. Significant differences were in potency were determined by ANOVA ($p = .000$). Newman-Keuls analysis (.05) suggested differences between the following: 23,000 = 17,450 = native heparin $\neq$ 13,300 $\neq$ 9,000 $\neq$ 5,100 = Cy 216.

b. FPA Generation In Antithrombin III Deficient Plasma

Plasma from the same source used in the previous in vitro assays was made deficient in antithrombin III using the heparin affinity chromatography technique described in methods. Antithrombin III levels were determined to be 6.2 % of normal using a functional method (ACA). The plasma was supplemented at 10 ug/ml with the test fractions and assayed in the same thromboplastin activated FPA generation assay. The results illustrating the effects of the molecular weight fractions and a saline control, in this plasma are presented in table 10.

In the plasma deficient in antithrombin III, addition of the test fractions did not result in the inhibition of FPA generation. In the saline control, thromboplastin activation resulted in the generation of 1216 ng/ml FPA over the 2 minute assay period. When the test fractions were supplemented to the antithrombin III deficient plasma, the generated FPA was similar to control values (ANOVA value p = .73).

c. FPA Generation In Platelet Rich Plasma

The effects of the test fractions on FPA generation were compared in platelet poor and platelet rich normal human plasma at a concentration of 2.5 ug/ml. The results are seen in figure 4. All fractions inhibited the generation of thromboplastin activated FPA generation in both the platelet rich and platelet poor plasmas. Using paired t-tests, no difference was observed between the effects of any of the test fractions in the platelet rich or platelet poor plasma. The individual probabilities were as follows: 23,000 p = .104; 17,450 p = .072; 13,300 p = .60; 9,000 p = .60; 5,100 p = .83; heparin p = .51; CY 216 p = .80.

Although no significant differences were detectable between the two plasmas, slightly more FPA was consistently generated in the platelet rich plasma.

d. FPA Generation In Whole Blood

Five fractions were screened in the whole blood FPA generation assay exactly following the procedure described in methods. The results are illustrated in table 11. All test fractions displayed concentration dependent inhibition of FPA generation. These concentration dependent effects were different in terms of potency response between the test agents. At 10 ug/ml the most potent test fractions produced complete inhibition of FPA generation. In contrast in the presence of the least potent fraction (5,100 M.W.), 12.6 ng/ml FPA was generated over the 2 minute assay period. The apparent potency rank order was as follows: 13,300 > 23,000 > Heparin > CY 216 > 5,100. Using Newman-Keuls analysis (.05), significant differences were observed between the following: 13,300 = 23,300 = Heparin $\neq$ Cy 216 = 5,100 was achieved.

It was interesting to note that unlike the thromboplastin activated assays, the potency of the fractions did not appear to be related to molecular weight.

7. VII - Thromboplastin Assay

The enhanced potency of high molecular weight fractions observed in thromboplastin activated assays suggested high molecular weight heparin may inhibit the actions of thromboplastin. To investigate this relation-

ship, a assay using thromboplastin and factor VII to activate factor X to Xa was developed. The assay endpoint was determined by monitoring the release of pNA from a Xa specific substrate. In this assay factor VII and thromboplastin were both necessary to activate factor X. The activation of factor X was directly related to factor VII and thromboplastin concentrations (fig. 5).

Figure 6 shows the effect of the test fractions on this assay system. Although compared at an identical concentration of 1.25 ug/ml, large differences in the potency of the individual fractions were observed. The potency appeared to be related to the molecular weight of the individual fractions. The 23,000 M.W. fraction was the most potent inhibitor of the assay. In the presence of this fraction, p-NA was generated at a rate of .55 uM/min. In contrast, the 5,100 M.W. fraction produced the least inhibition. In its presence, p-NA was generated at a rate of 2.75 uM/min. The potency of the remaining fractions followed the following rank order: 23,000 > 17,450 > 13,300 > Heparin > 9,000 > CY 216 > 5,100. Significant differences in potency, as determined by Newman-Keuls analysis (.05) were detected between the following fractions: 23,000 $\neq$ 17,450 $\neq$ 13,300 $\neq$ Heparin $\neq$ 9,000 = CY 216 $\neq$ 5,100.

As with other assays using thromboplastin activation, a high correlation between potency and

molecular weight was observed (r = .97).

To distinguish whether the pattern of molecular weight dependent inhibition was due actions of the test fractions inhibiting factor Xa, the original assay was modified. Factor's VII and X were eliminated and replaced by a concentration of factor Xa which generated an amount of p-NA equivalent to the antithrombin III control in the original assay. The effect of the test fractions on this system are shown in figure 7. In the presence of the test fractions higher rates of p-NA release were observed compared to the original assay. All fractions inhibited the Xa, however no direct molecular weight dependent inhibition was observed as before. The 23,000 ; 17,450 ; 13,300 ; 9,000 and native heparin preparations all resulted in similar rates of p-NA release between 5 - 6 uM/min. In contrast to the results with the presence of factor VII, Newman-Keuls multiple comparison (.05) suggested that 23,000 = 17,450 = 13,300 = Heparin $\neq$ 9,000 $\neq$ CY 216 = 5,100.

To further investigate the mechanism of the molecular weight dependent potency differences between the fractions, two systems isolating the various fractions with either factor VII-thromboplastin or factor X were used. The centrifugal rotors were designed with two wells which allowed initial isolation with subsequent mixing of all reagents after acceleration. This design allowed the

test fractions to incubate either in the well containing the factor VII - thromboplastin or with the factor X. Figure 8 shows the result of the test fractions in this experiment. Paired t-tests revealed significant differences between the actions of the test fractions in the two incubation systems for only the 23,000 ; 17,450 M.W. fractions and unfractionated heparin (p = .000, .000 and .001 respectively). For these three fractions, incubation with factor VII - thromboplastin resulted in greater inhibition than incubation with factor X. The 13,300 ; 9,100 ; 5,100 M.W. fractions and CY 216 showed no difference between the two incubation systems (probabilities .084, .935, .101 and .95 respectively).

The % differences between the two incubation systems were calculated to provide a better indication of the molecular weight differences between the two incubation systems. These values were 64 %, 48 %, and 23 % for the 23,000, 17,450 and unfractionated heparin respectively. These results demonstrated that in this assay the 23,000 M.W. fraction produced the most inhibition followed by the 17,450 M.W. fraction and unfractionated heparin respectively.

a. VII - Thromboplastin in Plasma

The original VII - Thromboplastin assay was modified to examine the actions of the test fractions in a plasma

matrix. Fibrinogen deficient plasma was used to prevent clot formation and was the source factors VII and X. This assay was performed exactly as described in methods using both Xa and thrombin specific substrates. Figure 9 shows the effects of the molecular weight fractions at a concentration of 5 ug/ml in the plasma system with a Xa substrate. In this system the potency response of the fractions resembled that of the pure Xa system. The 23,000 ; 17,450 ; 13,300 and native heparin all exhibited similar potency. Significant differences, determined by Newman-Keuls analysis (.05) were observed between the following 13,300 = 17,450 = Heparin = 23,000 ≠ 9,000 = CY 216 ≠ 5,100. This observation was in strong contrast to other thromboplastin activated assays, where potency consistently increased with increasing molecular weight.

Figure 10 shows the results of the same assay using a 2.5 ug/ml concentration of the test fractions. A similar pattern of molecular weight dependent effects was observed. At this concentration, the 13,300 M.W. fraction appeared to be the most potent inhibitor of the assay. This observation was even more evident at 1.25 ug/ml (figure 11). The apparent potency rank order for both concentrations was 13,300 > 17,450 > Heparin > 23,000 > 9,000 > Cy 216 > 5,100. Newman-Keuls (.05) suggested significant differences between the following: 13,300 ≠ 17,450 = Heparin = 23,000 ≠ 9,000 = CY 216 ≠ 5,100.

The effects of the test fractions at a concentration of 5 ug/ml, in the plasma system using thrombin substrate are shown in figure 12. The pattern of molecular weight related potency in this system was similar to that observed with the Xa substrate. The apparent potency order of the fractions was as follows: 13,300 > 17,450 > 23,000 > Heparin > 9,000 > CY 216 > 5,100. Significant differences were detected using Newman-Keuls (.05) between all fractions except CY 216 = 5,100 M.W.

At a test fraction concentration of 2.5 ug/ml, a similar pattern of molecular weight dependent effects were observed. Consistent with the lower concentrations of the test fractions, less inhibition of the assay was seen (figure 13).

At a concentration of 1.25 ug/ml, additional concentration dependent decreases in the actions of all test fractions were observed (figure 14). At this concentration, the 13,300 M.W. fraction appeared to be the most potent inhibitor of the assay, however it was not significantly different from the 23,000 and 17,450 M.W. fractions.

D. <u>In Vivo Results (Pharmacodynamic Concentration / Time Course)</u>

The intravenous and subcutaneous pharmacodynamic concentration / time course of CY 216, unfractionated

heparin the 23,000 ; 13,300 and 5,100 M.W. fractions were studied in the primate Macaca mulatta as described in methods. Plasma concentrations of the test fractions were determined using 5 assays (Heptest , dilute PT, anti Xa, anti IIa and FPAGT). These assays were performed exactly as previously described. Gravimetric concentrations were extrapolated from calibration curves made by supplementing the test agents to autologous plasmas. The resulting concentrations were plotted as a semi-log function of time and used to estimate the time course parameters. The formulas used in these calculations are described in methods.

1. Intravenous Administration

Figure 15 shows the semi-log concentration / time plot for the 23,000 M.W. fraction calculated from data obtained using the Heptest assay. After injection, plasma concentrations declined at a constant rate throughout all sampling times. The apparent distribution volume was .049 L/Kg suggesting that this fraction remained within the plasma. The half-life was calculated at 29 ± 2.2 minutes.

The semi-log concentration / time plot for the 13,300 M.W. fraction calculated from data obtained using the Heptest assay is seen in figure 16. After injection, an initial rapid decrease in plasma concentration was observed through the 5, 10 and 15 minute sampling times.

Afterward, a slower rate of decrease persisted until the drug could no longer be detected. These differences in elimination rates demonstrated that for this agent both alpha and beta phases of distribution and elimination occurred. The half-life during the alpha phase was 25 ± 3 minutes compared to the 34.5 ± 4.5 minutes during the beta phase. The observation of a biphasic elimination curve suggested distribution of this fraction within two compartments.

Figure 17 shows the concentration / time plot for the 5,100 M.W. fraction calculated from plasma concentrations determined using the Heptest assay. A bi-phasic elimination pattern was again observed. The half-life calculated from the alpha slope was 25 ± 2 minutes compared to 51 ± 6.5 minutes during the beta phase. The kinetic behavior of this fraction suggested distribution to two compartments.

Figure 18 shows the concentration / time plot for CY 216 calculated from heptest assay data. The concentration / time course of this agent was similar to the 5,100 M.W. fraction. The alpha phase persisted throughout the 5,10 and 15 minute sampling times. The half-life during this phase was 22.5 ± 4 minutes. The beta phase half-life was 58 ± 4.8 minutes. A slightly larger distribution volume (.006 L/Kg) was calculated for this agent compared to the other test fractions.

The concentration / time course of the unfractionated heparin calculated using the Heptest assay is shown in figure 19. After injection, the plasma concentrations rapidly decreased over the first 10 minutes. Between 10 and 15 minutes, plasma concentrations decreased at a greatly reduced rate compared to the initial 10 minutes. Beyond this point, elimination increased to a rate similar to that initially observed. These different rates suggested distribion to more that two compartments. The half-life values for the elimination phases were 25 ± 3.5 and 31 ± 2.2 minutes for the alpha and beta phases respectively.

a. Pharmacodynamic Concentration / Time Course of the Test Fractions Using Different Assays

i. Heptest

A summary table for the intravenous plasma concentration / time course of the test fractions calculated using the heptest assay is seen in table 12. Comparison of the half-life values demonstrated an inverse relationship between this parameter and molecular weight. Half-life increased with decreasing molecular weight from 29 minutes for the 23,000 M.W. fraction, to 58 minutes for CY 216 (M.W. 4,500). This response was consistent for all test fractions, however significant differences were only observed between the highest and lowest molecular weighs.

The overall conclusion based upon ANOVA and Newman-Keuls (.05) multiple comparison for similarity in half-life was: 23,000 = 13,300 = Heparin ≠ CY 216 = 5,100. Clearance values were consistent with the half-life results.

The apparent distribution volume of all test fractions approximated the plasma volume (.05 -.06). With the exception of CY 216, no difference was observed in this parameter among the test agents (p = .01) It is interesting to note that the lowest molecular weight fraction (CY 216) had the greatest apparent volume of distribution.

Comparison of the molecular weight fractions for their response in terms of area under the plasma concentration / time curve (AUC) were similar to the half-life results. AUC increased with increasing molecular weight. This observation was consistent for all test fractions, however only the lowest M.W. fractions were significantly different. Comparison using Newman-Keuls (.05) suggested an overall conclusion of CY 216 = 5,100 ≠ 13,300 = heparin = 23,000.

ii. Anti Xa Assay

The results of the intravenous concentration / time course for the anti Xa activity of the test fractions is shown in table 13. The curves from which these values were calculated are given in appendix XVIa. No signif-

icant difference between the time course observed using the Heptest or anti Xa method was calculated (for Vd p = .03 ; for T 1/2 p = .26 ; for Cl p = .35 and for AUC p = .67).

iii. Anti IIa Assay

The results of the time course for the anti IIa activity of the fractions is seen in table 14. The curves from which these values were calculated are shown in appendix XVIb. No increase in half-life was observed with decreasing M.W. All test fractions displayed a similar plasma half-life which ranged from 30 - 32 minutes. These slight differences were not significantly different (p = .28). In addition, no molecular weight dependent effects were observed for clearance or apparent distribution volume. A molecular weight related effect was observed for AUC. CY216 and the 5,100 M.W. fraction displayed significantly greater values (4.0 & 4.73 respectively) compared to the higher molecular weight fractions (3.31, 3.53 and 3.5 for 23,000 ; 17,450 and heparin respectively.

iv. Dilute Thromboplastin Assay

The time course values of the test fractions calculated from the dilute thromboplastin assay are illustrated in table 15. The concentration / time plots from which these values were calculated are shown in appendix XVIc. Molecular weight dependent effects were

observed for all parameters. Half-life, apparent Vd and AUC all increased with decreasing molecular weight. Accordingly, plasma clearance decreased in a similar manner.

The concentration / time course of the test fractions calculated using this assay, showed a similar pattern of molecular weight dependence to those calculated from other assays. These values were less compared to those calculated using the heptest and anti Xa assays. The values ranged from 26 minutes for the 23,000 M.W. fraction, to 39 minutes for CY 216.

v. FPAGT

Table 16 shows the summary of the intravenous concentration / time course for the test agents calculated from the FPAGT. The concentration / time plots from which these values were calculated are shown in appendix XVId. As with previous assays, significant differences in the kinetic parameters of the molecular weight fractions were observed. Half-life and AUC increased as the molecular weight of the test fraction decreased. Clearance was inversely related to half-life.

When the time course as determined in this assay was compared to those calculated by other methods, significant differences were observed. Half-life values were longer for all fractions compared to those calculated

from other methods. These values ranged from 30 minutes for the 23,000 M.W. fraction, to 61 minutes for CY 216. Accordingly, AUC values for all fractions increased relative to their increased halflives.

2. Subcutaneous Administration

Figure 20 shows the semi-log concentration / time plot for the 23,000 M.W. fraction after subcutaneous injection. The concentrations used in this plot were determined using the Heptest assay. Poor absorption from the subcutaneous injection site was observed for this agent. After injection, plasma concentrations rose to a average peak of .06 ug/ml at 4 hours. From this point, plasma levels slowly declined until 12 hours when no drug was detectable.

The area under the plasma concentration time curve (AUC) was similarly low (.47 ug hr/ml). A half-life of 205 minutes was observed.

The semi-log concentration / time plot for the 13,300 M.W. fraction after subcutaneous injection is seen in figure 21. The plasma concentrations used in this plot were determined using the Heptest assay. A peak plasma level of about .25 ug/ml was observed at 4 hours. From this point, plasma levels slowly declined at varying rates. At 12 hours, a slight amount of drug was detectable in the circulation. No drug was detectable at 24 hours. The half-life and AUC values observed for this

fraction were 144 minutes and 1.54 ug hr/ml respectively.

When compared to the 23,000 and 13,300 M.W. fractions, the 5,100 M.W. fraction displayed much greater absorption from the subcutaneous injection site (figure 22). A peak plasma concentration (determined using the Heptest assay) of about 4.0 ug/ml was observed at 4 hours post injection. Plasma concentrations declined to a level of 1.5 ug/ml after 12 hours. No drug was detectable at 24 hours.

The greater absorption of this fraction was also reflected in the AUC, which was more that 15 X greater than either the 13,300 or 23,000 M.W. fractions (23.7 ug hr/ml). The half-life for this fraction was 108 minutes.

The semi-log concentration / time plot for CY 216 after subcutaneous administration determined from Heptest assay data, is seen in figure 23. The pharmacodynamic time course of this fraction was similar to that of the 5,100 M.W. fraction. A peak absorption of about 3.7 ug/ml was observed at 4 hours. The AUC and half-life were 22 ug hr/ml and 144 minutes respectively.

Figure 24 shows the subcutaneous pharmacodynamic concentration / time course of the unfractionated heparin. The data points were plotted using plasma concentrations determined from the Heptest assay. A peak plasma concentration of 1.5 ug/ml was observed at 4 hours post administration. From this peak, plasma levels steadily declined

to minimally detectable levels at 12 hours. No drug was detectable at 24 hours. The half-life of this agent was the shortest of all the test fractions (94 minutes). The AUC was 6.8 ug hr/ml.

a. Pharmacodynamic Time Course of the Molecular Weight Fractions After Subcutaneous Administration, Determined in Different Assays

i. Heptest

A comparison of the subcutaneous concentration / time course for the molecular weight fractions calculated from the heptest assay are seen in table 17. A direct relationship was observed between the molecular weight of the fractions and relative absorption. Absorption and AUC increased with decreasing molecular weight. Comparison of the absorption of the test fractions using ANOVA followed by Newman-Keuls analysis suggested significant differences between some of the test agents. The overall conclusion ranked in order of increasing absorption was: 23,000 $\neq$ 13,300 $\neq$ Heparin $\neq$ CY 216 $\neq$ 5,100.

ii. Anti Xa

The summary table for the subcutaneous pharmacodynamic concentration / time course of the test fractions calculated from the anti Xa assay are seen in table 18. These values were determined from the semi-log concentra-

tion / time plots shown in appendix XVIIa. As with the results calculated using the Heptest assay, relative absorption and AUC increased with decreasing molecular weight. The absorption of all fractions as calculated from this method was slightly greater when compared to that calculated from Heptest. The relative absorption of CY 216 and the 5,100 M.W. fraction approached 100 % (96 and 94 % respectively). The 23,000 M.W. fraction displayed the least absorption at 10 %.

iii. Anti IIa

Table 19 shows the subcutaneous pharmacodynamic concentration / time course for the test fractions calculated from the anti IIa assay. The plots from which these values were determined are shown in appendix XVIIb. As with previous assays, relative absorption increased with decreasing molecular weight. As determined by ANOVA and Newman-Keuls, the differences in absorption between the fractions was significant. The overall conclusion, ranked by decreasing absorption was: 5,100 $\neq$ Cy 216 $\neq$ Heparin $\neq$ 13,300 = 23,000. These values were lower than those observed using the heptest and anti Xa assays. Values ranged from a low of 6 %, to a high of 60 % for 23,000 and 5,100 M.W. fractions respectively.

iv. Dilute Thromboplastin

The results of the subcutaneous plasma concentration / time course for the test fractions calculated from the dilute thromboplastin assay are seen in table 20. These values were determined from concentration / time curves shown in appendix XVIIc. When compared to the relative absorption values calculated from other assays, these results were much lower. In contrast to the 96 % absorption observed with CY 216 using the anti Xa assay (table 17), the absorption of CY 216 calculated from this assay was 38 %. As with other assays, the relative absorption increased with decreasing molecular weight.

v. FPAGT

The concentration / time course values for subcutaneous administration of the test fractions calculated from the FPAGT are seen in table 21. The plots from which these values were determined are shown in appendix XVIId. The absorption increased with decreasing molecular weight. The 23,000 M.W. fraction displayed 28 % absorption compared to 100 % for CY 216. Significant differences were determined using Newman-Keuls analysis between all fractions except CY 216 and the 5,100 M.W. fraction.

Half-life also appeared to be different between the test fractions. Half-life increased with decreasing molecular weight however, a significant difference was only observed for CY 216. The overall conclusion for

equivalence in half-life based on Newman-Keuls analysis in order of decreasing values was: CY 216 ≠ 5,100 M.W. = 13,300 M.W. = heparin = 23,000 M.W.

E. In Vivo Results (Antithrombotic Actions)

The test fractions were compared for their antithrombotic actions in a rabbit stasis thrombosis model. The model used stasis, combined with prothrombin complex concentrate and Russell's viper venom as a thrombogenic challenge. The antithrombotic effects of the test agents were compared using both intravenous and subcutaneous routes of administration.

1. Intravenous (25 ug/kg)

The results obtained after intravenous administration of the test fractions at 25 ug/kg, compared to a saline control are seen in figure 25. The control rabbits averaged stasis clot scores of 6.5. Scores between 2 and 3 were observed for all agents except for the 5,100 M.W. fraction which resulted in a score of 5.5. When tested using the Kruskal Wallis nonparametric ANOVA and appropriate multiple comparison test, significant differences were detected between all test agents and control, except for the 5,100 M.W. fraction. Between the 23,000 and 13,300 M.W. fractions, CY 216 and unfractionated heparin, no difference in the antithrombotic effects were observed.

The overall conclusion determined by multiple comparison analysis (.05) ranked in order of decreasing antithrombotic potency was: 13,300 M.W. = 23,000 M.W. = Heparin = CY 216 ≠ 5,100 M.W. = control.

The circulating pharmacodynamic effects of the molecular weight fractions in the test rabbits are shown in table 22. These effects were measured in the clotting and amidolytic assays described in methods. The baseline values were determined prior to drug administration. Pre-challenge values indicate the circulating drug actions immediately prior to administration of the thrombogenic challenge.

Comparison of the pre-challenge and baseline values demonstrated minimal anticoagulant actions for all fractions in all assays. These observations were consistent with the low dose at which the fractions were administered. As with the in vitro studies, the potency of the individual fractions was dependent upon the assay method. However, due to the low plasma concentrations, significant differences between the effects of the fractions in different assays were not observed.

To determine whether the circulating pharmacodynamic actions were related to the antithrombotic effects, correlations coefficients were calculated. Poor correlation between the activity in the heptest assay and antithrombotic actions was observed ($r = -.47$). Activated

partial thromboplastin time (PTT), anti Xa and IIa amidolytic assays demonstrated better correlations (r = .65, .68 and .60 respectively).

2. Intravenous (50 ug/kg)

Figure 26 shows the antithrombotic effects after intravenous administration of the test agents at a concentration of 50 ug/kg. All fractions displayed stasis scores which were significantly different from the control score of 6.5. These values ranged from 0.25 for the unfractionated heparin, to 2.5 for the 5,100 M.W. fraction. Comparison for similarity between the test agents, suggested significant differences in their antithrombotic actions. The overall conclusion based upon multiple comparison analysis, ranked in order of potency was: Heparin $\neq$ 13,300 = 23,000 = CY 216 $\neq$ 5,100 $\neq$ control.

The circulating pharmacodynamic actions of the test fractions in this experiment are shown in table 23. All fractions produced inhibition of the various assays relative to baseline values. Compared to the 25 ug/kg dose, the circulating pharmacodynamic actions of the test fractions were only minimally increased.

Correlations were calculated as before to determine relationships between the circulating pharmacodynamics and the observed antithrombotic actions. Poor correlations, (r = .50 - .24) were observed in all assays.

3. Intravenous (100 ug/kg)

The antithrombotic actions of the test fractions after intravenous administration at 100 ug/kg are illustrated in figure 27. All fractions displayed antithrombotic actions which were significantly different from control. At this concentration, the unfractionated heparin resulted in complete inhibition of thrombosis. The other test agents resulted in stasis clot scores ranging from 0.25 for the 13,300 M.W. fraction to 2.75 for the 5,100 M.W. fraction. The variation in the stasis scores produced by the various agents were significantly different. The overall conclusion for similarity in antithrombotic actions, ranked in order of potency, determined using multiple comparison analysis was: Heparin = 13,300 M.W. = 23,000 M.W. = CY 216 $\neq$ 5,100 M.W. $\neq$ control.

The circulating pharmacodynamics of the test fractions for this experiment are shown in table 24. Anticoagulant actions were observed for all agents at the time of thrombogenic challenge. These anticoagulant actions were elevated, consistent with the higher dose at which the agents were compared. As with the lower dosages, the degree of anticoagulant potency differed between the individual fractions and assay methods. The correlation of these activities to antithrombotic actions was also higher than observed for the lower dosages. The

correlation values for heptest, anti Xa and anti IIa were $r = .89$, .87 and .82 respectively.

4. Subcutaneous (1 mg/kg)

The antithrombotic effects of the test fractions after subcutaneous administration at a concentration of 1.0 mg/kg are illustrated in figure 28. Significant antithrombotic actions were observed for all agents with the exception of the highest molecular weight fraction (23,000 M.W.). This fraction resulted in a stasis score of 6.0 compared to a control value of 6.5. CY 216 displayed the greatest potency (stasis score 1.25). When compared for similarity using non parametric ANOVA and multiple comparison testing, significant differences in antithrombotic potency were calculated between the test agents. The overall conclusion ranked in order of decreasing potency was: CY 216 = 13,300 M.W. = 5,100 M.W. = unfractionated heparin $\neq$ 23,000 M.W. = control.

The circulating pharmacodynamic activities of the individual fractions observed after subcutaneous administration are shown in table 25. A good correlation between the circulating anticoagulant and antithrombotic actions was observed in all assays. Correlation values for heptest, anti Xa, anti IIa, and PTT were r = .76, .81, .63 and .78 respectively.

CHAPTER V

DISCUSSION

A. Molecular Weight Fractionation and Determination

To study the effect of molecular weight on the anticoagulant, antithrombotic and pharmacokinetic actions of heparin, fractions differing in molecular weight were used. These fractions were obtained from one source of native porcine mucosal heparin using a gel - filtration technique. This technique has previously been used for studying the molecular weight dependent effects of heparin (Bergqvist et al., 1985 ; Losito et al., 1981 ; Thunberg et al., 1979). In these studies, gel filtration was shown to be useful since it relied almost exclusively on size for separation. For this reason, differences between the pharmacological properties of gel-filtered fractions are primarily due to characteristics related to or associated with molecular size.

The elution profiles of the gel-filtered fractions used in the current studies, illustrated that each fraction was composed of a bell-shaped distribution of molecular weight components. For each fraction, the average of these components determined the mean molecular weight. For most fractions, the molecular size range of the individual components was narrow, however the lower molecular weight fractions contained a broader molecular weight distribution range. The difference in molecular

weight range was due to the size exclusion properties of the Ultrogel, which lost resolution at lower molecular weights. The broader molecular weight distribution range of the low molecular weight fractions also reflected the composition of the unfractionated heparin, which contained a greater percentage of low molecular weight components.

The molecular weights of all experimental agents were determined using HPLC - gel permeation chromatography. This was an accurate method for determining molecular weight since well defined calibration standards of similar molecular composition were used (Harenberg and De Vries, 1983 ; Rodriquez, 1976).

HPLC - gel permeation chromatography also demonstrated the frequency distribution and range of molecular weight components within each fraction. This allowed evaluation of the results relative to both mean molecular weight and molecular weight distribution. Characterization relative to both these parameters was important since an almost unlimited number of molecular weight distributions could result in an identical mean molecular weight.

B. Comparison of the Pharmacological Actions of the Molecular Weight Fractions

For all experimental studies, gravimetric expressions (ug/ml or ug/Kg) were primarily used to indicate

test fraction concentrations. These expressions were selected primarily for reasons of accuracy and reproducability. Alternatively, concentrations could have been expressed in molar or unit amounts. No clear advantage existed for using any of these methods. To express the concentrations of the test agents in terms of units, standardization against a reference heparin preparation such as the International Standard would have been required. While this preparation was suitable for standardizing unfractionated heparins, it appeared unsuitable for fractionated heparins. In an international collaborative study, fractionated heparins did not parallel the International Standard Heparin in any of four assay systems used (Barrowcliffe et al., 1985). Since no suitable standard for cross-referencing the test agents was available, unit expressions were not used.

Molar expressions of concentration were used when they contributed to the explanation of molecular weight dependent differences between the test fractions. These expressions were not used more frequently since they could not be precisely determined but only approximated from the average molecular weight.

1. In Vitro Analysis

a. Clotting and Amidolytic Assays

Profiling the effects of the test agents, in

different clotting and amidolytic assays, revealed distinct differences in their molecular weight related anticoagulant potency in all assays. Interestingly, the pattern of these molecular weight dependent effects was different between assays of the intrinsic and extrinsic coagulation network.

In the APTT, Heptest, anti Xa and anti IIa assays, the 13,300 M.W. fraction consistently displayed the most potent anticoagulant and antiprotease actions. Similar, but slightly less potent effects were observed from the 17,450 and 23,000 molecular weight fractions respectively. The 9,000 and 5,100 M.W. fractions displayed the least anticoagulant and antiprotease actions. The potency of these two fractions decreased in direct proportion to their molecular weights. The anticoagulant potency of both CY 216 and the unfractionated heparin was consistent with other fractions of similar molecular weight.

The differences in potency between the various molecular weight fractions in the aforementioned assays were best explained through differences in affinity to antithrombin III, neutralization by anti-heparin proteins and molar ratios.

Heparin's affinity to antithrombin III has been shown to be related to its molecular weight. High affinity binding of heparin to this protein is dependent upon a specific tetrasaccharide sequence within the

heparin. The affinity of heparin to antithrombin III increases with the relative abundance of this sequence (Rosenberg et al., 1978). The probability of this sequence occurring, increases with molecular weight (Laurent et al., 1978). For these reasons, heparin of higher molecular weight generally displays greater affinity to antithrombin III compared to its low molecular weight counterparts.

The decreases in the anticoagulant potency observed between the 13,300 ; 9,000 and 5,100 molecular weight fractions were most likely due to molecular weight related decreases in antithrombin III affinity. These potency differences were even more dramatic when compared in terms of molar ratios. Since the fractions were compared at equal gravimetric concentrations, the 5,100 M.W. fraction had almost a three-fold higher molar ratio relative to the 13,300 M.W. fraction. If the test fractions had been compared at equal molar concentrations, much greater potency differences would have been observed between the high and low molecular weight fractions. At equal gravimetric concentrations, the low molecular weight fractions had more molecules available to bind to antithrombin III, however less anticoagulant actions were observed due to the decreased affinity of these components to this important heparin cofactor.

There was no possibility that the decreased anti-

coagulant actions of the lower molecular weight fractions were due to saturation of antithrombin III. This protein was always present in at least 5 - 10 X molar excess for all concentrations at which the test agents were compared (table 30).

The potency differences between the 23,000 ; 17,450 and 13,300 M.W. fractions most likely were not due to differences in antithrombin III affinity. Between these three fractions, potency decreased with increasing molecular weight. The molar ratio between the respective fractions and antithrombin III also decreased with increasing molecular weight. This observation suggested that the potency differences between these three fractions may have been due to relative molarity differences. Since heparin interacts with antithrombin in a 1 : 1 stoichiometric manner (Jordan et al., 1979), the greater molarity of the 13,300 M.W. fraction resulted in more molecules free to interact with antithrombin III.

It has been reported that in plasma, high molecular weight heparin is more readily neutralized by anti-heparin factors compared to lower molecular weight fractions (Andersson et al., 1979 ; Hubbard and Jennings, 1985). This differential neutralization along with the less favorable molar ratio of the high molecular weight heparin, most likely resulted in the decreased potency observed between the high molecular weight fractions.

As previously mentioned, the potency rank order of the molecular weight fractions was similar in the Heptest, APTT, anti Xa and anti IIa assays. These results were not surprising since these assays primarily reflect heparin's actions upon either factor Xa, thrombin or both.

When the test fractions were screened in the prothrombin time assay (PT), the relationship between potency and molecular weight was different from that observed in the previous assays. The 23,000 M.W. fraction displayed the greatest anticoagulant effects. The anticoagulant actions of all other fractions decreased in direct proportion to molecular weight. The potency differences between the individual fractions were significant and were even greater when molar ratios were considered. The different pattern of molecular weight dependent potency suggested that heparin was possibly acting through a different mechanism in the PT assay.

The prothrombin time differed from the other coagulation assays used to compare the fractions in that it reflected the extrinsic pathway of coagulation. The distinguishing factors of this pathway were factor VII and tissue thromboplastin. (Nemerson, 1983). Since these factors were not reflected by assays such as the APTT and heptest, it was possible that the different pattern of molecular weight dependence was due to effects by the test fractions on these factors.

It is important to mention that due to the insensitivity of the prothrombin time assay to the actions of heparin, two-fold higher concentrations of the test agents (5-20 ug/ml) were used in comparison to the other screening assays.

At high heparin concentrations (> 20 ug/ml), heparin cofactor II has been suggested to contribute to the overall anticoagulant actions of heparin (Ofosu et al., 1985). A molecular weight dependent activation of this factor has also been reported (Choay et al., 1986 ; Yamagishi et al., 1986). For these reasons, it was interesting to investigate whether heparin cofactor II played a role in the molecular weight dependent effects displayed by the fractions in the prothrombin time assay. This was accomplished by supplementing the various molecular weight fractions to antithrombin III deficient plasma and performing identical prothrombin time assays.

The antithrombin III deficient plasma was prepared using heparin affinity chromatography. This method resulted in plasma with functional antithrombin III levels which were 6 % of normal. Heparin cofactor II levels in plasma prepared using this method have been reported to be unchanged (personal communication).

If the molecular weight dependent effects of the test agents in the PT assay were mediated exclusively through antithrombin III, they should have been diminished

or eliminated in plasma deficient in this protein. However, if the effects were due to heparin cofactor II activity, they should have remained in the antithrombin III deficient plasma. As shown in table 5, removal of antithrombin III resulted in the loss of the molecular weight dependent potency observed in the PT assay in normal plasma. These results suggested that in this assay, the increased potency of the high molecular weight fractions were mediated by antithrombin III.

b. Fibrinopeptide-A Generation Tests

The fibrinopeptide - A generation test was developed for the purpose of studying the collective actions of heparin on the proteases of the coagulation network. The assay is based upon quantitation of a 16 amino acid peptide known as fibrinopeptide - A (FPA), after activation of citrated blood or plasma. This peptide is released from fibrinogen by the action of thrombin, and has been shown to be a specific indicator of thrombin's coagulant actions (Emanuele et al., 1986). Any event, either intrinsic or extrinsic, which results in thrombin generation will result in FPA release. FPA release is the final endpoint of coagulation prior to fibrin monomer polymerization. For this reason FPA release can be used to assess the collective anticoagulant activity of any agent which acts prior to this step. Since the assay uses

an immunoquantitation technique to measure the generated FPA, it has great sensitivity to any anticoagulant action. In addition to its sensitivity, this assay can be performed in a variety of matrices, provided thrombin and fibrinogen are present. This feature allowed comparison of the molecular weight fractions in platelet rich and platelet poor plasma as well as whole human blood. The efficacy of these tests in different matrices has previously been discussed (Emanuele et al., 1985).

In order to further profile the effects of the different molecular weight fractions, four variations of the FPA generation assay were used. The results of these assays were dependent upon the method of activation and matrix used.

When the test agents were studied in normal human plasma using the thromboplastin activated system, the results were similar to those obtained in the PT assay. The 23,000 M.W. fraction displayed the greatest potency in terms of inhibiting FPA generation. The potency of the other fractions decreased in direct proportion to their molecular weights. The similar pattern of molecular weight dependence observed in the PT and FPA generation assays was not surprising since both methods utilized thromboplastin activation. Both systems were reflecting the action of heparin along the extrinsic pathway. The similarity between the two systems implied that similar

mechanisms were responsible for the pattern of molecular weight dependent potency. These observations further reinforced the notion that high molecular weight heparin was inhibiting either factor VII or thromboplastin. The results from this assay were particularly interesting since they occurred at lower heparin concentrations (2.5 & 5 ug/ml) compared to the PT assay.

The thromboplastin activated FPA generation assay was also performed in antithrombin III (AT III) deficient plasma. The effects of the test agents in this assay were consistent with those observed in the PT assay performed in AT III deficient plasma. All agents failed to produce any inhibition of FPA generation although high heparin concentrations were used (10 ug/ml). No significant differences in the effects between any of the test agents were observed. This observation further reinforced the previous hypothesis, that the molecular weight dependent anticoagulant effects, displayed by the test fractions in thromboplastin activated plasma, were mediated through antithrombin III. Furthermore, at these concentrations, it was unlikely that Heparin cofactor II contributed to these molecular weight dependent effects.

Several reports have suggested that interactions between platelets and heparin may be important in terms of an overall hemostatic effect (Salzman et al., 1980). Additional reports have demonstrated that these interactions

are dependent upon heparin's molecular weight (Salzman et al., 1982 ; Brace and Fareed, 1986). Platelets also contain a small peptide (platelet factor 4) within their alpha granules which has been shown to neutralize the anticoagulant actions of heparin (Dawes et al., 1982).

To study whether the presence of platelets affected the molecular weight dependent responses of the test agents, they were studied in platelet rich and platelet poor plasma using the thromboplastin activated FPA generation assay. No significant difference was observed in the actions of any test fractions between the two plasmas (figure 4). These results suggested that the presence of platelets did not affect the molecular weight dependent actions of these agents.

It was interesting to note that in all cases, the amount of FPA generated was slightly higher in the platelet rich plasma. Since these slight differences were present between the controls of both plasmas, they were not attributed to neutralization of the test agents by platelet factor 4.

Phospholipoproteins (platelet factor 3) from the platelet membrane are known to accelerate two critical steps of blood coagulation (factor Xa activation and prothrombin conversion) (Ofosu et al., 1981). It was likely that the platelet rich plasma contained a greater concentration of these factors compared to the platelet

poor plasma. Thus, the greater FPA generation in the platelet rich plasma was probably due to accelerated factor X and prothrombin conversion due to phospholipoproteins contributed by the platelets.

The molecular weight fractions were also tested in a whole blood FPA generation assay. This test differed from the previous FPA assays in both the generation matrix and method of activation. The assay utilized contact between the glass surface of a test tube and the whole blood for activation. This procedure was intended to mimic intrinsic or contact activation of whole blood.

The molecular weight dependent effects of the test fractions were different in this assay compared to the thromboplastin activated system. Although not significantly different from the 23,000 M.W. fraction, the 13,300 M.W. fraction appeared to be the most potent inhibitor of FPA generation. The unfractionated heparin also displayed strong inhibitory actions. The two low molecular weight fractions were significantly less effective in inhibiting FPA generation compared to the higher molecular weight agents. These observations were consistent with results obtained in previous assays of the intrinsic pathway. It was likely that in this assay, the different potencies of the test fractions resulted from similar differences in the molar ratios and susceptibility to neutralization previously discussed for the other

assays utilizing intrinsic activation.

c. VII-Thromboplastin Assay

The different pattern of potency observed for the molecular weight fractions between assays of the intrinsic and extrinsic pathway, suggested possible inhibition of factor VII by heparin. Previous investigators have suggested this may occur (Dahl et al., 1982 ; Godal et al.,1974 ; Osterud et al., 1976). However, these reports were controversial and other investigations concluded that factor VII was not inhibited by heparin (Jesty, 1978 ; Rosenberg, 1977).

In order to more accurately investigate the interactions between factor VII and heparin, the test fractions were screened in an amidolytic VII-thromboplastin assay. This assay was performed on a Multistat centrifugal analyzer. The advantages of using this instrument in coagulation testing have been previously reviewed (Hills et al., 1983). In the assay, factor VII-thromboplastin was used to activate factor X to Xa. The assay endpoint was then determined by measuring the absorbance change due to Xa chromophore release. A direct relationship between this endpoint and factor VII concentration was established (figure 5). Since thrombin was not a necessary component of the assay, any effects by heparin could only be due to factor VII, thromboplastin or factor Xa.

When the test agents were screened in this assay, similar molecular weight dependent effects as seen in the PT and thromboplastin activated FPA generation assays were observed. The highest molecular weight fraction (23,000) displayed the greatest inhibition of the assay. The potency of the other fractions decreased in direct relation to their molecular weight. These observations were consistent with the hypothesis that high molecular weight fractions of heparin were inhibiting either factor VII or thromboplastin. The VII-thromboplastin assay relied on the actions of factor Xa for endpoint determination. The inhibition of this factor by heparin has been shown to be dependent upon molecular weight (Lane et al.,1978 ; Andersson et al., 1978 ; Thunberg et al., 1979). To study the possibility that the pattern of molecular weight dependence in this assay was due to effects on factor Xa, an identical assay substituting Xa for factors VII and X was used. Thromboplastin was also present in this system. Under these conditions, the 23,000 ; 17,450 and 13,300 M.W. fractions all displayed similar effects and no difference in potency was observed. These results suggested that the increased potency of 23,000 and 17,450 M.W. fractions observed in the VII-thromboplastin assay were not due to the inhibition of factor Xa.

To demonstrate that the increased potency of the

high molecular weight fractions were due to actions occurring prior to the activation of factor X, the test fractions were studied in two systems of the VII-thromboplastin assay. The test fractions were incubated either with VII-thromboplastin or factor X. These two incubation systems differed only in the incubation step and otherwise were identical in the volumes and concentrations of all reactants. The X - Xa activation catalyzed by VII-thromboplastin occurred in the presence of identical heparin-antithrombin III concentrations. Under these conditions, any difference between the two systems could only be due to inhibition by heparin-antithrombin III on VII-thromboplastin.

When the test agents were screened in this assay, only the 23,000 and 17,450 M.W. fractions, along with the unfractionated heparin, produced significant differences between the two incubation systems. These results suggested that only these fractions were inhibiting factor VII-thromboplastin. The potency of these three agents decreased with decreasing molecular weight implying that inhibition of VII-thromboplastin was influenced by molecular weight. These results further suggested that the increase potency of the high molecular weight fractions observed in all assays reflecting the extrinsic pathway were due to a molecular weight dependent inhibition of either factor VII or thromboplastin.

It was interesting that the unfractionated heparin [(12,575 M.W) demonstrated inhibition of factor VII-t[hromboplastin while the 13,300 M.W. fraction did not. This appeared contradictory since other observations indicated that the inhibition of VII-thromboplastin was influenced by molecular weight. The apparent discrepancy was explained by the molecular weight distribution range of the two fractions. The unfractionated heparin was composed of a range of molecular weight components from 1,500 - 44,000 while the 13,300 M.W. fraction contained components ranging from 7,000 - 22,000 (Table 1). The different molecular weight distributions of both agents resulted in similar mean molecular weights. The presence of high molecular weight components in the unfractionated heparin and their absence in the 13,300 M.W. fraction most likely contributed to the inhibition observed with the unfractionated heparin.

The VII-thromboplastin assay was modified to study the effects of the test agents in a plasma matrix. As before the assay was performed using a centrifugal analyzer, however factors VII, X and antithrombin III were replaced by fibrinogen deficient plasma. The fibrinogen deficient plasma was used to prevent the formation of a clot. Either a Xa or thrombin specific substrate was added for the purpose of monitoring p-NA release as an endpoint. Both assays used dilute (1:20) thromboplastin

to activate the plasma. For this reason it was thought that the actions of the test agents would be similar to those observed in other assays using extrinsic activation. However, the actions of these agents in this system were very different. In the assay using the Xa substrate, the 13,300 M.W. fraction displayed the most potent anti-protease actions. Above and below this molecular weight, the potency of the test agents decreased with increasing or decreasing molecular weight respectively. With the thrombin substrate, similar but slightly less potent effects were observed for all fractions. These results did not resemble those obtained in other assays of the extrinsic pathway, but rather were similar to results obtained with intrinsic and anti Xa and IIa assays. The similarity suggested that these assays were reflecting the actions of the test fractions on the enzyme directly cleaving the substrate (Xa and thrombin) and not on the initial activation process.

It was difficult to explain the failure of the test heparins in the plasma VII-thromboplastin system to reflect any inhibition of the initial activation process as observed with other extrinsic activated assays. A possible explanation was attributed to differences between the assay systems. The plasma VII-thromboplastin assay differed from the other extrinsic assays in two important properties. Unlike the pure VII-thromboplastin assay,

this assay was performed in plasma. Thus, in addition to factors VII and X, all other coagulation proteases and inhibitors were present. Also, compared to a conventional prothrombin time assay, the incubation and assay time period were much longer. It is known that extrinsic activation is a fast system compared to the longer activation time period involved in the intrinsic network (Thompson and Harker, 1983). It was possible that the longer time interval and the additional factors provided by the plasma VII-thromboplastin assay, resulted in the activation of intrinsic coagulation factors. Thus, the plasma VII-thromboplastin assay actually did not reflect the actions of the test fractions on extrinsic but rather intrinsic proteases.

i. Possible Significance of Factor VII or Thromboplastin Inhibition by Heparin

The observation that factor VII or thromboplastin may be inhibited by high molecular weight heparin may have relevance to the hemorrhagic side effects of this agent. Reports have suggested that low molecular weight may have less bleeding tendencies compared to higher molecular weight heparin (Salzman, 1986).

Factor VII has been shown to be a key factor in the regulation of hemostasis (Nemerson, 1983). The results presented in this dissertation have demonstrated the

inhibition of factor VII or thromboplastin only by high molecular weight heparin. If factor VII or its activation by thromboplastin are important to hemostasis, then their inhibition by high molecular weight heparin would compromise a key hemostatic mechanism and contribute to heparin's hemorrhagic effects. Conversely, low molecular weight heparin may have less hemorrhagic tendencies due to its lack of inhibition on factor VII or thromboplastin.

2. In Vivo Actions (Pharmacodynamic Concentration / Time Course)

a. Intravenous Administration

The pharmacodynamic concentration / time course of the test agents was studied in the primate Macaca mulatta. The validity of studying the plasma concentration / time course of heparin in this species has been demonstrated for both fractionated and native heparins (Fareed et al., 1985). Kinetic values were calculated from plasma concentrations determined at different time intervals. The clotting and protease assays used in the in vitro profiling were used in these determinations. Individual calibration curves for each test agent and primate were used to transform the individual assay parameters into gravimetric amounts. This technique was a accurate method for quantitation of the circulating heparin levels since individual variations were nullified by the separate

calibration curves.

The results of the concentration / time course studies indicated that heparins of different molecular weight displayed significantly different kinetic behavior. Differences between the test agents were observed in their absorption, half-life and distribution characteristics.

Upon introduction into the circulation, all test agents, with the exception of the 23,000 M.W. fraction, displayed bi-phasic rates of elimination (figures 16-19). The 23,000 M.W. fraction displayed a constant elimination rate (figure 15). The bi-phasic elimination consisted of an initial rapid decrease in plasma activity, followed by a reduced elimination rate. These two phases were due to an initial equilibration within the vascular compartment and subsequent distribution to a second tissue compartment. Differences in the rates of elimination were observed between the test fractions showing bi-phasic elimination. The higher molecular weight fractions were removed from the circulation at a greater rate compared to those of lower molecular weight. These observations were consistent with the molecular weight dependent differences in apparent volume of distribution and half-life.

All test agents were compared at equal gravimetric dosages. Since these agents differed in molecular weight, the circulating molar concentrations were of these agents were not equal. To test whether the different molar

concentrations resulted in different plasma concentration / time profiles, these values were calculated using both molar and gravimetric concentrations. The profiles were similar whether calculated from either value. (table 26).

To explain the differences between the concentration / time profiles of the molecular weight fractions it is important to review the mechanisms involved in the elimination of heparin. It has been suggested that heparin is removed from the circulation through a combination of saturable and non-saturable mechanisms. Only a combination of these mechanisms can account for the non-linear, dose dependent elimination of heparin from the circulation (de Swart et al., 1982).

The non-saturable mechanism is thought to be due to the renal elimination of heparin (Estes, 1980 ; Cocchetto and Bjornsson, 1984). The saturable mechanisms are more complex and involve neutralization of heparin molecules through a variety of processes. It is known that desulfation of heparin by various desulfating enzymes results in the loss of heparin's anticoagulant activity. Heparin desulfamidases have been isolated from lymphoid tissue. For this reason, the reticuloendothelial system has been associated with the saturable processes of heparin elimination (Estes, 1980). Inactivation of heparin by desulfation is thought to be saturable since increasing doses of heparin, result in greater amounts of intact (non

desulfated) drug in the urine (de Swart et al., 1982).

Heparin has been shown to bind to endothelial cells both in vitro and in vivo (Mahadoo et al., 1977 ; Barzu et al., 1984). The affinity of this binding has been shown to increase with increasing molecular weight (Choay et al., 1986 ; Boneu et al., 1985). The binding of heparin to endothelial cells effectively neutralizes its anti-coagulant activity (van Rijn et al., 1987). For these reasons endothelial cell binding has been implicated in the saturable mechanism involved in the elimination of heparin (van Rijn et al., 1987).

The similarity between heparin's molecular weight dependent plasma concentration time profile and endothelial cell binding characteristics suggested that these phenomena were related. The binding of heparin to endothelial cells may explain the short half-life of the high molecular weight and relatively longer half-life of the lower molecular weight fractions. The high molecular weight heparin may have been effectively neutralized by high affinity binding to endothelial cells. This process would be less effective for low molecular weight fractions due to their reduced affinity for these cells. In addition, the binding of low molecular weight components to endothelial cells may have acted as a tissue reservoir. The relative low affinity of the molecules to their endothelial binding sites may have allowed for the slow

release of these components into the circulation. This mechanism was consistent with the two compartment distribution and elimination behavior displayed by the lower molecular weight test fractions.

Since the loss of heparin's anticoagulant activity, due to endothelial cell binding, was a saturable process, the relative molar differences between the high and low molecular weight fractions may also have contributed to the differences in the kinetic behavior.

b. Comparison of the Intravenous Concentration / Time Profile in Different Assays

The intravenous plasma concentration / time course of the test fractions were calculated using five different ex vivo assays. Consistent with reports of other investigators (Cocchetto and Bjornsson, 1984), the concentration / time profile of all test fractions were assay dependent. Plasma concentrations determined from identical plasma samples yielded different heparin concentrations when different assays were used. These results were not surprising since the various assays were sensitive to different pharmacodynamic actions.

The sensitivities of the individual assays to the different molecular weight fractions were reflected in the concentration / time profiles. Those assays sensitive primarily to high molecular weight fractions (the anti IIa

and dilute PT assays) indicated short half-lives and small distribution volumes. Assays with greater sensitivity to low molecular weight fractions (anti Xa, heptest and FPAGT) suggested longer half-lives and greater distribution volumes. Thus, the assay dependent concentration / time profiles directly reflected the molecular weight dependent time course.

The different assay methods were useful to study the kinetics of the biological response of the individual fractions. The various assays reflected specific anticoagulant and antiprotease actions. For this reason, the time course calculated from different assays reflected the kinetics of specific pharmacodynamic actions. Thus, the time course observed using the anti IIa assay represented the kinetics of heparin components inhibiting this factor. Similarly, the anti Xa assay represented the kinetics of anti Xa components. Based on this reasoning, the different kinetic profiles of identical fractions calculated from different assays, were due to differences in the kinetics of the assay specific pharmacodynamic action.

The results demonstrated that those components expressing anti factor Xa actions displayed the longest halflives. These actions were reflected in the anti Xa assay and Heptest. The long halflives calculated from the FPAGT were also due to anti Xa actions. The association between low molecular weight and anti Xa activity, sug-

gested that the prolonged half-life of the anti Xa actions were a molecular weight related effect. Thus the mechanisms contributing to the increased half-life of low molecular weight heparin, were also responsible for the prolonged anti Xa actions.

The half-life of those components inhibiting thrombin, as indicated by the anti IIa assay, were significantly shorter than those exhibiting anti Xa effects. The short half-lives and distribution volumes observed in the dilute PT assay, suggested that components inhibiting extrinsic activation also have a short half-life. The associations of these actions with high molecular weight heparin, suggested that the short half-lives of these components were also molecular weight related.

c. Subcutaneous Administration

The subcutaneous concentration / time course study was carried out primarily for the purpose of comparing the effect of molecular weight on the absorption of heparin. This was accomplished by comparing the AUC of the individual fractions after intravenous and subcutaneous administration. After intravenous injection, 100 % absorption was observed for the test fractions. This was demonstrated by the recovery of plasma levels predicted from injection of a prescribed mg/kg dose. Differences in plasma concentrations of identical fractions after

subcutaneous injection were attributed to differences in the rate and extent of absorption from the subcutaneous site. This conclusion was only valid if the rate constant of elimination after both routes of administration was similar. Heparin has been shown to display both dose dependent elimination and reduced absorption after subcutaneous administration. For these reasons, a larger dosage of the test fractions was used in the subcutaneous route for the purpose of achieving similar plasma concentrations after both routes of administration. The dose adjusted AUC values were then used to calculate relative absorption.

It should be mentioned that since the absorption was calculated from anticoagulant data, only components of the test fractions expressing these actions were reflected. Thus, the exact gravimetric concentrations may or may not have been directly reflected. Also, the net absorption may have been different if a different endpoint (antithrombotic) was used.

Profound differences were observed in the absorption of the test fractions after subcutaneous injection. These differences were attributed to molecular weight dependent effects. Comparison of the AUC values for the test fractions demonstrated that absorption increased with decreasing molecular weight. Poor absorption was observed with the 23,000 M.W. fraction indicating that very little

of this agent actually reached the circulation after subcutaneous injection (figure 20). Slightly better absorption was observed with the 13,300 M.W. fraction (attributed to its lower molecular weight) (figure 21). CY 216 and the 5,100 M.W. fraction displayed much better absorption characteristics. At these low molecular weights almost all of the drug reached the circulation from the subcutaneous injection sites.

Since the absorption of the test agents was compared at equal gravimetric concentrations, the circulating molar concentrations were different. However, no difference was observed, whether the absorption was calculated from gravimetric or molar concentrations (table 27).

It was interesting that although the unfractionated heparin and the 13,300 M.W. fraction had similar mean molecular weights, significantly better absorption was displayed by the unfractionated heparin. This apparent contradiction to the molecular weight dependent differences in absorption observed with other fractions, probably was due to differences in the respective molecular weight distributions. The unfractionated heparin was composed of molecular weight components ranging from 44,000 - 1,500 M.W., while the 13,300 M.W. fraction contained components ranging from 22,000 - 7,000 (table 1). The presence of a greater percentage of low molecular weight components in the unfractionated heparin contributed in its greater

absorption.

The differences in absorption between the test fractions suggested a molecular weight dependent threshold for the absorption of heparin from subcutaneous sites. This threshold was determined to be about 10,000 M.W. based on comparisons between the relative absorption of the test fractions and their molecular weight distributions. A high correlation ($r = .99$) between the percentage of molecular weight components below 10,000 and relative absorption was observed (table 28). This data suggested that primarily those components of the test fractions with molecular weights less than 10,000 were absorbed from the subcutaneous site into the circulation.

It is important to mention that this absorption threshold may not have been exclusively determined by molecular size exclusion. It is well known that charge plays an important role in the absorption characteristics of most therapeutic agents. Since heparin is a highly charged molecule, it is reasonable to assume that the charge characteristics of the test fractions also may have influenced their absorption.

The observation of a molecular weight dependent threshold for the absorption of heparin may be important relative to its therapeutic use. If the absorption of subcutaneously administered heparin is limited by molecular weight, then administration by this route may

restrict some of its molecular weight dependent actions. The data presented in this study suggests that only components of molecular weight of about 10,000 or less are absorbed after subcutaneous injection. Thus, actions of heparin exclusively associated with molecular weights above 10,000 should not be displayed after subcutaneous administration.

Furthermore, this observation suggests that subcutaneous administration of native heparin may be inefficient and uneconomical since a significant portion of the drug never reaches the circulation. Heparins whose molecular weight range does not exceed 10,000 may be better suited for this route of administration.

d. Comparison of the Pharmacodynamic Concentration / Time Course After Subcutaneous Injection Using Different Assays

As with the intravenous study, the pharmacodynamic concentration / time course of the test fractions after subcutaneous injection was assay dependent and consistent with the sensitivities of the assay methods to molecular weight. Assays reflecting low molecular weight components demonstrated greater absorption, compared to those reflecting the actions of higher molecular weight components.

The different assay methods also reflected the differences in absorption relative to pharmacodynamic

response (tables 16 - 20). The highest absorption was associated with components inhibiting factor Xa. This was due to the potent anti Xa actions of low molecular weight components. The high absorption observed in the Heptest and the FPAGT also were attributed to anti Xa effects.

Consistent with the association between efficient antithrombin inhibition and higher molecular weight heparin, poor absorption was observed for all test fractions using the anti IIa assay. This observation suggested that after subcutaneous administration, components of the fractions with high antithrombin actions were not efficiently absorbed into the circulation. Similar results were observed with the dilute thromboplastin clotting time. The low overall absorption observed in this assay was due to poor absorption of high molecular weight components which were necessary to efficiently inhibit this assay. These results suggested that components necessary for the inhibition of extrinsic proteases are not effectively absorbed after subcutaneous administration.

3. In Vivo Antithrombotic Actions

a. Intravenous Administration

The antithrombotic effects of the molecular weight fractions were studied in a rabbit stasis model. This model utilized a complex of human factors II, VII, IX, X

and Russell's viper venom (a factor X activator) along with stasis to produce a localized clot. This thrombogenic procedure, produced high factor Xa levels which subsequently generated thrombin to produce a clot. Since the method relied on both factor Xa and thrombin for thrombus formation, inhibitory actions of the test fractions directed toward either of these factors should have resulted in antithrombotic actions.

It is important to mention that the results obtained using this model were relative to the thrombogenic challenge. The use of a different challenge may have resulted in a different antithrombotic profile for the test agents. As previously stated, this model primarily relied upon the actions of factor Xa to initiate thrombus production. Thus, this model may have resulted in a more favorable antithrombotic profile for the lower molecular weight fractions by virtue of their high anti factor Xa actions. A less favorable antithrombotic profile may have resulted for these fractions if thromboplastin or thrombin had been used as the thrombogenic challenge.

Using the PCC/RVV challenge, control animals consistently produced a similar degree of thrombus formation. Administration of a low dose (25 ug/Kg) of the test agents resulted significant antithrombotic actions for all but the 5,100 M.W. fraction. The experimental design included quantitation of the thrombus size. This

design made it possible to compare the antithrombotic potencies of the individual test fractions. At the 25 ug/kg dose, differences in antithrombotic potency were observed. These differences were reflected the in vitro anticoagulant potency profiles observed in the APTT, Heptest, anti Xa and anti IIa assays. As previously discussed, these assays primarily reflected the effects of the fractions on factors Xa and thrombin. The similarity between the anticoagulant and antithrombotic potency profiles suggested that the antithrombotic effects observed in the rabbit model were primarily due to the inhibition of these two proteases.

When a higher dosage of the test agents was used in the rabbit model, greater antithrombotic effects were observed. These results demonstrated that as with the in vitro anticoagulant effects, the antithrombotic effects were dose dependent. At these higher dosages, the antithrombotic effects correlated relatively well with the in vitro anticoagulant and circulating pharmacodynamic effects for most of the molecular weight fractions. However, a poor correlation was observed with the unfractionated heparin. After intravenous administration of both the 50 and 100 ug/kg doses, the unfractionated heparin was the most effective antithrombotic agent. Only this agent resulted in the complete inhibition of thrombosis in all experimental animals. The antithrombotic

potency of this agent was not reflected by its in vitro anticoagulant or circulating pharmacodynamic actions since these activities were greater for both the 23,000 and 13,300 M.W. fractions.

The poor correlation between the anticoagulant and antithrombotic potency of fractionated heparins has been reported by other investigators (Merton et al., 1984 ; Barrowcliffe et al., 1984). These investigators have shown that heparin fractions with relatively higher anticoagulant actions, have been shown to be less effective antithrombotic agents compared to unfractionated heparin when administered at equivalent units. The enhanced antithrombotic effects of native heparin were attributed to the actions of low antithrombin III affinity components.

In this study, the disassociation between the anticoagulant and antithrombotic actions of the unfractionated heparin further suggested a role for the low antithrombin III affinity components in the antithrombotic effects of this agent. Speculation about the mechanisms by which the low antithrombin affinity components contribute to antithrombotic effects may involve pro-fibrinolytic actions, the inhibition of platelets or other cellular components and interactions resulting in a less thrombogenic endothelial surface (Barrowcliffe et al., 1984).

b. Subcutaneous Administration

After subcutaneous administration, apparent molecular weight dependent differences were observed in antithrombotic potencies of the test agents. With the exception of the 23,000 M.W. fraction, all test agents produced significant antithrombotic effects. Between the agents displaying antithrombotic effects, no statistically significant differences in potency were calculated. However, the lower molecular weight fractions did appear to be the more effective antithrombotic agents. The enhanced antithrombotic actions of these fractions was probably due to better absorption from the subcutaneous injection sites.

For most fractions, the antithrombotic effects increased in direct proportion to the circulating pharmacodynamic actions (table 29). These effects correlated well with the absorption of the respective agents observed in the primate model (table 29). Only the 13,300 M.W. fraction displayed a poor correlation between these actions. This agent resulted in good antithrombotic effects while displaying only minimal circulating pharmacodynamic actions. These results suggested that after subcutaneous administration of this fraction, components expressing antithrombotic but not anticoagulant effects were absorbed into the circulation. The differential absorption between the anticoagulant and antithrombotic

actions may have been due to the charge characteristics of the heparin. It has been shown that highly anionic heparin displays high anticoagulant actions (Sache et al., 1981). A highly charged heparin would also be less efficiently absorbed from a subcutaneous injection site. After subcutaneous injection of the 13,300 M.W. fraction, the highly charged high anticoagulant components did not appear to have been absorbed into the circulation. This may have been due to size and charge characteristics. Those components responsible for the antithrombotic actions may have been less charged since they displayed only minimal anticoagulant actions. The lower charge of these components may have allowed for absorption and the subsequent production of antithrombotic effects not mediated through anticoagulant actions.

Since the antithrombotic actions of the 13,300 M.W. fraction appeared to be independent of its anticoagulant actions, it was difficult to account for these effects. Similar observations by other investigators have been accounted for by increased fibrinolytic actions, effects on platelets or endothelium and by effects contributing to a reduction in blood viscosity (Merton et al., 1984 ; Ruggerio et al., 1983).

It was interesting that only the 13,300 M.W. fraction showed a disassociation between the anticoagulant and antithrombotic effects after subcutaneous injection.

One possible interpretation of this observation is that the low anticoagulant, high antithrombotic components of heparin are primarily of this molecular weight. Additional experimentation would be required to verify this hypothesis.

C. Integration of the Molecular Weight Dependent Anticoagulant, Antithrombotic and Concentration / Time Profile of Heparin

The results obtained from the anticoagulant, concentration / time course and antithrombotic studies using the different test agents, suggested that these pharmacological functions were influenced by molecular weight. Since the therapeutic effect of heparin is determined by these actions, integration of the molecular weight dependent anticoagulant, antithrombotic and pharmacodynamic properties should result in a comprehensive understanding of the molecular weight dependent effects of this agent.

The in vitro anticoagulant studies demonstrated that more potent anticoagulant actions were associated with the higher molecular weight heparins. The enhanced potency of these agents was primarily due to greater molecular weight dependent anti factor IIa actions. The inhibition of factor VII or thromboplastin by these agents, may also have contributed to their enhanced potency. These results

predicted that higher molecular weight heparins should be more effective antithrombotic agents compared to those of lower molecular weight. This prediction was based upon the assumption that heparin's antithrombotic effects were due primarily to its anticoagulant actions.

The antithrombotic studies verified that, for most agents, the in vitro anticoagulant potency was predictive of antithrombotic actions, however this only applied to the intravenous route of administration. In this route, all but the unfractionated heparin demonstrated good correlation between the in vitro anticoagulant and in vivo antithrombotic actions. After subcutaneous administration the in vitro anticoagulant activity was not predictive of antithrombotic effects. Molecular weight dependent differences in absorption resulted in the production of greater antithrombotic effects by fractions which had displayed the least in vitro anticoagulant actions. This observation demonstrated that route of administration, due to its influence on drug absorption, was an important characteristic for determining the antithrombotic effects of the different molecular weight heparins.

Significant differences in half-life were also observed between the molecular weight fractions. The lower molecular weight fractions, possessing the least anticoagulant actions, displayed the longest half-lives. The amount by which the half-life increased in the lower

molecular weight fractions, was dependent upon the method of assay.

It is interesting to speculate on whether the increased anticoagulant half-life of lower molecular weight heparins results in a similar prolongation of their antithrombotic effects. Intuitively, the prolonged presence of these agents in the circulation should contribute to a sustained antithrombotic response. However, half-life values of identical fractions were different when calculated in different assays. As previously discussed, these different values were interpreted to represent different half-lives for the assay specific pharmacodynamic response of the individual agents.

Different pharmacodynamic responses have been associated with different antithrombotic potencies. It is generally believed that the pharmacodynamic actions of heparin associated with its antithrombotic effects are the inhibition of factors Xa and IIa. Previous investigators have speculated on the relative importance of these two factors to the antithrombotic actions of this drug. A strong argument has been made for the necessity of anti factor IIa actions for efficient antithrombotic actions (Buchanan et al., 1985 ; Carter et al., 1982). However, Walenga et al (1986) have recently shown antithrombotic actions using a synthetic heparin like pentasaccharide

possessing only anti factor Xa actions. No clear answer is readily apparent for determining the contribution of the anti Xa or IIa actions of heparin to its antithrombotic actions. However, it seems likely that any contribution to a prolonged antithrombotic effect due to a molecular weight related increase in half-life, is relative to its pharmacodynamic action.

This dissertation has suggested that the increased half-life of the lower molecular weight heparins is primarily associated with anti factor Xa actions. When measured in terms of anti factor IIa actions, no similar increase in half-life was observed. Thus, if anti factor IIa effects are required for efficient antithrombotic actions, the greater half-lives of lower molecular weight heparins may not result in a concomitant prolongation in antithrombotic actions. However if the anti factor Xa actions are sufficient for effective antithrombotic actions, then the longer half-life should contribute to prolonged antithrombotic effects.

Due to the molecular weight dependent differences in the half-life of heparins anti Xa and IIa actions, it may be possible to identify heparins therapeutic actions as a function of its pharmacokinetic time coarse. Initially when both anti Xa and IIa effects are present, heparin may have therapeutic actions relative to inhibiting an ongoing thrombotic process. At a later time point, when primarily

only anti Xa actions are present, the actions of heparin may be better described as prophylactic relative to a similar thrombotic process. If this argument is true, then low molecular weight heparins by virtue of their high anti Xa / IIa ratio and long half-lives, may be more appropriately indicated as prophylactic agents. Clearly such a distinction could only be determined through large scale clinical trials.

CHAPTER VI

SUMMARY AND CONCLUSIONS

By studying the in vitro anticoagulant, in vivo antithrombotic and concentration / time profile of different molecular weight fractions of heparin, several molecular weight dependent actions were observed.

1. The in vitro potency of the test fractions increased with increasing molecular weight from 5,100 through 13,300 molecular weight in assays reflecting the intrinsic pathway. Beyond 13,300 no further increases in potency were observed. The potency differences between the fractions were attributed to the molecular weight dependent inhibition of factor Xa and thrombin.

2. It was interesting to note that all low molecular weight heparins used in this study (including CY 216) displayed similar anti Xa to IIa ratios. Although anti Xa activity was slightly higher in the lower molecular weight fractions, the ratio was not significantly greater than 1.0 as reported by other investigators. This low ratio was probably due to the relatively wide molecular weight distributions of these fractions.

3. In assays of the extrinsic pathway, potency increased in direct relation to molecular weight for all agents tested (M.W.'s 5,100 - 23,000). The enhanced potency of the high molecular weight fractions was due to

either the inhibition of factor VII or thromboplastin. The direct inhibition of this enzyme or cofactor was observed only by fractions containing high molecular weight components.

4. It was not possible to distinguish the point of inhibition by the high molecular weight fractions between factor VII or thromboplastin. The observation that the inhibition was dependent upon antithrombin III implicated factor VII, since the inhibition of this serine protease was consistent with the mechanism of action for antithrombin III.

5. The inhibition of factor VII and thromboplastin by high molecular weight heparin may be of importance relative to the side effects of this agent. Reports have suggested greater hemorrhagic tendencies for high compared to low molecular weight heparin. Factor VII has been shown to be a key hemostatic enzyme (Nemerson, 1983). The results of this thesis suggest that this key enzyme is inhibited only by high molecular weight heparin. This observation, along with the association between high molecular weight heparin and increased hemorrhagic tendencies, suggests a possible association between these two events. Further studies investigating factor VII or thromboplastin inhibition with the bleeding tendencies of different molecular weight fractions would be required to test this theory.

6. Molecular weight proved to be an important determinant of the pharmacodynamic time course of heparin. The highest molecular weight fraction exhibited distribution and elimination characteristics consistent with a one compartment model. Fractions of lower molecular weight clearly fit a two compartment model. These molecular weight related differences in distribution and elimination may have been due to different molecular weight dependent interactions with endothelial cells.

7. Significant differences were observed in the half-life of the different molecular weight fractions. Half-life increased with decreasing molecular weight. The half-life of the lowest molecular weight fractions was almost 100 percent greater than the highest molecular weight fraction.

8. The half-life values of the individual fractions were assay dependent. Assays with greater sensitivity to low molecular weight components demonstrated longer half-lives. Similarly, assays reflecting higher molecular weight components resulted in shorter half-life values. These results also demonstrated the differences in the kinetics of the pharmacodynamic actions of the different molecular weight fractions.

9. Absorption studies suggested that molecular weight was an important determinant of this characteristic. Absorption increased with decreasing molecular

weight. The high absorption of low molecular weight heparins suggests that subcutaneous administration may be an efficient route by which to administer these agents.

10. The high correlation between absorption and percent content of molecular weight components under 10,000 M.W. suggested that only components of about 10,000 molecular weight or less reached the circulation after subcutaneous administration. It is important to mention that this molecular weight dependent absorption threshold was relative only to the anticoagulant actions of the fractions and may have been different for other pharmacodynamic effects.

11. After intravenous injection, the antithrombotic potency of most agents was directly related to the in vitro anticoagulant potency. Only the unfractionated heparin displayed a dissociation between its anticoagulant and antithrombotic effects. For this agent, the antithrombotic effects were greater than the anticoagulant effects suggested. After subcutaneous administration, the antithrombotic effects of most agents were similarly related to absorption. However, the 13,300 M.W. fraction exhibited strong antithrombotic effects with poor absorption. These two observations suggested that not all antithrombotic effects of heparin are reflected by anticoagulant actions.

12. For all molecular weight dependent actions of

heparin, the effect was influenced not only by mean molecular weight but also by the percent distribution of molecular weight components.

TABLES

Table 1

HPLC-GPC Molecular Weight (MW) Determinations for Fractions Obtained by Gel Filtration

Fraction	MW	Mw Distribution Range	Peak MW
I	23,000	44,000 - 12,500	22,000
II	17,450	30,000 - 11,000	16,780
III	15,000	24,000 - 8,000	14,470
IV	13,300	22,000 - 7,000	13,000
V	11,750	21,000 - 5,500	11,200
VI	10,400	20,000 - 4,300	9,500
VII	9,000	20,000 - 2,600	7,600
VIII	7,400	18,000 - 2,400	5,600
IX	5,100	15,000 - 1,500	3,800
heparin	12,575	44,000 - 1,500	13,550
CY 216	5,400	14,000 - 1,000	4,400

Table 1. The molecular weight characteristics of the fractions obtained from the gel - filtration procedure were determined by HPLC - GPC. The molecular weight values were calculated by comparing column retention times to those obtained from standards of known molecular weight. The values in the table represent the averages calculated from two columns of different porosity.

Table 2

Viscosity of the Test Fractions

Heparin preparation	Viscosity (mPa's)
unfractionated	1.02
CY 216	0.95
23,000	1.28
17,450	1.18
15,000	1.10
13,300	1.05
11,750	0.98
10,400	0.97
9,000	0.94
7,400	0.92
5,100	0.76

Table 2. The viscosity of the gel - filtered fractions was measured using a Wells-Brookfield viscometer. The viscometer was calibrated with oils of known viscosity and 10 mg/ml solutions of the test fractions were measured at 25° C at three shear rates. Values in centipoise were obtained by multiplying the readings from the viscometer by a constant based upon the geometry of the cone plate. The values in the table represent averages of two determinations at the three rates.

Table 3
Effect of Molecular Weight on APTT

Molecular Weight	Concentration ug/ml	Clotting Time seconds
23,000	10	> 200
	5	163 ± 9
	2.5	55 ± 4.3
	1.25	36 ± 2.4
	.625	31 ± .9
17,450	10	> 200
	5	183 ± 12.4
	2.5	61 ± 5.7
	1.25	38 ± 2
	.625	31 ± .7
13,300	10	> 200
	5	> 200
	2.5	82 ± 4
	1.25	47 ± 2.7
	.625	34 ± 1.1
9,000	10	> 200
	5	121 ± 7.7
	2.5	57 ± 3.4
	1.25	38 ± 2.1
	.625	32 ± 1
5,100	10	64 ± 2.5
	5	44 ± 1.4
	2.5	36 ± 1.6
	1.25	30 ± .4
	.625	29 ± 1.2
Native Heparin	10	> 200
	5	> 200
	2.5	78 ± 7.6
	1.25	52 ± 2.3
	.625	36 ± 3
CY 216	10	85 ± 4.8
	5	67 ± 3.3
	2.5	55 ± 2.6
	1.25	36 ± 3
	.625	30 ± 1.3
Control		27 ± .4
N = 5		

Table 3. The molecular weight fractions were supplemented to pooled normal human platelet poor plasma at concentrations ranging from 0.625 through 10.0 ug/ml. The APTT assay was performed on five separate occasions exactly as described in appendix VII. The results represent the mean and the standard deviation of the five determinations.

Table 4

Effect of Molecular Weight on PT

Molecular Weight	Concentration (ug/ml)	Clotting Time
23,000	20	55 ± 2.7
	10	23 ± 1.3
	5	16 ± 1.4
	2.5	14 ± .8
	1.25	13 ± .3
17,450	20	47 ± 3.6
	10	21 ± 1.7
	5	16 ± .9
	2.5	14 ± 1
	1.25	13 ± .3
13,300	20	36 ± 1.5
	10	22 ± 1.3
	5	17 ± .8
	2.5	14 ± .6
	1.25	13 ± .4
9,000	20	21 ± .7
	10	18 ± .7
	5	15 ± .6
	2.5	14 ± .7
	1.25	13 ± .6
5,100	20	15 ± .9
	10	14 ± .6
	5	13 ± .6
	2.5	13 ± .4
	1.25	13 ± .7
Native heparin	20	23 ± 3.3
	10	20 ± 1.9
	5	17 ± 1.4
	2.5	14 ± .8
	1.25	13 ± .9
CY 216	20	18 ± 1.4
	10	16 ± .8
	5	15 ± .7
	2.5	14 ± .9
	1.25	13 ± .4
Control		13 ± .5
N = 5		

Table 4. The molecular weight fractions were supplemented to pooled normal human platelet poor plasma at concentrations ranging from 0.625 through 10.0 ug/ml. The PT assay was performed on five separate occasions exactly as described in appendix VI. The results represent the mean and the standard deviation of the five determinations.

Table 5

The Effect of Molecular Weight Fractions of Heparin on PT in Antithrombin III Deficient Plasma

Fraction	Clotting Time (seconds)
23,000 M.W.	16.3 ± .3
17,450 M.W.	16.4 ± .5
13,300 M.W.	16.4 ± .4
9.000 M.W.	16.0 ± .7
5,100 M.W.	15.9 ± .8
CY 216	16.1 ± .4
unfractionated heparin	16.2 ± .2
control	13.8 ± .3

Table 5. Antithrombin III deficient plasma was prepared as described in methods. The antithrombin content of this plasma was determined to be 6.2 % of normal. The test fractions were supplemented to the anthhrombin deficient plasma at a concentration of 20 ug/ml and the PT assay was performed exactly as described in appendix VI.

Table 6

Effect of Molecular Weight on Heptest

Molecular Weight	Concentration (ug/ml)	Clotting Time
23,000	10	290 ± 16
	5	145 ± 3.2
	2.5	70 ± 3
	1.25	43 ± 8.2
	.625	35 ± 2.4
17,450	10	256 ± 12
	5	135 ± 6.5
	2.5	64 ± 5
	1.25	52 ± 3.4
	.625	38 ± 1.9
13,300	10	346 ± 14.7
	5	170 ± 9.6
	2.5	82 ± 2.8
	1.25	62 ± .5
	.625	41 ± 3.9
9,000	10	235 ± 18.6
	5	120 ± 8
	2.5	72 ± 9.4
	1.25	54 ± 5.4
	.625	38 ± 1.3
5,100	10	128 ± 11.7
	5	69 ± 17.6
	2.5	56 ± 2.2
	1.25	42 ± 2.5
	.625	29 ± 2.7
Native Heparin	10	353 ± 9.4
	5	160 ± 12.3
	2.5	84 ± 4.6
	1.25	50 ± 3.4
	.625	39 ± 1.7
CY 216	10	120 ± 5.6
	5	86 ± 6.6
	2.5	64 ± 4.3
	1.25	48 ± 4.5
	.625	31 ± 2
Control		16 ± .9
N = 5		

Table 6. The molecular weight fractions were supplemented to pooled normal human platelet poor plasma at concentrations ranging from 0.625 through 10.0 ug/ml. The heptest assay was performed on five separate occasions exactly as described in appendix VIII. The results represent the mean and the standard deviation of the five determinations.

Table 7

Effect of Molecular Weight on Xa amidolytic activity

Molecular Weight	Concentration (ug/ml)	% inhibition
23,000	10	87 ± 2.2
	5	87 ± 1.7
	2.5	76 ± 1.3
	1.25	51 ± 2.2
	.625	28 ± .7
17,450	10	89 ± 1.7
	5	86 ± 1.4
	2.5	75 ± .7
	1.25	51 ± 2.9
	.625	26 ± 1.5
13,300	10	88 ± 2.7
	5	88 ± 1.3
	2.5	77 ± 1.1
	1.25	53 ± 2.2
	.625	29 ± 1.1
9,000	10	88 ± 2.3
	5	72 ± 1
	2.5	48 ± 1.9
	1.25	38 ± .6
	.625	13 ± 1
5,100	10	69 ± 1.3
	5	45 ± .9
	2.5	24 ± 1.3
	1.25	13 ± 1.1
	.625	8 ± .6
Native Heparin	10	87 ± 2.0
	5	86 ± 1.4
	2.5	77 ± 1.2
	1.25	50 ± 2.2
	.625	28 ± 1.7
CY 216	10	80 ± 3.2
	5	59 ± 1.6
	2.5	37 ± 1.4
	1.25	21 ± 1.3
	.625	13 ± 1.3
Control		6 ± 1.2

N - 5

Table 7. The molecular weight fractions were supplemented to pooled normal human platelet poor plasma at concentrations ranging from 0.625 through 10.0 ug/ml. The anti factor Xa assay was performed on five separate occasions exactly as described in methods. The results represent the mean and the standard deviation of the five determinations.

Table 8

Effect of Molecular Weight on Anti IIa Activity

Molecular Weight	Concentration (ug/ml)	% Inhibition
23,000	10	91 ± 2.2
	5	86 ± 3.5
	2.5	82 ± 3.1
	1.25	62 ± 3.7
	.625	40 ± 4.3
17,450	10	90 ± 1.3
	5	89 ± 2.7
	2.5	79 ± 3.4
	1.25	68 ± 4.7
	.625	44 ± 2.6
13,300	10	93 ± 2.4
	5	88 ± 1.7
	2.5	79 ± 1.6
	1.25	74 ± 3.1
	.625	41 ± 4.6
9,000	10	88 ± 1.8
	5	77 ± 3.2
	2.5	59 ± 2.1
	1.25	42 ± 2.9
	.625	15 ± .7
5,100	10	68 ± 2.1
	5	44 ± 1.9
	2.5	24 ± 1.7
	1.25	13 ± 3.5
	.625	2 ± 1.7
Native Heparin	10	87 ± 2.1
	5	84 ± 3.1
	2.5	76 ± 1.6
	1.25	64 ± 2.4
	.625	36 ± 4.2
CY 216	10	58 ± 3.1
	5	34 ± 3.2
	2.5	14 ± 4.6
	1.25	9 ± 3.1
	.625	3 ± 2.7
Control		3 ± 1.4

N - 5

Table 8. The molecular weight fractions were supplemented to pooled normal human platelet poor plasma at concentrations ranging from 0.625 through 10.0 ug/ml. The anti factor IIa assay was performed on five separate occasions exactly as described in methods. The results represent the mean and the standard deviation of the five determinations.

Table 9

Effect of Molecular Weight on Thromboplastin Activated FPA Generation

Molecular Weight	Concentration (ug/ml)	FPA (ng/ml)
23,000	5	42 ± 22
	2.5	64 ± 19
	1.25	425 ± 60
	.625	713 ± 50
17,450	5	63 ± 30
	2.5	87 ± 21
	1.25	528 ± 76
	.625	836 ± 110
13,300	5	75 ± 30
	2.5	146 ± 29
	1.25	509 ± 115
	.625	823 ± 82
9,000	5	221 ± 89
	2.5	332 ± 44
	1.25	686 ± 76
	.625	947 ± 147
5,100	5	763 ± 116
	2.5	915 ± 60
	1.25	1,023 ± 143
	.625	1,357 ± 276
Native Heparin	5	81 ± 15
	2.5	66 ± 18
	1.25	475 ± 62
	.625	762 ± 123
CY 216	5	756 ± 110
	2.5	820 ± 134
	1.25	997 ± 138
	.625	1,059 ± 106
Control		1,275 ± 186

N = 5

Table 9. The molecular weight fractions were supplemented to pooled normal human platelet poor plasma at concentrations ranging from 0.625 through 5.0 ug/ml. The fibrinopeptide - A generation assay was performed on five separate occasions exactly as described in methods. The results represent the mean and the standard deviation of the five determinations.

Table 10

Comparison of Molecular Weight Fractions of Heparin on FPA Generation in At III Deficient Plasma

Heparin concentration: 10 ug/ml

HEPARIN	FPA GENERATED (ng/ml) AT III DEFICIENT
Control	1216 ± 167
23,000 M.W.	1254 ± 181
17,450 M.W.	1183 ± 139
13,300 M.W.	1180 ± 124
9,000 M.W.	1100 ± 162
5,100 M.W.	1138 ± 121
Native Heparin	1118 ± 109
CY 216	1156 ± 182

N = 5

Table 10. Antithrombin III deficient plasma was prepared as described in methods. The antithrombin content of this plasma was determined to be 6.2 % of normal. The test fractions were supplemented to the anthhrombin deficient plasma at a concentration of 10 ug/ml and the fibrinopeptide-A generation assay was performed exactly as described in methods.

Table 11

The Effect of Molecular Weight Fractions of Heparin on FPA Generation in Whole Human Blood

Heparin Fraction	Concentration (ug/ml)	FPA Generated (ng/nl)
23,000 M.W.	10	0.66 ± .3
	5	3.5 ± 2.7
	2.5	18.3 ± 9.0
13,300 M.W.	10	0.5 ± .5
	5	0.37 ± .5
	2.5	6.0 ± 3.6
5,100 M.W.	10	12.6 ± 3
	5	14.2 ± 4.4
	2.5	42.6 ± 6.4
Native Heparin	10	0.0 ± 0
	5	6.0 ± 3.0
	2.5	12.0 ± 6.8
CY 216	10	4.3 ± 1.1
	5	12.5 ± 3.3
	2.5	24.0 ± 3.4
control		50 ± 10.0
N = 4		

Table 11. Three concentrations of the individaul test fractions were added to freshly drawn human whole blood. The whole blood fibrinopeptide - A generation assay was performed on these samples exactly as described in methods. The results represent the mean and standard deviation from assays performed in the whole blood of four individuals.

Table 12
Time Course Values Obtained Using Heptest

FRACTION	KINETIC PARAMETER	VALUE
23,000 M.W.	Vd (L/Kg)	.05 ± .0005
	t 1/2 (min.)	29 ± 2.2
	Cl_p (ml/min/Kg)	1.18 ± .1
	AUC (ug hr/ml)	3.99 ± .2
13,300 M.W.	Vd (L/Kg)	.05 ± .003
	t 1/2 (min.)	34.5 ± 4.5
	Cl_p (ml/min/Kg)	1.12 ± .11
	AUC (ug hr/ml)	4.36 ± .53
5,100 M.W.	Vd (L/Kg)	.055 ± .004
	t 1/2 (min.)	50.8 ± 6.5
	Cl_p (ml/min/Kg)	.76 ± .09
	AUC (ug hr/ml)	6.38 ± .61
CY 216 4,500 M.W.	Vd (L/Kg)	.061 ± .008
	t 1/2 (min.)	58 ± 4.8
	Cl_p (ml/min/Kg)	.72 ± .06
	AUC (ug hr/ml)	6.25 ± .57
Native Heparin	Vd (L/Kg)	.05 ± .0001
	t 1/2 (min.)	31 ± 2.2
	Cl_p (ml/min/Kg)	1.14 ± .08
	AUC (ug hr/ml)	4.26 ± .3

Table 12. The intravenous plasma time course of the individual test fractions was calculated exactly as described in methods. The data in this table represents the time course parameters calculated from data obtained with the heptest assay. All values indicate the mean and standard deviation obtained from administration of the individual fractions to five primates.

Table 13
Time Course of plasma Anti Xa Activity

FRACTION	KINETIC PARAMETER	VALUE
23,000 M.W.	Vd (L/Kg)	.050 ± .005
	t 1/2 (min.)	29 ± 2.2
	Cl_p (ml/min/Kg)	1.18 ± .03
	AUC (ug hr/ml)	4.10 ± .07
13,300 M.W.	Vd (L/Kg)	.054 ± .005
	t 1/2 (min.)	34.4 ± 4.3
	Cl_p (ml/min/Kg)	1.15 ± .08
	AUC (ug hr/ml)	4.36 ± .35
5,100 M.W.	Vd (L/Kg)	.05 ± .004
	t 1/2 (min.)	50.6 ± 7.7
	Cl_p (ml/min/Kg)	.77 ± .58
	AUC (ug hr/ml)	6.08 ± .35
CY 216 4,500 M.W.	Vd (L/Kg)	.064 ± .005
	t 1/2 (min.)	55 ± 4.0
	Cl_p (ml/min/Kg)	.77 ± .13
	AUC (ug hr/ml)	5.9 ± .57
Native Heparin	Vd (L/Kg)	.05 ± .004
	t 1/2 (min.)	31 ± 2.0
	Cl_p (ml/min/Kg)	1.06 ± .06
	AUC (ug hr/ml)	4.36 ± .43

Table 13. The intravenous plasma time course of the individual test fractions was calculated exactly as described in methods. The data in this table represents the time course parameters calculated from data obtained with the anti factor Xa assay. All values indicate the mean and standard deviation obtained from administration of the individual fractions to five primates.

Table 14
Time Course of Plasma Anti IIa Activity

FRACTION	PHARMACOKINETIC PARAMETER	VALUE
23,000 M.W.	Vd (L/Kg)	.05 ± .009
	t 1/2 (min.)	30 ± 1.5
	Cl_p (ml/min/Kg)	1.4 ± .03
	AUC (ug hr/ml)	3.31 ± .2
13,300 M.W.	Vd (L/Kg)	.06 ± .006
	t 1/2 (min.)	32 ± 1.3
	Cl_p (ml/min/Kg)	1.46 ± .05
	AUC (ug hr/ml)	3.53 ± .04
5,100 M.W.	Vd (L/Kg)	.05 ± .004
	t 1/2 (min.)	32 ± 1.9
	Cl_p (ml/min/Kg)	1.08 ± .08
	AUC (ug hr/ml)	4.73 ± .14
CY 216 4,500 M.W.	Vd (L/Kg)	.05 ± .005
	t 1/2 (min.)	32 ± 1.4
	Cl_p (ml/min/Kg)	1.1 ± .07
	AUC (ug hr/ml)	4.0 ± .5
Native Heparin	Vd (L/Kg)	.06 ± .01
	t 1/2 (min.)	32 ± 1.5
	Cl_p (ml/min/Kg)	1.4 ± .22
	AUC (ug hr/ml)	3.5 ± .52

Table 14. The intravenous plasma time course of the individual test fractions was calculated exactly as described in methods. The data in this table represents the time course parameters calculated from data obtained with the anti factor IIa assay. All values indicate the mean and standard deviation obtained from administration of the individual fractions to five primates.

Table 15
Time Course Values Obtained From PT Assay

FRACTION	KINETIC PARAMETER	VALUE
23,000 M.W.	Vd (L/Kg)	.1 ± .01
	t 1/2 (min.)	26.2 ± 2.0
	Cl_p (ml/min/Kg)	2.68 ± .39
	AUC (ug hr/ml)	2.4 ± .3
13,300 M.W.	Vd (L/Kg)	.11 ± .004
	t 1/2 (min.)	35 ± 3.4
	Cl_p (ml/min/Kg)	2.14 ± .15
	AUC (ug hr/ml)	2.42 ± .16
5,100 M.W.	Vd (L/Kg)	.09 ± .005
	t 1/2 (min.)	36 ± 2.1
	Cl_p (ml/min/Kg)	1.8 ± .19
	AUC (ug hr/ml)	2.6 ± .2
CY 216 4,500 M.W.	Vd (L/Kg)	.06 ± .007
	t 1/2 (min.)	39 ± 7.2
	Cl_p (ml/min/Kg)	1.2 ± .14
	AUC (ug hr/ml)	3.80 ± .58
Native Heparin	Vd (L/Kg)	.07 ± .01
	t 1/2 (min.)	29 ± 5.4
	Cl_p (ml/min/Kg)	1.76 ± .16
	AUC (ug hr/ml)	2.78 ± .34

Table 15. The intravenous plasma time course of the individual test fractions was calculated exactly as described in methods. The data in this table represents the time course parameters calculated from data obtained using a dilute PT assay. All values indicate the mean and standard deviation obtained from administration of the individual fractions to five primates.

Table 16
Time Course of Plasma FPA Inhibiting Actions

FRACTION	KINETIC PARAMETER	VALUE
23,000 M.W.	Vd (L/Kg)	.07 ± .008
	t 1/2 (min.)	30 ± 2.7
	Cl_p (ml/min/Kg)	2.05 ± .5
	AUC (ug hr/ml)	2.56 ± 1.1
13,300 M.W.	Vd (L/Kg)	.10 ± .1
	t 1/2 (min.)	45.6 ± 5
	Cl_p (ml/min/Kg)	1.9 ± .3
	AUC (ug hr/ml)	3.13 ± .1
5,100 M.W.	Vd (L/Kg)	.07 ± .01
	t 1/2 (min.)	52 ± 7
	Cl_p (ml/min/Kg)	1.0 ± .23
	AUC (ug hr/ml)	5.1 ± .49
CY 216 4,500 M.W.	Vd (L/Kg)	.09 ± .01
	t 1/2 (min.)	61 ± 6.8
	Cl_p (ml/min/Kg)	1.21 ± .1
	AUC (ug hr/ml)	3.9 ± .14
Native Heparin	Vd (L/Kg)	.08 ± .02
	t 1/2 (min.)	37 ± 1.9
	Cl_p (ml/min/Kg)	1.6 ± .2
	AUC (ug hr/ml)	3.57 ± .3

Table 16. The intravenous plasma time course of the individual test fractions was calculated exactly as described in methods. The data in this table represents the time course parameters calculated from data obtained using a the fibrinopeptide - A generation assay. All values indicate the mean and standard deviation obtained from administration of the individual fractions to five primates.

Table 17

Subcutaneous Time Course Obtained Using Heptest Assay

FRACTION	KINETIC PARAMETER	VALUE
	t 1/2 (min.)	205 ± 40
23,000 M.W.	AUC (ug hr/ml)	.47 ± .38
	Absorption	3 %
	t 1/2 (min.)	144 ± 32
13,300 M.W.	AUC (ug hr/ml)	1.54 ± .2
	Absorption	9 %
	t 1/2 (min.)	108 ± 20
5,100 M.W.	AUC (ug hr/ml)	23.7 ± .9
	Absorption	93 %
	t 1/2 (min.)	144 ± 26
CY 216 4,500 M.W.	AUC (ug hr/ml)	21.9 ± 2.2
	Absorption	88 %
	t 1/2 (min.)	94 ± 32
Native Heparin	AUC (ug hr/ml)	6.8 ± 1.4
	Absorption	40 %

Table 17. The plasma time course of the test fractions were calculated after subcutaneous injection at a concentration of 1.0 mg/kg. The blood sampling protocal and time course calculations were performed exactly as previously described. These results were calculated from data obtained using the heptest assay. All values represent the mean and standard deviation of calculations from five individual primates.

Table 18
Subcutaneous Time Course of Plasma Anti Xa Activity

FRACTION	KINETIC PARAMETER	VALUE
	t 1/2 (min.)	106 ± 12
23,000 M.W.	AUC (ug hr/ml)	1.5 ± .4
	Absorption	10 %
	t 1/2 (min.)	163 ± 19
13,300 M.W.	AUC (ug hr/ml)	2.9 ± .4
	Absorption	14 %
	t 1/2 (min.)	200 ± 18
5,100 M.W.	AUC (ug hr/ml)	21.4 ± 1.7
	Absorption	94 %
	t 1/2 (min.)	201 ± 17
CY 216 4,500 M.W.	AUC (ug hr/ml)	22 ± 2
	Absorption	96 %
	t 1/2 (min.)	154 ± 17.5
Native Heparin	AUC (ug hr/ml)	5.7 ± 1.3
	Absorption	32 %

Table 18. The plasma time course of the test fractions was calculated after subcutaneous injection at a concentration of 1.0 mg/kg. The blood sampling protocal and time course calculations were performed exactly as previously described. These results were calculated from data obtained using the anti factor Xa assay. All values represent the mean and standard deviation of calculations from five individual primates.

Table 19
Subcutaneous Time Course of Plasma Anti IIa Activity

FRACTION	KINETIC PARAMETER	VALUE
	t 1/2 (min.)	104 ± 16
23,000 M.W.	AUC (ug hr/ml)	0.9 ± .3
	Absorption	6 %
	t 1/2 (min.)	147 ± 28.3
13,300 M.W.	AUC (ug hr/ml)	1.4 ± .63
	Absorption	10 %
	t 1/2 (min.)	139 ± 22
5,100 M.W.	AUC (ug hr/ml)	12.2 ± 2.1
	Absorption	60 %
	t 1/2 (min.)	167 ± 15
CY 216 4,500 M.W.	AUC (ug hr/ml)	7.5 ± 1
	Absorption	47 %
	t 1/2 (min.)	119 ± 19
Native Heparin	AUC (ug hr/ml)	3.5 ± .7
	Absorption	24 %

Table 19. The plasma time course of the test fractions was calculated after subcutaneous injection at a concentration of 1.0 mg/kg. The blood sampling protocal and time course calculations were performed exactly as previously described. These results were calculated from data obtained using the anti factor IIa assay. All values represent the mean and standard deviation of calculations from five individual primates.

Table 20
Subcutaneous Time Course Determined Using A PT Assay

FRACTION	KINETIC PARAMETER	VALUE
	t 1/2 (min.)	N.A.
23,000 M.W.	AUC (ug hr/ml)	N.A.
	Absorption	0
	t 1/2 (min.)	167 ± 27
13,300 M.W.	AUC (ug hr/ml)	1.2 ± .4
	Absorption	13 %
	t 1/2 (min.)	121 ± 17
5,100 M.W.	AUC (ug hr/ml)	3.3 ± .6
	Absorption	30 %
	t 1/2 (min.)	135 ± 14
CY 216 4,500 M.W.	AUC (ug hr/ml)	5.8 ± 1.1
	Absorption	38 %
	t 1/2 (min.)	117 ± 14
Native Heparin	AUC (ug hr/ml)	3.7 ± .5
	Absorption	36 %

Table 20. The plasma time course of the test fractions were calculated after subcutaneous injection at a concentration of 1.0 mg/kg. The blood sampling protocal and time course calculations were performed exactly as previously described. These results were calculated from data obtained using a dilute PT assay. All values represent the mean and standard deviation of calculations from five individual primates.

Table 21
Subcutaneous Time Course Determined Using the FPAGT

FRACTION	KINETIC PARAMETER	VALUE
	t 1/2 (min.)	89 ± 21
23,000 M.W.	AUC (ug hr/ml)	2.8 ± .5
	Absorption	28 %
	t 1/2 (min.)	131 ± 20
13,200 M.W.	AUC (ug hr/ml)	5.7 ± .5
	Absorption	44 %
	t 1/2 (min.)	158 ± 19.2
5,100 M.W.	AUC (ug hr/ml)	19.4 ± 2.1
	Absorption	95 %
	t 1/2 (min.)	196 ± 36
CY 216 4,500 M.W.	AUC (ug hr/ml)	19.9 ± 2.5
	Absorption	100 %
	t 1/2 (min.)	120 ± 24
Native Heparin	AUC (ug hr/ml)	8.2 ± 1.4
	Absorption	66 %

Table 21. The plasma time course of the test fractions was calculated after subcutaneous injection at a concentration of 1.0 mg/kg. The blood sampling protocal and time course calculations were performed exactly as previously described. These results were calculated from data obtained using the fibrinopeptide - A generation assay. All values represent the mean and standard deviation of calculations from five individual primates.

Table 22
Circulating Pharmacodynamic Effects of the Molecular Weight Fractions in the Rabbit Stasis Thrombosis Model

Dose: 25 ug/Kg
Route: Intravenous

	Clotting Time in Seconds			/ Percent Inhibition	
Test Fraction	PTT	TT	Heptest	/ Anti Xa	Anti IIa
23,000 M.W.					
Baseline	78 ± 6	37 ± 7	27 ± 4	/ 5 ± 3	4 ± 2
Pre-challenge	85 ± 9	39 ± 9	31 ± 3	/ 14 ± 6	16 ± 10
13,300 M.W.					
Baseline	83 ± 7	33 ± 4	32 ± 6	/ 4 ± 1	3 ± 1
Pre-challenge	88 ± 8	44 ± 6	40 ± 6	/ 13 ± 4	13 ± 5
5,100 M.W.					
Baseline	84 ± 7	34 ± 6	31 ± 5	/ 3 ± 2	5 ± 3
Pre-challenge	82 ± 9	35 ± 6	39 ± 7	/ 8 ± 3	10 ± 7
CY 216					
Baseline	80 ± 9	32 ± 5	29 ± 3	/ 4 ± 2	4 ± 2
Pre-challenge	83 ± 8	37 ± 6	39 ± 6	/ 15 ± 6	8 ± 3
Unfractionated Heparin					
Baseline	82 ± 14	35 ± 6	30 ± 6	/ 4 ± 3	3 ± 1
Pre-challenge	92 ± 17	38 ± 5	35 ± 6	/ 12 ± 5	13 ± 7

Table 22. The circulating pharmacodynamic actions of the test fractions were measured during the stasis thrombosis experiments using the PTT, PT, heptest, anti Xa and anti IIa assays. The baseline values represent the coagulation profile prior to drug administration. The pre-challenge levels represent the circulating pharmacodynamic actions immediately prior to administration of the thrombogenic challenge. All results represent the mean and standard deviation obtained from five experimental animals.

Table 23
Circulating Pharmacodynamic Effects of the Molecular Weight Fractions in the Rabbit Stasis Thrombosis Model

Dose: 50 ug/Kg
Route: Intravenous

	Clotting Time in Seconds			/ Percent Inhibition	
Test Fraction	PTT	TT	Heptest	/ Anti Xa	Anti IIa
23,000 M.W.					
Baseline	84 ± 11	36 ± 3	29 ± 4	/ 5 ± 3	4 ± 3
Pre-challenge	105 ± 15	109 ± 40	42 ± 6	/ 23 ± 6	32 ± 6
13,300 M.W.					
Baseline	84 ± 7	33 ± 3	28 ± 7	/ 5 ± 2	5 ± 3
Pre-challenge	100 ± 16	55 ± 12	37 ± 4	/ 23 ± 6	29 ± 14
5,100 M.W.					
Baseline	87 ± 14	33 ± 3	33 ± 3	/ 6 ± 3	4 ± 2
Pre-challenge	93 ± 12	37 ± 6	38 ± 3	/ 12 ± 6	7 ± 3
CY 216					
Baseline	83 ± 9	32 ± 4	31 ± 4	/ 5 ± 3	5 ± 1
Pre-challenge	96 ± 13	39 ± 7	40 ± 7	/ 17 ± 7	10 ± 5
Unfractionated Heparin					
Baseline	86 ± 8	30 ± 2	30 ± 2	/ 6 ± 3	4 ± 3
Pre-challenge	90 ± 16	70 ± 15	37 ± 4	/ 23 ± 6	29 ± 12

Table 23. The circulating pharmacodynamic actions of the test fractions were measured during the stasis thrombosis experiments using the PTT, PT, heptest, anti Xa and anti IIa assays. The baseline values represent the coagulation profile prior to drug administration. The pre-challenge levels represent the circulating pharmacodynamic actions immediately prior to administration of the thrombogenic challenge. All results represent the mean and standard deviation obtained from five experimental animals.

Table 24
Circulating Pharmacodynamic Effects of the Molecular Weight Fractions in the Rabbit Stasis Thrombosis Model

Dose: 100 ug/Kg
Route: Intravenous

	Clotting Time in Seconds		/ Percent Inhibition		
Test Fraction	PTT	TT	Heptest /	Anti Xa	Anti IIa
23,000 M.W.					
Baseline	86 ± 10	38 ± 2	30 ± 5 /	5 ± 2	4 ± 3
Pre-challenge	> 150	> 150	63 ± 14 /	40 ± 11	47 ± 14
13,300 M.W.					
Baseline	82 ± 8	34 ± 5	27 ± 3 /	4 ± 3	5 ± 2
Pre-challenge	> 150	> 150	63 ± 9 /	50 ± 9	47 ± 7
5,100 M.W.					
Baseline	83 ± 7	35 ± 5	33 ± 6 /	6 ± 2	4 ± 2
Pre-challenge	85 ± 5	47 ± 7	48 ± 17 /	20 ± 9	19 ± 12
CY 216					
Baseline	82 ± 9	33 ± 3	31 ± 3 /	6 ± 3	3 ± 2
Pre-challenge	86 ± 3	50 ± 9	53 ± 7 /	27 ± 12	22 ± 7
Unfractionated Heparin					
Baseline	84 ± 9	36 ± 4	28 ± 5 /	7 ± 4	4 ± 1
Pre-challenge	123 ± 30	>150	57 ± 12 /	43 ± 13	47 ± 17

Table 24. The circulating pharmacodynamic actions of the test fractions were measured during the stasis thrombosis experiments using the PTT, PT, heptest, anti Xa and anti IIa assays. The baseline values represent the coagulation profile prior to drug administration. The pre-challenge levels represent the circulating pharmacodynamic actions immediately prior to administration of the thrombogenic challenge. All results represent the mean and standard deviation obtained from five experimental animals.

Table 25
Circulating Pharmacodynamic Effects of the Molecular Weight Fractions in the Rabbit Stasis Thrombosis Model

Dose: 1mg/Kg
Route: Subcutaneous

	Clotting Time in Seconds			/ Percent Inhibition	
Test Fraction	PTT	TT	Heptest	/ Anti Xa	Anti IIa
23,000 M.W.					
Baseline	79 ± 9	32 ± 2	27 ± 2	/ 3 ± 2	3 ± 6
Pre-challenge	84 ± 11	36 ± 3	28 ± 3	/ 3 ± 2	2 ± 4
13,300 M.W.					
Baseline	80 ± 7	34 ± 2	30 ± 2	/ 3 ± 2	4 ± 4
Pre-challenge	90 ± 9	42 ± 6	33 ± 5	/ 12 ± 6	10 ± 8
5,100 M.W.					
Baseline	84 ± 10	32 ± 3	29 ± 6	/ 4 ± 3	5 ± 3
Pre-challenge	94 ± 12	44 ± 16	55 ± 13	/ 17 ± 8	23 ± 8
CY 216					
Baseline	81 ± 9	31 ± 4	27 ± 3	/ 4 ± 2	4 ± 2
Pre-challenge	96 ± 10	47 ± 7	62 ± 9	/ 23 ± 9	19 ± 12
Unfractionated Heparin					
Baseline	79 ± 10	35 ± 3	24 ± 5	/ 3 ± 2	2 ± 2
Pre-challenge	88 ± 12	37 ± 5	33 ± 8	/ 19 ± 5	4 ± 5

Table 25. The circulating pharmacodynamic actions of the test fractions were measured during the stasis thrombosis experiments using the PTT, PT, heptest, anti Xa and anti IIa assays. The baseline values represent the coagulation profile prior to drug administration. The pre-challenge levels represent the circulating pharmacodynamic actions immediately prior to administration of the thrombogenic challenge. All results represent the mean and standard deviation obtained from five experimental animals.

Table 26
Comparison of Half-life Calculated from
Molar and Gravimetric Concentrations

Fraction	Time post Injection	concentration (μM)	concentration (ug/ml)
23,000	5	.207	4.78
	10	.18	4.2
	15	.16	3.7
	30	.10	2.5
	60	.05	1.15
	180	.001	.02
		t 1/2 = 27 min.	t 1/2 = 29 min.
13,300	5	.35	4.72
	10	.30	4.0
	15	.26	3.5
	30	.18	2.4
	60	.09	1.3
	180	.01	.13
		t 1/2 = 33 min.	t 1/2 = 34 min.
5,100	5	.93	4.77
	10	.81	4.14
	15	.70	3.61
	30	.59	3.05
	60	.42	2.14
	180	.078	.40
		t 1/2 = 52 min.	t 1/2 = 50 min.
CY 216	5	.89	4.81
	10	.79	4.29
	15	.70	3.8
	30	.54	2.9
	60	.35	1.9
	180	.09	.47
		t 1/2 = 60 min.	t 1/2 = 58 min.
Heparin	5	.375	4.72
	10	.329	4.14
	15	.304	3.82
	30	.227	2.85
	60	.10	1.27
	180	.002	.02
		t 1/2 = 30 min.	t 1/2 = 31 min.

Table 26. The gravimetric concentrations from the primate kinetic study were converted to molar amounts using the mean molecular weights in table 1. Semi - log concentration / time plots were generated for both values from which half-life values were calculated. These values were similar for both molar and gravimetric concentrations.

Table 27

Comparison of Relative Absorption Calculated From Molar and Gravimetric Concentrations

Test Fraction	Molar Absorption	Gravimetric Absorption
23,000	2.89 %	3 %
13,300	8.85 %	9 %
5,100	92 %	93 %
CY 216	86 %	88 %
Heparin	41 %	40 %

Table 27. Absorption was calculated using the AUC data obtained from the heptest assay exactly as described in methods. The ug hr/ml concentrations were transformed to uM values using the mean molecular weights of the individual fractions (table 1).

Table 28

Relationship Between Absorption Distribution and Molecular Weight

Fraction	% of components less than 10,000	approximate Absortpion
Native Heparin	38	40 %
23,000 M.W.	1 - 2	6 %
13,300 M.W.	7	11 %
5,100 M.W.	96	93
CY 216	97	91

correlation between absorption and the % content of molecular weight components less than 10,000 molecular weight (r = .99)

Table 29

Relationship Between Absorption , Pharmacodynamic Effects and Antithrombotic Actions After Subcutaneous Administration

Fraction	Antithrombotic Actions	Absorption (Primate) (%)	Pharmacodynamic Effects
23,000	7.7	3.0	3.7
13,300	62.0	9.0	9.9
5,100	47.0	93.0	48.0
CY 216	81.0	88.0	57.0
Heparin	27.0	40.0	28.0

correlation between antithrombotic actions and absorption r = .55

correlation between antithrombotic actions and circulating anticoagulant actions r = .63

Table 29. The antithrombotic actions represent the percent reduction in thrombus formation in the rabbit model compared to control. The absorption was calculated from the primate heptest data exactly as previously described. The pharmacodynamic effects indicate the circulating drug actions in the rabbit model prior to injection of the thrombogenic challenge. These values represent the percent difference from baseline values.

Table 30

Molar Concentration of Antithrombin III in Normal Human Plasma and the Test Fractions at a gravimetric concentration of 5 ug/ml

Test Fraction	Concentration (uM)
23,000 M.W.	.217
17,450 M.W.	.286
13,300 M.W.	.375
9,000 M.W.	.555
5,100 M.W.	.98
CY 216	.92
Heparin	.397
Antithrombin III	4.53

Table 30. The molar concentrations of the test fractions were based on the molecular weights determined by HPLC-GPC (table 1). The molar concentration of antithrombin III was based upon a molecular weight of 64,000 and a normal plasma concentration of 29 mg/dL. The molar ratio between the test fractions and antithrombin III was even greater than the calculations indicate since only about one third of a given heparin preparation possesses affinity to antithrombin III.

FIGURES

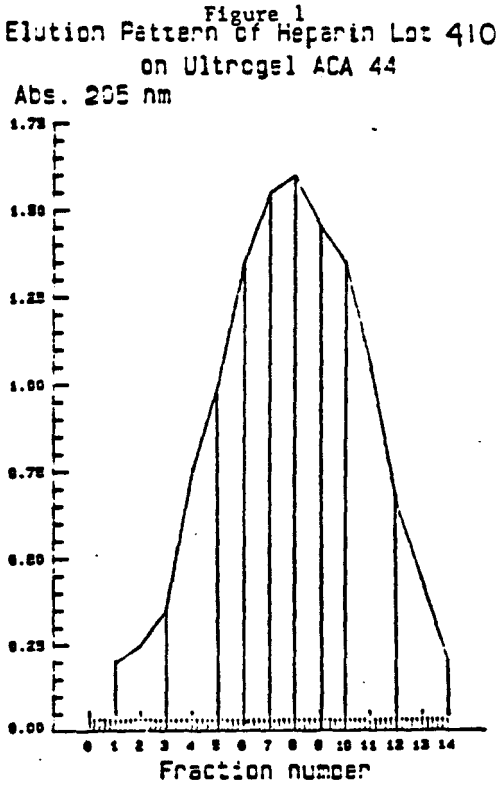

Figure 1. Five grams of porcine sodium heparin were fractionated on an Ultro-gel ACA-44 column. Fourteen fractions were collected at 35 minute intervals and the absorbance at 205 nm was recorded to determine heparin content. Initial and latter fractions were pooled in order to achieve a sufficient quantity for experimental work. The elution profile demonstrated a normally distributed population of molecular weight components within the native heparin.

Figure 2
Comparison of the Glycosaminoglycan Content of the Various Test Heparins Using a Toluidine Blue Assay

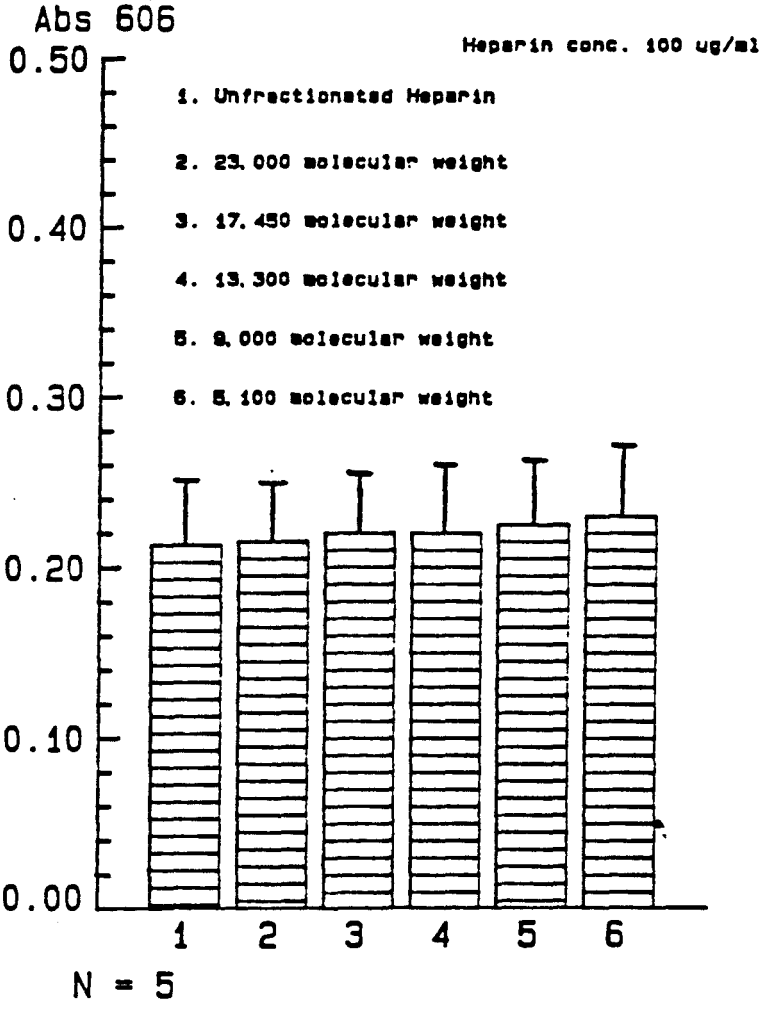

Figure 2. The glycosaminoglycan content of the gel-filtered fractions was measured using a toluidine blue assay. The dye reacted with the sulfate groups of the heparin molecules to produce a color change which was measured in a spectrophotometer. The absorbance at 606 nm was directly proportional to the heparin content of the individual frations. All fractions displayed similar heparin content.

Figure 3
Relationship Between Viscosity and the Molecular Weight of the Test Heparins Determined by HPLC - GPC

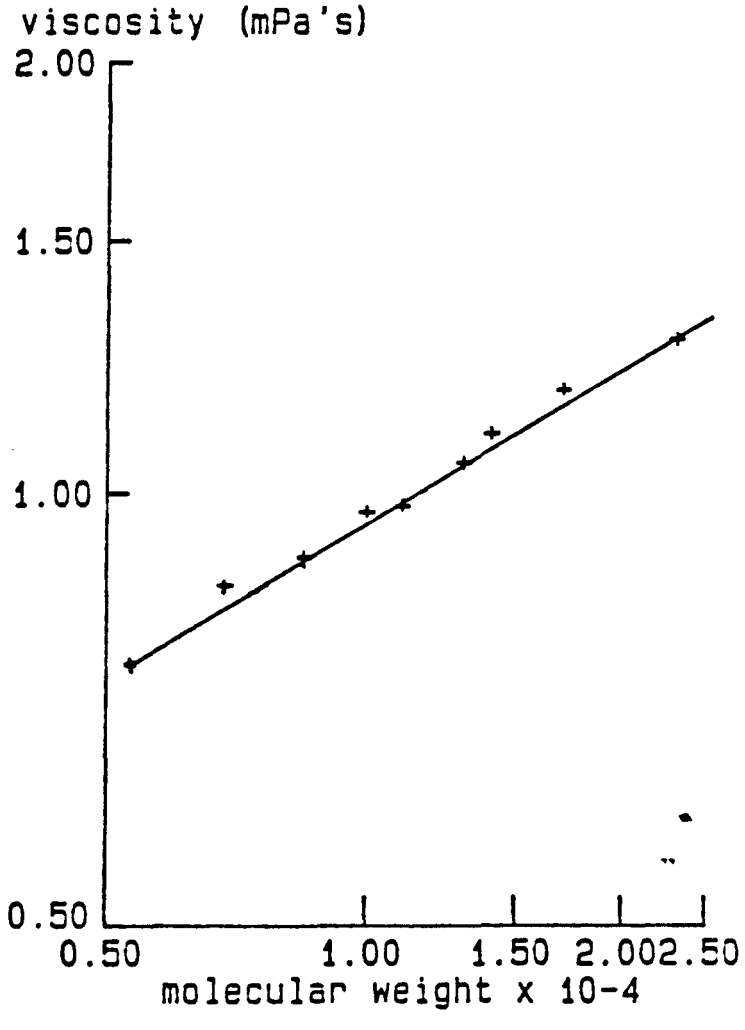

Figure 3. Viscosity measurements were made on 10 mg/ml solutions of the gel-filtered fractions using a cone-plate viscometer. The figure shows the relationship between the resulting viscosity and the molecular weight determined by HPLC-GPC. A linear relationship was observed between viscosity and molecular weight.

Figure 4
Effect of Molecular Weight on Thromboplastin Activated FPA Generation in Platelet Poor and Platelet Rich Plasma

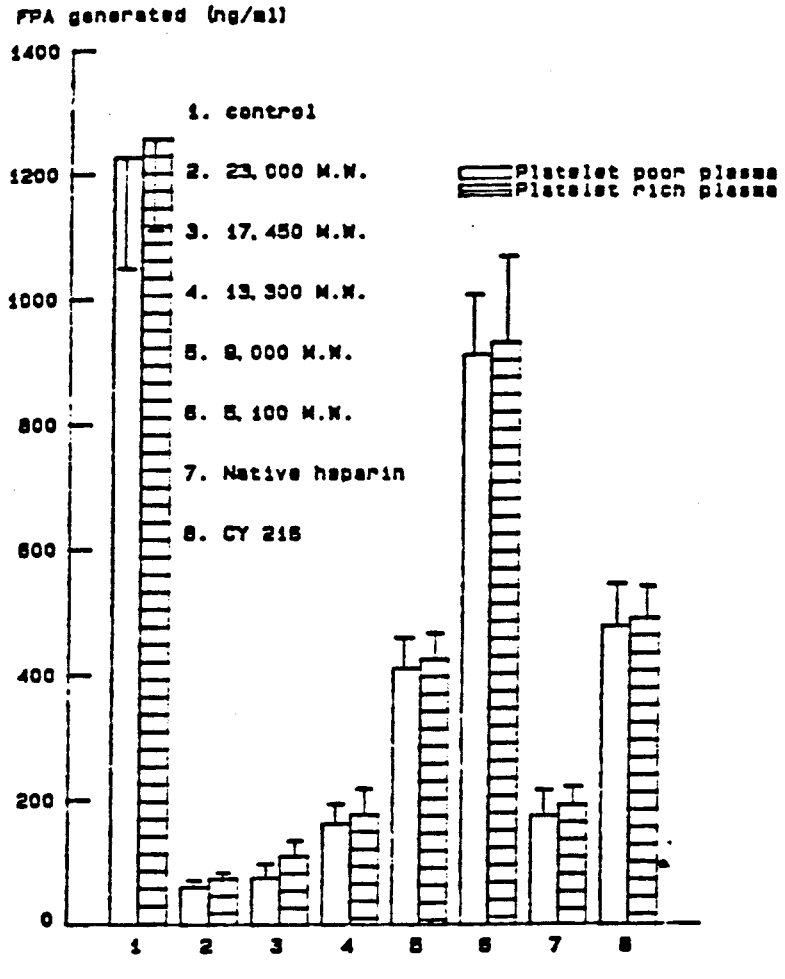

Figure 4. The molecular weight fractions were compared in the thromboplastin activated FPA generation assay in both platelet rich and platelet poor plasmas. Both plasmas were prepared from the same donor (N = 5) and supplemented with the individual test fractions at 2.5 ug/ml. FPA generation was performed exactly as described in methods. The effects of all fractions were similar in both platelet rich and platelet poor plasmas.

Figure 5
Effect of Factor VII Concentration
on VII-Thromboplastin Activation

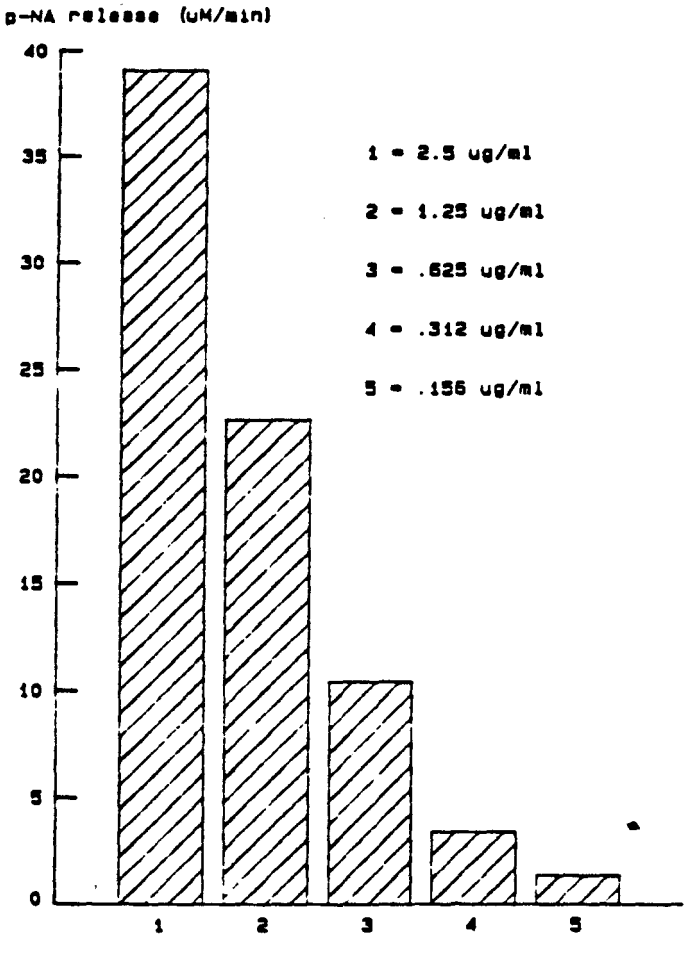

Figure 5. The factor VII-thromboplastin assay was performed as described in methods using five different concentrations of factor VII. The concentration of all other reactants was kept constant. The rate of p-NA release was directly related to the concentration of factor VII.

Figure 6
Effect of the Test Fractions on Factor VII - Thromboplastin Activated Factor Xa in an Amidolytic Assay

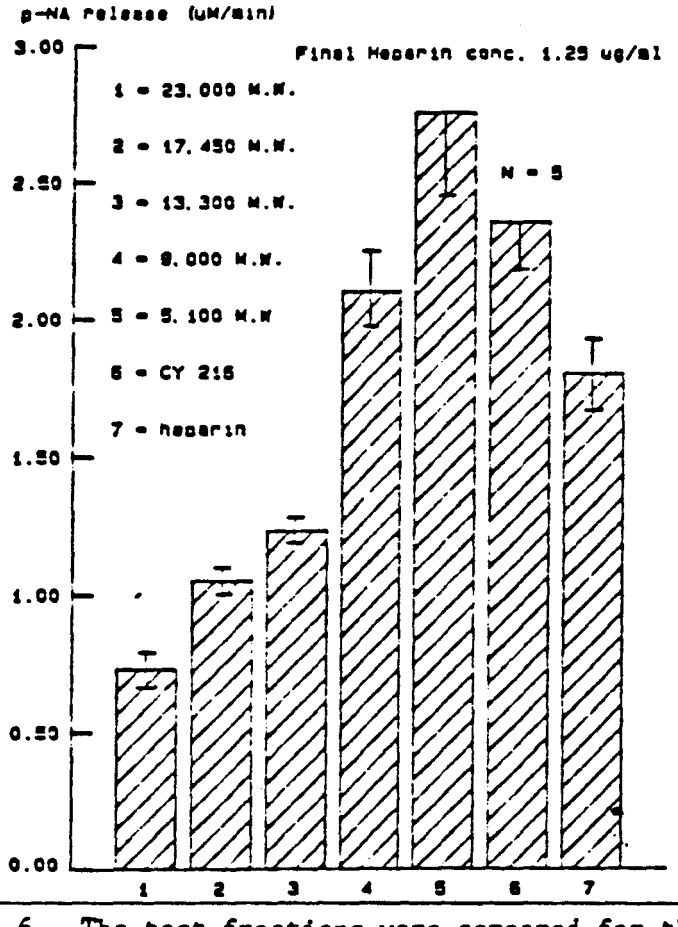

Figure 6. The test fractions were compared for their effect in the factor VII-Thromboplastin assay. The assay used factor VII-Thromboplastin to activate factor X to Xa. The assay endpoint was measured by recording the kinetics of p-NA release from a Xa specific substrate using a kinetic analyzer (IL Multistat). The effects of the M.W. fractions were studied using 25 ug/ml solutions of the indivual test fractions diluted 1:2 in 1.25 U/ml antithrombin III and comparing the resulting p-NA release.

Figure 7
Effect of the Test Fractions on Factor Xa as Studied in an Amidolytic Assay

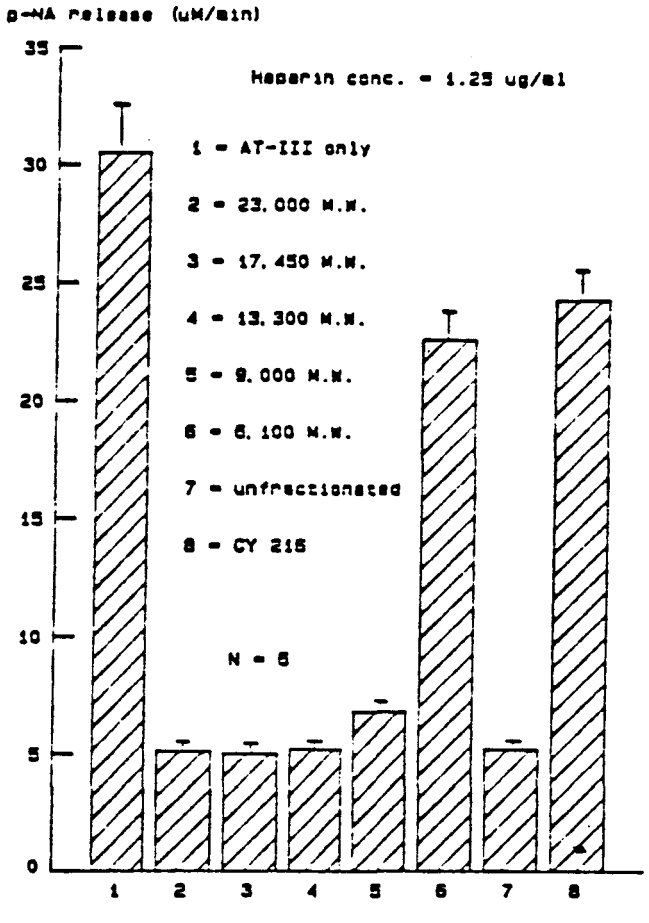

Figure 7. The factor VII-Thromboplastin assay was modified to study the effects of the M.W. fractions on factor Xa in this assay. The assay was performed by replacing factor's VII and X with an equivalent amount of Xa. Thromboplastin remained in the reaction mixture. 25 ug/ml concentrations of the test fractions were diluted 1:2 in 1.25 U/ml antithrombin III and compared for their effect on p-NA release from a Xa specific substrate. With the exception of the two low molecular weight fractions, no difference in potency was observed.

Figure 8
Differential Effect of the Test Fractions When Incubated With Factor VII - Thromboplastin or Factor X in the VII - Thromboplastin Activated Amidolytic Assay

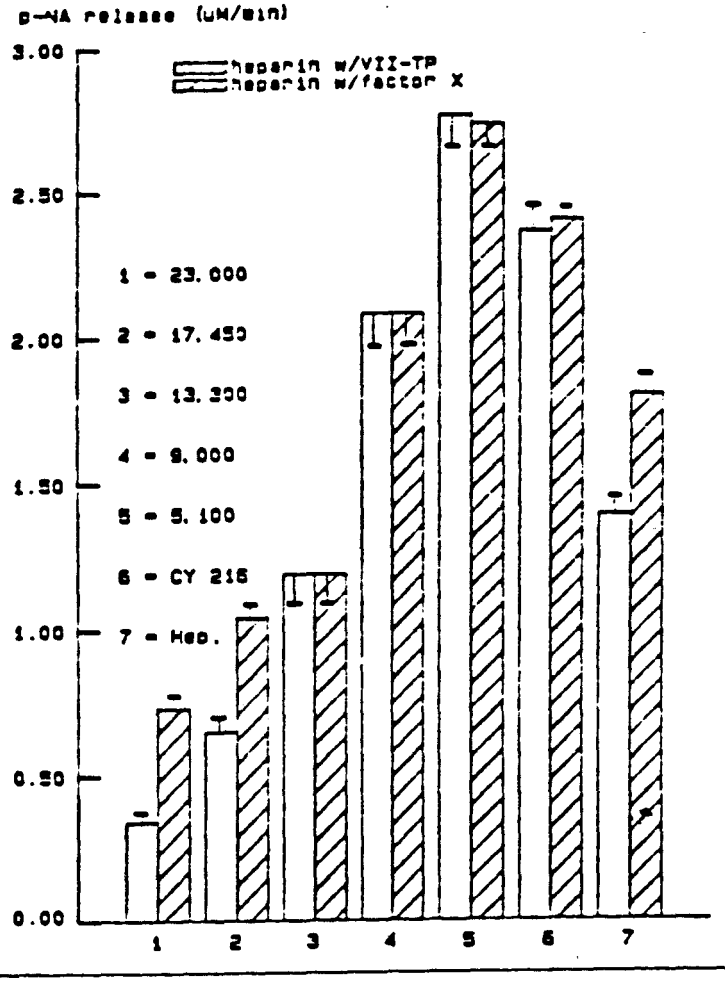

Figure 8. The factor VII-Thromboplastin assay was modified to distinguish the effects of the M.W. fractions on factor VII. 25 ug/ml concentrations of the test fractions were diluted 1:2 in 1.25 U/ml antithrombin III and incubated either with factor VII-thromboplastin or factor X. Differences in p-NA release were compared from the two incubation systems (N = 5). Significant differences between the two systems were only observed for the 23,000 and 17,450 M.W. fractions.

Figure 9
Effect of the Test Fractions on a Thromboplastin Activated Amidolytic Assay in Plasma

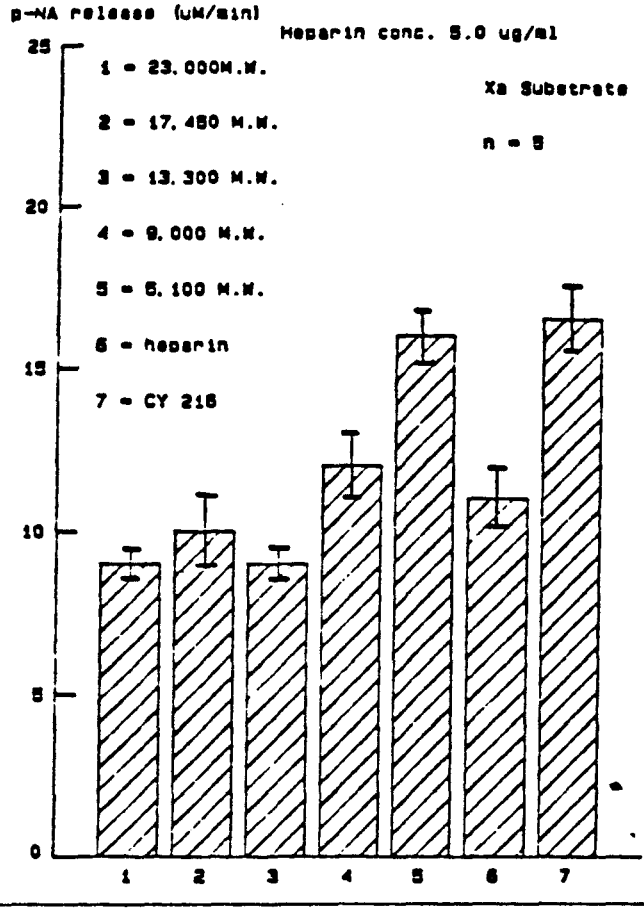

Figure 9. The test fractions were compared for their effects in the plasma VII-Thromboplastin assay. In this assay, factor's VII, X and antithrombin III were provided by the plasma. The assay used dilute thromboplastin (1:20) to activate dilute (1:2), factor II deficient plasma supplemented with 5 ug/ml of each test fraction. p-NA release from a Xa specific substrate was compared to determine the effects of the test fractions. The 23,000 ; 17,450 and 13,300 M.W. fractions displayed the most inhibition in this assay.

Figure 10
Effect of the Test Fractions on a Thromboplastin Activated Amidolytic Assay in Plasma

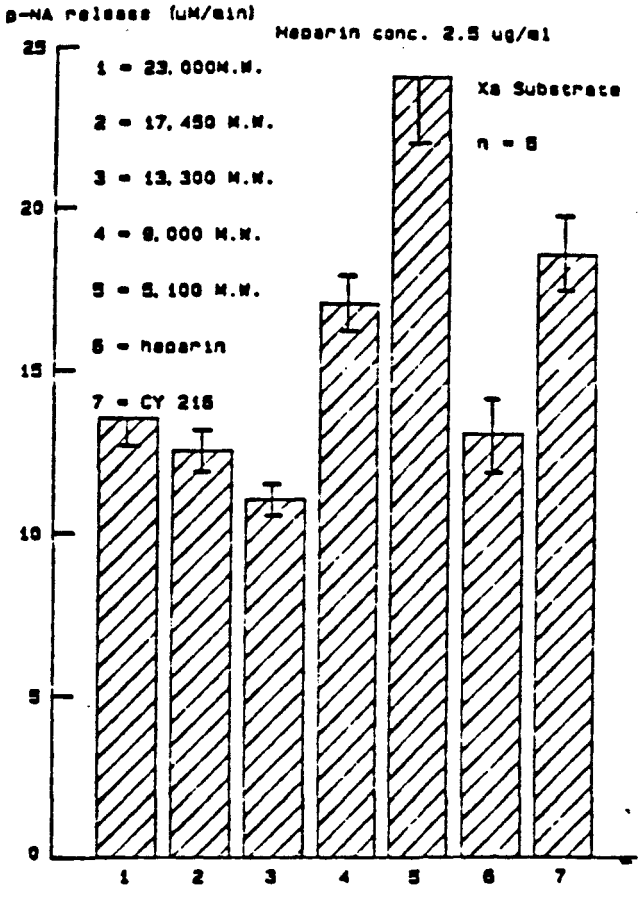

Figure 10. The test fractions were compared for their effects in the plasma VII-Thromboplastin assay. In this assay, factor's VII, X and antithrombin III were provided by the plasma. The assay used dilute thromboplastin (1:20) to activate dilute (1:2), factor II deficient plasma supplemented with 2.5 ug/ml of each test fraction. p-NA release from a Xa specific substrate was compared to determine the effects of the test fractions. In contrast to the results observed at 5 ug/ml concentrations, the 13,300 M.W. fraction displayed the greatest potency.

Figure 11
Effect of the Test Fractions on a Thromboplastin Activated Amidolytic Assay in Plasma

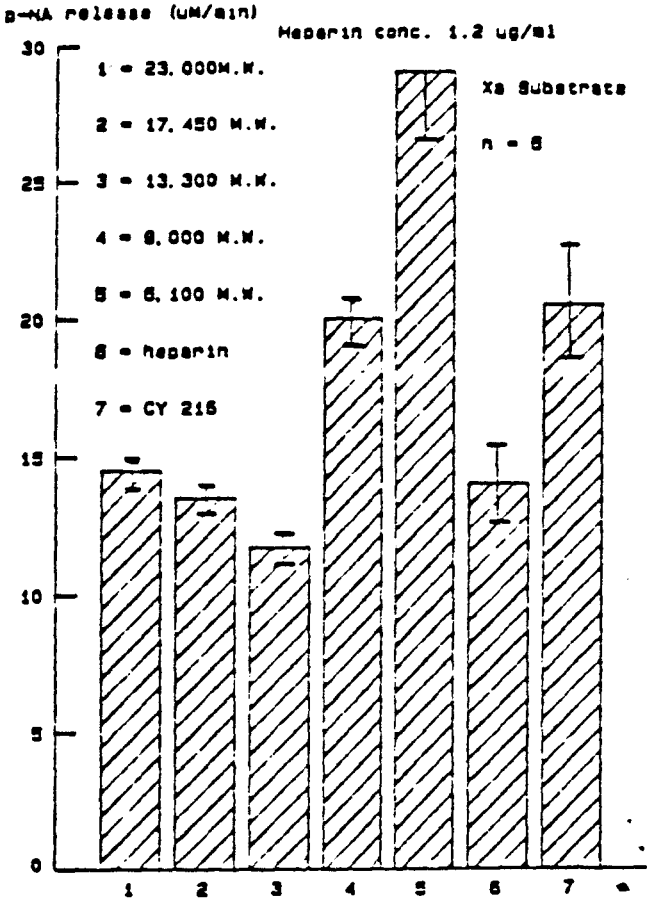

Figure 11. The test fractions were compared for their effects in the plasma VII-Thromboplastin assay. In this assay, factor's VII, X and antithrombin III were provided by the plasma. The assay used dilute thromboplastin (1:20) to activate dilute (1:2), factor II deficient plasma supplemented with 1.25 ug/ml of each test fraction. p-NA release from a Xa specific substrate was compared to determine the effects of the test fractions. Less inhibition was observed compared to the 2.5 and 5 ug/ml concentrations.

Figure 12
Effect of the Test Fractions on a Thromboplastin Activated Amidolytic Assay in Plasma

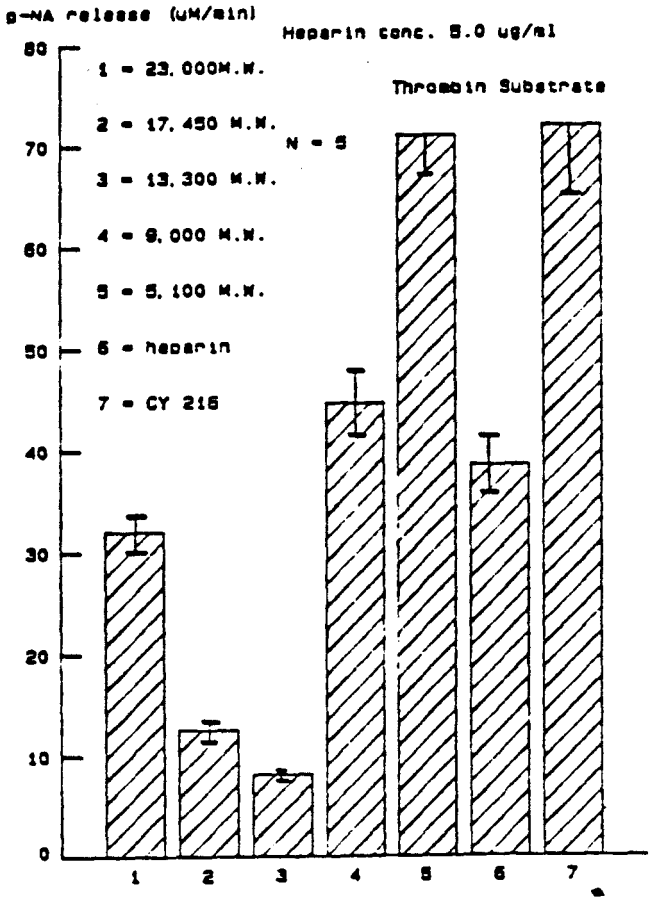

Figure 12. The test fractions were compared for their effects in the plasma VII-Thromboplastin assay. In this assay, factor's VII, X and antithrombin III were provided by the plasma. The assay used dilute thromboplastin (1:20) to activate dilute (1:2), factor II deficient plasma supplemented with 5 ug/ml of each test fraction. p-NA release from a thrombin specific substrate was compared to determine the effects of the test fractions. Similar with the results observed with the Xa substrate, the 13,300 M.W. fraction displayed the greatest potency.

Figure 13
Effect of the Test Fractions on a Thromboplastin Activated Amidolytic Assay in Plasma

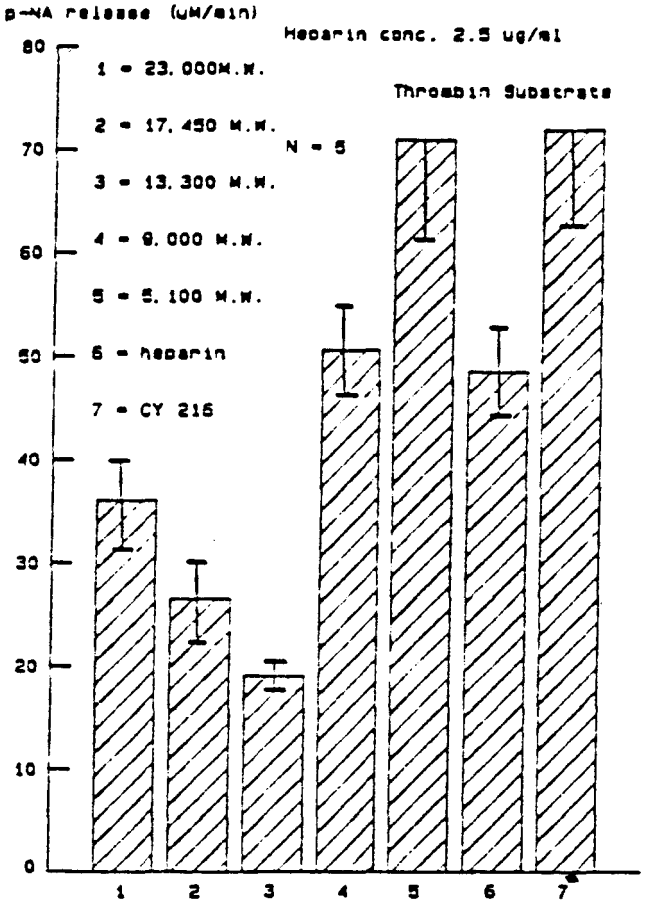

Figure 13. The test fractions were compared for their effects in the plasma VII-Thromboplastin assay. In this assay, factor's VII, X and antithrombin III were provided by the plasma. The assay used dilute thromboplastin (1:20) to activate dilute (1:2), factor II deficient plasma supplemented with 2.5 ug/ml of each test fraction. p-NA release from a thrombin specific substrate was compared to determine the effects of the test fractions.

Figure 14
Effect of the Test Fractions on a Thromboplastin Activated Amidolytic Assay in Plasma

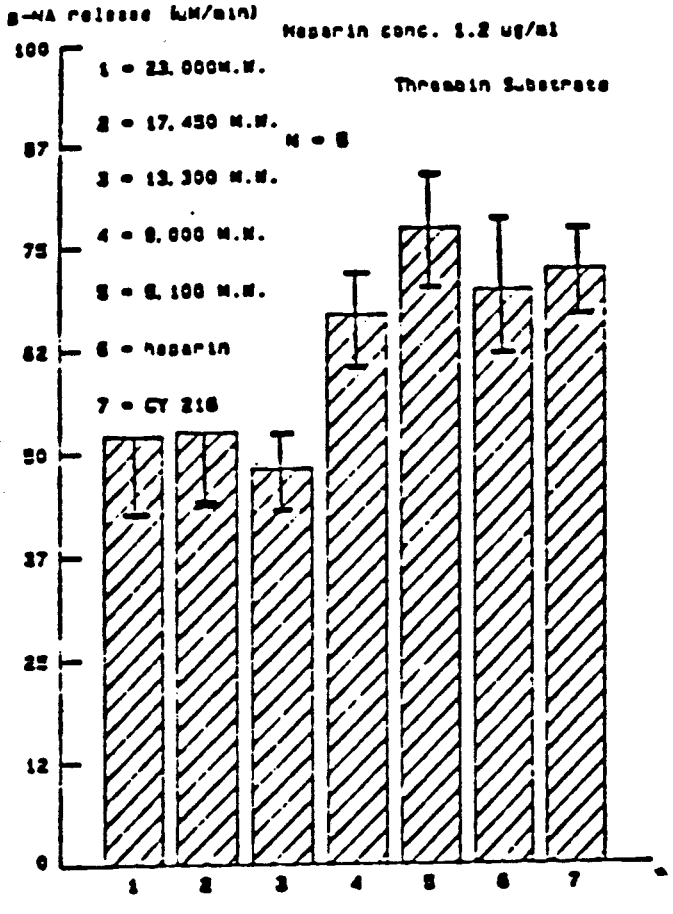

Figure 14. The test fractions were compared for their effects in the plasma VII-Thromboplastin assay. In this assay, factor's VII, X and antithrombin III were provided by the plasma. The assay used dilute thromboplastin (1:20) to activate dilute (1:2), factor II deficient plasma supplemented with 1.25 ug/ml of each test fraction. p-NA release from a thrombin specific substrate was compared to determine the effects of the test fractions. At this concentration, minimal effects were observed for all test fractions.

Figure 15
Effect of Molecular Weight
on Plasma Concentration Time Course

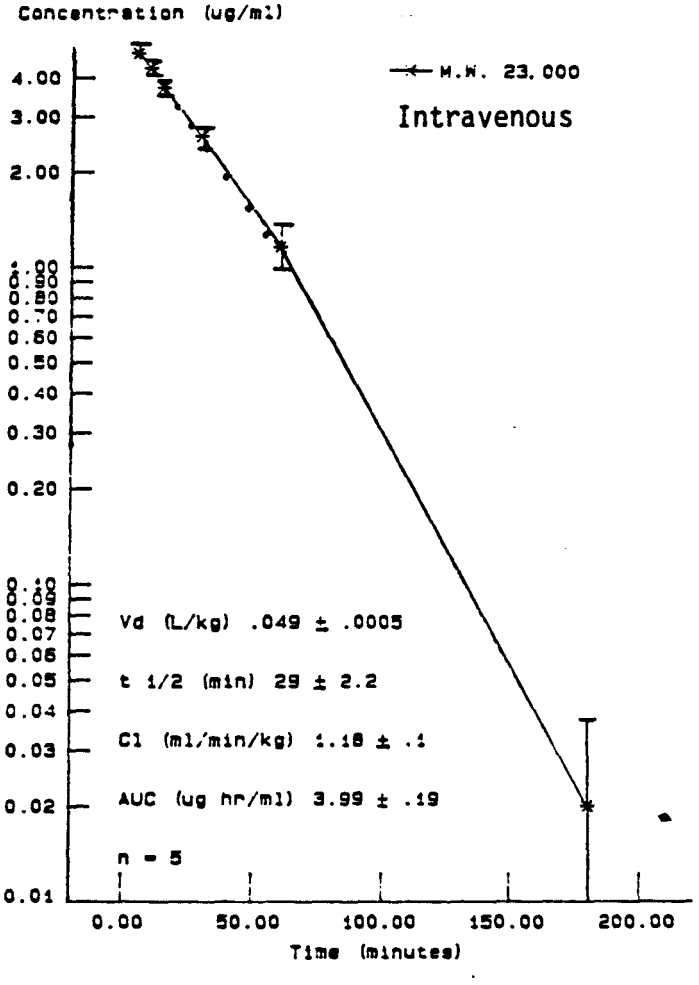

Figure 15. Five primates were administered a 250 ug/Kg dose of the 23,000 M.W. fraction. Blood samples were taken at 5, 10, 15, 30, 60, 120 and 180 minutes post injection and plasma concentrations were determined using the Heptest heparin assay. Semi-log plots were generated using the mean plasma concentrations of the five primates at each time interval. The error bars represent the standard deviation.

Figure 16
Effect of Molecular Weight
on Plasma Concentration Time Course

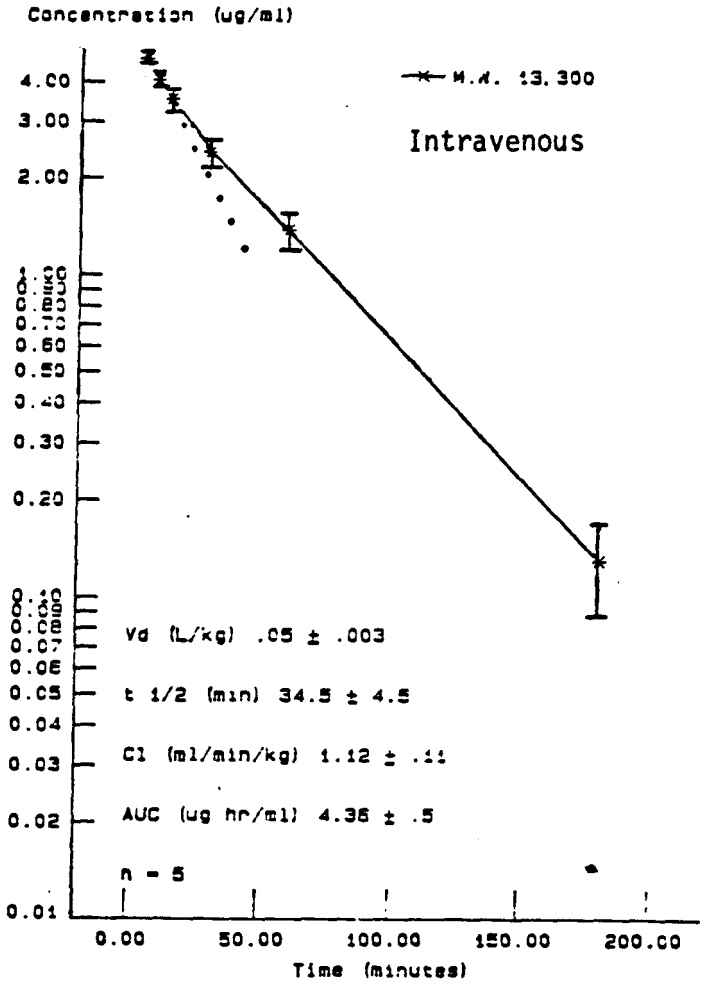

Figure 16. Five primates were administered a 250 ug/Kg dose of the 13,300 M.W. fraction. Blood samples were taken at 5, 10, 15, 30, 60, 120 and 180 minutes post injection and plasma concentrations were determined using the Heptest heparin assay. Semi-log plots were generated using the mean plasma concentrations of the five primates at each time interval. The error bars represent the standard deviation.

Figure 17
Effect of Molecular Weight
on Plasma Concentration Time Course

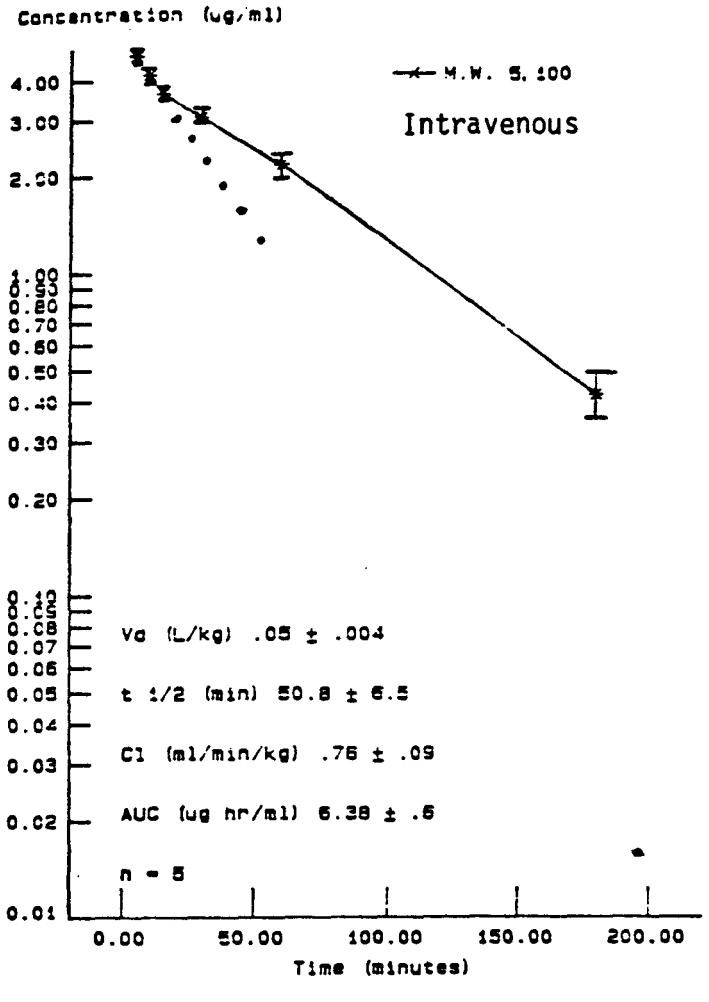

Figure 17. Five primates were administered a 250 ug/Kg dose of the 5,100 M.W. fraction. Blood samples were taken at 5, 10, 15, 30, 60, 120 and 180 minutes post injection and plasma concentrations were determined using the Heptest heparin assay. Semi-log plots were generated using the mean plasma concentrations of the five primates at each time interval. The error bars represent the standard deviation.

Figure 18
Effect of Molecular Weight
on Plasma Concentration Time Course

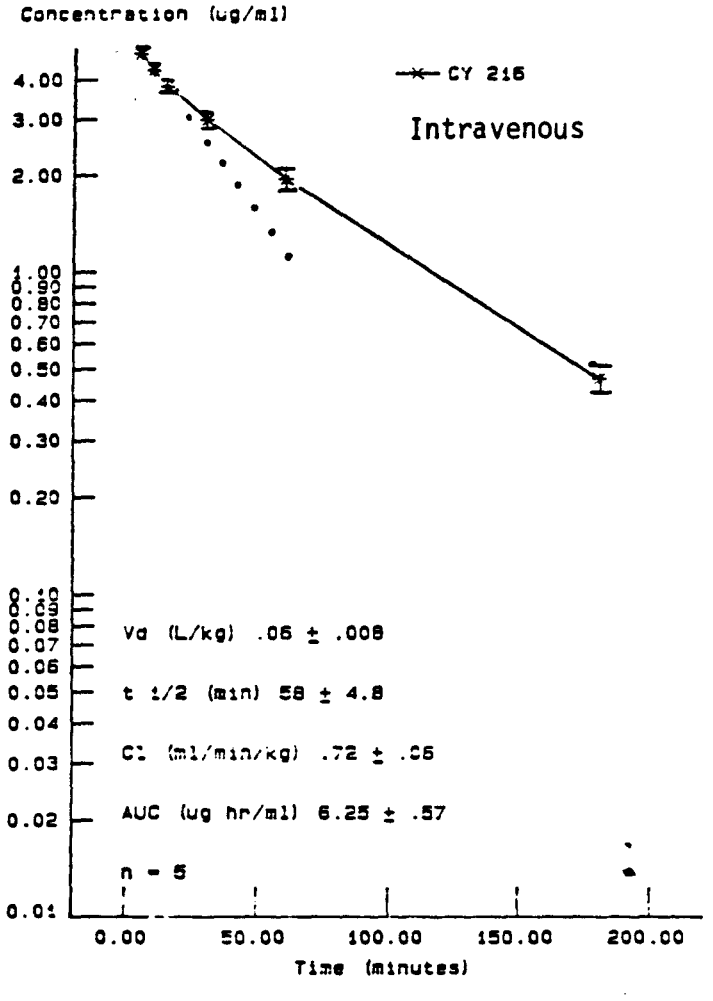

Figure 18. Five primates were administered a 250 ug/Kg dose of CY 216. Blood samples were taken at 5, 10, 15, 30, 60, 120 and 180 minutes post injection and plasma concentrations were determined using the Heptest heparin assay. Semi-log plots were generated using the mean plasma concentrations of the five primates at each time interval. The error bars represent the standard deviation.

Figure 19
Effect of Molecular Weight
on Plasma Concentration Time Course

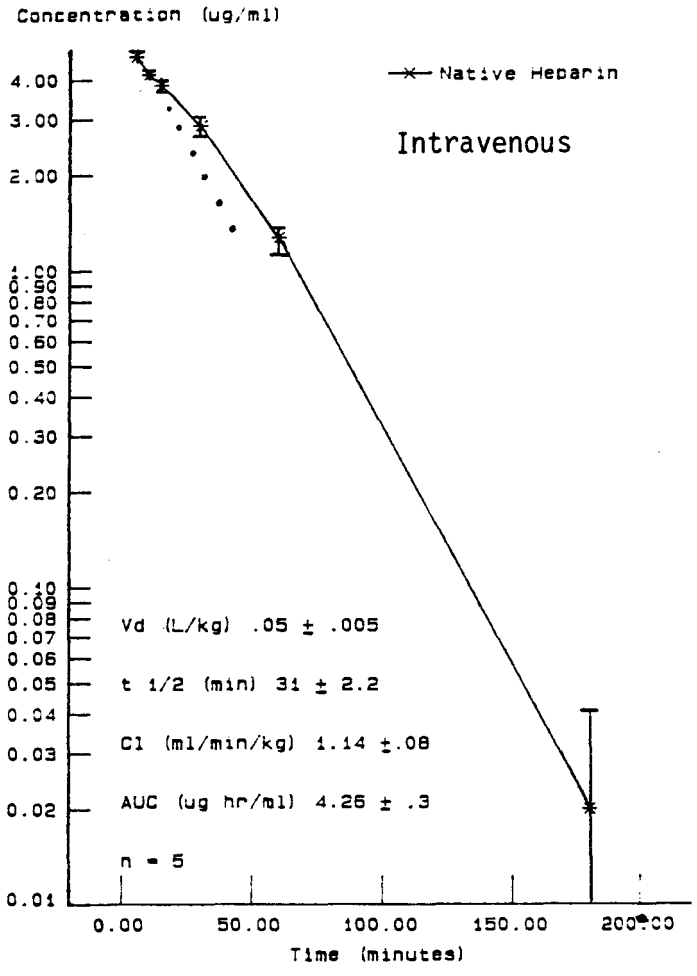

Figure 19. Five primates were administered a 250 ug/Kg dose of the unfractionated heparin. Blood samples were taken at 5, 10, 15, 30, 60, 120 and 180 minutes post injection and plasma concentrations were determined using the Heptest heparin assay. Semi-log plots were generated using the mean plasma concentrations of the five primates at each time interval. The error bars represent the standard deviation.

Figure 20
Effect of Molecular Weight
on Plasma Concentration Time Course

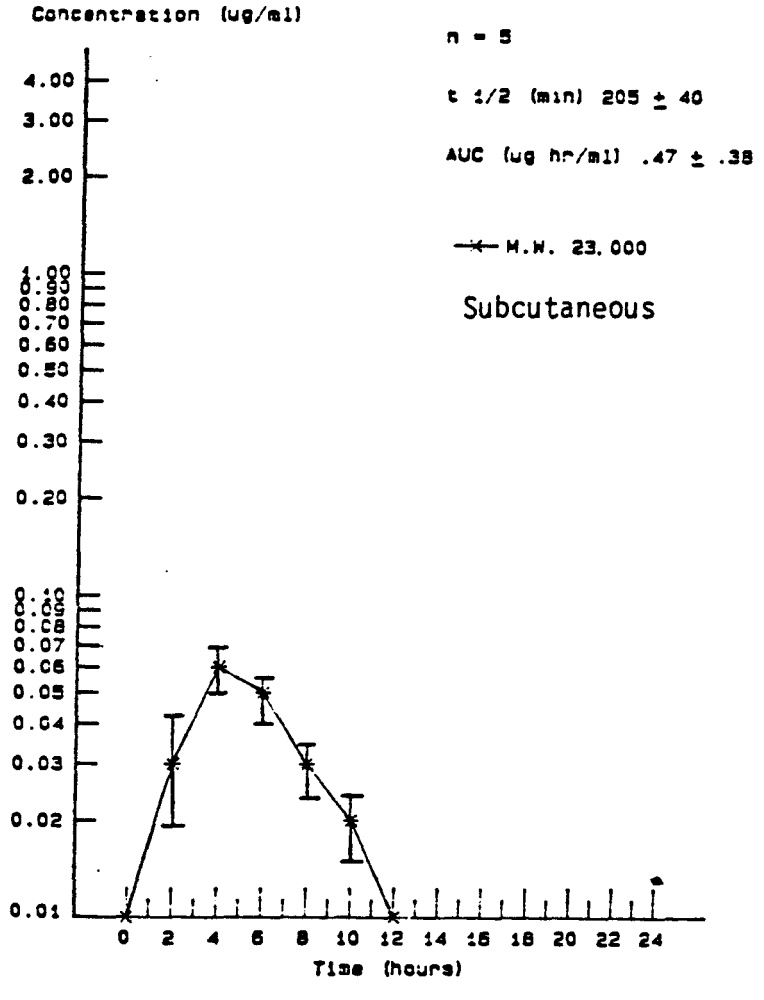

Figure 20. Five primates were administered a 1.0 mg/Kg dose of the 23,000 M.W. fraction. Blood samples were taken at 0, 2, 4, 6, 8, 10 and 12 hours post injection and plasma concentrations were determined using the Heptest heparin assay. Semi-log plots were generated using the mean plasma concentrations of the five primates at each time interval. The error bars represent the standard deviation.

Figure 21
Effect of Molecular Weight
on Plasma Concentration Time Course

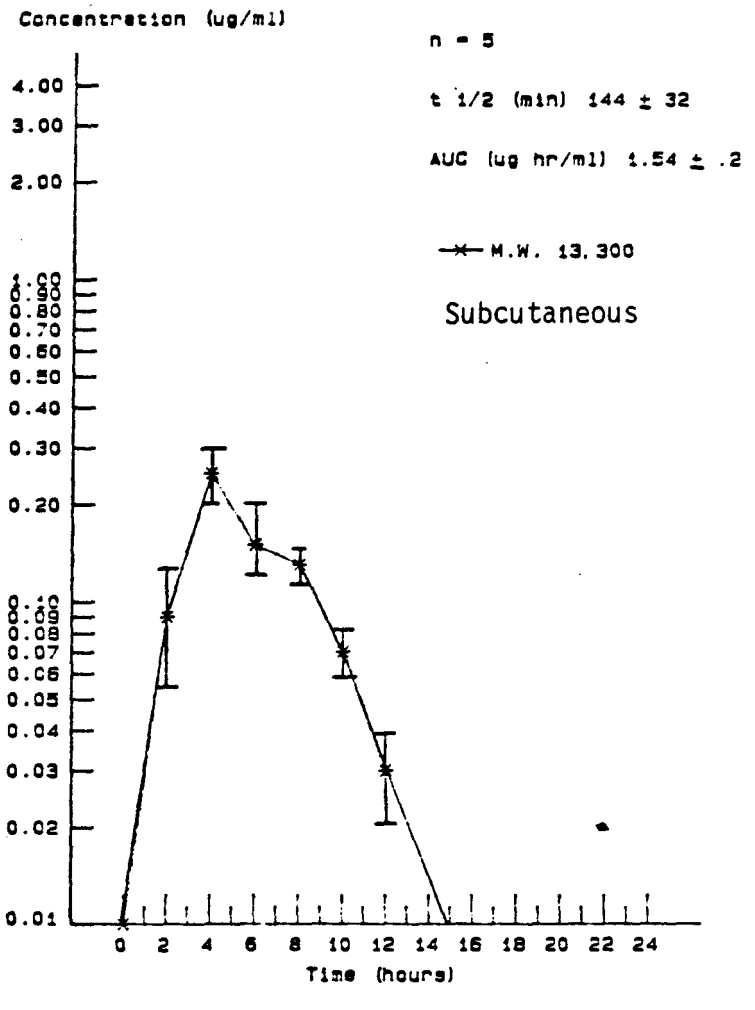

Figure 21. Five primates were administered a 1.0 mg/Kg dose of the 13,300 M.W. fraction. Blood samples were taken at 0, 2, 4, 6, 8, 10 and 12 hours post injection and plasma concentrations were determined using the Heptest heparin assay. Semi-log plots were generated using the mean plasma concentrations of the five primates at each time interval. The error bars represent the standard deviation.

Figure 22
Effect of Molecular Weight
on Plasma Concentration Time Course

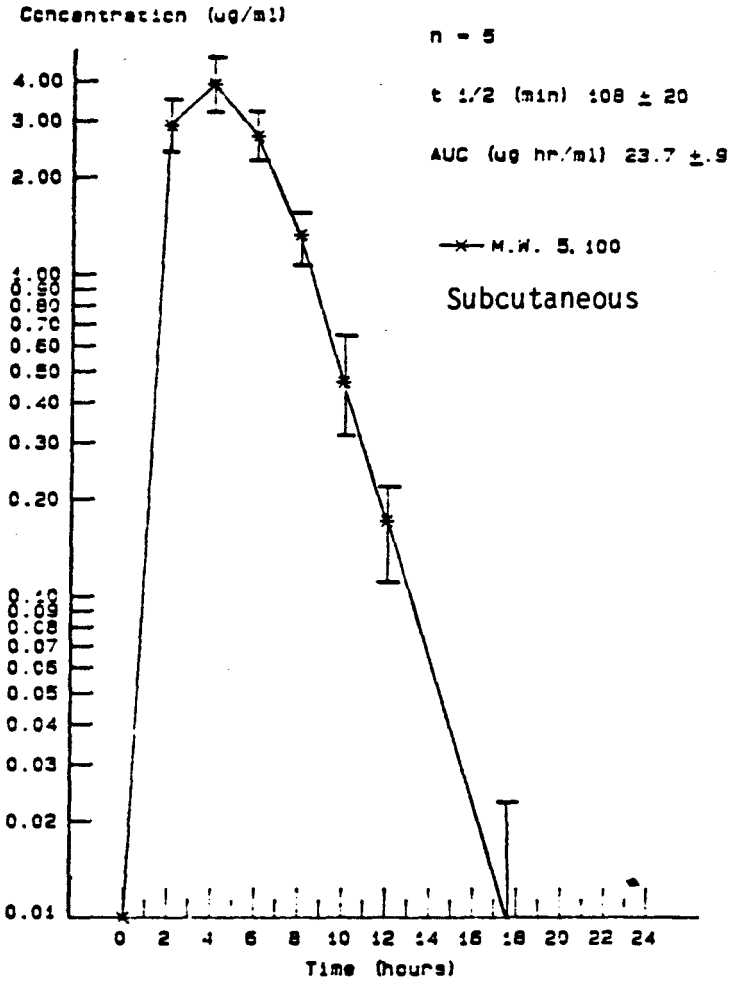

Figure 22. Five primates were administered a 1.0 mg/Kg dose of the 5,100 M.W. fraction. Blood samples were taken at 0, 2, 4, 6, 8, 10 and 12 hours post injection and plasma concentrations were determined using the Heptest heparin assay. Semi-log plots were generated using the mean plasma concentrations of the five primates at each time interval. The error bars represent the standard deviation.

Figure 23
Effect of Molecular Weight
on Plasma Concentration Time Course

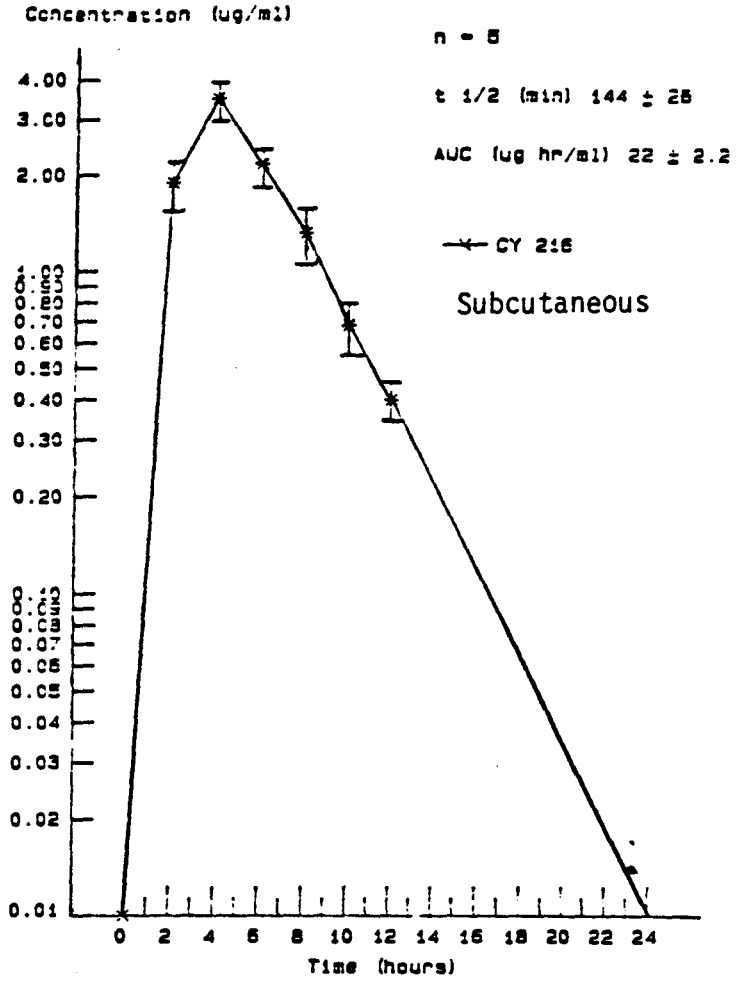

Figure 23. Five primates were administered a 1.0 mg/Kg dose of CY 216. Blood samples were taken at 0, 2, 4, 6, 8, 10 and 12 hours post injection and plasma concentrations were determined using the Heptest heparin assay. Semi-log plots were generated using the mean plasma concentrations of the five primates at each time interval. The error bars represent the standard deviation.

Figure 24
Effect of Molecular Weight
on Plasma Concentration Time Course

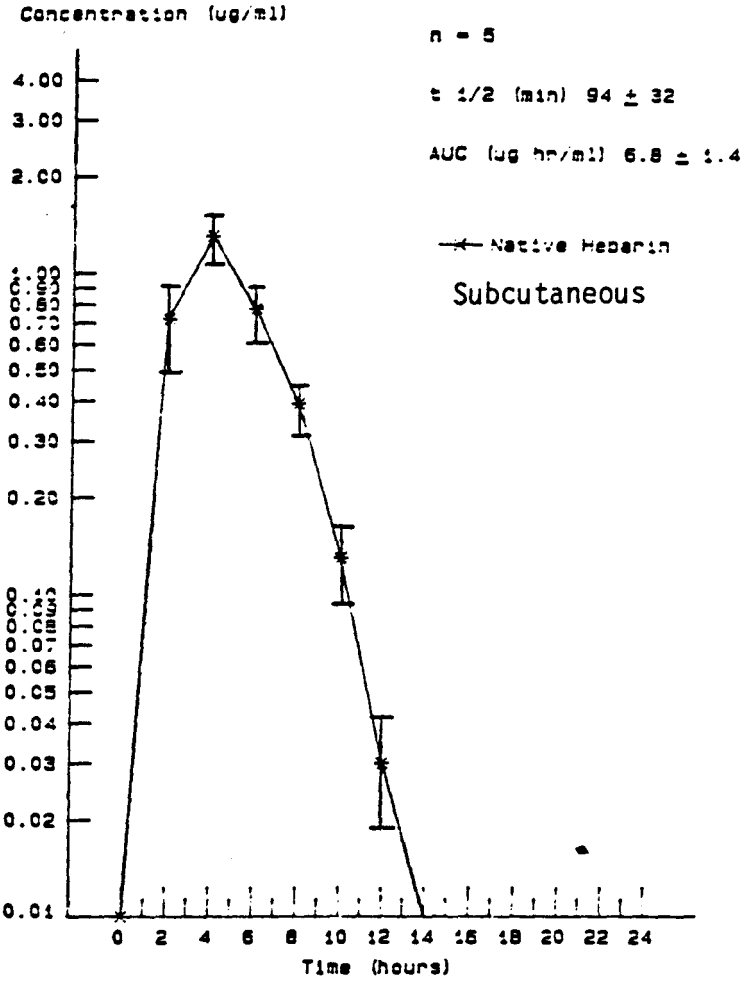

Figure 24. Five primates were administered a 1.0 mg/Kg dose of the unfractionated heparin. Blood samples were taken at 0, 2, 4, 6, 8, 10 and 12 hours post injection and plasma concentrations were determined using the Heptest heparin assay. Semi-log plots were generated using the mean plasma concentrations of the five primates at each time interval. The error bars represent the standard deviation.

Figure 25
Effect of Molecular Weight on PCC/RVV
Activated Stasis Thrombosis

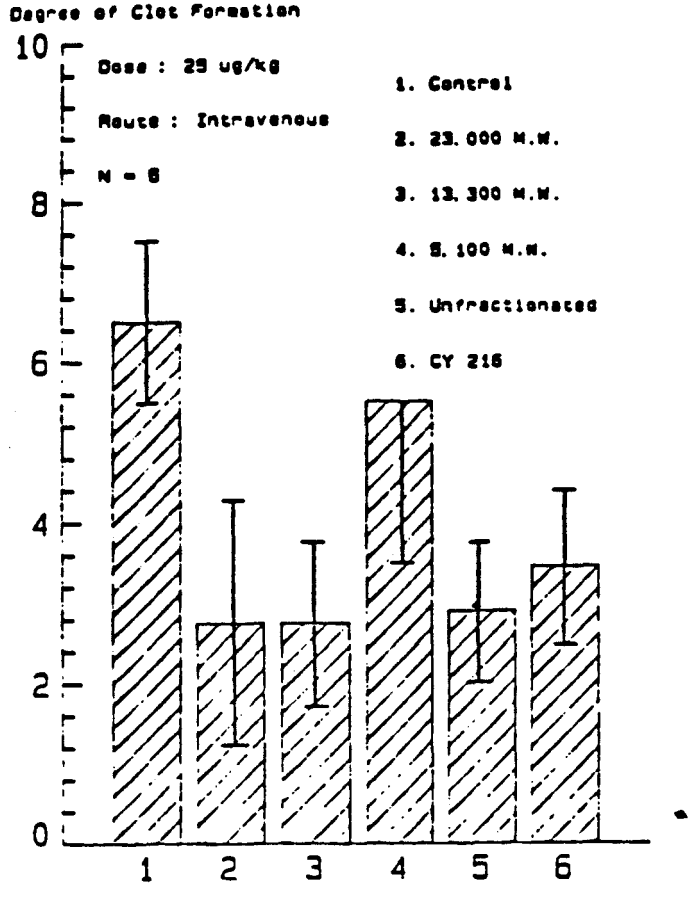

Figure 25. Male rabbits were anesthetized and segments of both jugular veins surgically exposed. Each test fraction was administered to five rabbits and allowed to circulate for five minutes. A thrombogenic challenge of prothrombin complex concentrate and Russells viper venom was given. After 20 seconds both jugular veins were ligated for exactly 10 minutes and the jugular vein segments removed and evaluated for degree of clot formation. With the exception of the 5,100 M.W. fraction, all agents produced significant inhibition of clot formation compared to control ($p = .001$).

Figure 26
Effect of Molecular Weight on PCC/RVV
Activated Stasis Thrombosis

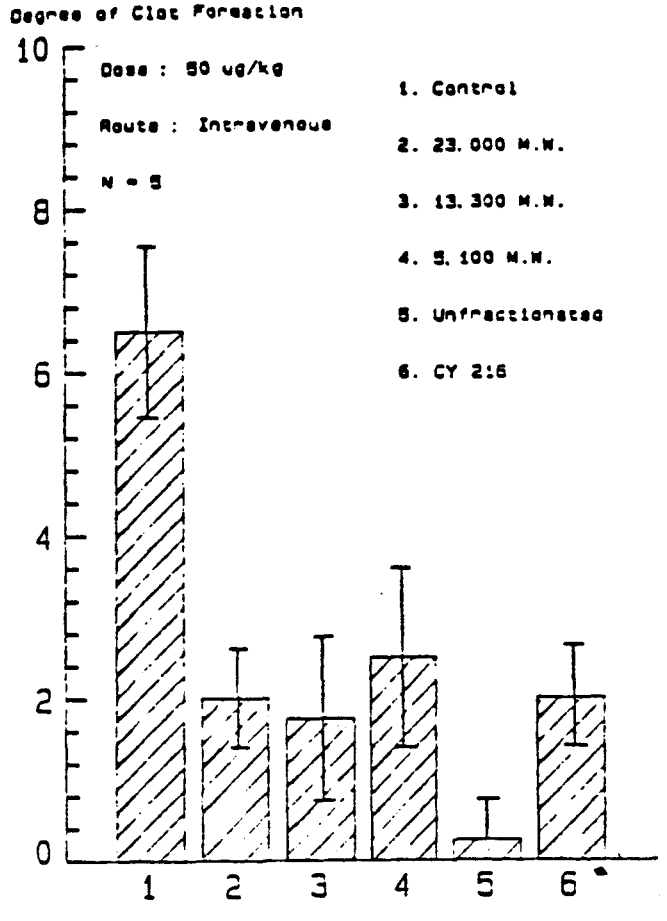

Figure 26. Male rabbits were anesthetized and segments of both jugular veins surgically exposed. Each test fraction was administered to five rabbits and allowed to circulate for five minutes. A thrombogenic challenge of prothrombin complex concentrate and Russells viper venom was given. After 20 seconds both jugular veins were ligated for exactly 10 minutes and the jugular vein segments removed and evaluated for degree of clot formation. All fractions produced significant antithrombotic actions compared to control.

Figure 27
Effect of Molecular Weight on PCC/RVV
Activated Stasis Thrombosis

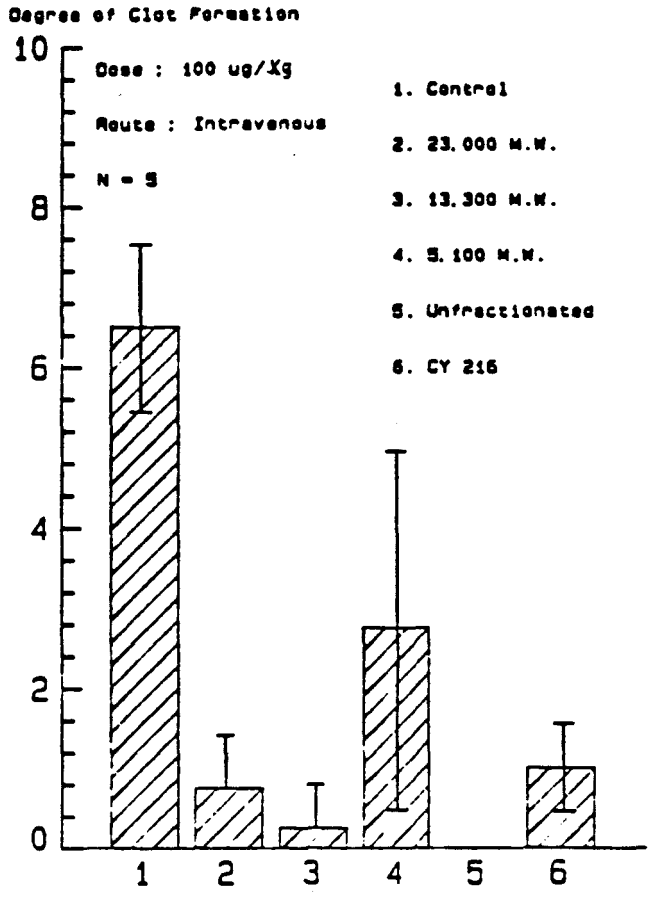

Figure 27. Male rabbits were anesthetized and segments of both jugular veins surgically exposed. Each test fraction was administered to five rabbits and allowed to circulate for five minutes. A thrombogenic challenge of prothrombin complex concentrate and Russells viper venom was given. After 20 seconds both jugular veins were ligated for exactly 10 minutes and the jugular vein segments removed and evaluated for degree of clot formation. All fractions produced significant antithrombotic actions compared to control. The unfractionated heparin was the only agent to produce complete inhibition in all five animals.

Figure 28

Effect of Molecular Weight on PCC/RVV Activated Stasis Thrombosis

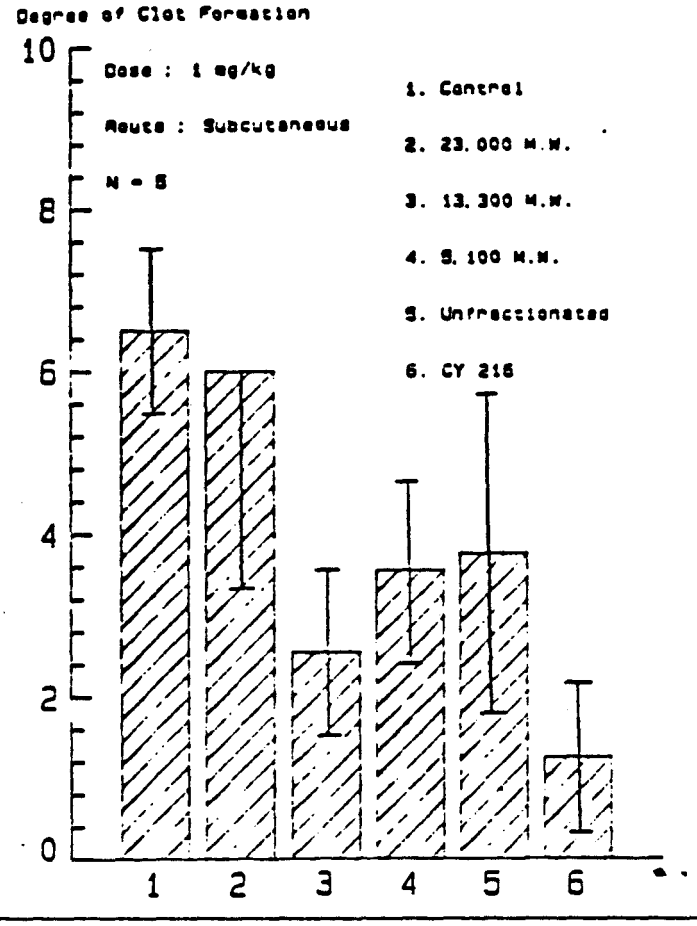

Figure 28. Male rabbits (N = 5) were given subcutaneous injections of the test fractions at a dose of 1.0 mg/Kg. After four hours the rabbits were anesthetized and segments of both jugular veins surgically exposed. A thrombogenic challenge of prothrombin complex concentrate and Russells viper venom was given. After 20 seconds both jugular veins were ligated for exactly 10 minutes and the jugular vein segments removed and evaluated for degree of clot formation. With the exception of the 23,000 M.W. fraction, all agents produced significant antithrombotic effects compared to control. Error bars represent standard deviation

CHAPTER IX

BIBLIOGRAPHY

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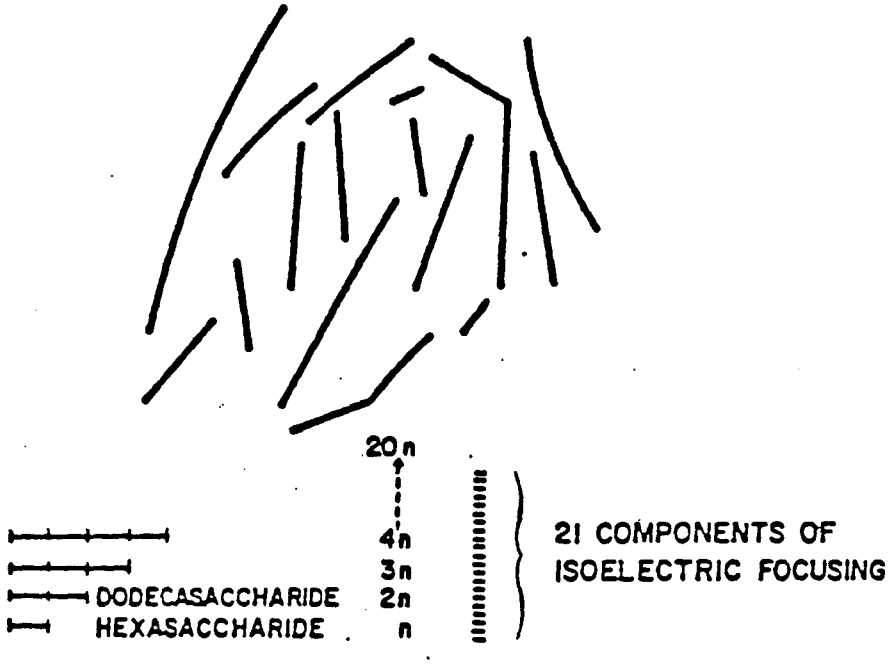
APPENDIX I

APPENDIX I

Conceptual Representation of the Molecular Diversity in Heparin

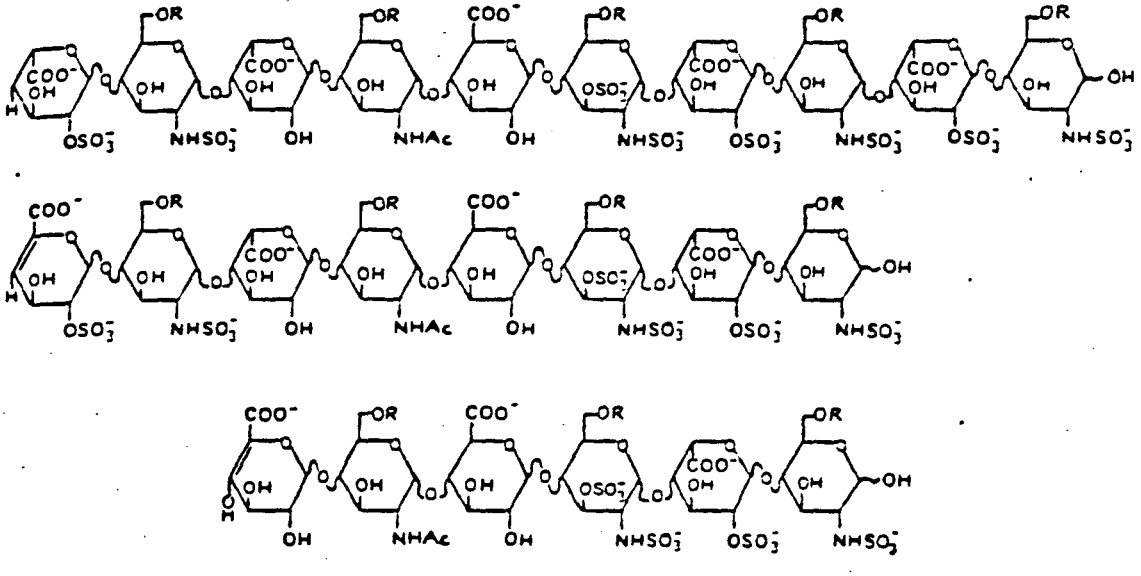


Dietrich et al., 1975

APPENDIX II

APPENDIX II

The Chemical Structure of Heparin



R = H or SO_3^-

(Adapted from Choay et al, Fareed, 1985)

APPENDIX III

APPENDIX III
HUMAN COAGULATION PROTEINS

Clotting Factors

Name	Molecular Weight	Plasma Conc.	Active Form
Factor XII	80,000	30 ug/ml	serine protease
Prekallikrein	80,000	50 ug/ml	serine protease
High Molecular Weight Kininogen	120,000	70 ug/ml	cofactor
Factor XI	160,000	4 ug/ml	serine protease
Factor IX	57,000	4 ug/ml	serine protease
Factor VII/ von Willebrand factor	2,000,000	7 ug/ml	cofactor
Factor VII	47,000	1 ug/ml	serine protease
Tissue Factor	45,000	0	cofactor
Factor X	59,000	5 ug/ml	serine protease
Factor V	330,000	5 - 10 ug/ml	cofactor
Factor II	70,000	100 ug/ml	serine protease
Fibrinogen	340,000	250 mg/dl	clot structure
Factor XIII	300,000	10 ug/ml	transaminase

Regulatory Factors

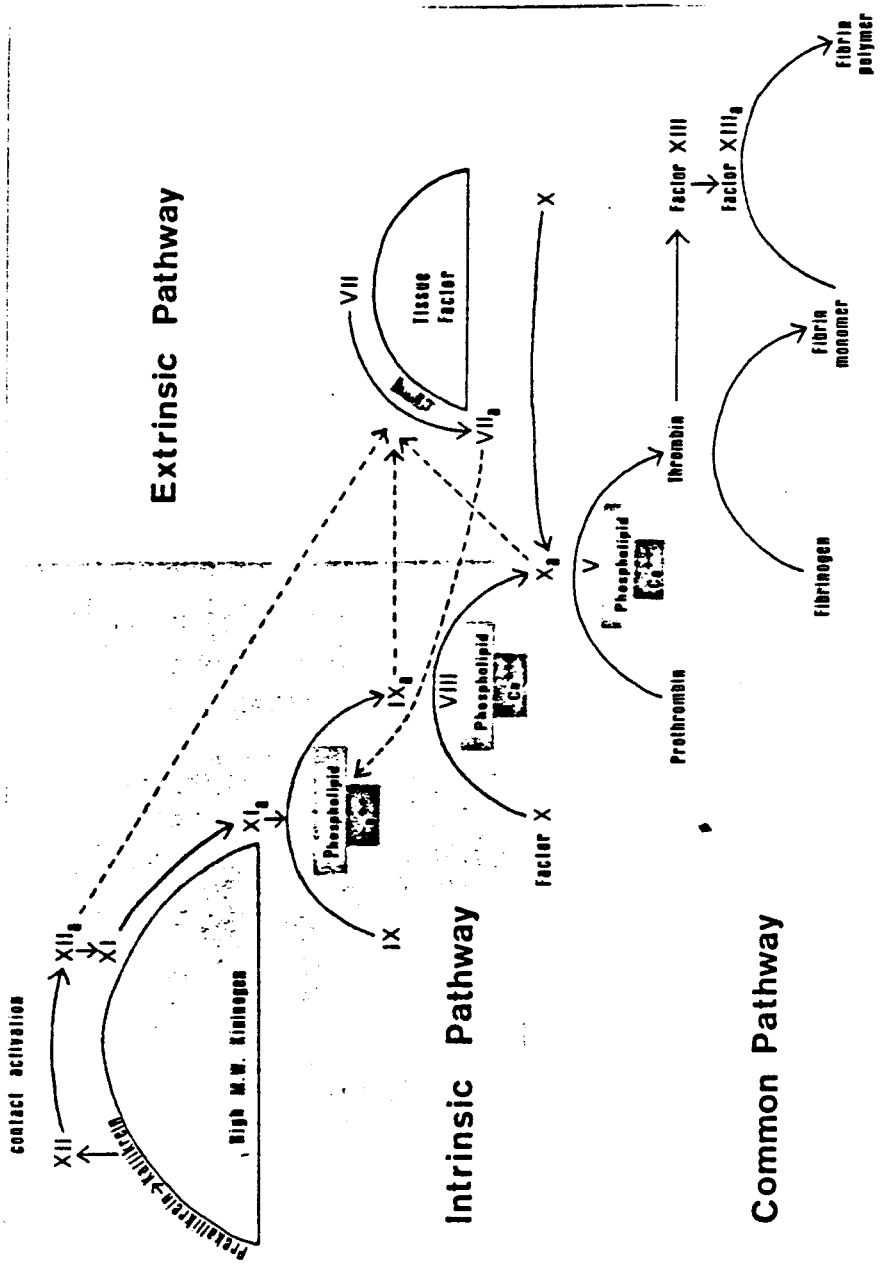
Antithrombin III	64,000	29 mg/dl	Inhibitor
Protein C	62,000	5 ug/ml	Serine protease
Protein S	65,000	?	Regulates Protien C

Thompson and Harker, 1983

APPENDIX IV

APPENDIX IV

Conceptual Representation of the Coagulation Network



APPENDIX V

Analysis Of The Unfractionated Heparin And A Low Molecular Weight Heparin (CY 216)

	Unfractionated Heparin	Low Molecular Weight Heparin
Lot number	H-410	XH 46
Description	Powder	Powder
Color	White	White
Origin, Intestinal Mucosa ..	Porcine	Porcine
Molecular Weight	12,500	5,400
Chemical Data		
USP Potency, as is ...	160 U/mg	58 U/mg
AXa Potency, as is ...	154 U/mg	96 U/mg
Loss on Drying	3.1 %	2.9 %
Residue on Ignition ..	38.7 %	29 %
Nitrogen Content	2.2 %	2.3 %
Heavy Metals	< 10 ppm	< 10 ppm
pH @ 1 %	6.94	6.86
Protein Content	Negative	Negative
Solubility @ 20,000 U/ml	Clear	-
Absorbance @ 20,000 U/ml at 400 nm	0.015	-
Uronic Acid (%)	24.5	23.9
SO_3^-/COO^- (meq)	2.44 Neg.	2.23 Neg.
Pyrogen DY LAL	Negative	Negative

APPENDIX VI

APPENDIX VI
Method for Prothrombin Time Assay

Principle

The prothrombin time (PT) is a screening test for the extrinsic pathway of coagulation (factors VII, X, V, II, I) and for the monitoring of coumadin therapy. It is based on the measurement of time to clot after plasma has been activated by tissue thromboplastin and $CaCl_2$.

Reagents

1. Thromboplastin-calcium reagent (Dade, Miami, FL). Reconstituted according to manufacturer's instructions.

2. Citrated test plasma (platelet poor)

3. Normal plasma control

Procedure

Incubate 100 ul plasma at 37° C in fibrometer* for 3 minutes.

Add 200 ul of pre-warmed (37° C) thromboplastin-C and immediately record time to clot upon addition of activator.

* The fibrometer is a electro-mechanical device used to detect clot formation. It utilizes a probe arm which drops into a reaction vessel and alternately descends and rises in a sweeping motion to sense the formation of a clot. When the reagents are in a fluid phase, an electric current is transmitted. As clotting begins, the electrical conductance is reduced, the electrode and mechanical timer stop and the clotting time is read from the digital readout.

APPENDIX VII

APPENDIX VII
Method for Activated Partial Thromboplastin Time Assay

Principle

The activated partial thromboplastin time assay is a screening test for the intrinsic pathway of coagulation (factors XII, XI, IX, VIII, X, V, II, I) and for monitoring heparin therapy. It is based on the time for plasma to clot after activation by a platelet substitute (phospholipid) activator and $CaCl_2$.

Reagents

1. APTT reagent (General Diagnostics, Morris Plains, NJ) containing phospholipid and micronized silica is reconstituted as directed by the manufacturer.

2. $CaCl_2$ (0.025 M)

3. Citrated test plasma (platelet poor)

4. Normal plasma control

Procedure

Add 100 ul of APTT reagent to 100 ul of plasma. Incubate at 37° C for 5 minutes in fibrometer well.

Add 100 ul of prewarmed (37° C) .025 M $CaCl_2$ and record time to clot immediately upon adding the $CaCl_2$.

APPENDIX VIII

APPENDIX VIII
Method for Heptest Heparin Assay

Principle

The heptest assay is a clot based test for determining heparin concentrations in plasma. Inhibition of factor's Xa and IIa is accelerated by heparin bound to antithrombin-III. The amount of factor Xa/IIa activity neutralized during a specific time period is directly proportional to the concentration of heparin in the reaction mixture.

Reagents

1. Factor Xa (supplied by manufacturer) reconstituted in 2.0 ml distilled water. The factor Xa is kept at room temperature.

2. Recalmix (supplied by manufacturer) containing phospholipid and fibrinogen is reconstituted in 2.0 ml distilled water. The recalmix must be pre-warmed to 37° C for at least 10 minutes before using.

Procedure

100 ul of plasma is pre-warmed in the fibrometer for 3 minutes.

100 ul of factor Xa is added to the plasma and incubated for exactly 2 minutes.

After the 2 minute incubation, 100 ul of recalmix is added and the time to clot is recorded from this point using the fibrometer.

APPENDIX IX

APPENDIX IX

CONTINUING REVIEW OF APPROVED PROJECTS
(SIX MONTHS)

The IRB is available for continuing exchange of information and advice between itself and the activity director on any matters affecting the rights and welfare of human subjects who participate in the activity. Prior approval must be obtained if there are changes or additions to the originally approved protocol, or if there is a change in the number or kind of subject. The IRB must be consulted if adverse side effects or unanticipated problems arise that may affect any subject.

TITLE OF APPLICATION: "Consent form for Control Blood Specimens."

PRINCIPAL INVESTIGATOR OR RESPONSIBLE PHYSICIAN: Dr. Fareed

DATE OF INITIAL BOARD APPROVAL: 7/78 IRB NUMBER: 5f

This study will be reviewed at the January 16, 1985 IRB meeting

PLEASE CHECK THREE (3) OF THE FOLLOWING:

1. X STUDY STILL IN PROGRESS. This is to certify that the original protocol approved by the Institutional Review Board for Protection of Human Subjects has been fully complied with, and that no changes or additions have been made in the approved protocol. ITEMS #2 and #3 MUST BE CHECKED OFF ALSO.

2. 328 INDICATE NUMBER OF PATIENTS ENROLLED in this Protocol within the last 12 months. (if none - put 0).

3. 0 INDICATE NUMBER OF ADVERSE REACTIONS OR COMPLICATIONS, if any (including drug reactions) in this protocol. (If none - put 0).

4. ___ NO LONGER IN PROGRESS (only place a check mark here if this project or investigation is no longer in progress). ITEMS #2 and #3 MUST BE CHECKED OFF ALSO).

NOTE: ALL ADVERSE REACTIONS MUST BE REPORTED TO THE IRB.

Signature: [signature] Principal Investigator or Responsible Physician

1/7/85 Date

JAN 16 1984 RECEIVED

IRB approved 1/85

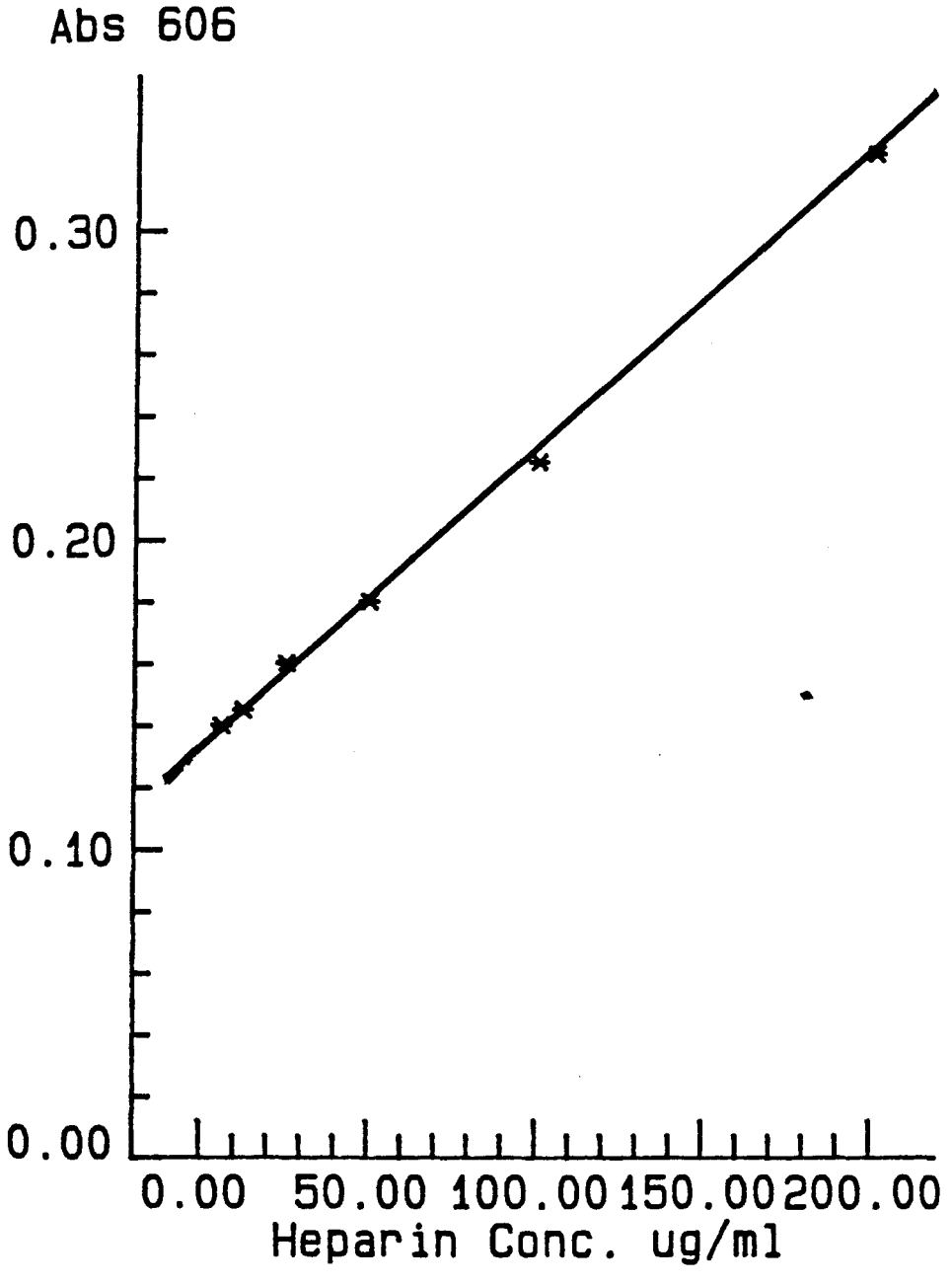
Principle copy

RETURN TO: Administrative Secretary
Institutional Review Board
Room 1067 - LUMC

APPENDIX X

APPENDIX X

Calibration Curve for Toluidine Blue Assay



APPENDIX XI

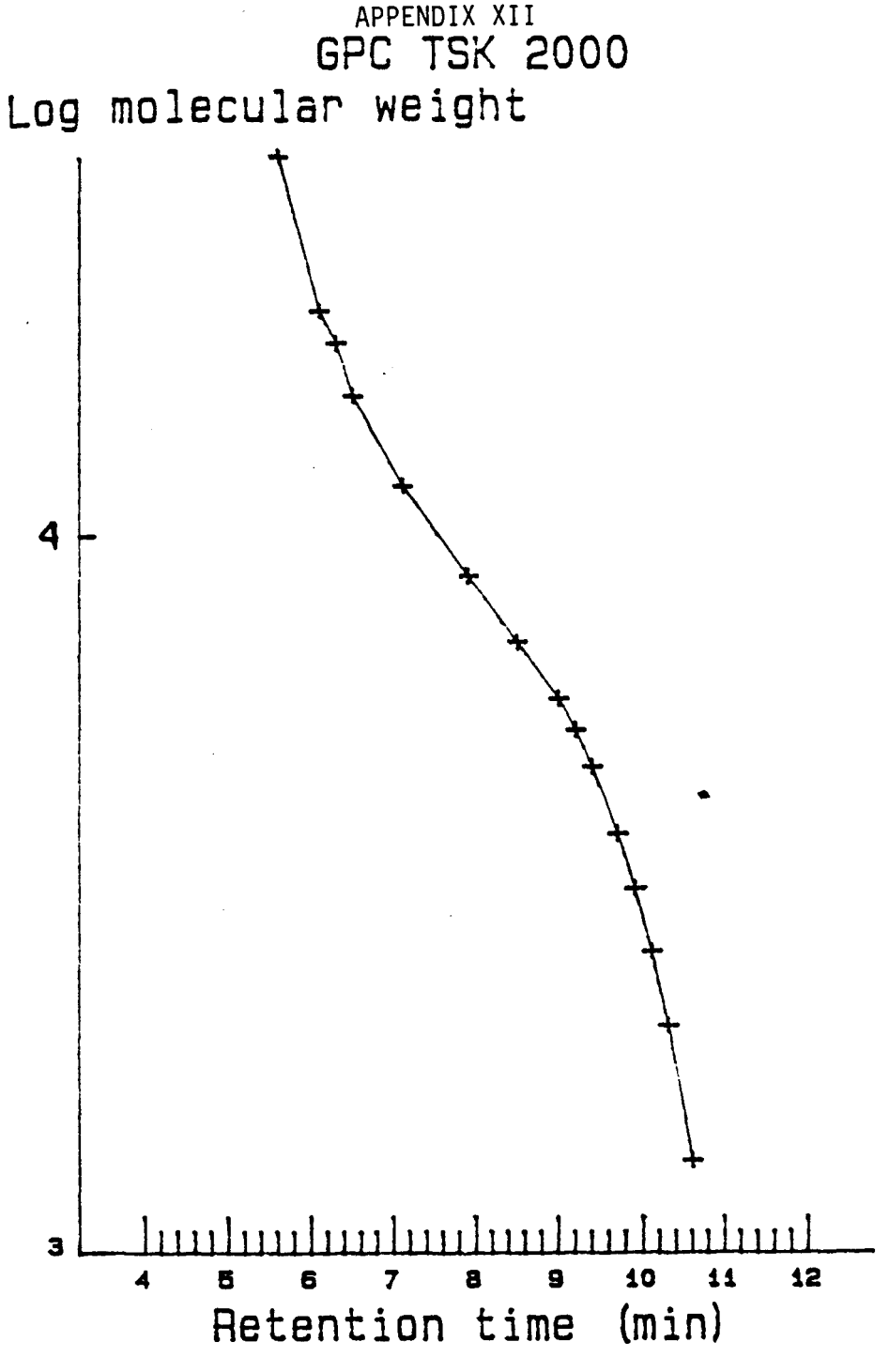
APPENDIX XI

Reference Standards For GAG Molecular Weight Determination

Reference Standard	Molecular Weight
Hyaluronate	230,000
CH-6-S	45,000
CH-2-S	29,000
Choay Stnd.#1 (heparin)	22,900
Barlow Stnd. #2 (heparin)	16,500
CH-4-S	15,000
Barlow Stnd. #3 (heparin)	13.280
Choay Stnd. #3 (heparin)	12,600
Barlow Stnd. #5	7,700
Choay Stnd. #5	7,570
Octodecasaccharide	5,706
Hexadecasaccharide	5,072
Tetradecasaccharide	4,438
Dodecasaccharide	3,804
Decasaccharide	3,170
Octosaccharide	2,536
Hexasaccharide	1,902
Pentasaccharide	1,585
Tetrasaccharide	1,268
Disaccharide	634

APPENDIX XII

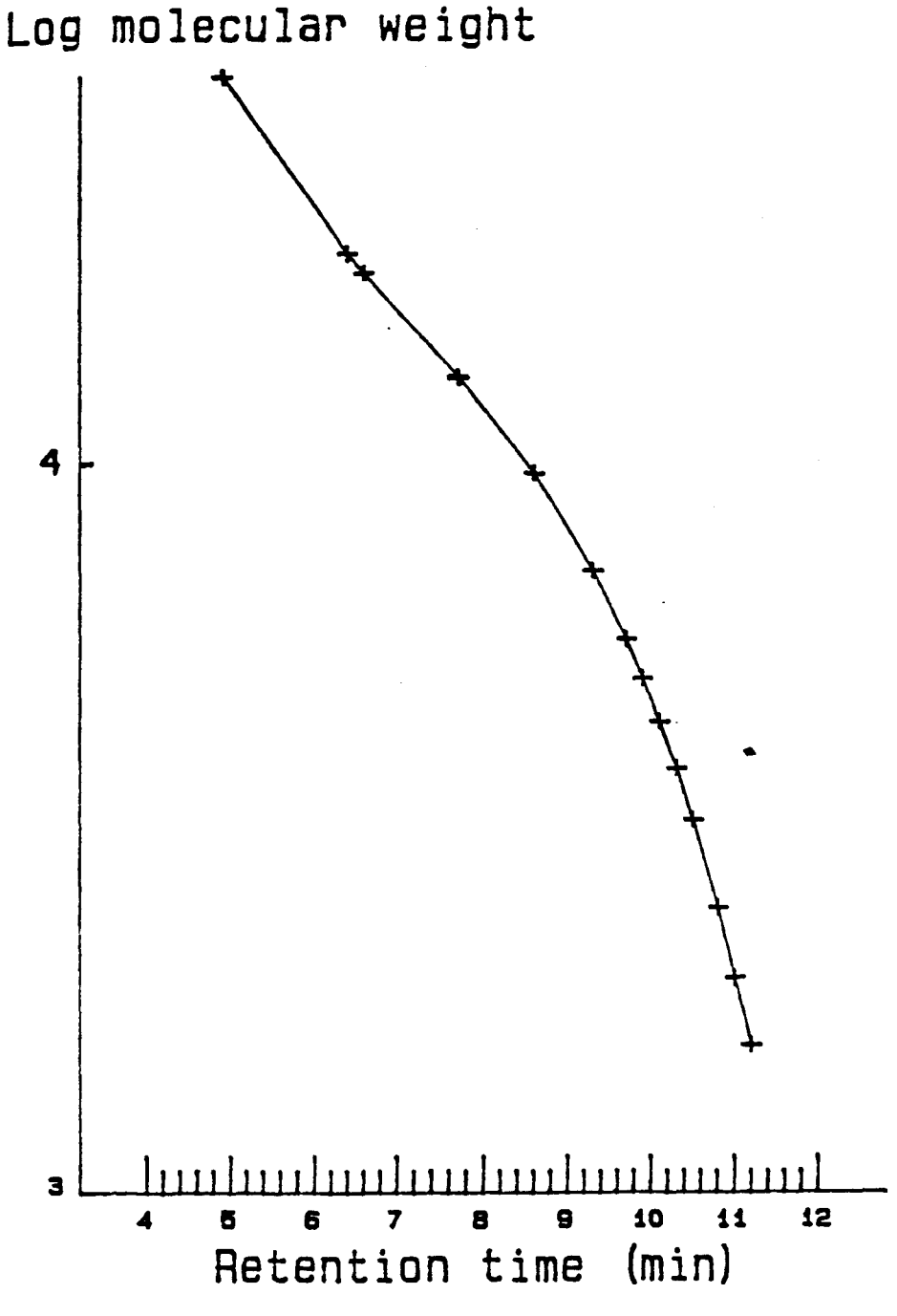
APPENDIX XII



APPENDIX XIII

APPENDIX XIII

GPC TSK 3000



APPENDIX XIV

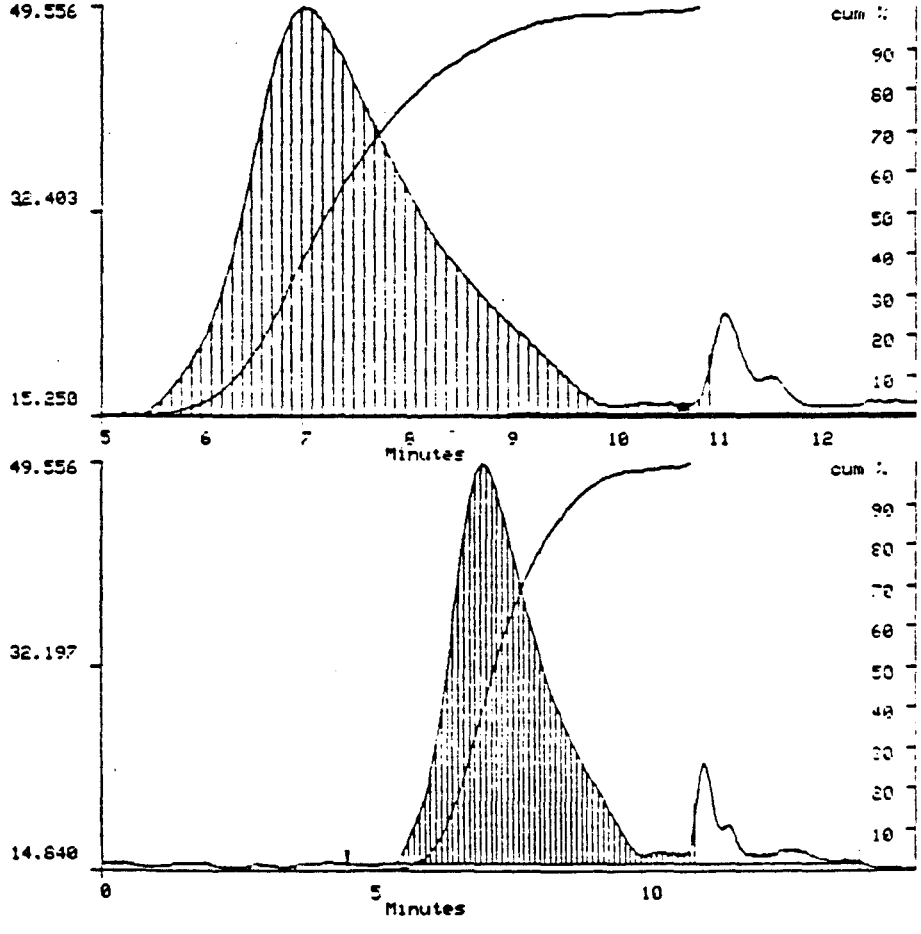
HPLC-GPC Profile of Unfractionated Heparin

Column TSK 2000 Solvent Na_2SO_4 0.5 M Detector UV 205

599941 4/9/86 17:43:17

Acquisition method	gagts2602	Quantitation method	gagts2602
Units	200ug	System number	1
Channel	1	Vial	1
Injection	1	Total injections	1
Run time	15.00 min	Sample rate	2 per sec
Injection volume	20 uL	Sample amount	
Internal standard amt		Scale factor	
Mode	Analysis	Response factors	Replace
Version	REV03.0	Channel to calibrate	1
Description			

COLONNE: TSK 2000SW VI SOLVANT: Na2SO4 0,5M DETECTEUR: UV 205



Molecular Weight Distribution Averages

Weight Average	12487	Number Average	9275
Z Average	16723	Dispersivity	1.346
Viscosity Average	12487	Intrinsic Viscosity	0.000
Z + 1 Average	33376	Peak Mol Weight	13108
Mz / Mw	1.339	Mz+1 / Mw	2.673

APPENDIX XIV a

Frequency Distribution of Molecular Weight Components

Column TSK 2000

Unfractionated heparin

Ret. time	Mol. Wt.	Cum. %	Slice area
5.500	52169	0.217	4947
5.600	45397	0.467	10119
5.700	39829	0.869	16347
5.800	35219	1.434	22961
5.900	31377	2.193	30830
6.000	28155	3.206	41153
6.100	25437	4.555	54775
6.200	23131	6.293	70616
6.300	21163	8.513	90183
6.400	19476	11.283	112498
6.500	18021	14.639	136353
6.600	16760	18.567	159559
6.700	15662	22.995	179456
6.800	14701	27.760	193969
6.900	13856	32.747	202554
7.000	13108	37.782	204548
7.100	12443	42.739	201339
7.200	11848	47.511	193859
7.300	11312	52.047	184263
7.400	10826	56.336	174206
7.500	10382	60.333	162332
7.600	9974	64.059	151342
7.700	9594	67.513	140302
7.800	9239	70.710	129946
7.900	8902	73.666	120076
8.000	8581	76.389	110609
8.100	8271	78.891	101672
8.200	7970	81.177	92823
8.300	7674	83.268	84942
8.400	7392	85.187	77960
8.500	7091	86.948	71533
8.600	6800	88.586	65750
8.700	6507	90.043	59963
8.800	6212	91.355	54509
8.900	5915	92.602	49437
9.000	5614	93.700	44599
9.100	5310	94.685	40042
9.200	5004	95.556	35356
9.300	4696	96.311	30666
9.400	4388	96.947	25934
9.500	4080	97.461	20868
9.600	3775	97.855	15996
9.700	3473	98.140	11607
9.800	3177	98.334	7878
9.900	2888	98.467	5407
10.000	2609	98.574	4341
10.100	2340	98.690	4685
10.200	2084	98.828	5625
10.300	1843	98.973	5969
10.400	1616	99.112	5674
10.500	1406	99.239	5132
10.600	1212	99.376	5603
10.700	1037	99.501	5076
10.800	878	99.649	5994
10.900	737	100.000	14259

APPENDIX XIV b

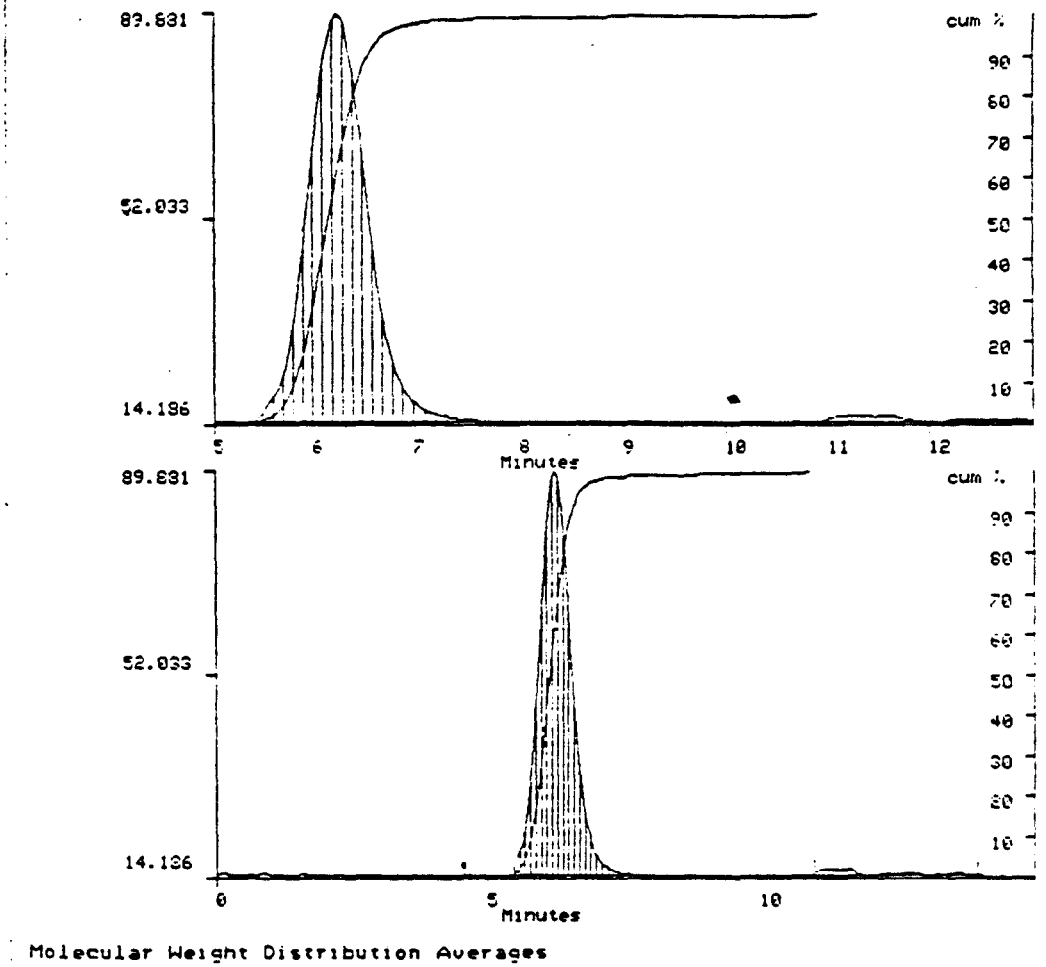
HPLC-GPC Profile of the 23,000 M.W. Fraction

Column TSK 2000 Solvent Na_2SO_4 0.5 M Detector UV 205

683HH1 4/9/86 18:00:05

Acquisition method	gagts2602	Quantitation method	gagts2602
Units	200ug	System number	1
Channel	1	Vial	2
Injection	1	Total injections	1
Run time	15.00 min	Sample rate	2 per sec
Injection volume	20 uL	Sample amount	
Internal standard amt		Scale factor	
Mode	Analysis	Response factors	Replace
Version	REV03.0	Channel to calibrate	1
Description			

COLONNE: TSK 2000SW VI SOLVANT: Na2SO4 0,5M DETECTEUR: UV 205



Molecular Weight Distribution Averages

Weight Average	24299	Number Average	21511
Z Average	28624	Dispersivity	1.130
Viscosity Average	24299	Intrinsic Viscosity	0.000
Z + 1 Average	49348	Peak Mol Weight	23131
Mz / Mw	1.178	Mz+1 / Mw	2.031

APPENDIX XIV b

Frequency Distribution of Molecular Weight Components

Column TSK 2000

23,000 M.W. Fraction

Ret. time	Mol. Wt.	Cum. %	Slice area
5.500	52169	1.002	20658
5.600	45397	2.201	38042
5.700	39829	4.579	75479
5.800	35219	9.280	149182
5.900	31377	17.156	249970
6.000	28155	28.219	351078
6.100	25437	41.601	424715
6.200	23131	55.774	449774
6.300	21163	68.936	417723
6.400	19476	79.643	339809
6.500	18021	87.245	241242
6.600	16760	92.082	153521
6.700	15662	94.931	90412
6.800	14701	96.565	51863
6.900	13856	97.513	30091
7.000	13108	98.087	18216
7.100	12443	98.446	11371
7.200	11848	98.682	7505

APPENDIX XIV c

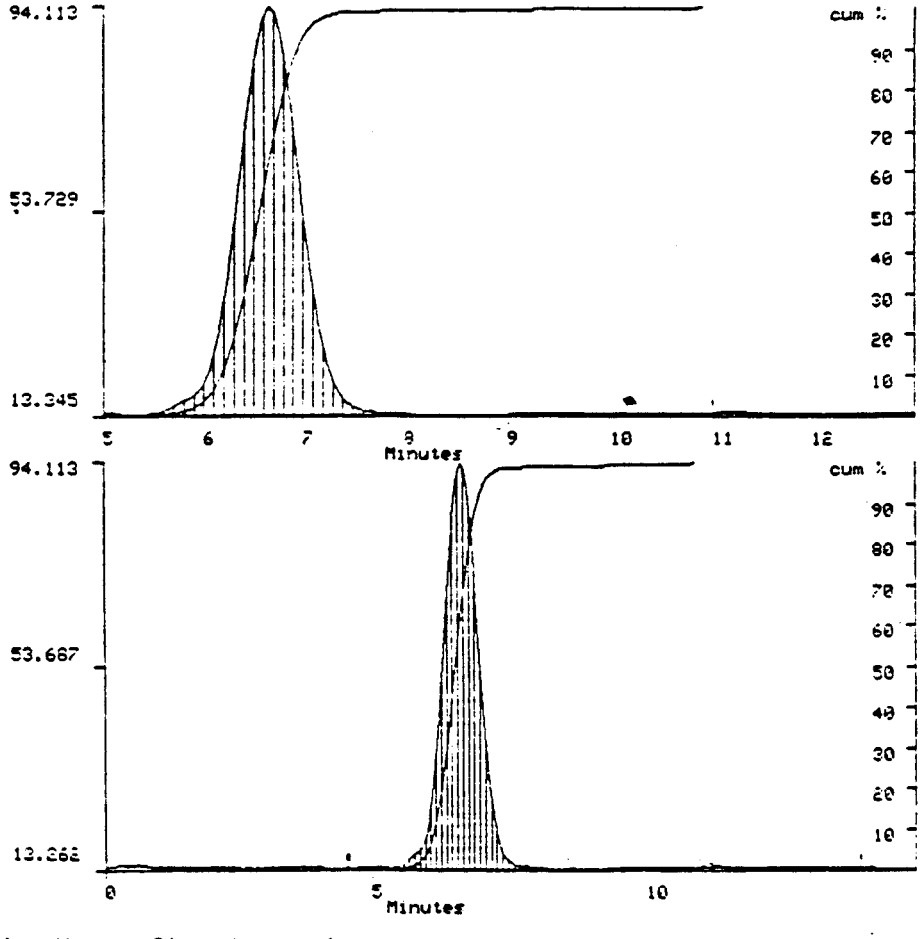
HPLC-GPC Profile of the 17,450 M.W. Fraction

Column TSK 2000 Solvent Na_2SO_4 0.5 M Detector UV 205

683HH2 4/9/86 18:16:49

Acquisition method	gagts2602	Quantitation method	gagts2602
Units	200ug	System number	1
Channel	1	Vial	3
Injection	1	Total injections	1
Run time	15.00 min	Sample rate	2 per sec
Injection volume	20 uL	Sample amount	
Internal standard amt		Scale factor	
Mode	Analysis	Response factors	Replace
Version	REV03.0	Channel to calibrate	1
Description			

COLONNE: TSK 2000SW UI SOLVANT: Na2SO4 0,5M DETECTEUR: UV 205



Molecular Weight Distribution Averages

Weight Average	17558	Number Average	16106
Z Average	19439	Dispersivity	1.090
Viscosity Average	17558	Intrinsic Viscosity	0.000
Z + 1 Average	27466	Peak Mol Weight.	16760
Mz / Mw	1.107	Mz+1 / Mw	1.564

APPENDIX XIV c

Frequency Distribution of Molecular Weight Components

Column TSK 2000

17,450 M.W. Fraction

Ret. time	Mol. Wt.	Cum. %	Slice area
5.500	52169	0.168	2862
5.600	45397	0.367	7100
5.700	39829	0.752	13742
5.800	35219	1.288	19158
5.900	31377	2.099	28954
6.000	28155	3.590	53240
6.100	25437	6.459	102451
6.200	23131	11.489	179593
6.300	21163	19.265	277679
6.400	19476	29.794	375964
6.500	18021	42.395	449951
6.600	16760	55.834	479893
6.700	15662	68.605	456014
6.800	14701	79.392	385161
6.900	13856	87.432	287099
7.000	13108	92.744	189697
7.100	12443	95.874	111748
7.200	11848	97.523	58888
7.300	11312	98.332	28893
7.400	10826	98.718	13771
7.500	10382	98.903	6632
7.600	9974	99.001	3486

APPENDIX XIV d

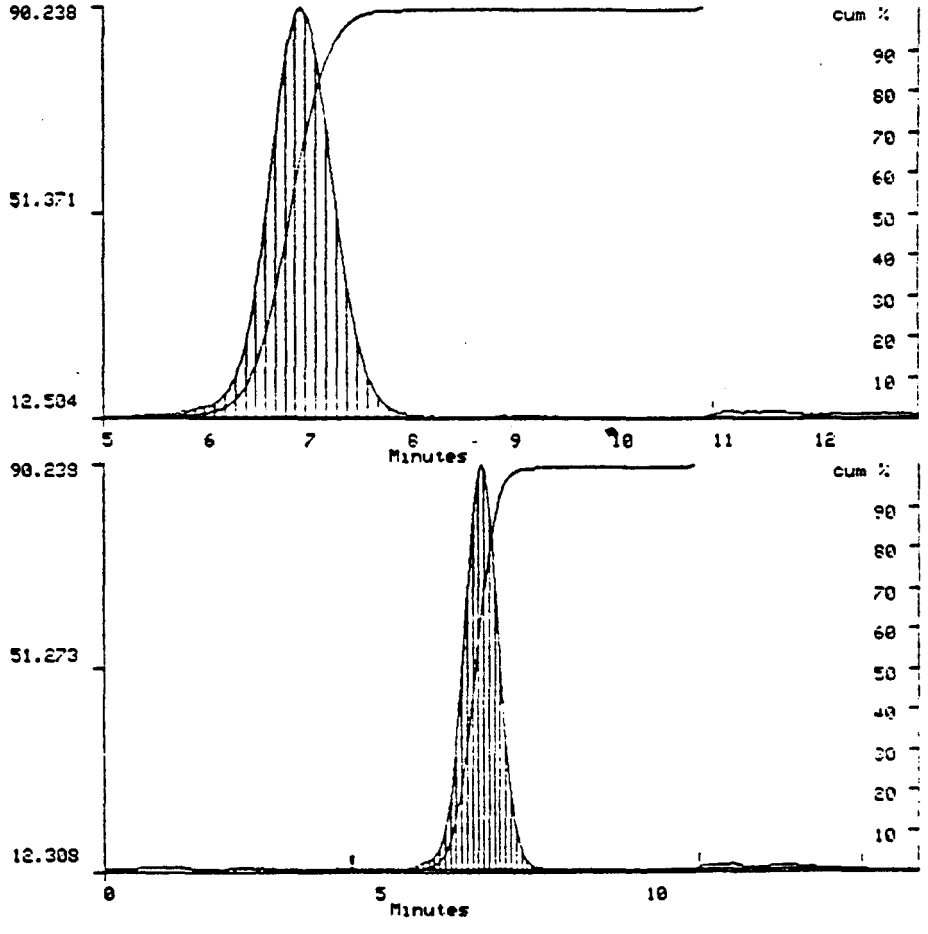
HPLC-GPC Profile of the 15,000 M.W. Fraction

Column TSK 2000 Solvent Na_2SO_4 0.5 M Detector UV 205

683HH3 4/9/86 18:33:38

Acquisition method	gagts2602	Quantitation method	gagts2602
Units	200ug	System number	1
Channel	1	Vial	4
Injection	1	Total injections	1
Run time	15.00 min	Sample rate	2 per sec
Injection volume	20 uL	Sample amount	
Internal standard amt		Scale factor	
Mode	Analysis	Response factors	Replace
Version	REV03.0	Channel to calibrate	1
Description			

COLONNE: TSK 2000SW VI SOLVANT: Na2SO4 0,5M DETECTEUR: UV 205



Molecular Weight Distribution Averages

Weight Average	14644	Number Average	13699
Z Average	17701	Dispersivity	1.069
Viscosity Average	14644	Intrinsic Viscosity	0.000
Z + 1 Average	43573	Peak Mol Weight	13856
Mz / Mw	1.209	Mz+1 / Mw	2.975

APPENDIX XIV d

Frequency Distribution of Molecular Weight Components

Column TSK 2000

15,000 M.W. Fraction

Ret. time	Mol. Wt.	Cum. %	Slice area
5.700	39829	0.354	4030
5.800	35219	0.541	6616
5.900	31377	0.806	9370
6.000	28155	1.160	12558
6.100	25437	1.692	18805
6.200	23131	2.650	33930
6.300	21163	4.455	63875
6.400	19476	7.669	113782
6.500	18021	12.944	186710
6.600	16760	20.739	275884
6.700	15662	31.093	366484
6.800	14701	43.400	435626
6.900	13856	56.464	462415
7.000	13108	68.875	439281
7.100	12443	79.384	371957
7.200	11848	87.331	281302
7.300	11312	92.686	189518
7.400	10826	95.953	115641
7.500	10382	97.770	64329
7.600	9974	98.722	33707
7.700	9594	99.190	16566
7.800	9239	99.408	7685
7.900	8902	99.509	3579

APPENDIX XIV e

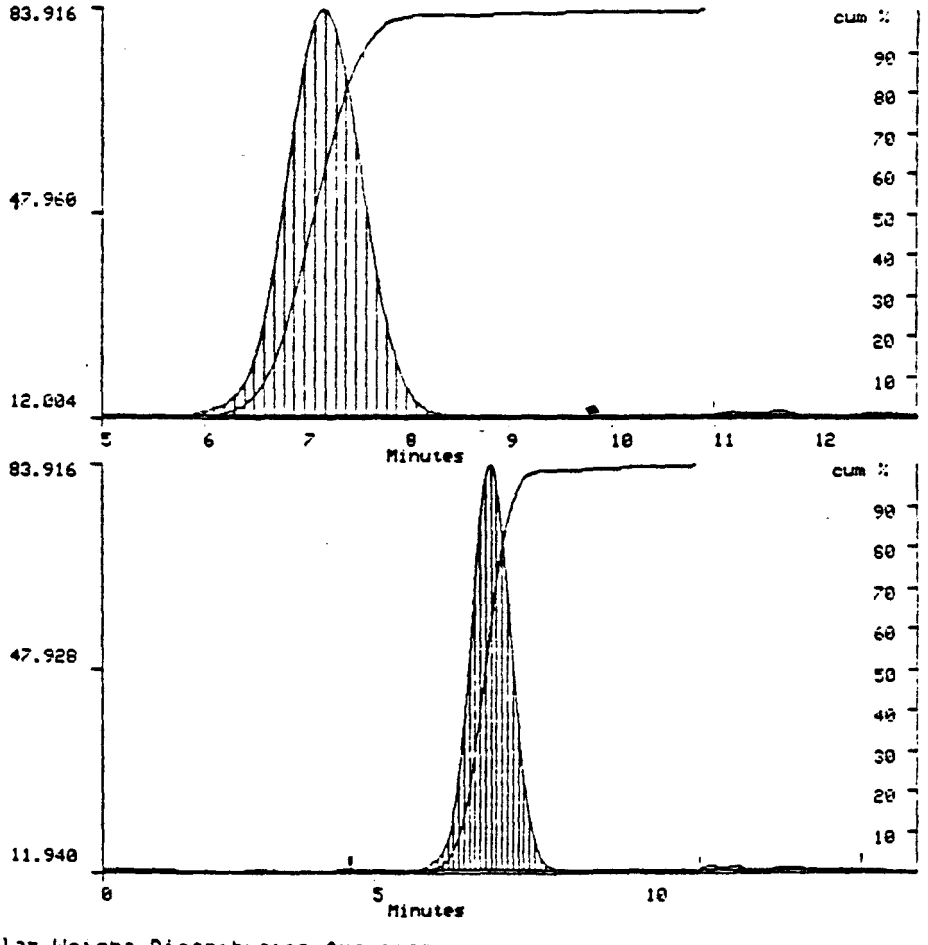
HPLC-GPC Profile of the 13,300 M.W. Fraction

Column TSK 2000 Solvent Na_2SO_4 0.5 M Detector UV 205

683HH4 4/9/86 18:50:26

Acquisition method	gagts2602	Quantitation method	gagts2602
Units	200ug	System number	1
Channel	1	Vial	5
Injection	1	Total injections	1
Run time	15.00 min	Sample rate	2 per sec
Injection volume	20 uL	Sample amount	
Internal standard amt		Scale factor	
Mode	Analysis	Response factors	Replace
Version	REV03.0	Channel to calibrate	1
Description			

COLONNE: TSK 2000SW VI SOLVANT: Na2SO4 0,5M DETECTEUR: UV 205



Molecular Weight Distribution Averages

Weight Average	12843	Number Average	11841
Z Average	15804	Dispersivity	1.085
Viscosity Average	12843	Intrinsic Viscosity	0.000
Z + 1 Average	42894	Peak Mol Weight	12443
Mz / Mw	1.231	Mz+1 / Mw	3.340

APPENDIX XIV e

Frequency Distribution of Molecular Weight Components

Column TSK 2000

13,300 M.W. Fraction

Ret. time	Mol. Wt.	Cum. %	Slice area
6.000	28155	0.601	6118
6.100	25437	0.857	9842
6.200	23131	1.263	15619
6.300	21163	1.939	25995
6.400	19476	3.128	45718
6.500	18021	5.191	79370
6.600	16760	8.549	129147
6.700	15662	13.608	194574
6.800	14701	20.603	269035
6.900	13856	29.484	341548
7.000	13108	39.815	397349
7.100	12443	50.881	425614
7.200	11848	61.815	420512
7.300	11312	71.792	383733
7.400	10826	80.231	324561
7.500	10382	86.831	253857
7.600	9974	91.617	184094
7.700	9594	94.814	122956
7.800	9239	96.792	76045
7.900	8902	97.910	43028
8.000	8581	98.478	21764
8.100	8271	98.731	9814
8.200	7970	98.836	4044
8.300	7674	98.867	1174

APPENDIX XIV f

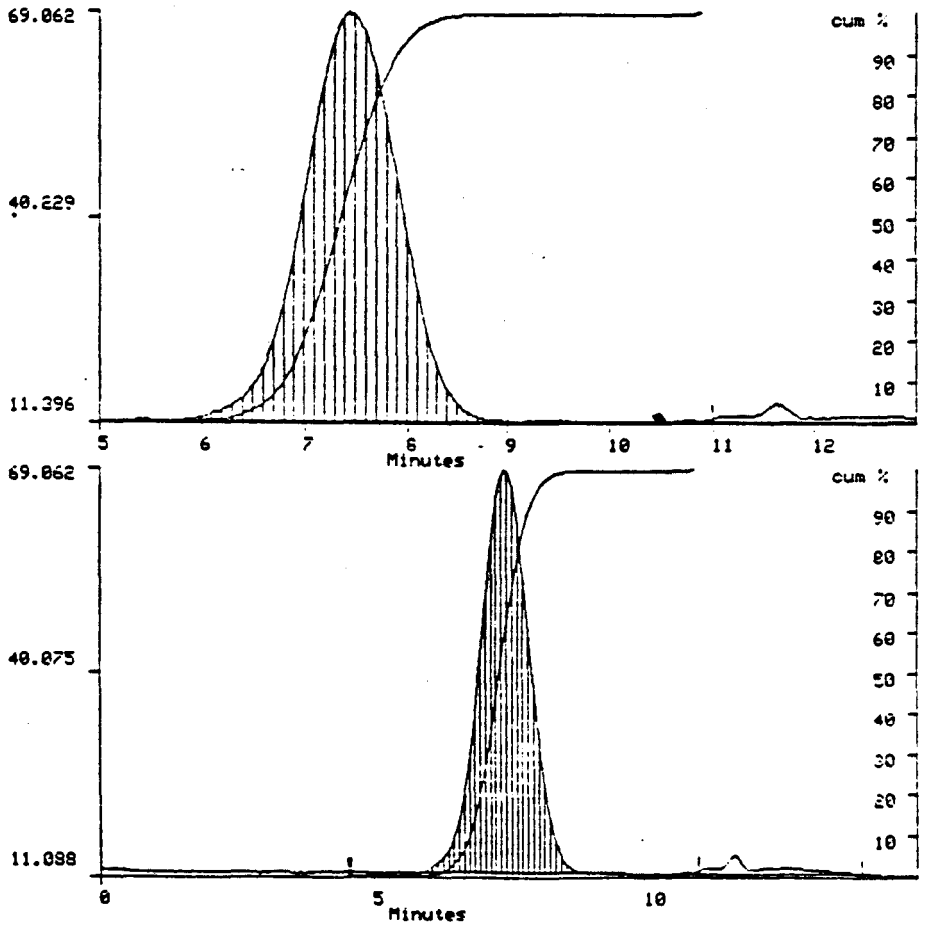
HPLC-GPC Profile of the 11,750 M.W. Fraction

Column TSK 2000 Solvent Na_2SO_4 0.5 M Detector UV 205

683HH5 4/9/86 19:07:09

Acquisition method	gagts2602	Quantitation method	gagts2602
Units	200ug	System number	1
Channel	1	Vial	6
Injection	1	Total injections	1
Run time	15.00 min	Sample rate	2 per sec
Injection volume	20 uL	Sample amount	
Internal standard amt		Scale factor	
Mode	Analysis	Response factors	Replace
Version	REV03.0	Channel to calibrate	1
Description			

COLONNE: TSK 2000SW VI SOLVANT: Na2SO4 0,5M DETECTEUR: UV 205



Molecular Weight Distribution Averages

Weight Average	11393	Number Average	10685
Z Average	14026	Dispersivity	1.066
Viscosity Average	11393	Intrinsic Viscosity	0.000
Z + 1 Average	44130	Peak Mol Weight	10826
Mz / Mw	1.231	Mz+1 / Mw	3.873

APPENDIX XIV f

Frequency Distribution of Molecular Weight Components

Column TSK 2000

11,750 M.W. Fraction

Ret. time	Mol. Wt.	Cum. %	Slice area
6.200	23131	0.806	11050
6.300	21163	1.241	16166
6.400	19476	1.901	24476
6.500	18021	2.897	36957
6.600	16760	4.412	56231
6.700	15662	6.679	84120
6.800	14701	9.929	120599
6.900	13856	14.374	164942
7.000	13108	20.159	214659
7.100	12443	27.256	263349
7.200	11848	35.477	305065
7.300	11312	44.451	332978
7.400	10826	53.717	343850
7.500	10382	62.801	337085
7.600	9974	71.258	313800
7.700	9594	78.709	276507
7.800	9239	84.921	230495
7.900	8902	89.822	181876
8.000	8581	93.450	134593
8.100	8271	95.966	93370
8.200	7970	97.581	59920
8.300	7674	98.552	36030
8.400	7382	99.114	20873
8.500	7091	99.416	11217
8.600	6800	99.569	5681
8.700	6507	99.641	2662

APPENDIX XIV g

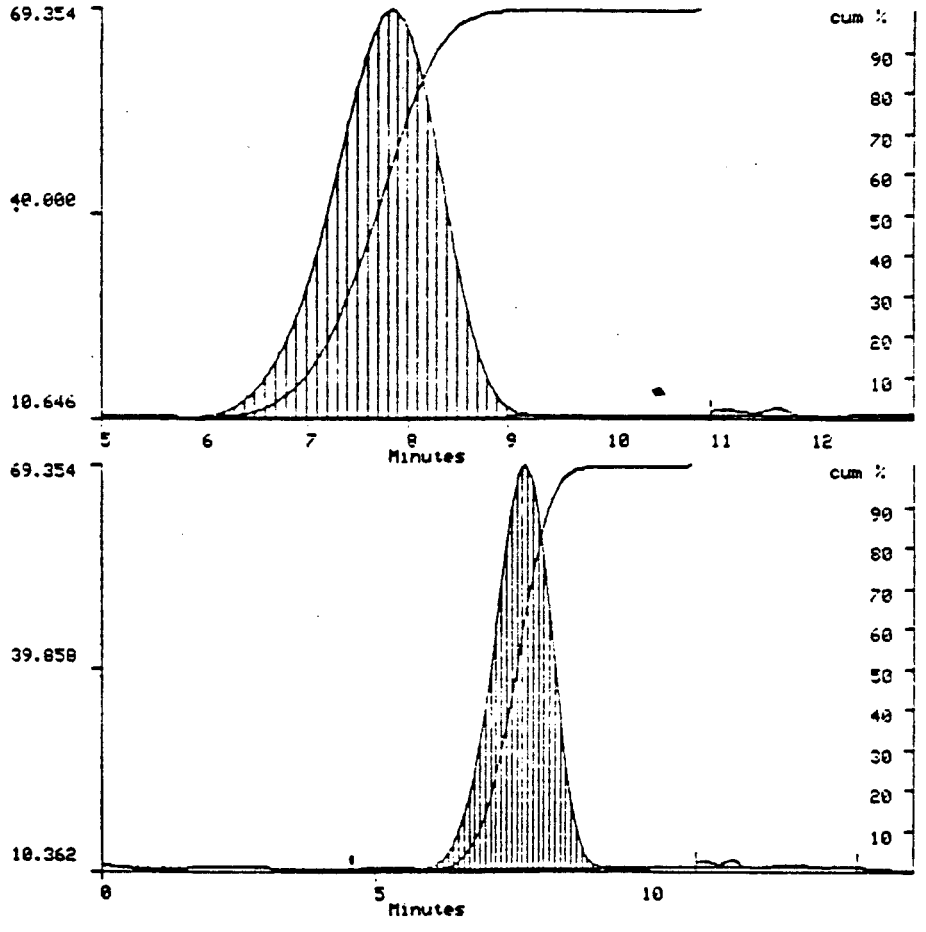
HPLC–GPC Profile of the 10,400 M.W. Fraction

Column TSK 2000 Solvent Na_2SO_4 0.5 M Detector UV 205

683HH6 4/9/86 19:23:55

Acquisition method	gagts2602	Quantitation method	gagts2602
Units	200ug	System number	1
Channel	1	Vial	7
Injection	1	Total injections	1
Run time	15.00 min	Sample rate	2 per sec
Injection volume	20 uL	Sample amount	
Internal standard amt		Scale factor	
Mode	Analysis	Response factors	Replace
Version	REV03.0	Channel to calibrate	1
Description			

COLONNE: TSK 2000SW UI SOLVANT: Na2SO4 0,5M DETECTEUR: UV 205



Molecular Weight Distribution Averages

Weight Average	10220	Number Average	9358
Z Average	14933	Dispersivity	1.092
Viscosity Average	10220	Intrinsic Viscosity	0.000
Z + 1 Average	63165	Peak Mol Weight	9239
Mz / Mw	1.461	Mz+1 / Mw	6.180

APPENDIX XIV g

Frequency Distribution of Molecular Weight Components

Column TSK 2000

10,400 M.W. Fraction

Ret. time	Mol. Wt.	Cum. %	Slice area
6.200	23131	0.549	6237
6.300	21163	0.797	11180
6.400	19476	1.207	18487
6.500	18021	1.830	28135
6.600	16760	2.727	40439
6.700	15662	3.968	56025
6.800	14701	5.639	75407
6.900	13856	7.831	98918
7.000	13108	10.638	126657
7.100	12443	14.148	158378
7.200	11848	18.434	193369
7.300	11312	23.549	230832
7.400	10826	29.475	267378
7.500	10382	36.126	300104
7.600	9974	43.371	326924
7.700	9594	51.006	344530
7.800	9239	58.770	350320
7.900	8902	66.379	343308
8.000	8581	73.541	323186
8.100	8271	79.996	291261
8.200	7970	85.542	250255
8.300	7674	90.044	203153
8.400	7382	93.475	154822
8.500	7091	95.930	110747
8.600	6800	97.557	73411
8.700	6507	98.556	45121
8.800	6212	99.123	25556
8.900	5915	99.418	13303
9.000	5614	99.561	6474

APPENDIX XIV h

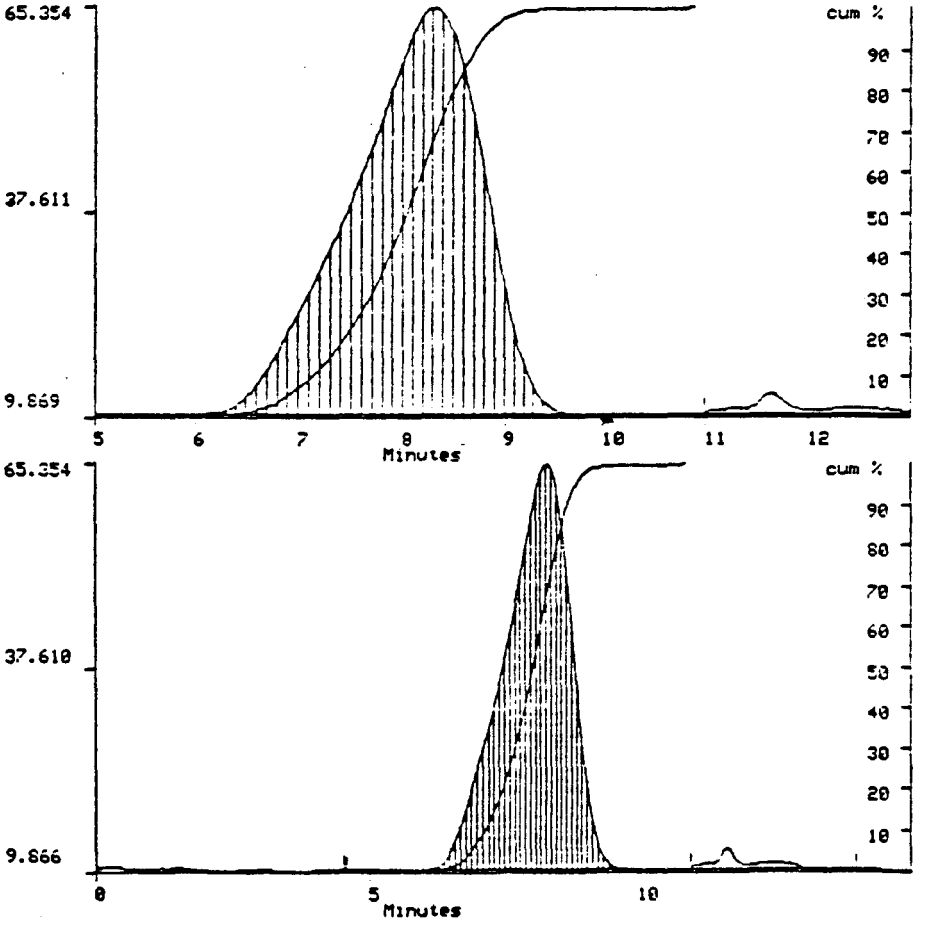
HPLC-GPC Profile of the 9,000 M.W. Fraction

Column TSK 2000 Solvent Na_2SO_4 0.5 M Detector UV 205

683HH7 4/9/86 19:40:42

Acquisition method	gagts2602	Quantitation method	gagts2602
Units	200ug	System number	1
Channel	1	Vial	8
Injection	1	Total injections	1
Run time	15.00 min	Sample rate	2 per sec
Injection volume	20 uL	Sample amount	
Internal standard amt		Scale factor	
Mode	Analysis	Response factors	Replace
Version	REV03.0	Channel to calibrate	1
Description			

COLONNE: TSK 2000SW VI SOLVANT: Na2SO4 0,5M DETECTEUR: UV 205



Molecular Weight Distribution Averages

Weight Average	9074	Number Average	8187
Z Average	14647	Dispersivity	1.108
Viscosity Average	9074	Intrinsic Viscosity	0.000
Z + 1 Average	78791	Peak Mol Weight	7674
Mz / Mw	1.614	Mz+1 / Mw	8.683

APPENDIX XIV h

Frequency Distribution of Molecular Weight Components

Column TSK 2000

9,000 M.W. Fraction

Ret. time	Mol. Wt.	Cum. %	Slice area
6.400	19476	0.671	10111
6.500	18021	1.043	18331
6.600	16760	1.642	29495
6.700	15662	2.515	43013
6.800	14701	3.699	58342
6.900	13856	5.214	74603
7.000	13108	7.063	91098
7.100	12443	9.254	107952
7.200	11848	11.799	125405
7.300	11312	14.709	143338
7.400	10826	18.010	162612
7.500	10382	21.749	184225
7.600	9974	25.920	205468
7.700	9594	30.546	227929
7.800	9239	35.661	251962
7.900	8902	41.262	275944
8.000	8581	47.318	298347
8.100	8271	53.750	316880
8.200	7970	60.409	328089
8.300	7674	67.103	329742
8.400	7382	73.604	320286
8.500	7091	79.663	298499
8.600	6800	85.034	264603
8.700	6507	89.528	221412
8.800	6212	93.058	173893
8.900	5915	95.642	127314
9.000	5614	97.393	86275
9.100	5310	98.491	54074
9.200	5004	99.122	31097
9.300	4696	99.457	16490
9.400	4388	99.619	7994
9.500	4080	99.685	3249

APPENDIX XIV i

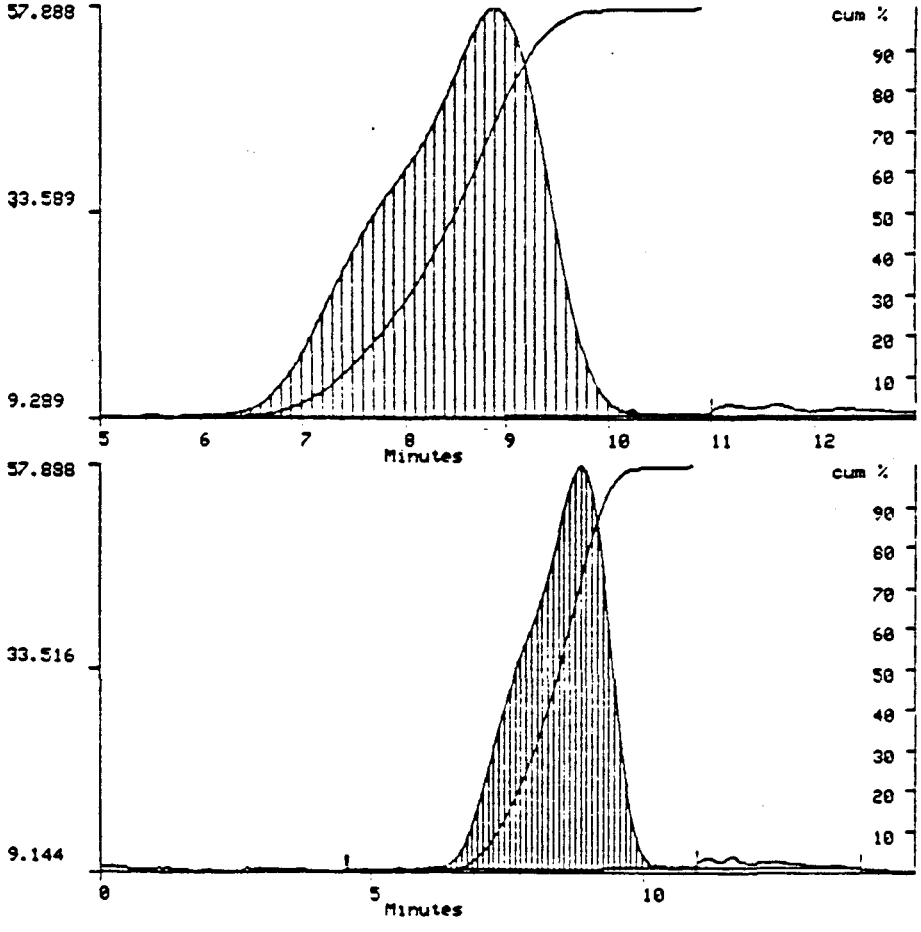
HPLC-GPC Profile of the 7,400 M.W. Fraction

Column TSK 2000 Solvent Na_2SO_4 0.5 M Detector UV 205

683HH8 4/9/86 19:57:32

Acquisition method	gagts2602	Quantitation method	gagts2602
Units	200ug	System number	1
Channel	1	Vial	9
Injection	1	Total injections	1
Run time	15.00 min	Sample rate	2 per sec
Injection volume	20 uL	Sample amount	
Internal standard amt		Scale factor	
Mode	Analysis	Response factors	Replace
Version	REV03.0	Channel to calibrate	1
Description			

COLONNE: TSK 2000SW VI SOLVANT: Na2SO4 0,5M DETECTEUR: UV 205



Molecular Weight Distribution Averages

Weight Average	7458	Number Average	6548
Z Average	10166	Dispersivity	1.139
Viscosity Average	7458	Intrinsic Viscosity	0.000
Z + 1 Average	45552	Peak Mol Weight	5915
Mz / Mw	1.363	Mz+1 / Mw	6.108

APPENDIX XIV i

Frequency Distribution of Molecular Weight Components

Column TSK 2000

7,400 M.W. Fraction

Ret. time	Mol. Wt.	Cum. %	Slice area
6.700	15662	1.093	18368
6.800	14701	1.630	27368
6.900	13856	2.380	38251
7.000	13108	3.384	51204
7.100	12443	4.671	65603
7.200	11848	6.250	80503
7.300	11312	8.119	95265
7.400	10826	10.270	109636
7.500	10382	12.687	123239
7.600	9974	15.355	135999
7.700	9594	18.249	147558
7.800	9239	21.357	158441
7.900	8902	24.664	168574
8.000	8581	28.179	179186
8.100	8271	31.910	190234
8.200	7970	35.891	202938
8.300	7674	40.157	217494
8.400	7382	44.741	233707
8.500	7091	49.663	250898
8.600	6800	54.906	267269
8.700	6507	60.41[illegible]	280662
8.800	6212	66.071	288549
8.900	5915	71.738	288882
9.000	5614	77.230	279995
9.100	5310	82.357	261398
9.200	5004	86.918	232500
9.300	4696	90.765	196150
9.400	4388	93.818	155629
9.500	4080	96.082	115427
9.600	3775	97.630	78910
9.700	3473	98.612	50039
9.800	3177	99.182	29092
9.900	2888	99.489	15620
10.000	2609	99.643	7854
10.100	2340	99.715	3654

APPENDIX XIV j

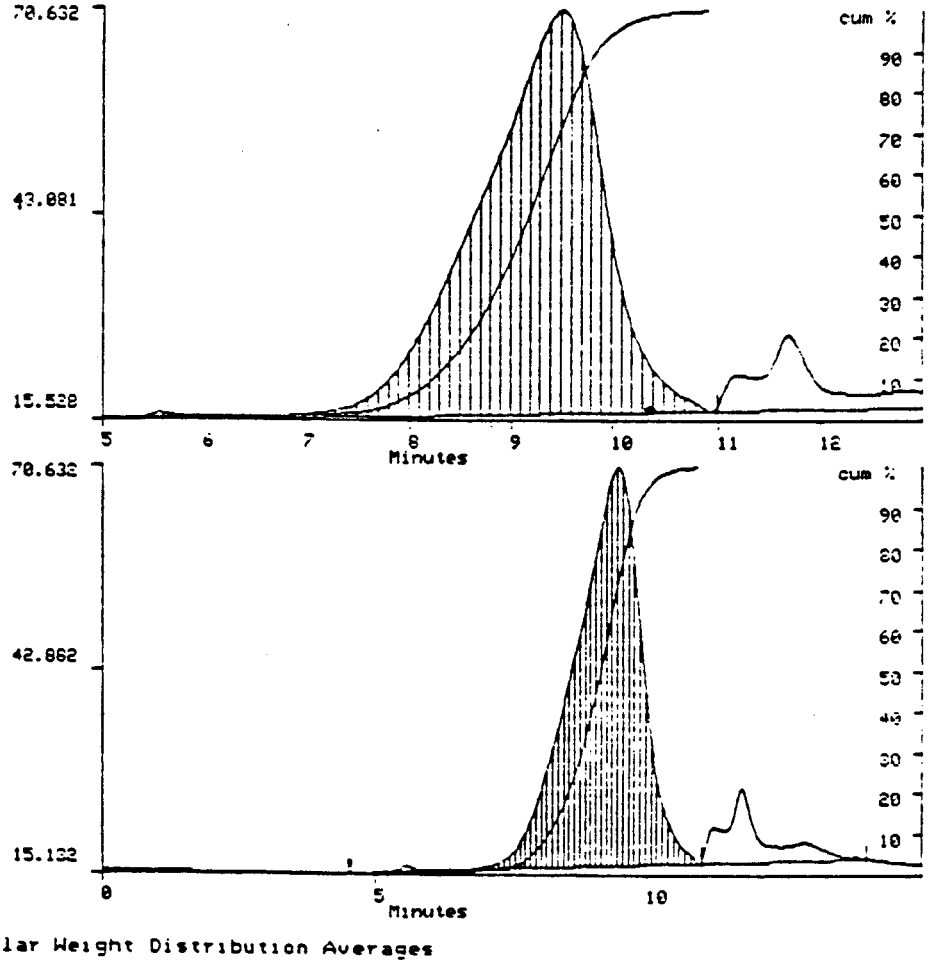
HPLC-GPC Profile of the 5,100 M.W. Fraction

Column TSK 2000 Solvent Na_2SO_4 0.5 M Detector UV 205

683HH9 4/10/86 9:29:10

Acquisition method	gagts2602	Quantitation method	gagts2602
Units	200ug	System number	1
Channel	1	Vial	1
Injection	1	Total injections	1
Run time	15.00 min	Sample rate	2 per sec
Injection volume	100 uL	Sample amount	
Internal standard amt		Scale factor	
Mode	Analysis	Response factors	Replace
Version	REV03.0	Channel to calibrate	1
Description			

COLONNE: TSK 2000SW VI SOLVANT: Na2SO4 0,5M DETECTEUR: UV 205



Molecular Weight Distribution Averages

Weight Average	5500	Number Average	4365
Z Average	12381	Dispersivity	1.260
Viscosity Average	5500	Intrinsic Viscosity	0.000
Z + 1 Average	78276	Peak Mol Weight	4080
Mz / Mw	2.251	Mz+1 / Mw	14.232

APPENDIX XIV j

Frequency Distribution of Molecular Weight Components

Column TSK 2000

5,100 M.W. Fraction

Ret. time	Mol. Wt.	Cum. %	Slice area
7.200	11848	1.194	5031
7.300	11312	1.340	6850
7.400	10826	1.540	9322
7.500	10382	1.815	12884
7.600	9974	2.200	18021
7.700	9594	2.735	25060
7.800	9239	3.465	34135
7.900	8902	4.427	45042
8.000	8581	5.669	58118
8.100	8271	7.223	72736
8.200	7970	9.120	88789
8.300	7674	11.396	106489
8.400	7382	14.063	124847
8.500	7091	17.140	144012
8.600	6800	20.628	163203
8.700	6507	24.519	182095
8.800	6212	28.813	200990
8.900	5915	33.526	220549
9.000	5614	38.689	241671
9.100	5310	44.333	264142
9.200	5004	50.475	287425
9.300	4696	57.055	307980
9.400	4388	63.940	322186
9.500	4080	70.861	323930
9.600	3775	77.438	307796
9.700	3473	83.264	272680
9.800	3177	88.041	223552
9.900	2888	91.684	170503
10.000	2609	94.294	122131
10.100	2340	96.100	84541
10.200	2084	97.349	58441
10.300	1843	98.240	41712
10.400	1616	98.884	30150
10.500	1406	99.342	21419
10.600	1213	99.655	14667
10.700	1037	99.870	10048
10.800	878	99.982	5234
10.900	737	100.000	851

APPENDIX XIV k

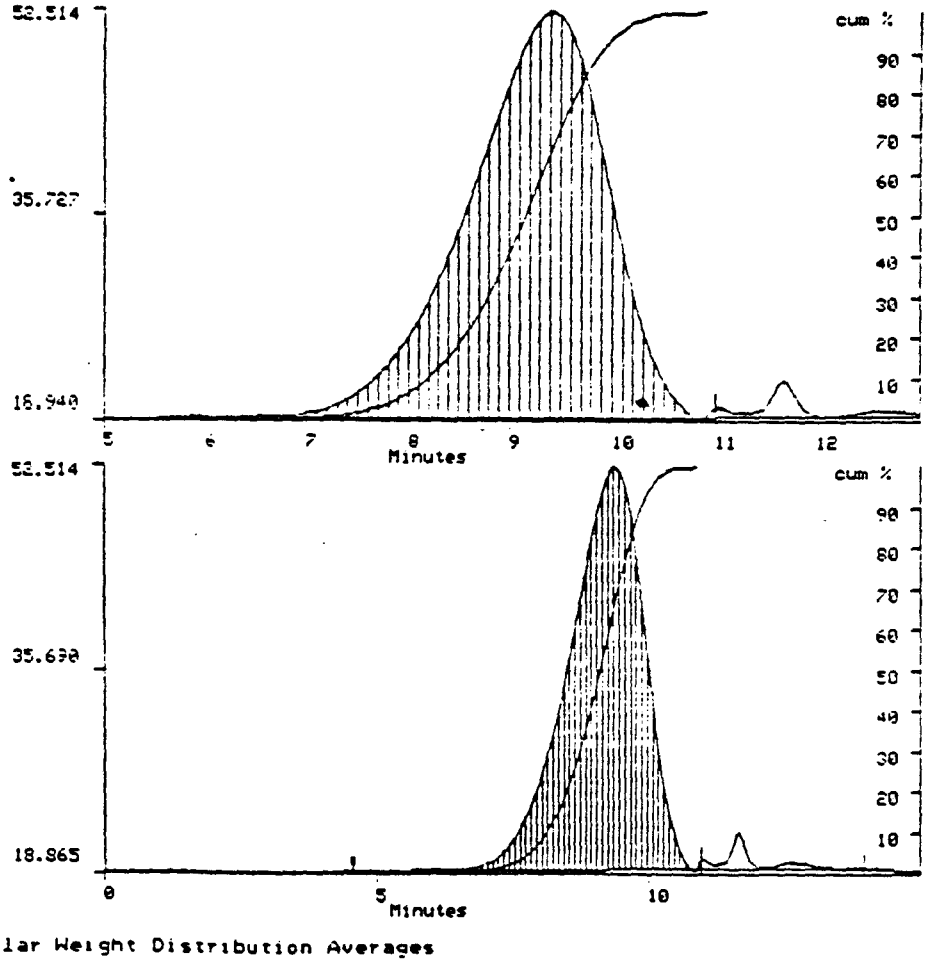
HPLC-GPC Profile of CY 216

Column TSK 2000 Solvent Na_2SO_4 0.5 M Detector UV 205

2162 4/4/86 11:17:27

Acquisition method	gagts2602	Quantitation method	gagts2602
Units	200ug	System number	1
Channel	1	Vial	1
Injection	1	Total injections	1
Run time	15.00 min	Sample rate	2 per sec
Injection volume	20 uL	Sample amount	
Internal standard amt		Scale factor	
Mode	Analysis	Response factors	Replace
Version	REV03.0	Channel to calibrate	1
Description			

COLONNE: TSK 2000SW VI SOLVANT: Na2SO4 0,5M DETECTEUR: UV 205



Molecular Weight Distribution Averages

Weight Average	5399	Number Average	4391
Z Average	10799	Dispersivity	1.230
Viscosity Average	5399	Intrinsic Viscosity	0.000
Z + 1 Average	86436	Peak Mol Weight	4388
Mz / Mw	2.000	Mz+1 / Mw	16.010

APPENDIX XIV k

Frequency Distribution of Molecular Weight Components

Column TSK 2000

CY 216

Ret. time	Mol. Wt.	Cum. %	Slice area
7.000	13108	0.631	3058
7.100	12443	0.753	3870
7.200	11848	0.931	5638
7.300	11312	1.173	7665
7.400	10826	1.497	10281
7.500	10382	1.944	14168
7.600	9974	2.522	18341
7.700	9594	3.258	23317
7.800	9239	4.175	29087
7.900	8902	5.309	35941
8.000	8581	6.688	43749
8.100	8271	8.344	52500
8.200	7970	10.315	62478
8.300	7674	12.636	73598
8.400	7382	15.336	85610
8.500	7091	18.444	98543
8.600	6800	21.987	112363
8.700	6507	25.999	127194
8.800	6212	30.467	141668
8.900	5915	35.398	156348
9.000	5614	40.758	169948
9.100	5310	46.507	182283
9.200	5004	52.565	192086
9.300	4696	58.814	198146
9.400	4388	65.104	199443
9.500	4080	71.244	194673
9.600	3775	77.037	183686
9.700	3473	82.302	166935
9.800	3177	86.850	144189
9.900	2888	90.608	119157
10.000	2609	93.590	94236
10.100	2340	95.809	70630
10.200	2084	97.402	50523
10.300	1843	98.481	34203
10.400	1616	99.187	22386
10.500	1406	99.610	13399
10.600	1213	99.829	6956
10.700	1037	99.893	2043
10.800	878	99.917	740
10.900	737	100.000	2637

APPENDIX XV

APPENDIX XV a

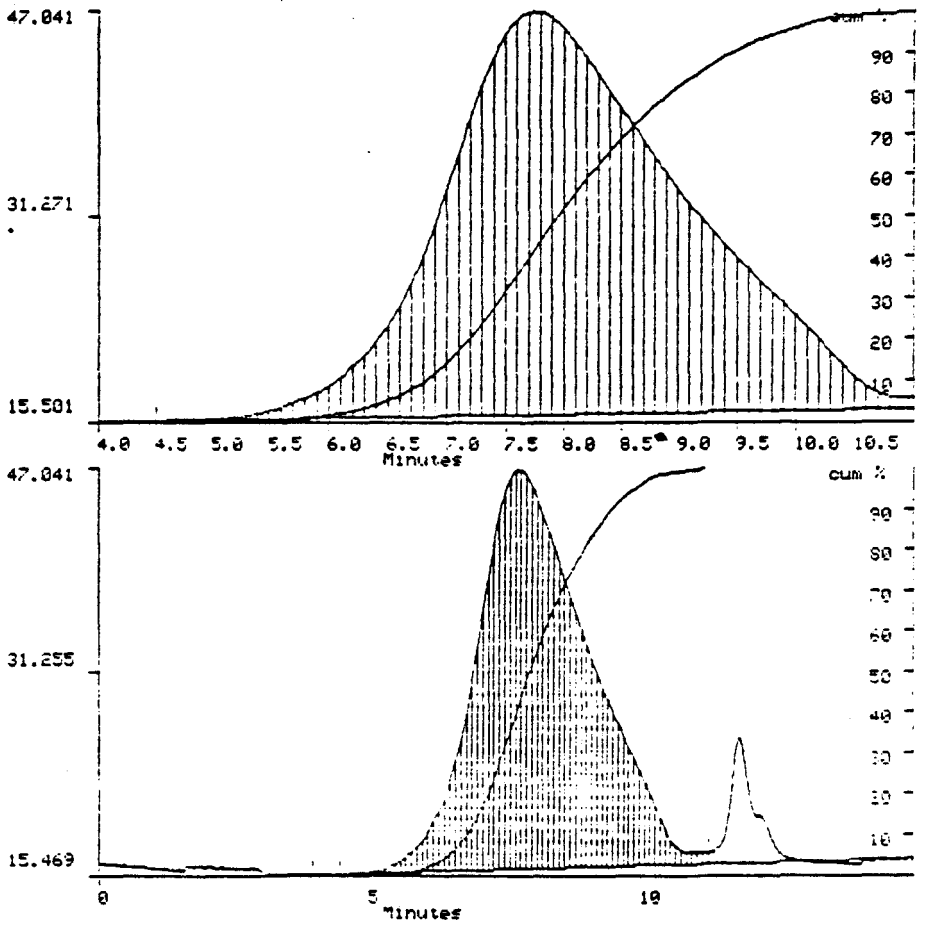
HPLC-GPC Profile of the unfractionated heparin

Column TSK 3000 Solvent Na_2SO_4 0.5 M Detector UV 205

599 4/10/86 18:48:15

Acquisition method	gagts3303	Quantitation method	gagts3303
Units	200ug	System number	1
Channel	1	Vial	1
Injection	1	Total injections	1
Run time	15.00 min	Sample rate	2 per sec
Injection volume	20 uL	Sample amount	
Internal standard amt		Scale factor	
Mode	Analysis	Response factors	Replace
Version	REV03.0	Channel to calibrate	1
Description			

COLONNE: TSK 3000SW III SOLVANT: NA2SO4 0,5M DETECTEUR: UV 205 nm



Molecular Weight Distribution Averages

Weight Average	12660	Number Average	10039
Z Average	14877	Dispersivity	1.261
Viscosity Average	12660	Intrinsic Viscosity	0.000
Z + 1 Average	17023	Peak Mol Weight	14011
Mz / Mw	1.175	Mz+1 / Mw	1.345

APPENDIX XV a

Frequency Distribution of Molecular Weight Components

Column TSK 3000

Unfractionated heparin

Ret. time	Mol. Wt.	Cum. %	Slice area
5.000	44432	0.039	757
5.100	41830	0.061	976
5.200	39559	0.097	1643
5.300	37438	0.151	2422
5.400	35436	0.226	3431
5.500	33718	0.333	4831
5.600	32082	0.473	6353
5.700	30573	0.659	8437
5.800	29179	0.893	10649
5.900	27887	1.187	13339
6.000	26687	1.554	16643
6.100	25568	2.029	21570
6.200	24524	2.609	26331
6.300	23546	3.304	31526
6.400	22627	4.164	39011
6.500	21762	5.204	47197
6.600	20945	6.455	56765
6.700	20171	7.956	68123
6.800	19435	9.757	81742
6.900	18734	11.899	97160
7.000	18064	14.324	113252
7.100	17422	17.253	129710
7.200	16805	20.458	145465
7.300	16210	23.991	159855
7.400	15635	27.761	171535
7.500	15079	31.730	180114
7.600	14538	35.806	185028
7.700	14011	39.916	186443
7.800	13499	43.999	185206
7.900	12996	47.992	181253
8.000	12504	51.862	175512
8.100	12022	55.592	166839
8.200	11549	59.144	161639
8.300	11084	62.538	153747
8.400	10627	65.747	145579
8.500	10177	68.789	139009
8.600	9734	71.662	130884
8.700	9299	74.368	122583
8.800	8869	76.824	114361
8.900	8447	79.061	107434
9.000	8031	81.178	100493
9.100	7624	83.136	93462
9.200	7223	85.051	89815
9.300	6831	87.023	89642
9.400	6447	88.977	74648
9.500	6071	90.825	88887
9.600	5705	91.795	69514
9.700	5349	93.074	53012
9.800	5003	94.286	52850
9.900	4668	95.285	47523
10.000	4343	96.211	42022
10.100	4031	97.015	36480
10.200	3730	97.692	30682
10.300	3442	98.239	24841
10.400	3167	98.661	19134
10.500	2905	98.969	14004
10.600	2656	99.189	9977
10.700	2420	99.352	7396
10.800	2198	99.487	6113
10.900	1990	99.616	5966
11.000	1794	99.746	5917
11.100	1612	99.875	5946
11.200	1443	100.000	5662

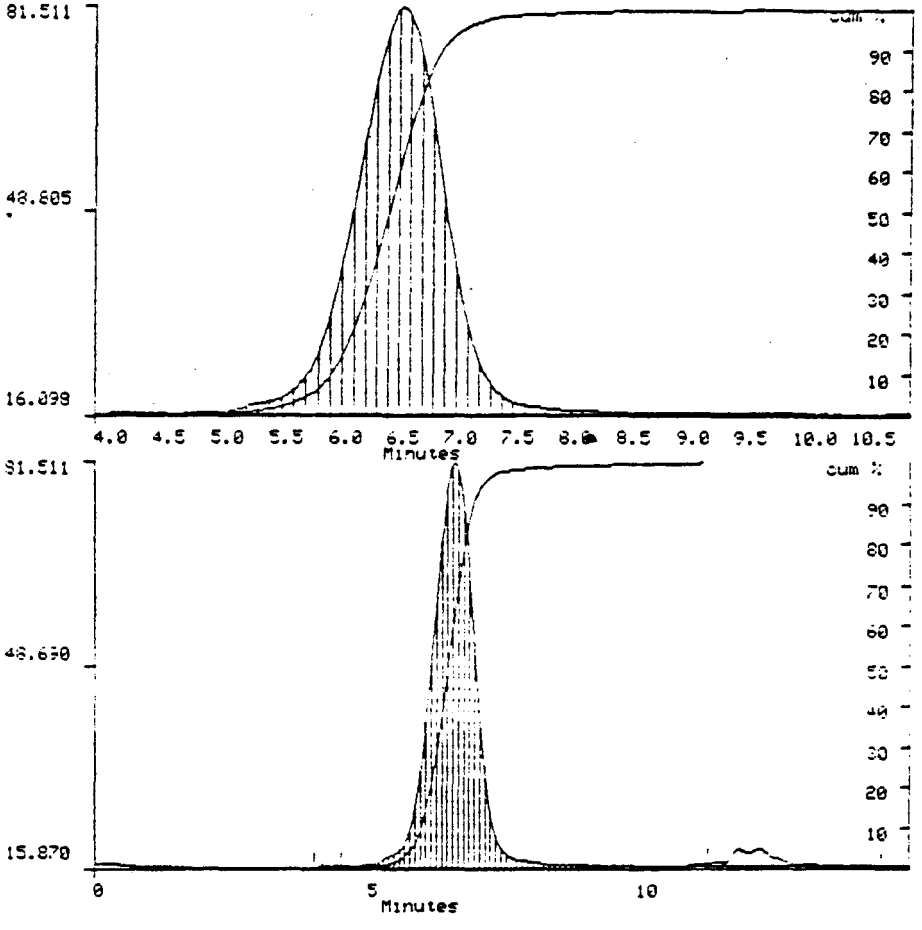
HPLC–GPC Profile of the 23,000 M.W. fraction

Column TSK 3000 Solvent Na_2SO_4 0.5 M Detector UV 205

683H1 4/10/86 19:04:53

Acquisition method	gagts3303	Quantitation method	gagts3303
Units	200ug	System number	1
Channel	1	Vial	2
Injection	1	Total injections	1
Run time	15.00 min	Sample rate	2 per sec
Injection volume	20 uL	Sample amount	
Internal standard amt		Scale factor	
Mode	Analysis	Response factors	Replace
Version	REV03.0	Channel to calibrate	1
Description			

COLONNE: TSK 3000SW III SOLVANT: NA2SO4 0,5M DETECTEUR: UV 205 nm



Molecular Weight Distribution Averages

Weight Average	21691	Number Average	20354
Z Average	22527	Dispersivity	1.066
Viscosity Average	21691	Intrinsic Viscosity	0.000
Z + 1 Average	23509	Peak Mol Weight	20945
Mz / Mw	1.039	Mz+1 / Mw	1.084

APPENDIX XV b

Frequency Distribution of Molecular Weight Components

Column TSK 3000

23,000 M.W. Fraction

Ret. time	Mol. Wt.	Cum. %	Slice area
5.200	39558	0.596	6522
5.300	37438	0.863	9652
5.400	35496	1.190	11771
5.500	33718	1.595	14613
5.600	32082	2.135	19486
5.700	30573	2.947	29308
5.800	29179	4.267	47620
5.900	27887	6.421	77669
6.000	26687	9.737	119647
6.100	25568	14.520	172512
6.200	24524	20.953	232072
6.300	23546	28.047	291993
6.400	22627	38.598	344543
6.500	21762	49.132	379994
6.600	20945	59.955	390422
6.700	20171	70.197	369464
6.800	19435	79.028	318577
6.900	18734	85.910	248232
7.000	18064	90.737	174149
7.100	17422	93.816	111061
7.200	16805	95.654	66300
7.300	16210	96.719	38423
7.400	15635	97.355	22932
7.500	15079	97.757	14492

APPENDIX XV c

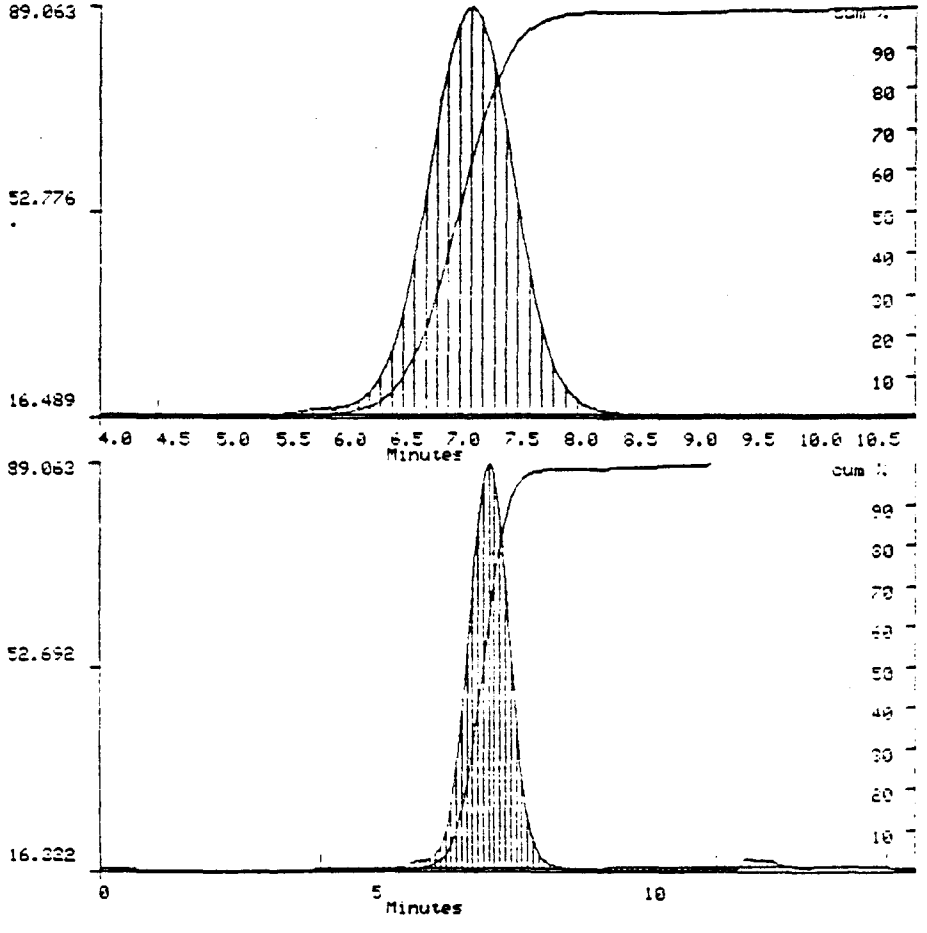
HPLC–GPC Profile of the 17,450 M.W. fraction

Column TSK 3000 Solvent Na_2SO_4 0.5 M Detector UV 205

683H2 4/10/86 19:21:37

Acquisition method	gagts3303	Quantitation method	gagts3303
Units	200ug	System number	1
Channel	1	Vial	3
Injection	1	Total injections	1
Run time	15.00 min	Sample rate	2 per sec
Injection volume	20 uL	Sample amount	
Internal standard amt		Scale factor	
Mode	Analysis	Response factors	Replace
Version	REV03.0	Channel to calibrate	1
Description			

COLONNE: TSK 3000SW III SOLVANT: NA2SO4 0.5M DETECTEUR: UV 205 nm



Molecular Weight Distribution Averages

Weight Average	17348	Number Average	16389
Z Average	17896	Dispersivity	1.059
Viscosity Average	17348	Intrinsic Viscosity	0.000
Z + 1 Average	18493	Peak Mol Weight	16805
Mz / Mw	1.032	Mz+1 / Mw	1.066

APPENDIX XV c

Frequency Distribution of Molecular Weight Components

Column TSK 3000

17,450 M.W. Fraction

Ret. time	Mol. Wt.	Cum. %	Slice area
5.500	33718	0.164	2253
5.600	32082	0.268	4121
5.700	30573	0.425	6269
5.800	29179	0.620	7753
5.900	27887	0.818	7849
6.000	26687	1.061	9665
6.100	25568	1.386	12938
6.200	24524	1.874	19415
6.300	23546	2.686	32275
6.400	22627	4.060	54655
6.500	21762	6.319	89885
6.600	20945	9.822	139325
6.700	20171	14.904	202139
6.800	19435	21.740	271884
6.900	18734	30.295	340294
7.000	18064	40.233	395290
7.100	17422	50.979	427419
7.200	16805	61.758	428793
7.300	16210	71.752	397504
7.400	15635	80.280	339211
7.500	15079	86.932	264575
7.600	14538	91.674	183630
7.700	14011	94.759	122665
7.800	13498	96.614	73806
7.900	12996	97.660	41585
8.000	12504	98.226	22505
8.100	12022	98.525	11896
8.200	11549	98.681	6291

APPENDIX XV d

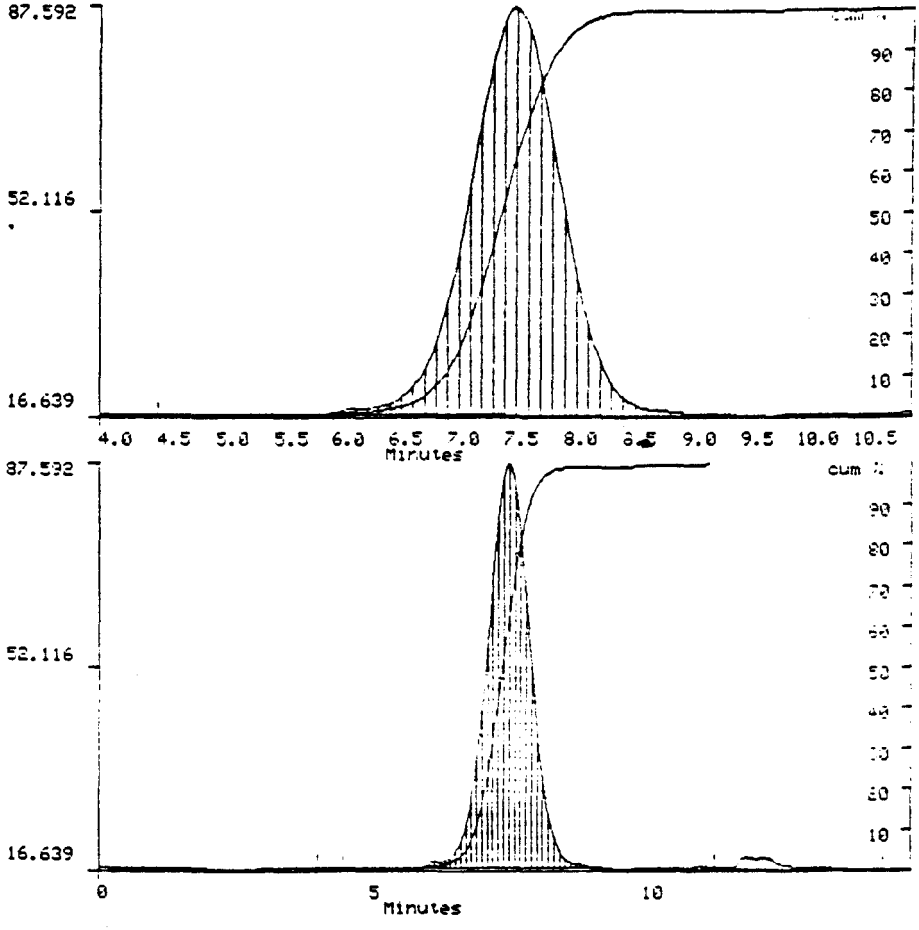
HPLC–GPC Profile of the 15,000 M.W. fraction

Column TSK 3000 Solvent Na_2SO_4 0.5 M Detector UV 205

683H3 4/10/86 13:38:16

Acquisition method	gagts3303	Quantitation method	gagts3303
Units	200ug	System number	1
Channel	1	Vial	4
Injection	1	Total injections	1
Run time	15.00 min	Sample rate	2 per sec
Injection volume	20 uL	Sample amount	
Internal standard amt		Scale factor	
Mode	Analysis	Response factors	Replace
Version	REV03.0	Channel to calibrate	1
Description			

COLONNE: TSK 3000SW III SOLVANT: NA2SO4 0,5M DETECTEUR: UV 205 nm



Molecular Weight Distribution Averages

Weight Average	15273	Number Average	14464
Z Average	16133	Dispersivity	1.056
Viscosity Average	15273	Intrinsic Viscosity	0.000
Z + 1 Average	17948	Peak Mol Weight	15079
Mz / Mw	1.056	Mz+1 / Mw	1.175

APPENDIX XV d

Frequency Distribution of Molecular Weight Components

Column TSK 3000

15,000 M.W. Fraction

Ret. time	Mol. Wt.	Cum. %	Slice area
6.100	25568	0.989	5687
6.200	24524	1.161	6706
6.300	23546	1.350	7358
6.400	22627	1.598	9706
6.500	21762	1.961	14138
6.600	20945	2.538	22520
6.700	20171	3.486	36984
6.800	19435	5.034	60404
6.900	18734	7.478	95382
7.000	18064	11.143	143035
7.100	17422	16.356	203423
7.200	16805	23.308	271280
7.300	16210	31.942	336915
7.400	15635	41.953	390649
7.500	15079	52.630	419002
7.600	14538	63.334	415348
7.700	14011	73.058	379441
7.800	13498	81.241	319326
7.900	12996	87.572	247071
8.000	12504	92.093	176417
8.100	12022	95.083	116658
8.200	11549	96.935	72261
8.300	11084	98.018	42294
8.400	10627	98.627	23751
8.500	10177	98.965	13208
8.600	9734	99.160	7602
8.700	9298	99.281	4730
8.800	8869	99.389	4223
8.900	8447	99.456	2592

APPENDIX XV e

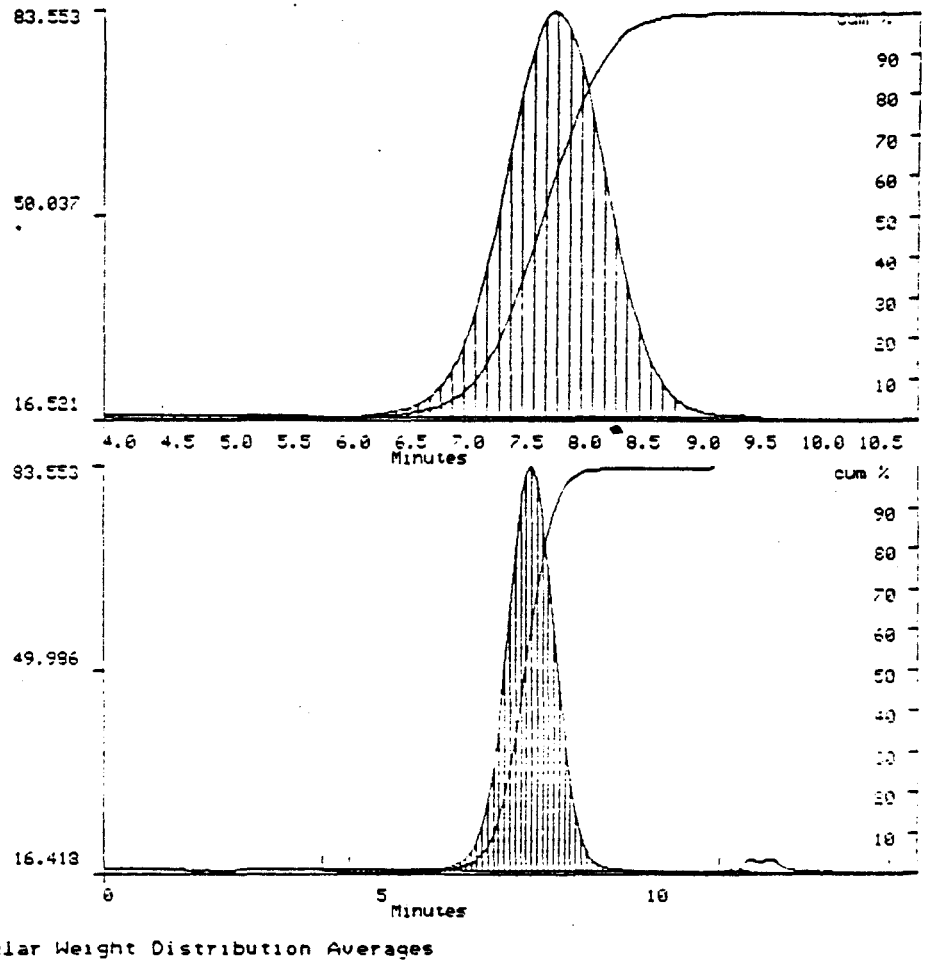
HPLC–GPC Profile of the 13,300 M.W. fraction

Column TSK 3000 Solvent Na_2SO_4 0.5 M Detector UV 205

683H4 4/10/86 19:55:03

Acquisition method	gagts3303	Quantitation method	gagts3303
Units	200ug	System number	1
Channel	1	Vial	5
Injection	1	Total injections	1
Run time	15.00 min	Sample rate	2 per sec
Injection volume	20 uL	Sample amount	
Internal standard amt		Scale factor	
Mode	Analysis	Response factors	Replace
Version	REV03.0	Channel to calibrate	1
Description			

COLONNE: TSK 3000SW III SOLVANT: NA2SO4 0,5M DETECTEUR: UV 205 nm



Molecular Weight Distribution Averages

Weight Average	13707	Number Average	13053
Z Average	14665	Dispersivity	1.050
Viscosity Average	13707	Intrinsic Viscosity	0.000
Z + 1 Average	16840	Peak Mol Weight	13498
Mz / Mw	1.070	Mz+1 / Mw	1.229

APPENDIX XV e

Frequency Distribution of Molecular Weight Components

Column TSK 3000

13,300 M.W. Fraction

Ret. time	Mol. Wt.	Cum. %	Slice area
6.200	24524	0.888	1687
6.300	23546	0.969	3474
6.400	22627	1.091	5209
6.500	21762	1.265	7416
6.600	20945	1.515	10660
6.700	20171	1.887	15869
6.800	19435	2.451	24065
6.900	18734	3.324	37260
7.000	18064	4.680	57836
7.100	17422	6.726	87265
7.200	16805	9.707	127161
7.300	16210	13.852	176835
7.400	15635	19.316	233074
7.500	15079	26.133	290800
7.600	14538	34.160	342436
7.700	14011	43.071	380149
7.800	13498	52.402	398038
7.900	12996	61.623	393354
8.000	12504	70.220	366741
8.100	12022	77.821	324259
8.200	11549	84.152	270102
8.300	11084	89.125	212131
8.400	10627	92.804	156923
8.500	10177	95.371	109535
8.600	9734	97.061	72063
8.700	9298	98.113	44901
8.800	8869	98.742	26819
8.900	8447	99.112	15809
9.000	8031	99.334	9459
9.100	7624	99.473	5936

APPENDIX XV f

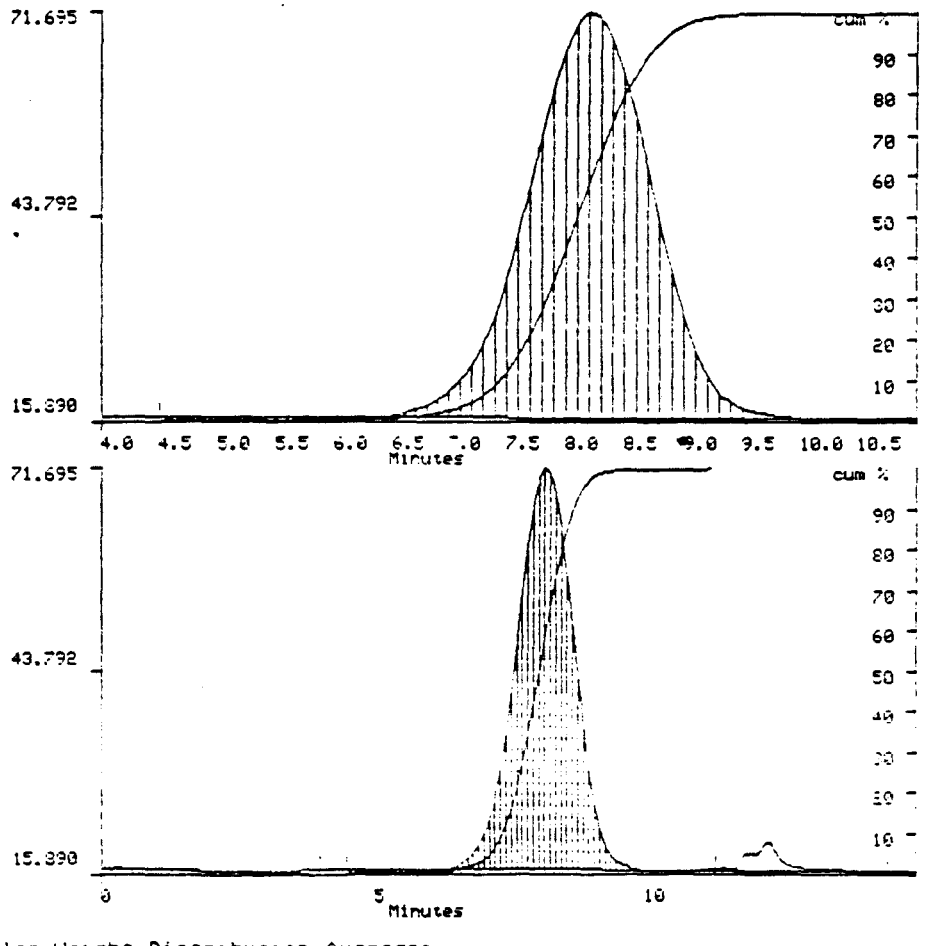
HPLC-GPC Profile of the 11,700 M.W. fraction

Column TSK 3000 Solvent Na_2SO_4 0.5 M Detector UV 205

683H5 4/10/86 20:11:46

Acquisition method	gagts3303	Quantitation method	gagts3303
Units	200ug	System number	1
Channel	1	Vial	6
Injection	1	Total injections	1
Run time	15.00 min	Sample rate	2 per sec
Injection volume	20 uL	Sample amount	
Internal standard amt		Scale factor	
Mode	Analysis	Response factors	Replace
Version	REV03.0	Channel to calibrate	1
Description			

COLONNE: TSK 3000SW III SOLVANT: NA2SO4 0,5M DETECTEUR: UV 205 nm



Molecular Weight Distribution Averages

Weight Average	12091	Number Average	11438
Z Average	12937	Dispersivity	1.057
Viscosity Average	12091	Intrinsic Viscosity	0.000
Z + 1 Average	14501	Peak Mol Weight	11549
Mz / Mw	1.070	Mz+1 / Mw	1.199

APPENDIX XV f

Frequency Distribution of Molecular Weight Components

Column TSK 3000

11,750 M.W. Fraction

Ret. time	Mol. Wt.	Cum. %	Slice area
6.500	21762	0.908	2535
6.600	20945	1.025	4759
6.700	20171	1.220	7988
6.800	19435	1.512	11885
6.900	18734	1.934	17246
7.000	18064	2.542	24831
7.100	17422	3.412	35506
7.200	16805	4.649	50502
7.300	16210	6.389	71004
7.400	15635	8.783	97712
7.500	15079	11.982	130595
7.600	14538	16.107	168368
7.700	14011	21.227	209014
7.800	13498	27.331	249146
7.900	12996	34.300	284492
8.000	12504	41.936	311628
8.100	12022	49.956	327356
8.200	11549	58.042	330085
8.300	11084	65.866	319358
8.400	10627	73.132	296613
8.500	10177	79.602	264093
8.600	9734	85.104	224581
8.700	9298	89.558	181624
8.800	8869	92.973	139389
8.900	8447	95.444	100875
9.000	8031	97.123	68739
9.100	7624	98.207	43995
9.200	7223	98.858	26575
9.300	6831	99.250	16017
9.400	6447	99.492	9846
9.500	6071	99.632	5745

APPENDIX XV g

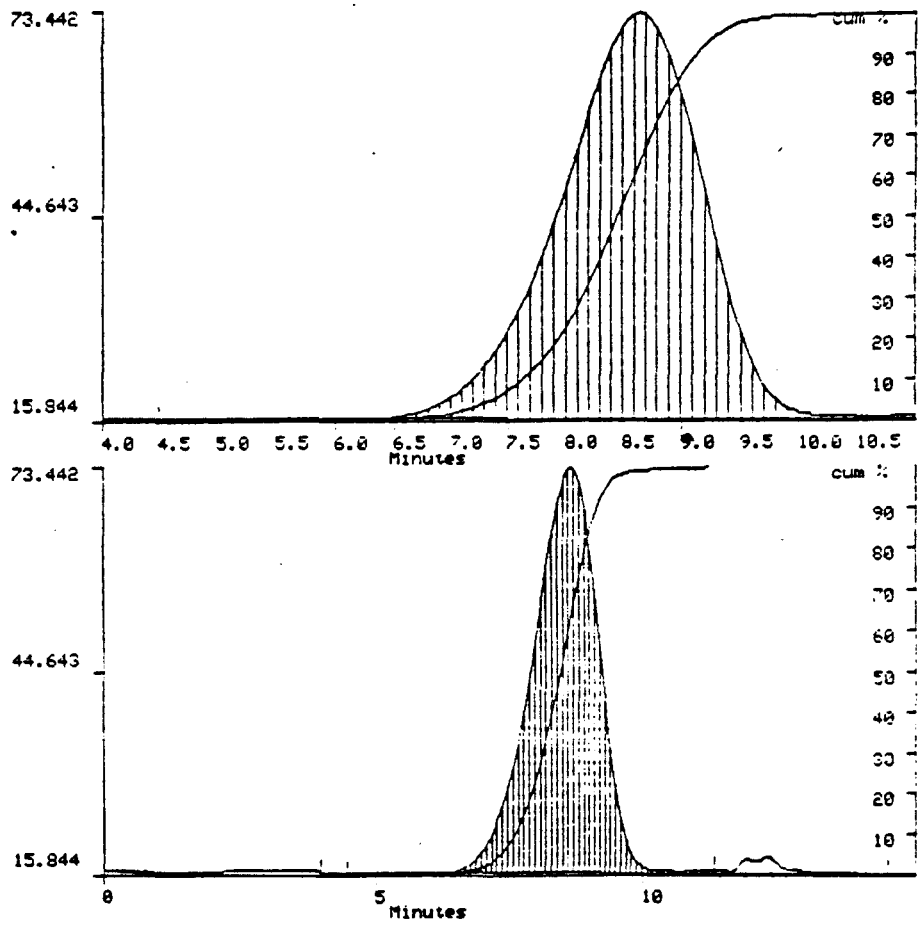
HPLC-GPC Profile of the 10,400 M.W. fraction

Column TSK 3000 Solvent Na_2SO_4 0.5 M Detector UV 205

683H6 4/10/86 20:28:33

Acquisition method	gagts3303	Quantitation method	gagts3303
Units	200ug	System number	1
Channel	1	Vial	7
Injection	1	Total injections	1
Run time	15.00 min	Sample rate	2 per sec
Injection volume	20 uL	Sample amount	
Internal standard amt		Scale factor	
Mode	Analysis	Response factors	Replace
Version	REV03.0	Channel to calibrate	1
Description			

COLONNE: TSK 3000SW III SOLVANT: NA2SO4 0,5M DETECTEUR: UV 205 nm



Molecular Weight Distribution Averages

Weight Average	10598	Number Average	9682
Z Average	11638	Dispersivity	1.094
Viscosity Average	10588	Intrinsic Viscosity	0.000
Z + 1 Average	14595	Peak Mol Weight	9734
Mz / Mw	1.118	Mz+1 / Mw	1.379

APPENDIX XV g

Frequency Distribution of Molecular Weight Components

Column TSK 3000

10,400 M.W. Fraction

Ret. time	Mol. Wt.	Cum. %	Slice area
6.500	21762	0.686	1815
6.600	20945	0.761	3693
6.700	20171	0.889	6310
6.800	19435	1.090	9896
6.900	18734	1.388	14618
7.000	18064	1.808	20648
7.100	17422	2.385	28415
7.200	16805	3.166	38376
7.300	16210	4.205	51071
7.400	15635	5.513	64334
7.500	15079	7.172	81536
7.600	14538	9.223	100856
7.700	14011	11.749	124224
7.800	13498	14.802	150091
7.900	12996	18.463	179982
8.000	12504	22.761	211313
8.100	12022	27.697	242711
8.200	11549	33.264	273710
8.300	11084	39.382	300777
8.400	10627	45.943	322598
8.500	10177	52.810	337635
8.600	9734	59.788	343103
8.700	9298	66.656	337665
8.800	8869	73.181	320817
8.900	8447	79.149	293397
9.000	8031	84.383	257376
9.100	7624	88.757	215057
9.200	7223	92.218	170169
9.300	6831	94.803	127094
9.400	6447	96.615	89082
9.500	6071	97.808	58644
9.600	5705	98.544	36206
9.700	5349	98.981	21468
9.800	5003	99.230	12242
9.900	4668	99.375	7155
10.000	4343	99.463	4318

APPENDIX XV h

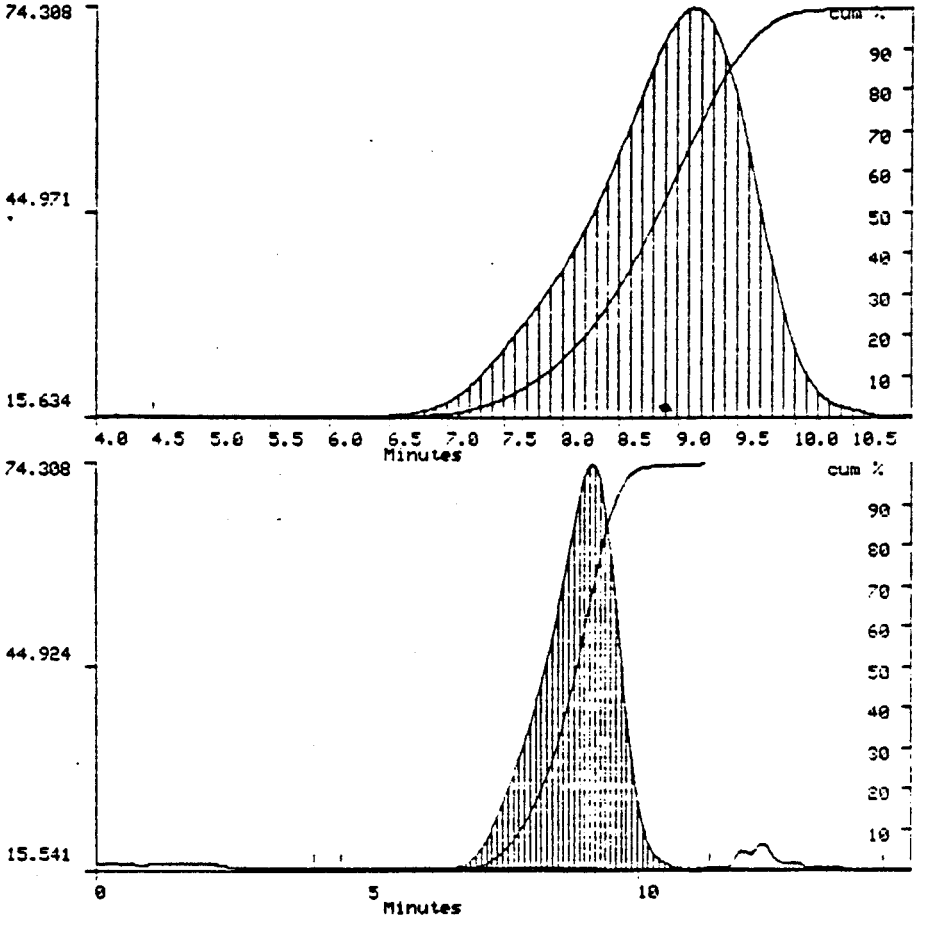
HPLC–GPC Profile of the 9,000 M.W. fraction

Column TSK 3000 Solvent Na_2SO_4 0.5 M Detector UV 205

683H7 4/10/86 20:45:13

Acquisition method	gagts3303	Quantitation method	gagts3303
Units	200ug	System number	1
Channel	1	Vial	8
Injection	1	Total injections	1
Run time	15.00 min	Sample rate	2 per sec
Injection volume	20 uL	Sample amount	
Internal standard amt		Scale factor	
Mode	Analysis	Response factors	Replace
Version	REV03.0	Channel to calibrate	1
Description			

COLONNE: TSK 3000SW III SOLVANT: NA2SO4 0,5M DETECTEUR: UV 205 nm



Molecular Weight Distribution Averages

Weight Average	8999	Number Average	8078
Z Average	10065	Dispersivity	1.114
Viscosity Average	8999	Intrinsic Viscosity	0.000
Z + 1 Average	11505	Peak Mol Weight	7624
Mz / Mw	1.118	Mz+1 / Mw	1.278

APPENDIX XV h

Frequency Distribution of Molecular Weight Components

Column TSK 3000

9,000 M.W. Fraction

Ret. time	Mol. Wt.	Cum. %	Slice area
6.500	21762	0.156	1170
6.600	20945	0.195	2149
6.700	20171	0.262	3712
6.800	19435	0.370	5946
6.900	18734	0.549	9859
7.000	18064	0.817	14847
7.100	17422	1.207	21516
7.200	16805	1.754	30191
7.300	16210	2.483	40269
7.400	15635	3.413	51345
7.500	15079	4.563	63535
7.600	14538	5.945	76329
7.700	14011	7.569	89671
7.800	13499	9.447	103739
7.900	12996	11.599	118753
8.000	12504	14.043	135058
8.100	12022	16.802	152327
8.200	11549	19.899	171037
8.300	11084	23.369	191631
8.400	10627	27.242	213885
8.500	10177	31.549	237874
8.600	9734	36.307	262749
8.700	9298	41.520	287899
8.800	8869	47.180	312536
8.900	8447	53.210	332999
9.000	8031	59.480	346246
9.100	7624	65.834	350933
9.200	7223	72.070	344406
9.300	6831	77.962	325336
9.400	6447	83.291	294315
9.500	6071	87.869	252853
9.600	5705	91.583	205090
9.700	5349	94.407	155940
9.800	5003	96.423	111353
9.900	4668	97.768	74252
10.000	4343	98.623	47259
10.100	4031	99.147	28910
10.200	3730	99.464	17507
10.300	3442	99.660	10842
10.400	3167	99.788	7069

APPENDIX XV i

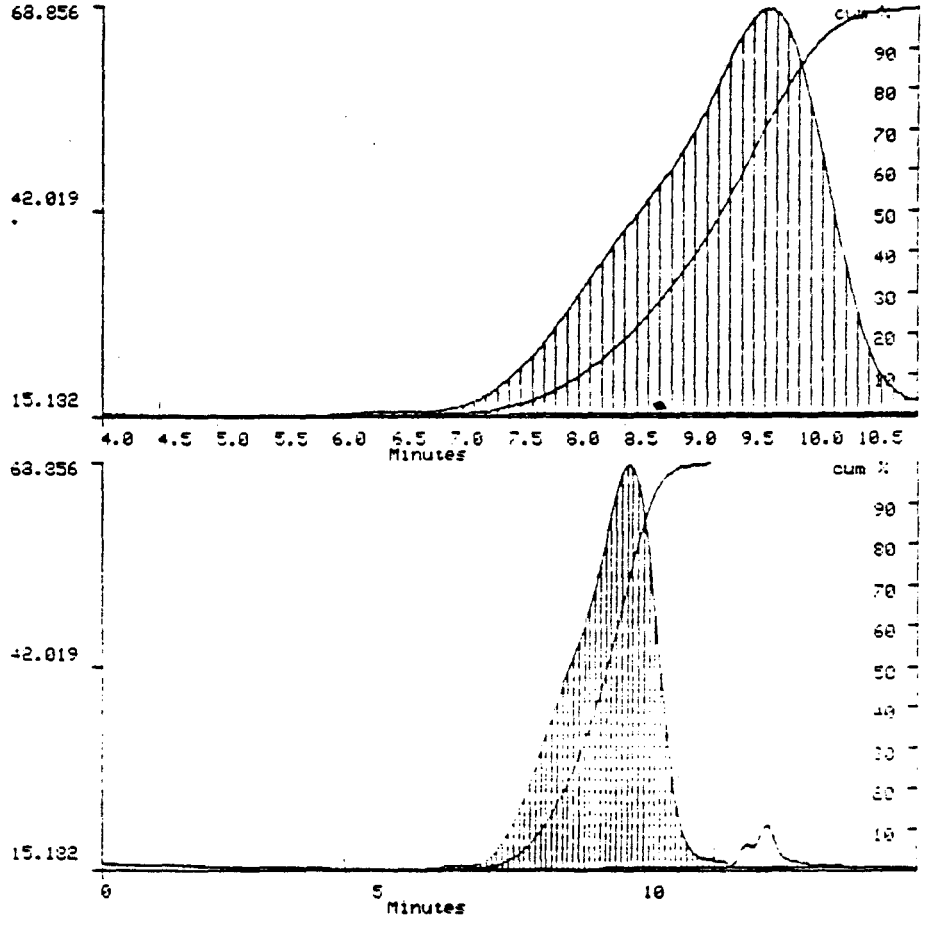
HPLC–GPC Profile of the 7,400 M.W. fraction

Column TSK 3000 Solvent Na_2SO_4 0.5 M Detector UV 205

683H8 4/10/86 21:02:00

Acquisition method	gagts3300	Quantitation method	gagts3300
Units	200ug	System number	1
Channel	1	Vial	9
Injection	1	Total injections	1
Run time	15.00 min	Sample rate	2 per sec
Injection volume	20 uL	Sample amount	
Internal standard amt		Scale factor	
Mode	Analysis	Response factors	Replace
Version	REV03.0	Channel to calibrate	1
Description			

COLONNE: TSK 3000SW III SOLVANT: NA2SO4 0,5M DETECTEUR: UV 205 nm



Molecular Weight Distribution Averages

Weight Average	7285	Number Average	6009
Z Average	8927	Dispersivity	1.212
Viscosity Average	7285	Intrinsic Viscosity	0.000
Z + 1 Average	11569	Peak Mol Weight	5349
Mz / Mw	1.225	Mz+1 / Mw	1.588

APPENDIX XV i

Frequency Distribution of Molecular Weight Components

Column TSK 3000

7,400 M.W. Fraction

Ret. time	Mol. Wt.	Cum. %	Slice area
7.100	17422	0.838	8758
7.200	16805	1.053	12252
7.300	16210	1.360	17565
7.400	15635	1.784	24177
7.500	15079	2.353	32486
7.600	14538	3.078	41352
7.700	14011	3.973	51044
7.800	13498	5.069	62593
7.900	12996	6.382	74923
8.000	12504	7.921	87795
8.100	12022	9.687	100783
8.200	11549	11.680	113733
8.300	11084	13.901	126709
8.400	10627	16.340	139214
8.500	10177	18.988	151063
8.600	9734	21.842	162889
8.700	9299	24.905	174754
8.800	8869	28.180	186890
8.900	8447	31.688	200203
9.000	8031	35.458	215141
9.100	7624	39.528	232228
9.200	7223	43.921	250708
9.300	6831	48.664	270638
9.400	6447	53.744	289863
9.500	6071	59.109	306177
9.600	5705	64.661	316787
9.700	5349	70.265	319793
9.800	5003	75.747	312222
9.900	4668	80.904	294271
10.000	4343	85.541	264606
10.100	4031	89.495	225656
10.200	3730	92.679	181708
10.300	3442	95.096	137925
10.400	3167	96.808	97668
10.500	2905	97.939	64517
10.600	2656	98.654	40844
10.700	2420	99.096	25211
10.800	2198	99.384	16413
10.900	1990	99.591	11831

APPENDIX XV j

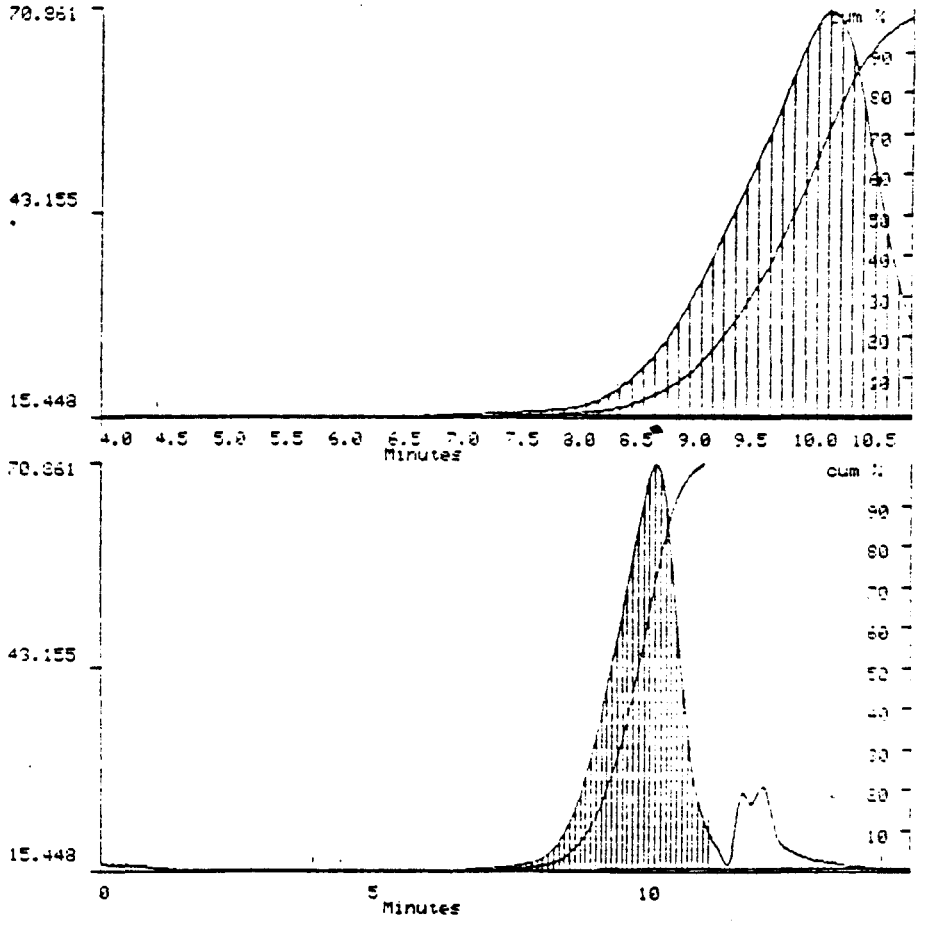
HPLC-GPC Profile of the 5,100 M.W. fraction

Column TSK 3000 Solvent Na_2SO_4 0.5 M Detector UV 205

683H9 4/10/86 21:18:39

Acquisition method	gagts3303	Quantitation method	gagts3303
Units	200ug	System number	1
Channel	1	Vial	10
Injection	1	Total injections	1
Run time	15.00 min	Sample rate	2 per sec
Injection volume	100 uL	Sample amount	
Internal standard amt		Scale factor	
Mode	Analysis	Response factors	Replace
Version	REV03.0	Channel to calibrate	1
Description			

COLONNE: TSK 3000SW III SOLVANT: NA2SO4 0,5M DETECTEUR: UV 205 nm



Molecular Weight Distribution Averages

Weight Average	4741	Number Average	3867
Z Average	6150	Dispersivity	1.226
Viscosity Average	4741	Intrinsic Viscosity	0.000
Z + 1 Average	9871	Peak Mol Weight	3442
Mz / Mw	1.297	Mz+1 / Mw	2.082

APPENDIX XV j

Frequency Distribution of Molecular Weight Components

Column TSK 3000

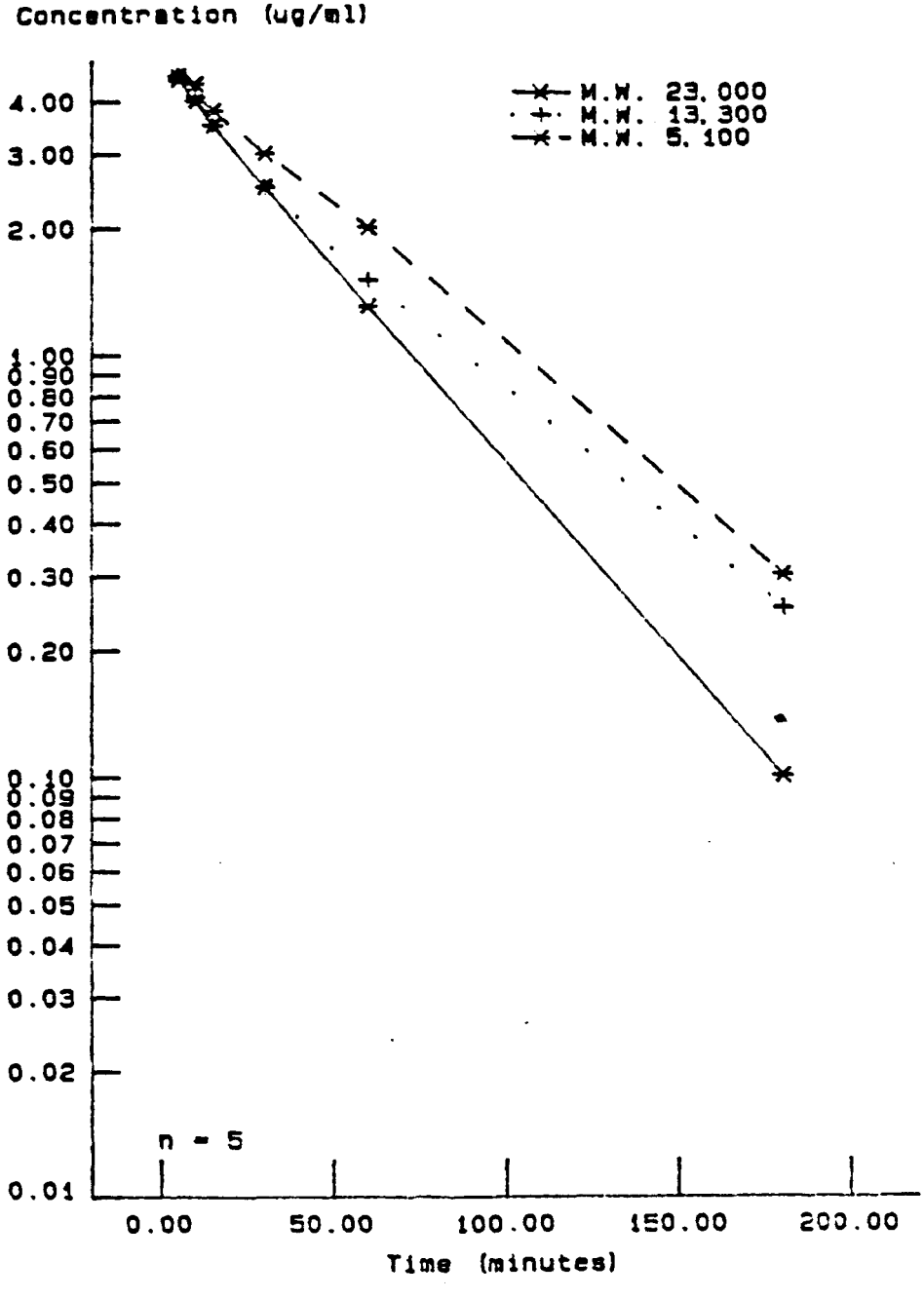
5,100 M.W. Fraction

Ret. time	Mol. Wt.	Cum. %	Slice area
7.000	18064	0.230	1084
7.100	17422	0.259	1345
7.200	16805	0.294	1657
7.300	16210	0.343	2266
7.400	15635	0.397	2540
7.500	15079	0.466	3244
7.600	14538	0.542	3553
7.700	14011	0.633	4229
7.800	13496	0.737	4864
7.900	12996	0.858	5689
8.000	12504	1.010	7135
8.100	12022	1.199	8832
8.200	11549	1.444	11461
8.300	11084	1.764	14991
8.400	10627	2.189	19910
8.500	10177	2.755	26461
8.600	9734	3.493	34559
8.700	9298	4.446	44622
8.800	8869	5.651	56417
8.900	8447	7.150	70170
9.000	8031	8.967	85034
9.100	7624	11.146	102003
9.200	7223	13.706	119963
9.300	6831	16.676	139053
9.400	6447	20.067	158717
9.500	6071	23.890	178975
9.600	5705	28.147	199285
9.700	5349	32.864	220812
9.800	5003	38.062	243315
9.900	4666	43.775	267446
10.000	4343	50.003	291583
10.100	4031	56.686	312822
10.200	3730	63.674	327139
10.300	3442	70.701	328987
10.400	3167	77.388	313003
10.500	2905	83.330	278186
10.600	2656	88.246	230130
10.700	2420	92.034	177325
10.800	2198	94.805	129697
10.900	1990	96.779	92411
11.000	1794	98.198	66458
11.100	1612	99.237	48638
11.200	1443	100.000	35710

APPENDIX XVI

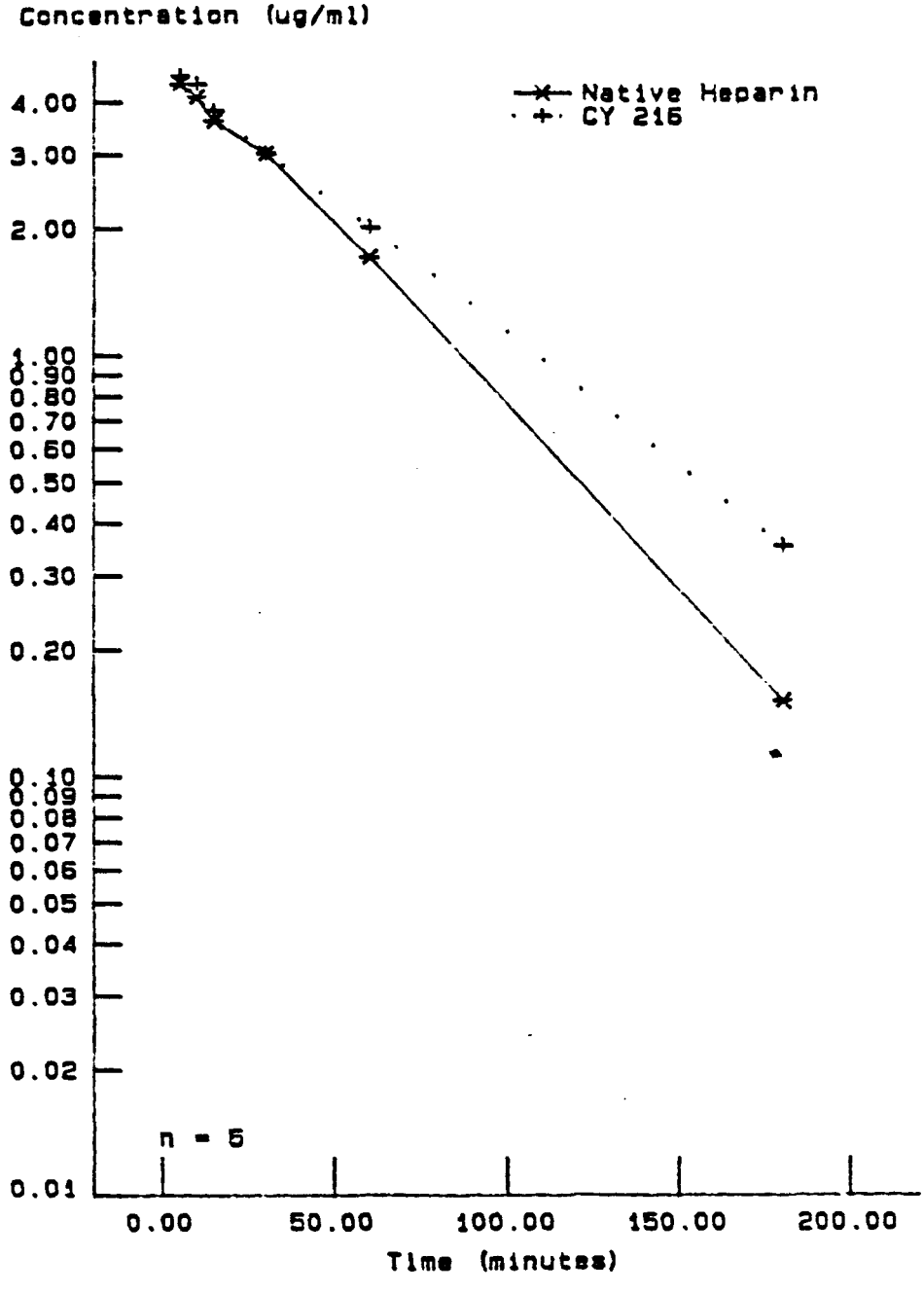
Appendix XVIa

Intravenous Time Course Determined Using the Anti Xa Assay



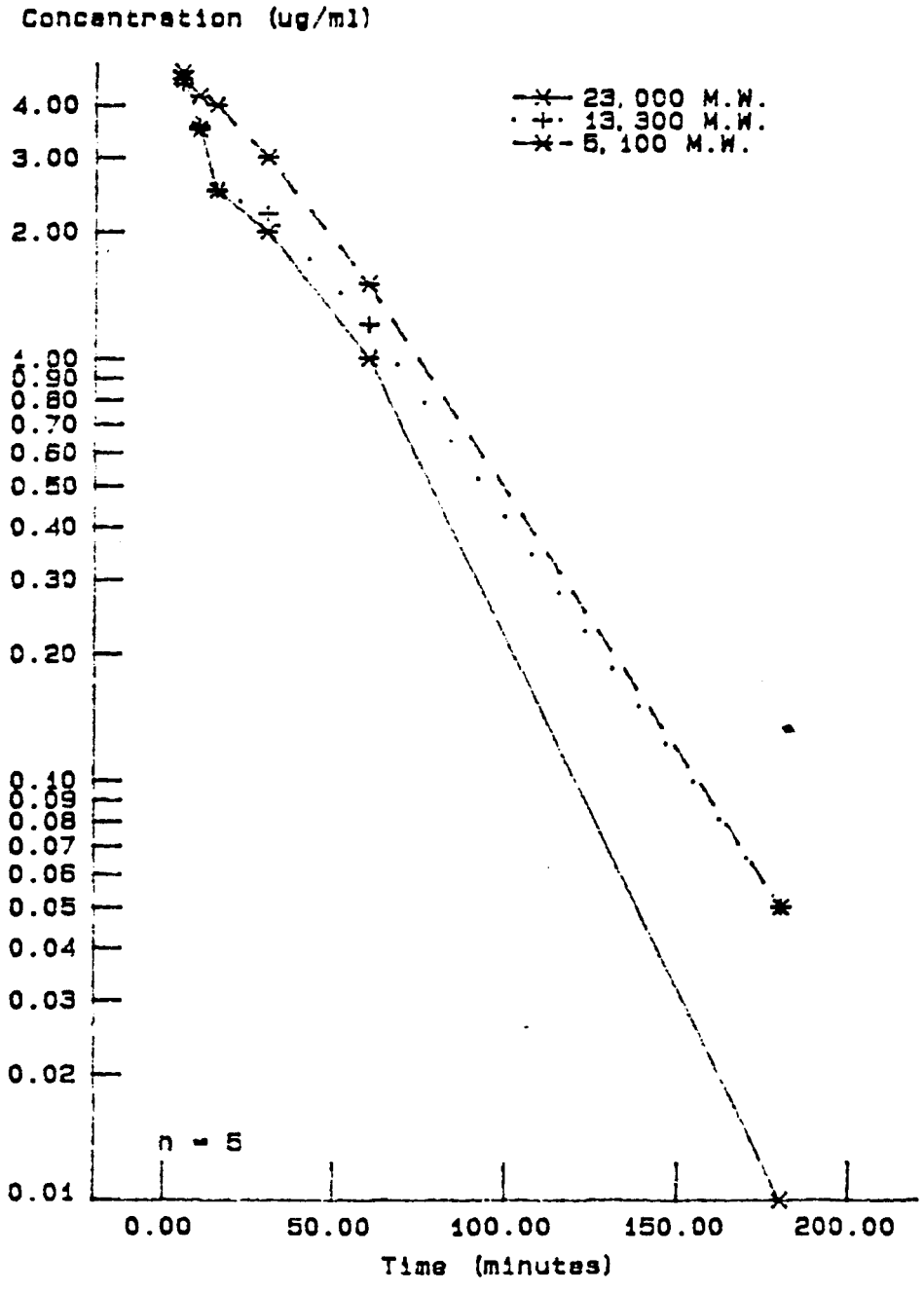
Appendix XVIa

Intravenous Time Course Determined Using the Anti Xa Assay



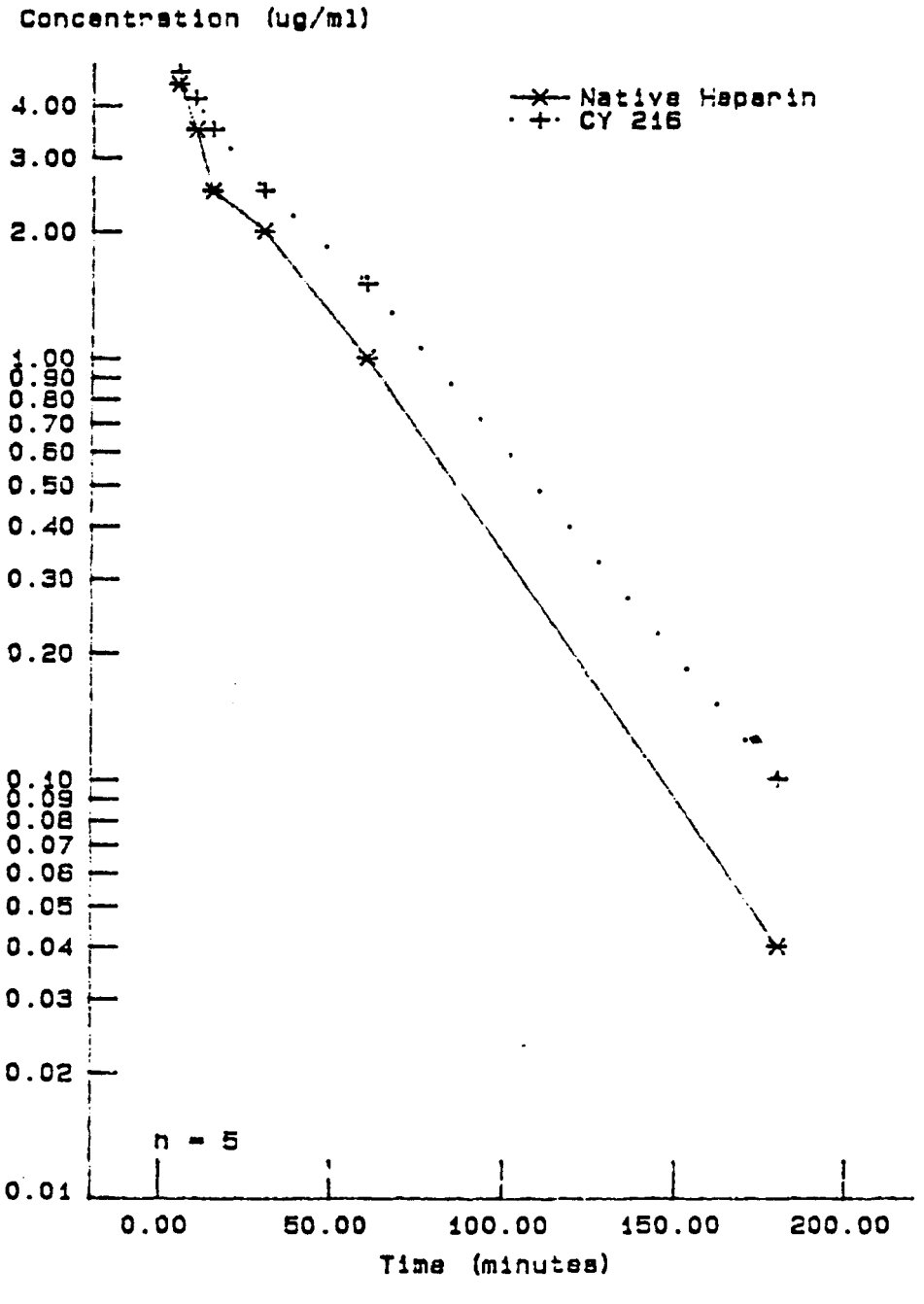
Appendix XVIb

Intravenous Time Course Determined Using the Anti IIa Assay

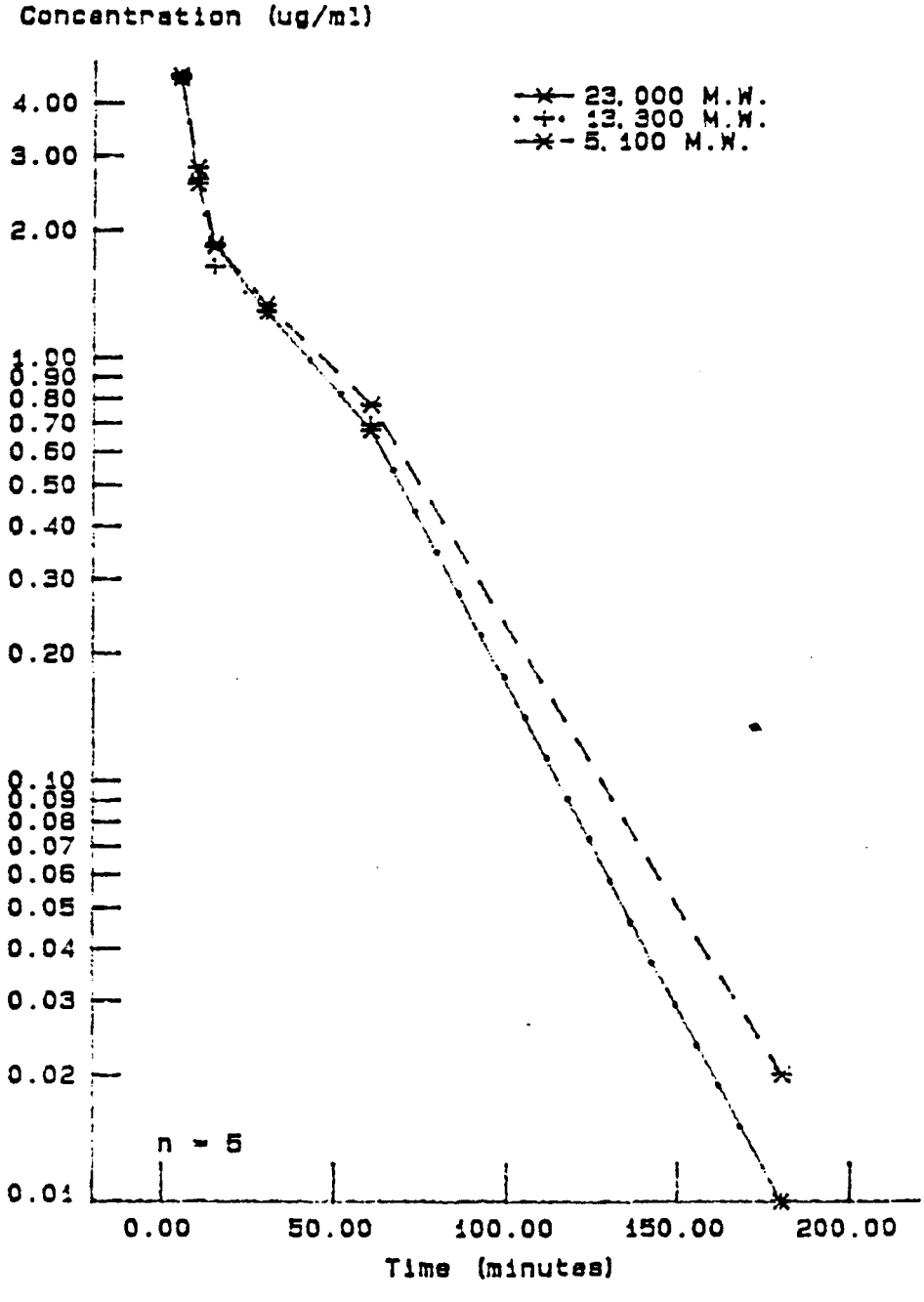


Appendix XVIb

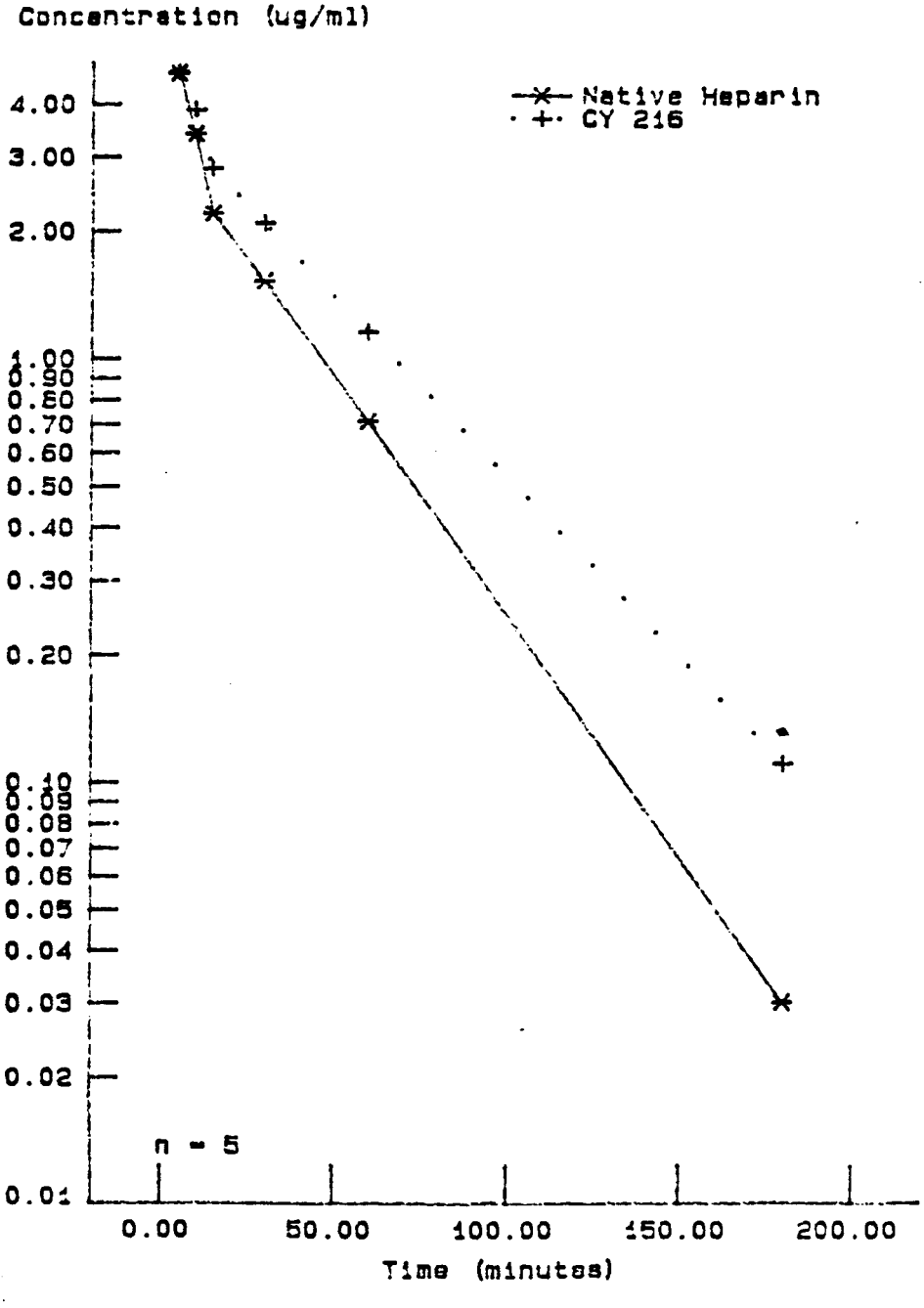
Intravenous Time Course Determined Using the Anti IIa Assay



Appendix XVI c
Intravenous Time Course Using A Dilute Prothrombin Time Assay

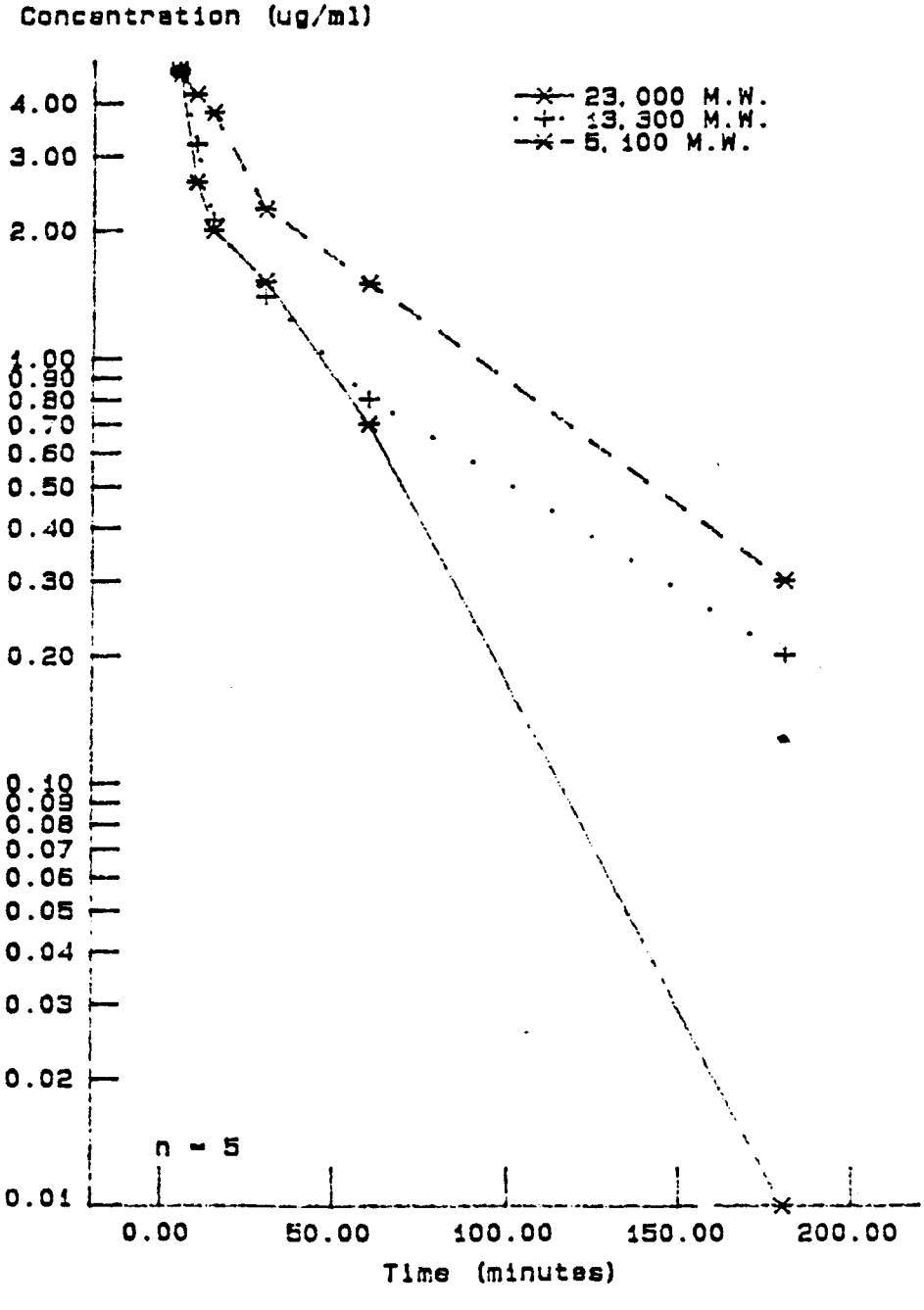


Appendix XVI c
Intravenous Time Course Using A Dilute Prothrombin Time Assay



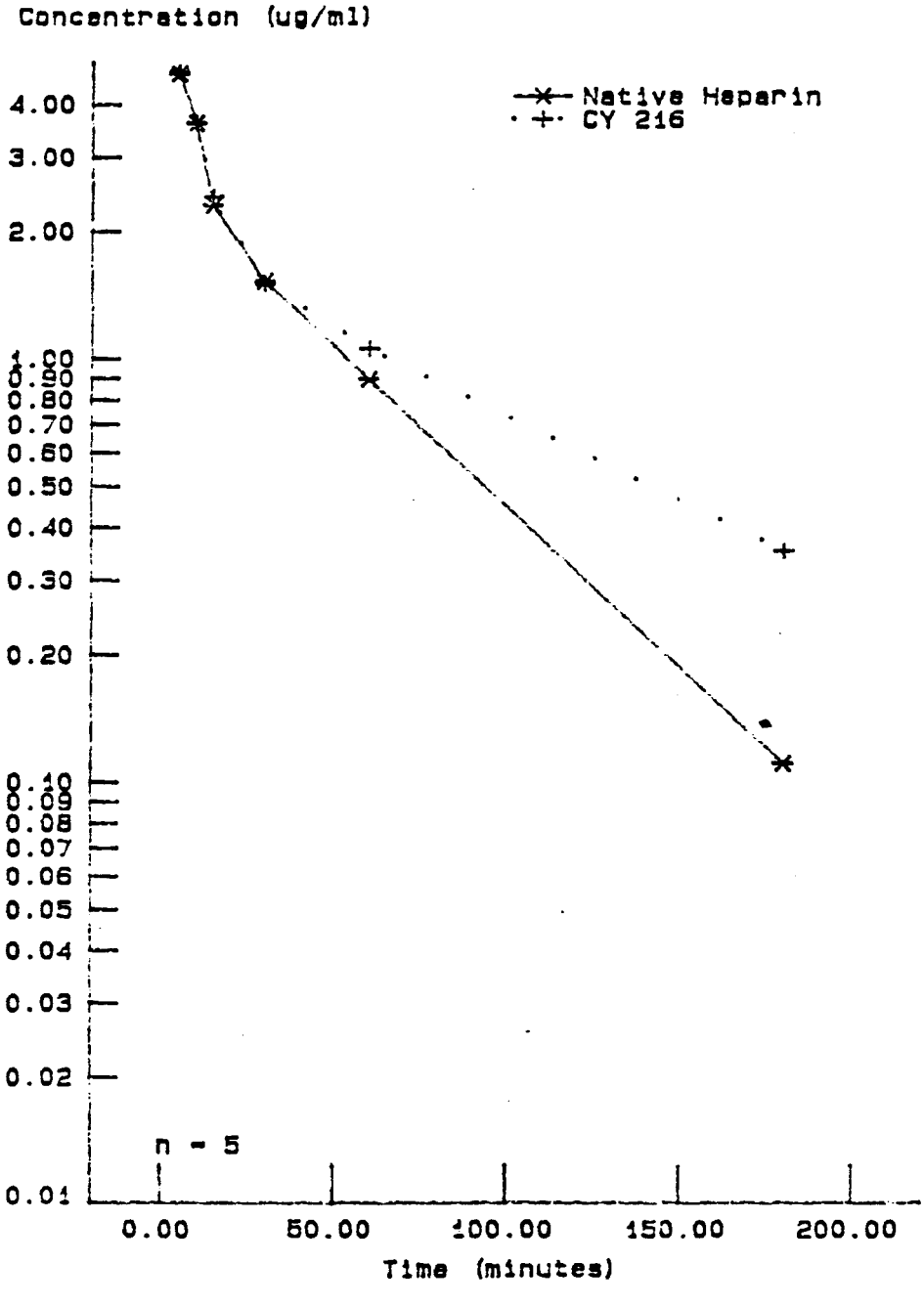
Appendix XVI d

Intravenous Time Course Using the Fibrinopeptide-A Assay



Appendix XVI d

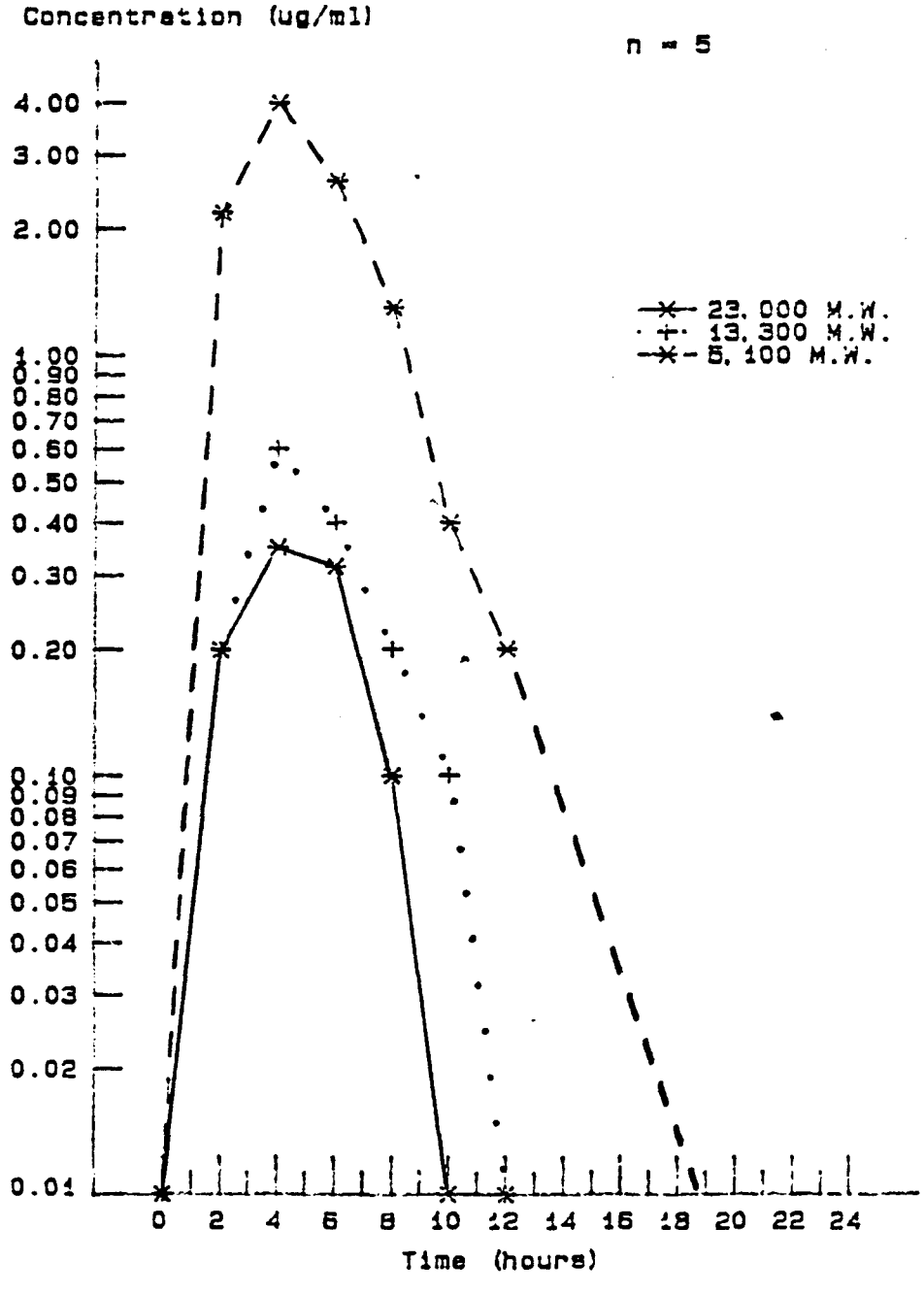
Intravenous Time Course Using the Fibrinopeptide-A Assay



APPENDIX XVII

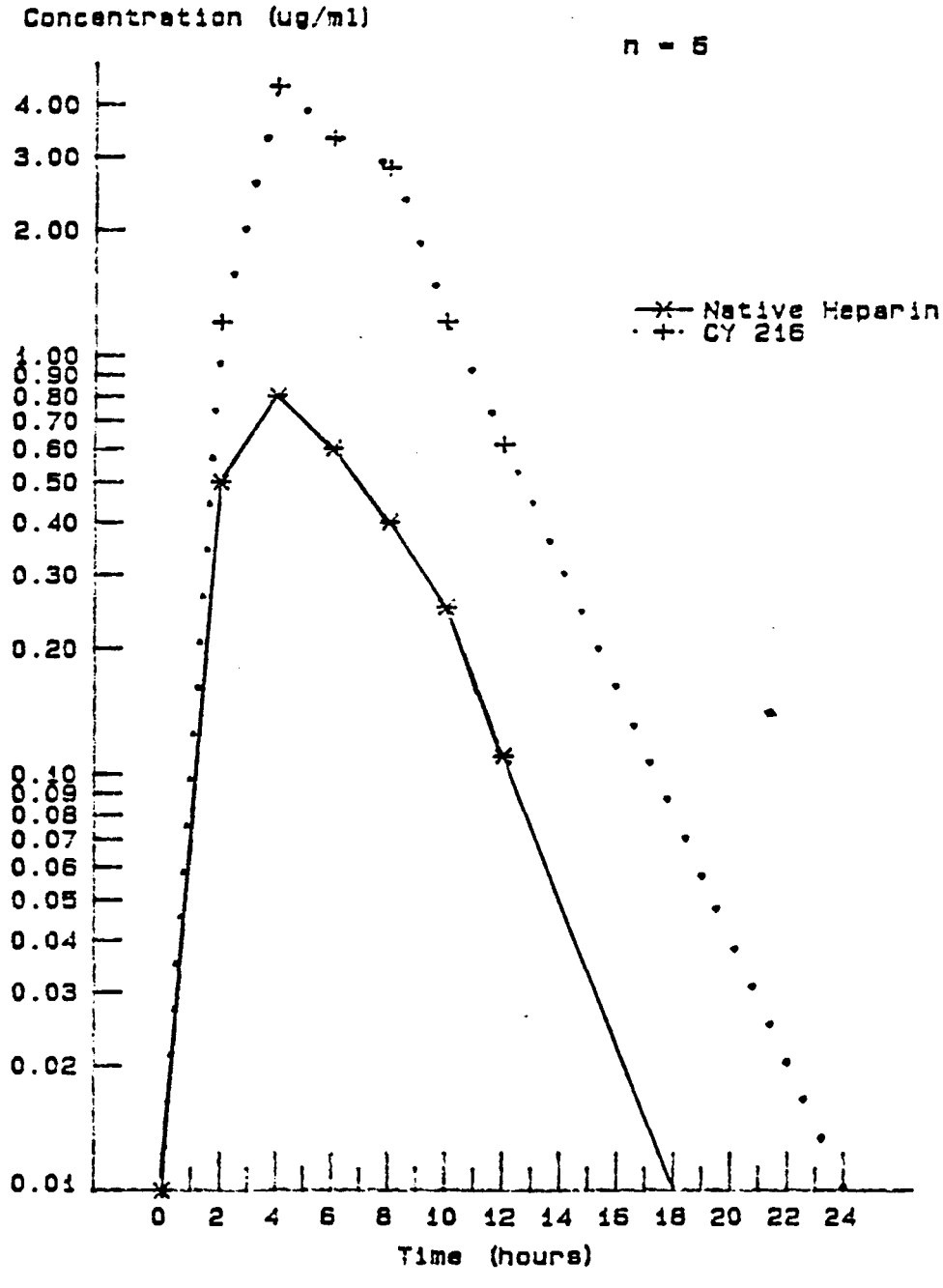
Appendix XVIIa

Subcutaneous Time Course Determined Using the Anti Xa Assay



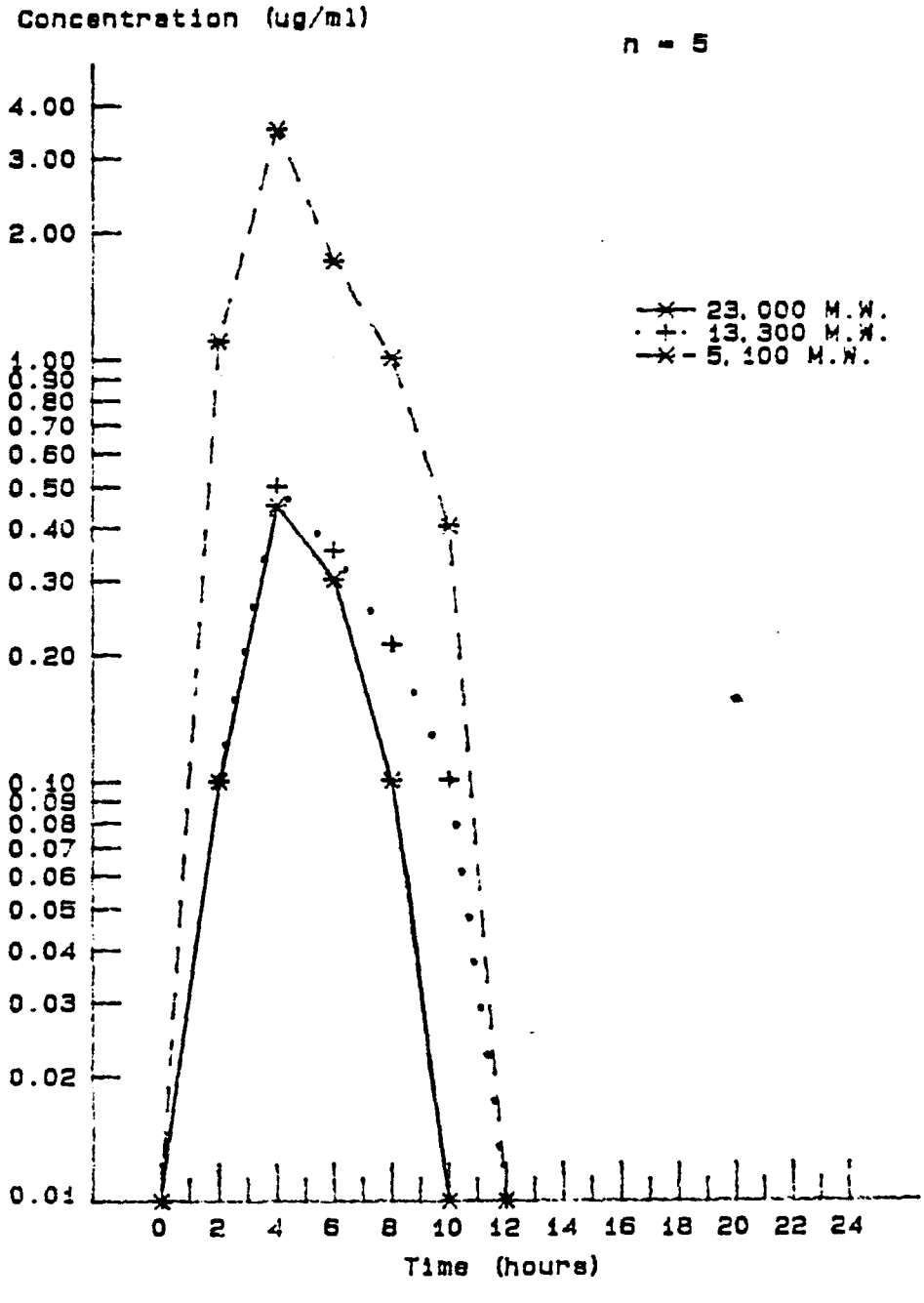
Appendix XVIIa

Subcutaneous Time Course Determined Using the Anti Xa Assay



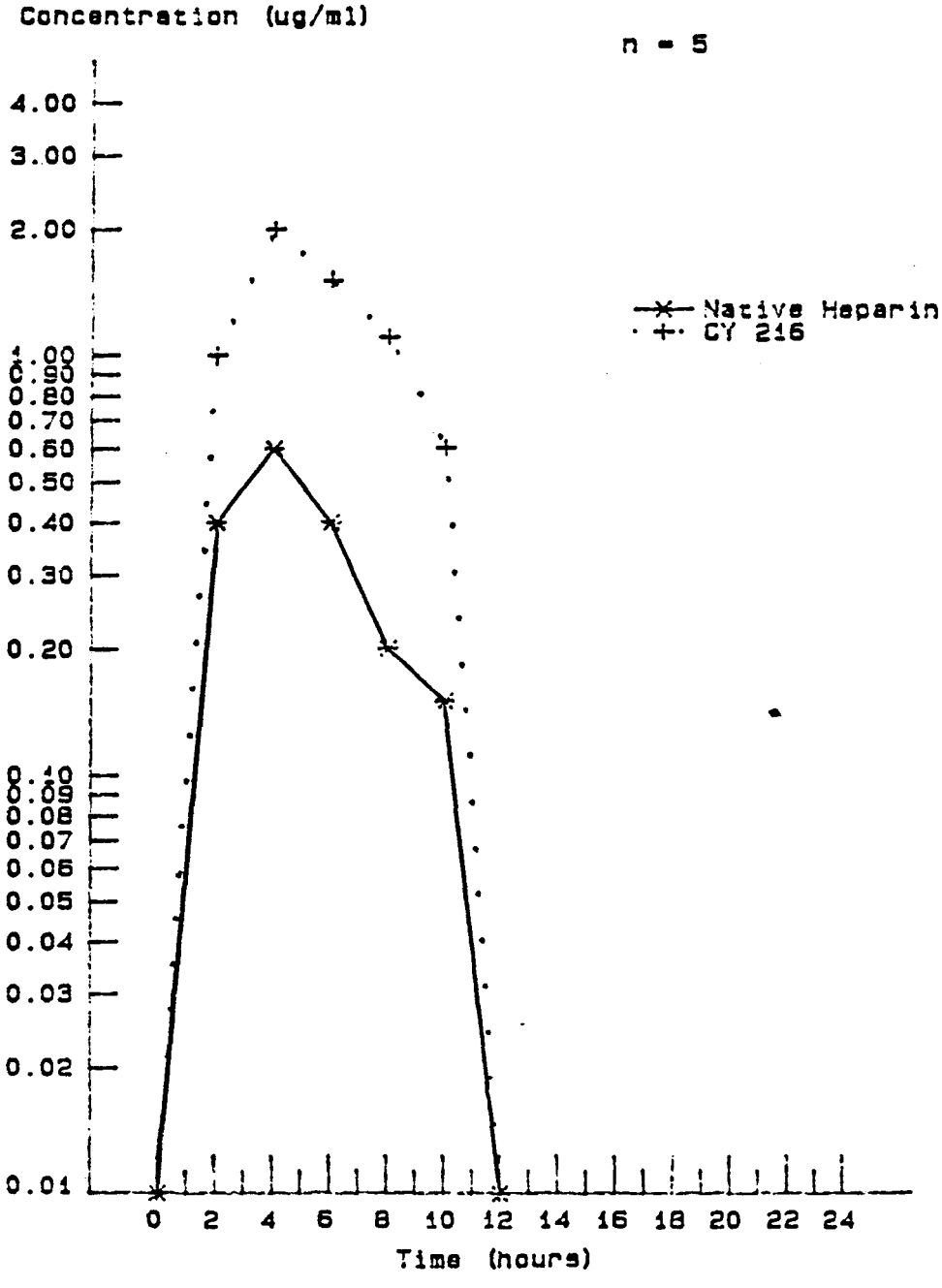
Appendix XVIIb

Subcutaneous Time Course Determined Using the Anti IIa Assay



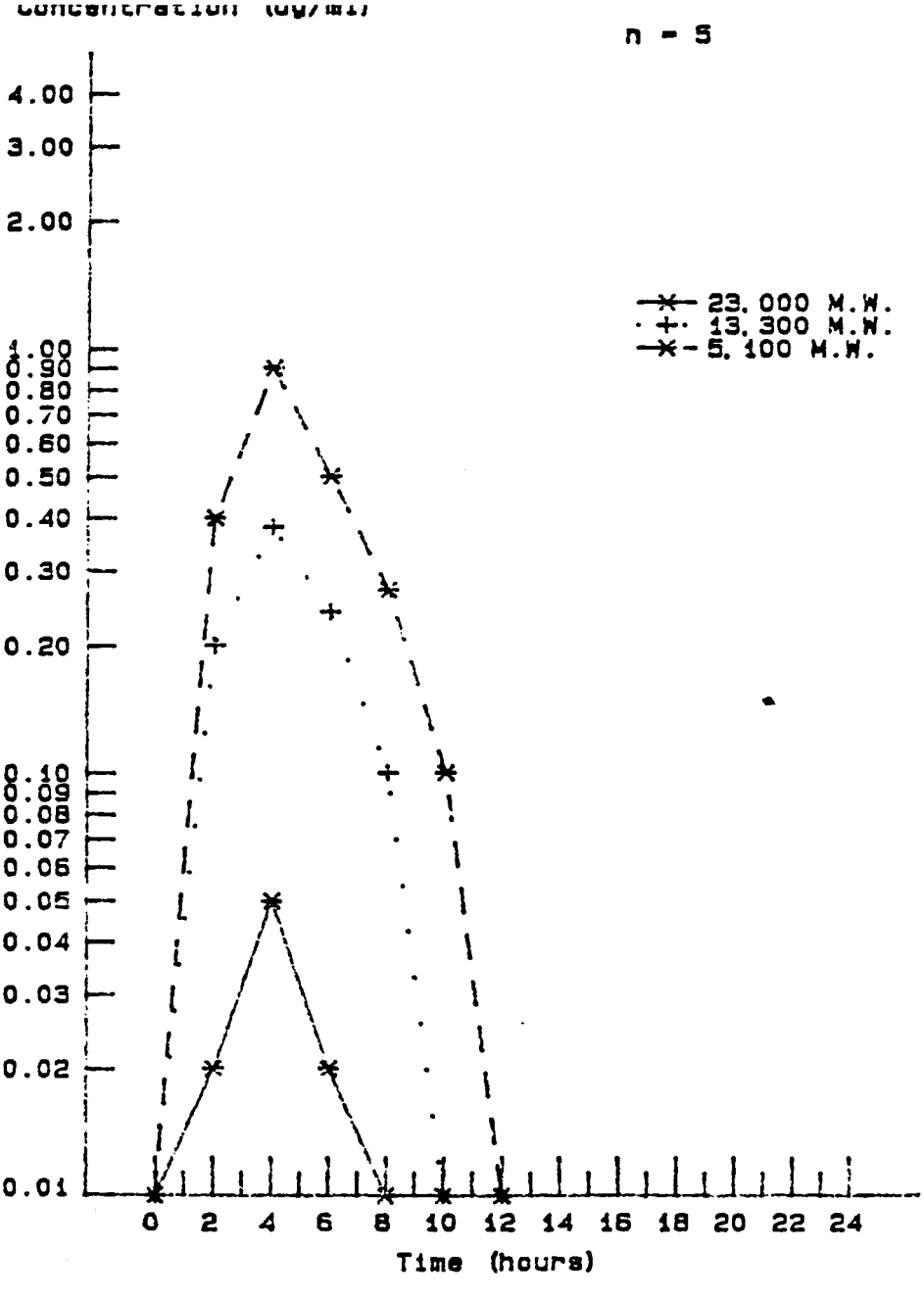
Appendix XVIIb

Subcutaneous Time Course Determined Using the Anti IIa Assay

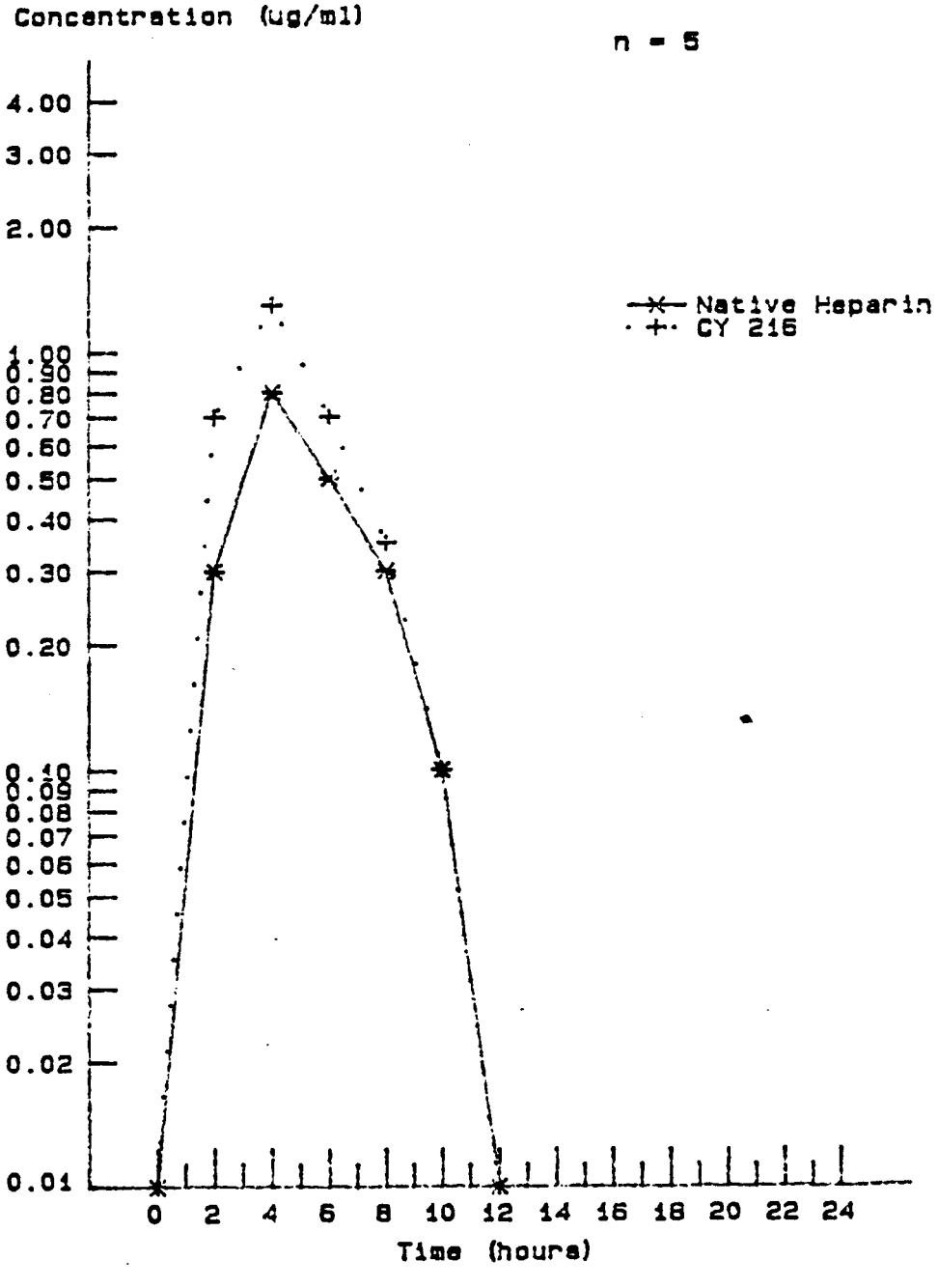


Appendix XVII c

Subcutaneous Time Course Using A Dilute Prothrombin Time Assay

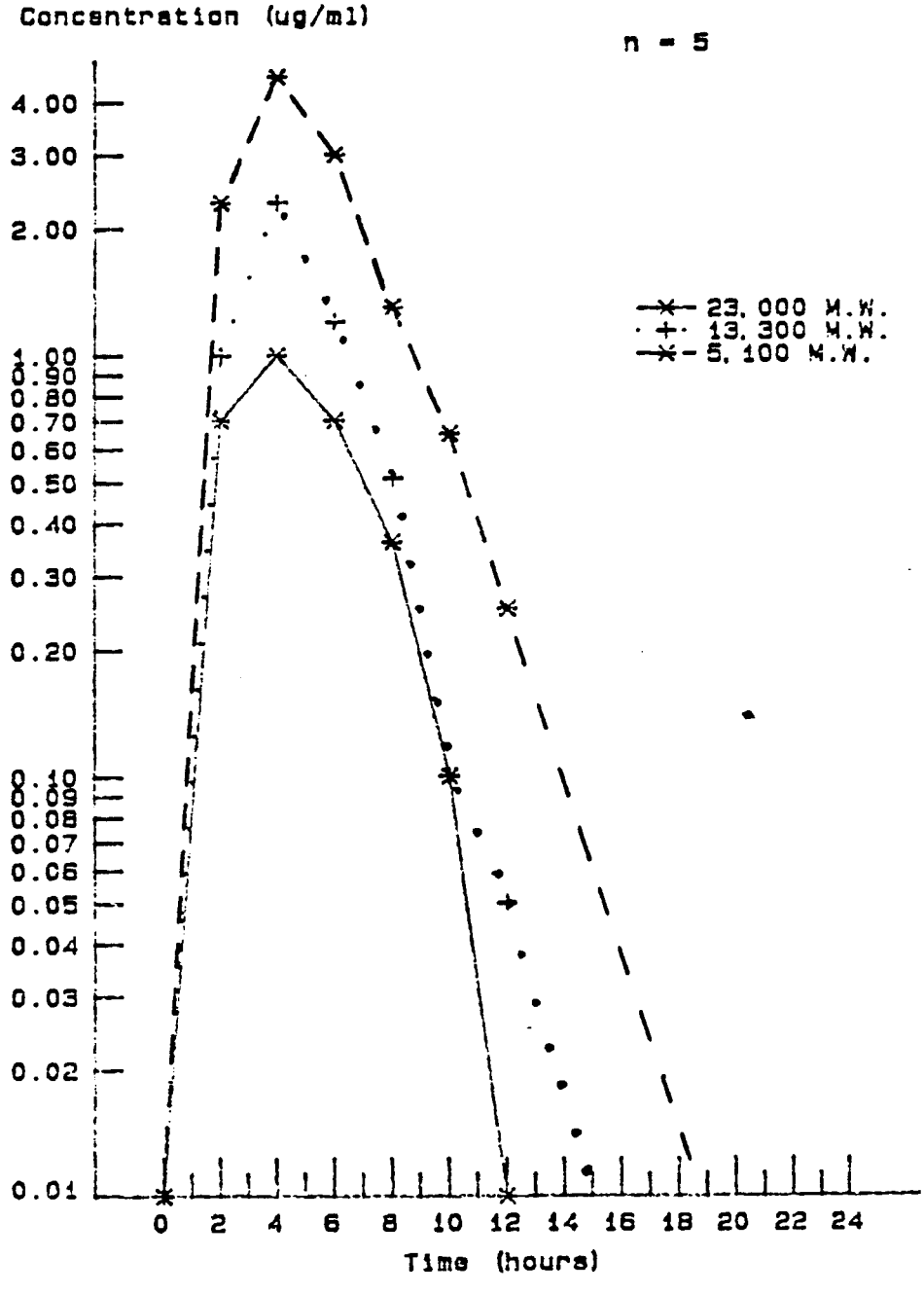


Appendix XVIIc
Subcutaneous Time Course Using A Dilute Prothrombin Time Assay



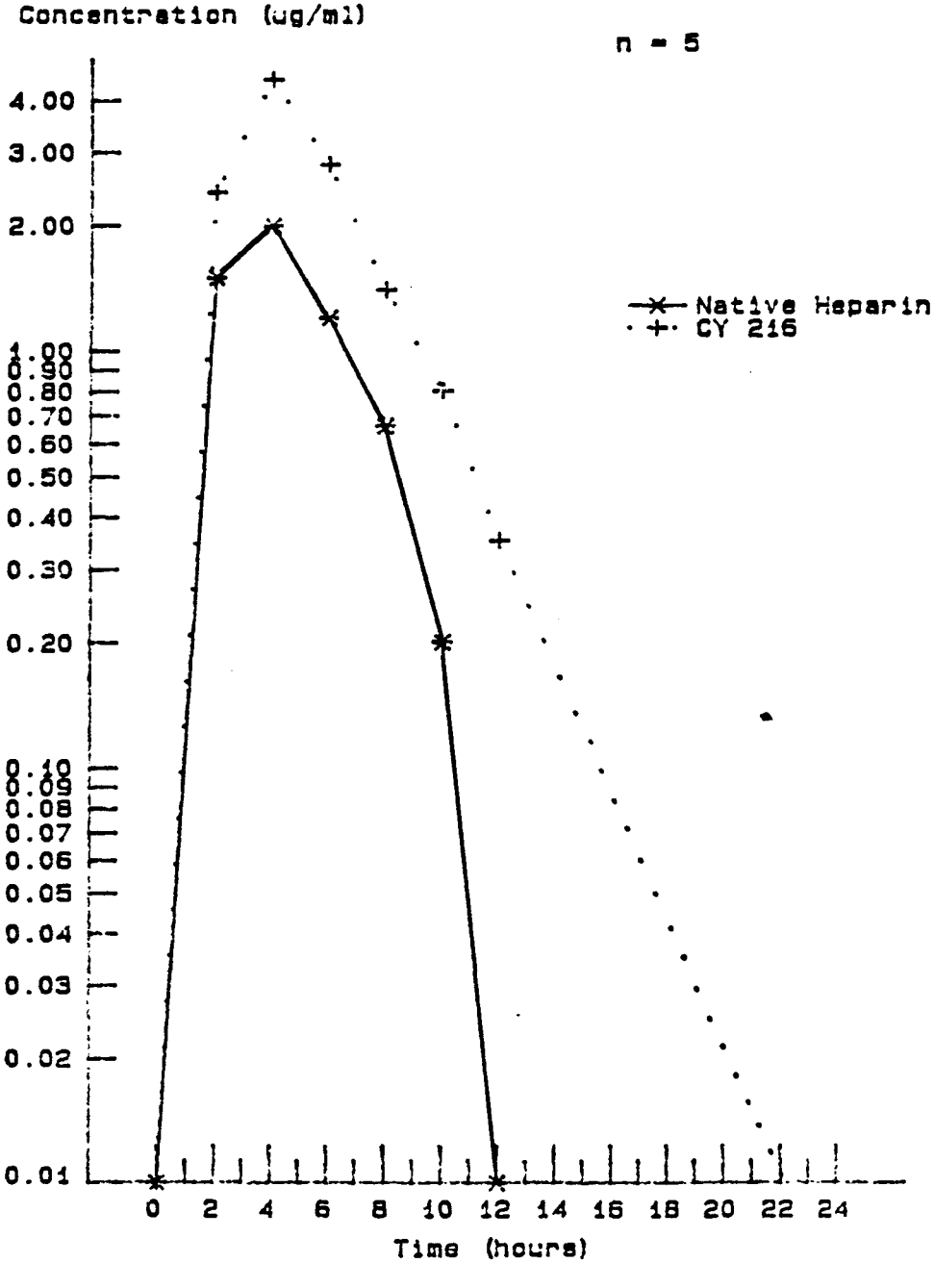
Appendix XVIId

Subcutaneous Time Course Using the Fibrinopeptide-A Assay



Appendix XVIId

Subcutaneous Time Course Using the Fibrinopeptide-A Assay



APPENDIX XVIII

MOLECULAR WEIGHT PROFILE OF TEST HEPARINS

M.W. Parameter	23,000	17,450	13,300	9,000	5,100	CY 216	Heparin
Weight Average	24,299	17,558	12,843	9,074	5,500	5,399	12,487
Z Average	28,624	19,439	15,804	14,647	12,381	10,799	14,877
Viscosity Average	24,299	17,558	12,843	9,074	5,500	5,399	12,487
Z+1 Average	49,348	27,466	42,894	78,791	78,276	86,436	17,023
MZ/ZW	1.178	1.107	1.231	1.614	2.251	2.000	1.175
Number Average	21,511	16,106	11,841	8,187	4,365	4,391	9,275
Dispersity	1.13	1.09	1.08	1.10	1.26	1.23	1.34
Peak MW	23,131	16,760	12,443	7,676	4,080	4,388	13,108
MZ+1 MW	2.673	1.654	3.340	8.683	14.232	16.010	1.345

APPENDIX XIX

APPENDIX XIX

Recovery of Fractions from Gel-Filtration Procedure

Fraction	Molecular Weight	% Recovered
I	23,000	5.0
II	17,450	14.5
III	15,000	11.5
IV	13,300	13.0
V	11,750	12.5
VI	10,400	11.0
VII	9,000	9.0
VIII	7,400	10.0
IX	5,100	2.0

Total Recovery = 88.5 %

Percent recovery was calculated by comparing the gravimetric yield of each fraction to the anticipated recovery of starting material. The percent of each fraction represents the percent of the total recovery.

APPENDIX XX

APPENDIX XX

Comparative Potency of Various Heparin Fractions

Agent	APTT D_2*	PT D_2*	Heptest D_3**	Anti Xa IC_{50}+	Anti IIa IC_{50}	FPAGT IC_{50}++
23,000	2.5	10.0	1.5	1.25	1.0	0.38
17,450	2.0	11.0	1.1	1.25	1.1	0.70
13,300	1.5	11.7	0.7	1.20	1.2	0.70
9,000	2.4	>20	1.0	2.65	2.0	1.20
5,100	6.5	>20	2.0	6.20	6.25	7.50
CY 216	2.5	>20	1.25	4.60	7.6	7.45
Heparin	1.3	20.0	1.20	1.25	0.8	0.5

* The concentration required to produce a doubling of the baseline time values. All values represent ug/ml concentrations.

** The concentration required to produce a tripling of the baseline time values. All values represent ug/ml concentrations.

\+ The concentration required to produce a 50 % inhibition in the activity of factor Xa and IIa respectively. All values represent ug/ml concentrations.

++ The concentration required to produce a 50 % inhibition of control FPA generation. All values represent ug/ml concentrations.

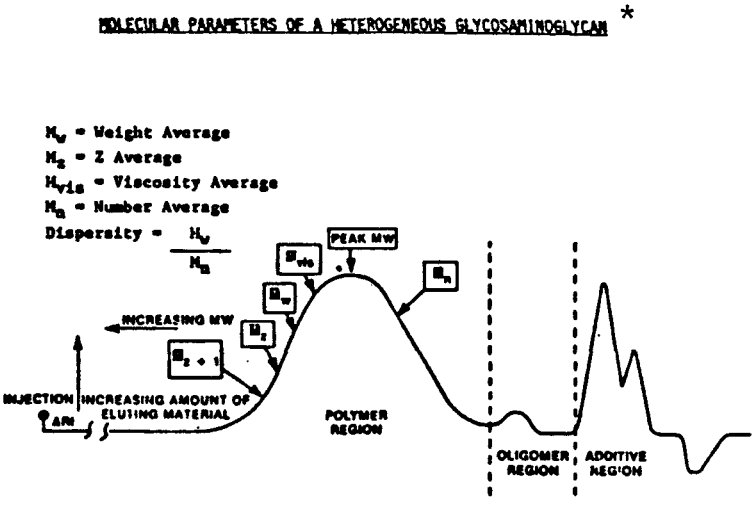
APPENDIX XXI

A Comparison of Unfractionated and Low Molecular Weight Heparin

	Heparin	Low Molecular Weight Heparin
Molecular Weight Distribution	1,000 - 40,000	1,000 - 15,000
Mean Molecular Weight	12,500	5,000
Anti Xa/IIa Ratio	1.0	> 2.0
Relative USP Potency	---→	< Heparin
Effect on Platelet Aggregation	----	< Heparin
Halflife	30 min.	60 min.
Bleeding tendency	----	< Heparin

APPENDIX XXII

Description of HPLC Elution Profile



Very slight changes in retention time can cause large changes in the mean molecular weight of polymers. For this reason it is important to interpret molecular weight data determined by HPLC relative to various parameters.

Peak Molecular Weight: Indicates the molecular weight of the greatest portion of the eluting material.

M_Z, M_W and $M_Z{}^{+}{}_1$: These values are affected by variations in the high molecular weight region of the eluting material. Thus they characterize the high molecular weight components.

M_n: Characterizes the low molecular weight portion of the elution curve. This value is sensitive to a long tail of low molecular weight components.

Dispersity: When the polymer is composed of a wide range of molecular weight components this value will be high. It indicates the range of molecular weights in the material.

* Taken from technical manual Waters 410 Chromatography system

APPROVAL SHEET

The dissertation submitted by R.Martin Emanuele has been read and approved by the following committee:

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Assistant Professor, Biochemistry & Pathology
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The final copies have been examined by the director of the dissertation and the signiture which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

8/21/87
Date

Jawed Fareed
Director's Signature